Implication des neurones exprimant NUCB2/nesfatine-1 dans la régulation de l'homéostasie énergétique

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Liste des abréviations

2-DG : 2-désoxyglucose

5-HT : sérotonine

5-TG : 5-thioglucose

ACTH : hormone corticotrope

AgRP : agouti-related peptide

AP : area postrema

APO : aire pré-optique

ARC : noyau arqué de l'hypothalamus

as-MON : oligonucléotides morpholinos antisens

BDNF : brain-derived neurotrophic factor

BHE : barrière hémato-encéphalique

BST : bed stria terminalis = noyau d'origine de la strie terminale

CART : cocaïne- and amphetamine-related transcript

CCK : cholécystokinine

CCK-A et B : récepteurs à la CCK

CeA : noyau central de l'amygdale

ChAT : choline acétyltransférase

COX : cyclooxygénase

cPGES : cytosolic PGES

CREB : cAMP response element binding protein

CRH : corticotropin-releasing hormone

CVD : complexe vagal dorsal

DBI : diazepam-binding inhibitor

DMH : noyau dorso-médian hypothalamique

DMNX : noyau dorsal moteur du nerf vague

DON : déoxynivalénol

EM : éminence médiane

EP : endogene prostanoid receptor

FSH : hormone folliculo-stimulante

GABA : acide γ-amino butyrique

GAD67 : glutamic acid decarboxylase 67

GE : glucose excited neurons

GFAP : glial fibrillary acidic protein

GFP : green fluorescent protein

GHS-R : growth hormone secretaguogue receptor = récepteur à la ghréline

GI : glucose inhibited neurons

GLP-1 : glucagon-like peptide 1

GLUT : transporteur du glucose

GnRH : gonadolibérine

GPR : gastrin-releasing peptide

icv : intracérébroventriculaire

IL : interleukine

IL-1ra : IL-1 receptor antagonist

IMC : indice de masse corporelle

ip : intrapéritonéale

IRS-1 : insulin receptor substrate 1

iv : intraveineuse

KO : knock out

LBP : LPS binding protein

LC : locus coeruleus

LCR : liquide céphalo-rachidien

LH : hormone lutéinisante

LHA : aire latérale hypothalamique

LPB : partie latérale du noyau parabrachial

LPS : lipopolysaccharide

M30 : mid-segment de 30 acides aminés

MC3/4-R : récepteurs aux mélanocortines de type 3 et 4

MCH : melanin-concentrating hormone

mPGES : microsomal PGES

NAPE : N-acylphosphoethanolamine

NLS : nerf laryngé supérieur

NPY : neuropeptide Y

NT4/5 : neurotrophine 4/5

NTS : noyau du tractus solitaire

NUCB2 : nucléobindine 2

Ob-R = LepR : récepteur à la leptine

ODN : octadécaneuropeptide

OEA : oleoylethanolamide

OMS : organisation mondiale de la santé

OVLT : organe vasculaire de la lame terminale

PACAP : pituitary adenylate cyclase activating polypeptide

PBN : noyau parabrachial

PG : prostaglandine

PGE2 : prostaglandine E2

PGES : prostaglandine E synthase

plg : plasminogène

POMC : pro-opiomélanocortine

PPAR-γ : peroxisome proliferator activated receptor-γ

PVN : noyau paraventriculaire de l'hypothalamus

PYY^{3-36} : peptide YY^{3-36}

RCPG : récepteur couplé à une protéine G

SF1 : steroidogenic factor 1

SFO : organe subfornical

SNC : sustème nesveux central

SON : noyau supra-optique

TH : tyrosine hydroxylase

TLR-4 : toll-like récepteur de type 4

TNF : tumor necrosis factor

TRH : thyrotropin-releasing hormone

TX : thromboxane

VEGF : vascular endothelial growth factor

VLM : medulla ventrolatérale

VMH : noyau ventro-médian hypothalamique

α-MSH : α-melanocyte stimulating hormone

AVANT PROPOS

Dans le cadre de mes études doctorales, je me suis intéressée à un nouveau peptide alimentaire : la nesfatine-1. J'ai plus particulièrement focalisé mon travail sur l'implication des neurones de l'hypothalamus et du complexe vagal dorsal (CVD) exprimant ce peptide dans la régulation de l'homéostasie énergétique.

Dans l'introduction de ce manuscrit, après avoir rappelé les différents troubles métaboliques, je traiterai du contrôle central de la prise alimentaire. Je m'attacherai particulièrement à décrire comment le cerveau intègre en permanence des signaux en provenance de la périphérie afin de maintenir l'homéostasie énergétique. Puis je montrerai comment les signaux inflammatoires impactent la circuiterie centrale de la prise alimentaire et modifient le comportement ingestif. Par la suite, je montrerai au travers de quelques exemples que les circuits neuronaux dévolus à la régulation de la prise alimentaire peuvent être la cible d'agents thérapeutiques. Mon introduction s'achèvera sur la présentation des données de la littérature relatives à la nesfatine-1. Nous verrons que ce peptide a initialement été décrit comme un peptide anorexigène exprimé au niveau de plusieurs organes périphériques ainsi que par le cerveau. J'expliquerai également que le spectre d'action de la nesfatine-1 dépasse largement le cadre de la régulation de la prise alimentaire puisqu'elle est impliquée dans plusieurs autres fonctions physiologiques dont la régulation de la glycémie.

Je présenterai ensuite les résultats obtenus au cours de ma thèse ayant conduit à la publication de cinq articles originaux de recherche. Ces travaux ont pour but d'analyser la sensibilité des neurones de l'hypothalamus et du CVD exprimant la nesfatine-1 à :

1. la glucoprivation provoquée par l'injection d'insuline ou d'un analogue non métabolisable du glucose : le 2-désoxyglucose (2-DG) ;
2. l'inflammation provoquée par l'administration de lipopolysaccharide (LPS), une endotoxine bactérienne mimant une infection aux bactéries à Gram négatif ;
3. l'inflammation provoquée par une intoxication orale au déoxynivalénol (DON), une mycotoxine contaminant les céréales.

Enfin, je discuterai ces résultats avant d'exposer quelques perspectives visant à :

1. approfondir le rôle de la nesfatine-1 dans les réponses à la glucoprivation et à l'inflammation en neutralisant la nesfatine-1 endogène.
2. Développer de nouveaux outils permettant d'améliorer les effets de la nesfatine-1. Ce projet, qui sera réalisé en collaboration avec des chercheurs en biochimie, consiste à synthétiser des peptides dérivés de la nesfatine-1 résistants à la protéolyse. Cette étude pourra à terme constituer des pistes pour améliorer le traitement de l'obésité et du diabète de type 2.
3. déterminer l'implication des cellules gliales de l'hypothalamus et du CVD dans la signalisation à la nesfatine-1. Cette deuxième perspective s'intègre dans un axe de recherche développé au sein du laboratoire : l'étude des cellules astrogliales du CVD, cellules présentant des caractéristiques morphologiques et phénotypiques originales **(Cf. : annexe 1)**.

INTRODUCTION

I Contrôle central de la prise alimentaire

Les déséquilibres de la balance énergétique et les troubles métaboliques tels que l'obésité, l'anorexie et le diabète constituent de nos jours un grave problème de santé publique aux conséquences économiques considérables. L'Organisation Mondiale de la Santé (OMS) définit le surpoids et l'obésité comme une accumulation anormale ou excessive de graisse corporelle qui peut nuire à la santé. En effet, un excès de poids peut entrainer des maladies cardio-vasculaires, respiratoires, articulaires, et même favoriser l'apparition de certains cancers (Calle et al., 2003; Han et al., 1997; Kopelman, 2000). La corpulence d'une personne est donnée par l'indice de masse corporelle (IMC) qui se calcule à partir de la taille et de la masse de l'individu : IMC = masse / taille^2 **(Figure 1)**.

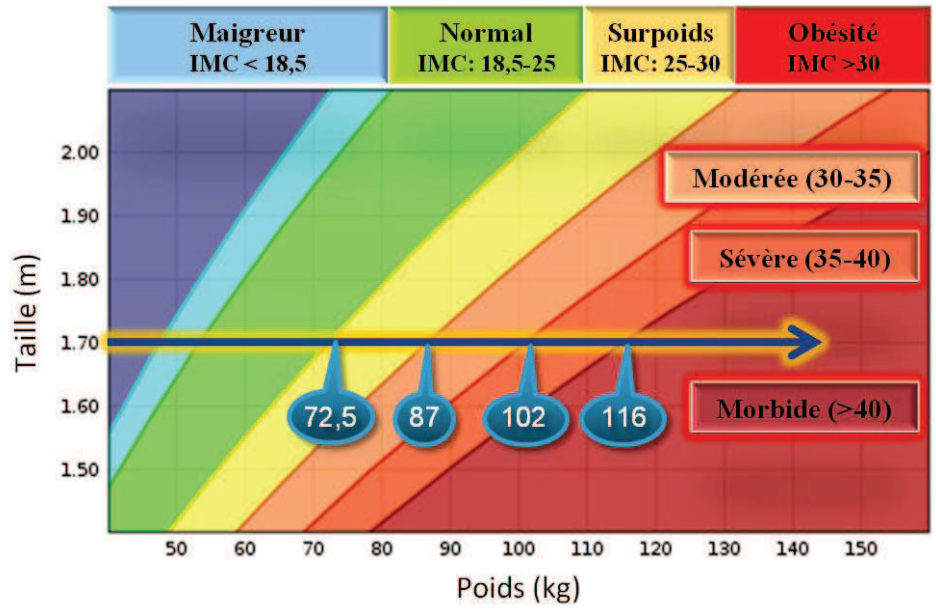


Figure 1 : Classification des IMC selon l'OMS.
L'indice de masse corporelle (IMC) est une grandeur qui permet d'estimer la corpulence d'une personne. Il se calcule à partir de la taille et de la masse de l'individu.

Bien qu'il ne s'agisse pas d'une maladie infectieuse, l'obésité est considérée comme une pandémie touchant en grande majorité les pays développés. D'après les estimations mondiales de l'OMS réalisées en 2008, environ 1,4 milliard de personnes adultes étaient en surpoids et plus de 500 millions étaient obèses. En 2011, environ 40 millions d'enfants de moins de cinq ans présentaient un surpoids. Les prédictions de l'OMS pour 2015 révèlent que

2,3 milliards d'adultes seront en surpoids et plus de 700 millions seront obèses. En France, actuellement, 32,3% des adultes sont en surpoids et 15% présentent une obésité. Ces chiffres alarmants ne cessent d'augmenter en raison de la sédentarisation de la société et de la facilité d'accès à la nourriture, en particulier aux aliments gras et sucrés **(Figure 2)**.

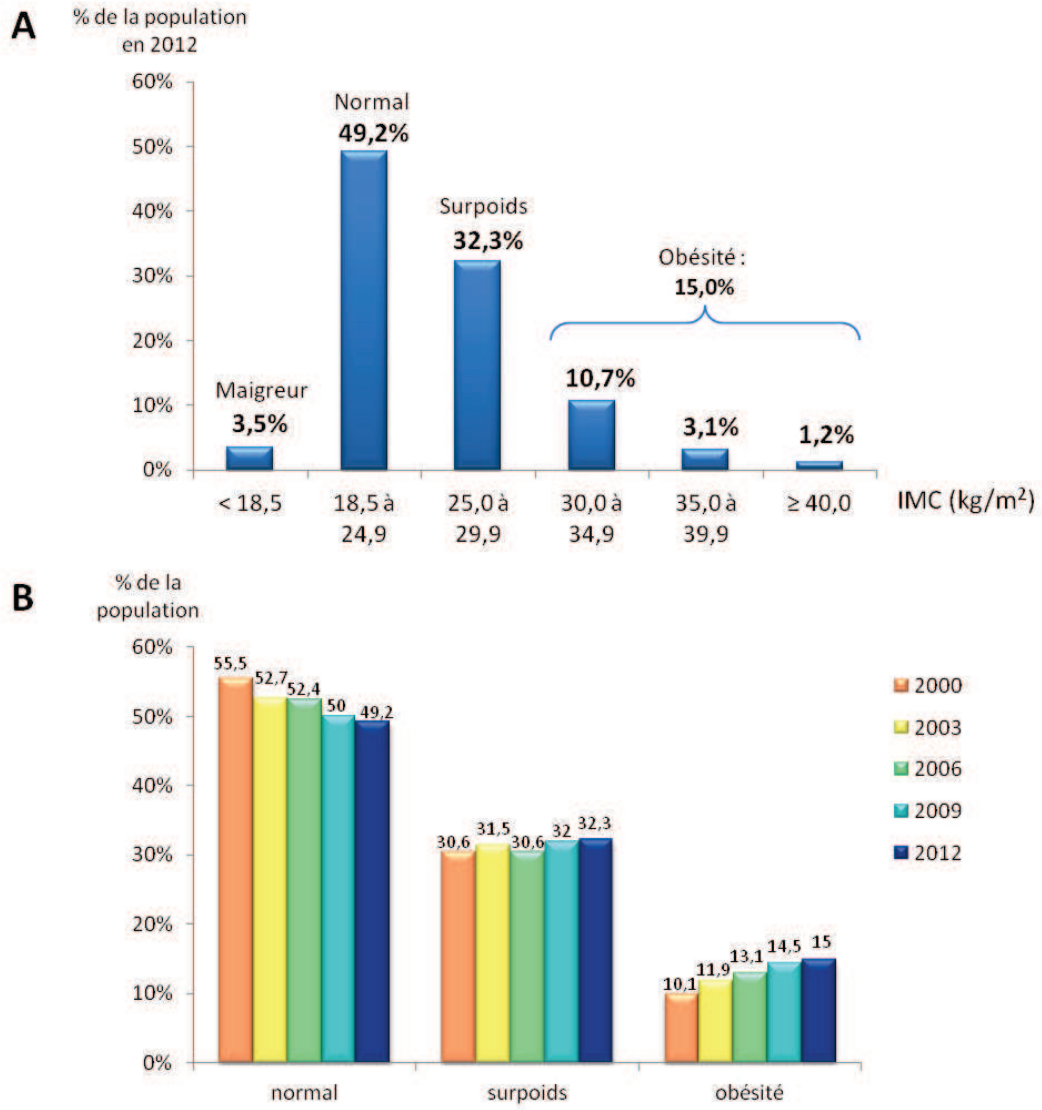


Figure 2 : Enquête épidémiologique nationale sur le surpoids et l'obésité (enquête ObEpi).
A. Répartition des individus de plus de 18 ans en fonction de leur IMC en France en 2012.
B. Evolution de la répartition des individus de plus de 18 ans en fonction de leur IMC en France depuis 2000.

L'obésité est souvent associée au diabète de type 2 puisque le surpoids est le facteur de risque majeur dans le développement de cette pathologie. Le diabète de type 2, encore appelé diabète non insulino-dépendant et diabète insulino-résistant, est une maladie métabolique

affectant la régulation de la glycémie et aboutissant à une hyperglycémie chronique. Cette hyperglycémie chronique est la conséquence d'une résistance à l'insuline, seule hormone hypoglycémiante de l'organisme. Bien que les facteurs génétiques doivent être pris en compte dans l'étiologie de cette pathologie, la principale cause du diabète de type 2 est environnementale (Hu, 2011). En effet, une alimentation riche en graisse et en sucre, ainsi que le manque d'activité physique favorisent le développement de cette maladie. On a ainsi constaté aux Etats-Unis et en Europe une nette corrélation entre la surcharge pondérale et le diabète de type 2. L'augmentation de l'obésité des enfants laisse craindre une augmentation des cas de diabète. A long terme, le diabète de type 2 peut entrainer des neuropathies, des rétinopathies et des néphropathies. De plus, comme dans le cas de l'obésité, les complications cardio-vasculaires sont fréquentes. En 2010, 285 millions de personnes dans le monde souffraient de diabète de type 2. L'OMS estime que ce nombre aura dépassé les 350 millions d'ici 2025 (Chen et al., 2012a; Shaw et al., 2010). En France, 2,9 millions de personnes sont aujourd'hui traitées pour cette maladie soit plus de 4% de la population.

A l'inverse, l'anorexie est caractérisée par une perte de poids excessive et elle est tout aussi dangereuse que l'obésité et le diabète. Il existe deux types d'anorexie : l'anorexie mentale et l'anorexie inflammatoire. L'anorexie mentale est une maladie psychiatrique aboutissant dans 10% des cas à la mort. Elle touche essentiellement les jeunes filles. Les données épidémiologiques sont encore rares en France. Aux États-Unis, où l'anorexie mentale est la troisième maladie chronique chez l'adolescente après l'obésité et l'asthme, la prévalence serait de 0,48 % dans la tranche des 15-19 ans. L'anorexie inflammatoire survient quant à elle lorsque l'organisme se défend contre une inflammation, qu'elle soit aiguë (infection par un virus ou une bactérie) ou chronique (par exemple un cancer). Ainsi, si la diminution de prise alimentaire est de courte durée, l'organisme ressentira simplement des signes de fatigue mais si elle se prolonge, les conséquences pourront être lourdes : dérèglement hormonal, décalcification, pertes de cheveux, hypotension, escarres… La mort peut survenir par arrêt cardiaque.

Ces quelques données succinctes illustrent clairement l'importance pour un individu d'équilibrer sa balance énergétique en jouant particulièrement sur son comportement alimentaire. En étudiant des effecteurs modulant la prise alimentaire et l'activité de réseaux neuronaux situés dans les structures centrales dédiées au contrôle du comportement ingestif, mon travail de thèse s'inscrit pleinement dans ce contexte général. Avant d'exposer plus

précisément l'objet de mon travail et les résultats obtenus, j'aborderai dans mon introduction les notions d'homéostasie énergétique et de contrôle central de la prise alimentaire.

1. Notion d'homéostasie énergétique

La stabilisation à long terme de notre poids corporel résulte d'un équilibre étroit entre les dépenses et les apports énergétiques dénommé « homéostasie énergétique ». Les dépenses sont continues puisqu'elles couvrent le métabolisme basal de l'organisme, ainsi que les besoins nécessaires à l'activité musculaire et cérébrale. Les apports énergétiques sont quant à eux assurés par le comportement de prise alimentaire qui fournit à l'organisme les nutriments nécessaires à son fonctionnement. La prise alimentaire est un phénomène discontinu réparti sous forme de repas permettant l'accomplissement d'autres comportements. Par conséquent, l'organisme doit faire face à la difficulté permanente d'équilibrer des dépenses continues à des apports discontinus. Pour contourner cet obstacle, il stocke une partie de l'énergie apportée par un repas et constitue des réserves sous la forme d'acides gras dans le tissu adipeux ou de glycogène dans le foie et les muscles squelettiques **(Figure 3)**. Ainsi, à l'âge adulte, si les apports énergétiques sont supérieurs aux dépenses, l'excédent est converti en graisse, et le poids corporel augmente. A l'inverse, si les dépenses sont supérieures aux apports, l'organisme est contraint d'utiliser ses réserves, ce qui induit une perte de poids **(Figure 4)**. Le contrôle de l'homéostasie énergétique nécessite donc une estimation permanente de l'état des réserves afin d'adapter la quantité d'aliments ingérés au cours d'un repas. Ces mécanismes sont sous le contrôle du système nerveux central (SNC).

C'est en 1840 que pour la première fois un lien entre le poids corporel et le SNC est proposé. En étudiant un patient atteint d'une tumeur de l'hypophyse, Mohr associe une forte obésité à un dysfonctionnement d'origine cérébrale (Mohr, 1840). Puis, en 1921, les travaux de Bailey et Bremer suggèrent que l'obésité ne serait pas d'origine hypophysaire mais hypothalamique. Ces auteurs montrent que des lésions de l'hypothalamus chez le chien entrainent une obésité (Bailey et Bremer, 1921). Quelques années plus tard, des travaux chez le rat semblent confirmer cette hypothèse et identifient une zone de l'hypothalamus à l'origine du contrôle de la balance énergétique. La lésion des noyaux ventro-médians hypothalamiques (VMH) entrainait systématiquement une obésité due à une hyperphagie,

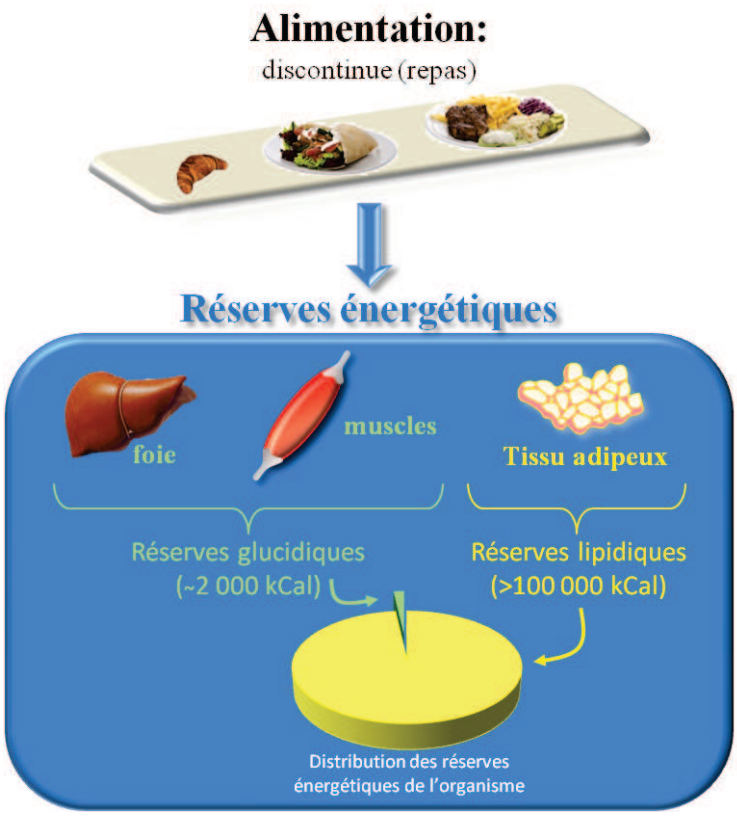


Figure 3 : Stockage de l'énergie apportée au cours des repas sous forme de réserves énergétiques.
L'énergie est stockée dans le foie, les muscles et le tissu adipeux. Les réserves adipeuses constituent la grande majorité des réserves énergétiques de l'organisme.

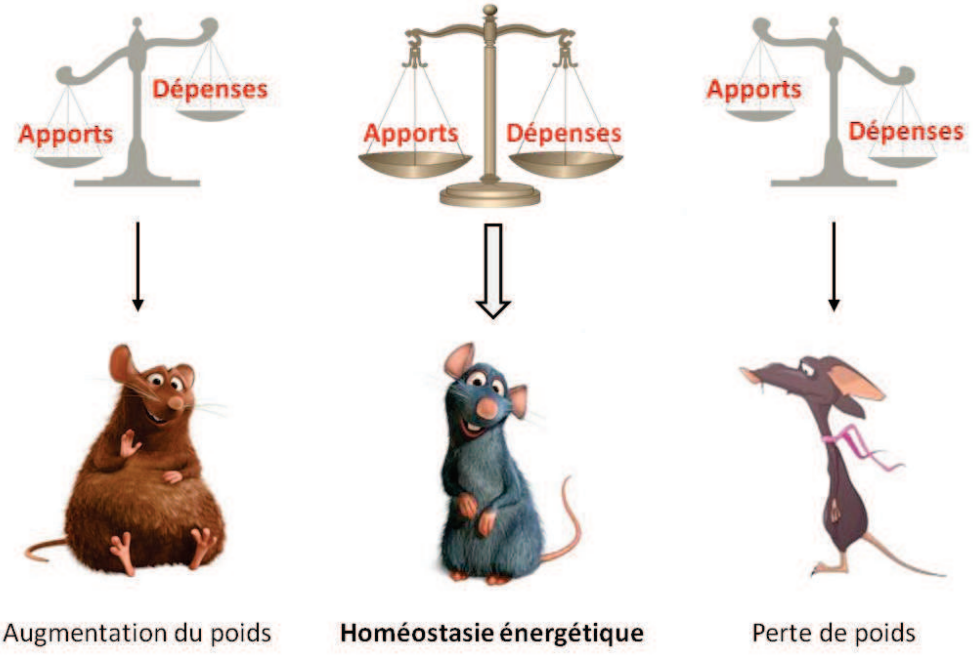


Figure 4 : Maintien de l'homéostasie énergétique.
Le maintien de l'homéostasie énergétique résulte d'un équilibre étroit entre les apports et les dépenses énergétiques. Tout déséquilibre dans cette balance entraine des modifications du poids corporel.

alors qu'une hypophysectomie ou des lésions d'autres zones hypothalamiques n'augmentaient pas le poids corporel (Brobeck et al., 1943; Hetherington, 1944; Hetherington et Ranson, 1940). A la lumière de ces résultats, le VMH fut qualifié de « centre cérébral de la satiété » (Kennedy, 1950), idée qui fut renforcée cinq ans plus tard lorsqu'Anand et Dua montrèrent que des stimulations électriques du VMH entraînaient une diminution de prise alimentaire (Anand et Dua, 1955). En parallèle, d'autres travaux montrèrent que la lésion de l'aire latérale hypothalamique (LHA) entrainait une diminution de la prise alimentaire et une perte de poids, alors que sa stimulation avait l'effet inverse (Anand et Brobeck, 1951). La LHA fut alors qualifiée de « centre cérébral de la faim ». Ainsi, cette « théorie des deux centres », bien qu'éloignée du modèle actuel de la régulation centrale de la prise alimentaire, fut la première théorie décrivant le système nerveux central comme moteur du comportement alimentaire. Elle a longtemps servi de base à de très nombreux travaux permettant d'aboutir à la conception actuelle du maintien de l'homéostasie énergétique que je décrirai dans la suite de cette introduction et qui consiste en deux grands mécanismes : l'estimation par le SNC des réserves énergétiques et l'adaptation de la fréquence et de la taille des repas selon l'état de ces réserves et les dépenses énergétiques.

2. Estimation de l'état des réserves énergétiques

Au sein de l'organisme, le tissu adipeux constitue la source majeure de réserves énergétiques. Kennedy, en 1953, est le premier à supposer qu'afin de maintenir l'homéostasie énergétique, le SNC est en permanence informé de l'état de ces réserves par des médiateurs périphériques anorexigènes appelés « signaux d'adiposité », émis proportionnellement à la quantité de masse adipeuse (Kennedy, 1953). Cette théorie « lipostatique » de Kennedy a été par la suite largement confirmée d'un point de vue expérimental. Pour illustrer ce point, j'aborderai d'abord la nature des signaux d'adiposité en donnant leurs caractéristiques principales puis je traiterai du rôle des structures centrales dans l'intégration de ces signaux.

2.1. Les signaux d'adiposité

- *L'insuline*

De façon paradoxale, l'insuline, hormone hypoglycémiante, a été la première hormone proposée comme signal d'adiposité (Woods et al., 1979). En effet, bien qu'elle ne soit pas synthétisée par le tissu adipeux mais par les cellules béta du pancréas, sa concentration plasmatique est proportionnelle à la masse adipeuse (Bagdade et al., 1967; Polonsky et al., 1988), phénomène pouvant s'expliquer par la diminution de la sensibilité à l'insuline lors d'un gain de poids aboutissant à une sécrétion accrue d'insuline (Schwartz et al., 1997a). Elle fut découverte en 1921 par Frederick Banting, Charles Best et John James Richard Macleod, ce qui leur valut un prix Nobel en 1923, mais sa structure fut déterminée en 1955 par Frederick Sanger qui fut également récompensé par un prix Nobel en 1958. L'insuline est constituée de 2 chaînes polypeptidiques reliées entre elles par 2 ponts disulfures : une chaîne A de 21 acides aminés et une chaîne B de 30 acides aminés. En plus de son action hypoglycémiante bien connue, plusieurs arguments montrent que l'insuline est capable d'agir au niveau central pour modifier la prise alimentaire **(Figure 5)**. D'une part l'insuline circulante peut rejoindre le cerveau par un transport saturable (Baura et al., 1993), et d'autre part, son injection intracérébroventriculaire (icv) engendre une diminution de la prise alimentaire et une perte de poids (Woods et al., 1979). Des récepteurs à l'insuline sont exprimés dans plusieurs noyaux centraux et particulièrement dans le noyau arqué de l'hypothalamus (ARC) (Havrankova et al., 1978). Des souris dépourvues de récepteurs à l'insuline uniquement dans les neurones sont hyperphagiques et obèses démontrant le rôle crucial de l'insuline sur le cerveau dans le contrôle de l'homéostasie énergétique (Brüning et al., 2000). Notons qu'il a été proposé que l'insuline puisse également être synthétisée directement dans le cerveau (Plata-Salamán, 1991). En effet, des expériences d'hybridation in situ menées chez le lapin et le rat ont montré la présence d'ARNm de l'insuline dans le bulbe olfactif, l'hippocampe, et le noyau périventriculaire de l'hypothalamus (Devaskar et al., 1994; Young, 1986). En microscopie électronique, la présence d'insuline a été démontrée dans des neurones de fœtus de rat, et plus précisément au niveau du réticulum endoplasmique, de l'appareil de Golgi, du cytoplasme, de l'axone et des dendrites (Schechter et al., 1996). Cependant, la synthèse neuronale d'insuline reste à ce jour controversée, certainement à cause de son niveau d'expression très faible dans le cerveau.

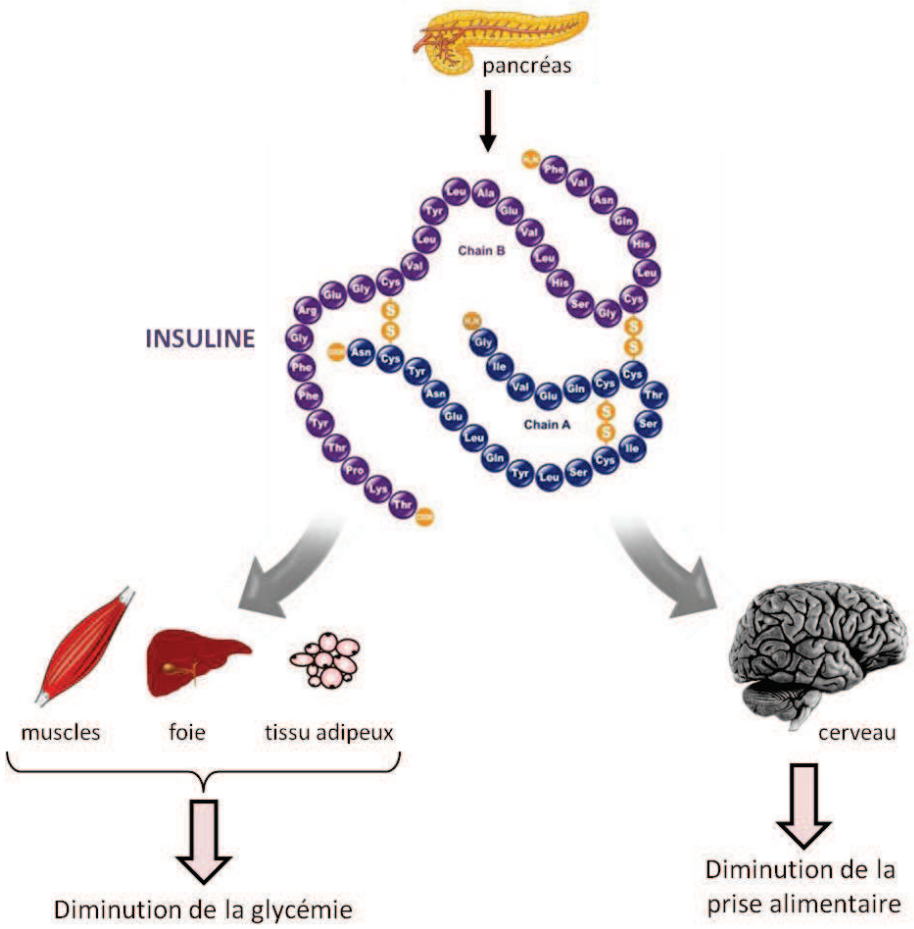


Figure 5 : Rôle de l'insuline.
L'insuline, libérée par les cellules béta du pancréas, peut agir en périphérie pour diminuer la glycémie, et en central comme signal d'adiposité pour diminuer la prise alimentaire.

- *La leptine*

La découverte de la leptine, du grec « leptos » qui signifie « maigre », en 1994 (Zhang et al., 1994) a rapidement suscité un très grand intérêt. Elle fut d'ailleurs immédiatement caractérisée comme le signal d'adiposité majeur car elle est synthétisée et sécrétée par les adipocytes eux-mêmes (Masuzaki et al., 1995). La leptine est une hormone polypeptidique de 146 acides aminés obtenue après clivage d'un propeptide de 167 acides aminés, produit du gène *ob* (pour *obese*). Comme l'insuline, la sécrétion de leptine est proportionnelle à la quantité de réserves adipeuses. Une perte de poids entraine donc une diminution de leptine plasmatique et inversement (Ahrén et al., 1997; Boden et al., 1996; Havel et al., 1996; Keim et al., 1998). Plusieurs travaux ont montré que l'administration périphérique mais aussi

centrale de leptine entraine un effet anorexigène et une perte de poids chez la souris et le rat (Campfield et al., 1995; Halaas et al., 1995; Rentsch et al., 1995; Seeley et al., 1996). La mutation ponctuelle du gène de la leptine aboutissant à une forme non fonctionnelle de cette hormone engendre une forte obésité et une hyperphagie, démontrant ainsi l'importance de la leptine dans le maintien de l'homéostasie énergétique (Zhang et al., 1994). Chez ces souris nommées *ob/ob*, l'administration de leptine exogène élimine l'hyperphagie et rétablit un phénotype non obèse (Pelleymounter et al., 1995). Chez l'homme également, des traitements à la leptine sont utilisés pour traiter des patients obèses atteints de déficience en leptine congénitale. Cependant il ne s'agit que d'une proportion minime des cas d'obésité, puisque la majorité des obèses ne présente pas de défaut de production de leptine mais au contraire souffre d'une hyperleptinémie contribuant à la résistance à la leptine (Farooqi et al., 1999; Paz-Filho et al., 2011).

Le récepteur à la leptine (Ob-R) est un membre de la famille des récepteurs aux cytokines et existe sous différentes isoformes dans le cerveau et certains organes périphériques comme le pancréas et les muscles squelettiques. Ces isoformes sont réparties en trois groupes : la forme longue, la forme courte et la forme soluble. C'est la forme longue de ce récepteur nommé Ob-Rb qui est responsable des effets centraux de la leptine. Les récepteurs Ob-Rb sont exprimés dans plusieurs structures cérébrales mais particulièrement dans l'hypothalamus et le complexe vagal dorsal (CVD) au niveau du tronc cérébral (Elmquist et al., 1998a; Mercer et al., 1996, 1998). Comme l'insuline, la leptine est capable de rejoindre le cerveau par un transport saturable (Banks et al., 1996). Certains récepteurs à forme courte (Ob-Ra et Ob-Rc) permettraient le passage de la leptine à travers la barrière hémato-encéphalique (BHE) (Kastin et al., 1999). Ainsi, dans le liquide céphalorachidien, la concentration de leptine est proportionnelle à sa concentration plasmatique et à la masse adipeuse (Schwartz et al., 1996).

Au niveau central, la fixation de la leptine sur le récepteur Ob-Rb conduit à la dimérisation de celui-ci, entrainant la phosphorylation des protéines JAK qui sont des kinases associées au domaine intracellulaire du récepteur. JAK permet alors le recrutement de la protéine STAT3 qui, une fois activée, est transloquée dans le noyau où elle agit comme facteur de transcription (Myers, 2004) **(Figure 6)**. Une altération du fonctionnement du récepteur à la leptine conduit à un phénotype obèse, hyperphagique, mais aussi diabétique : c'est le cas des souris *db/db* (pour *diabetes*) et des rats Zucker *fa/fa* (pour *fatty*) dont le

récepteur à la leptine est inactif. La réexpression du récepteur de la leptine dans les neurones de souris *db/db* restore un phénotype non obèse, non hyperphagique et non diabétique (de Luca et al., 2005; Kowalski et al., 2001).

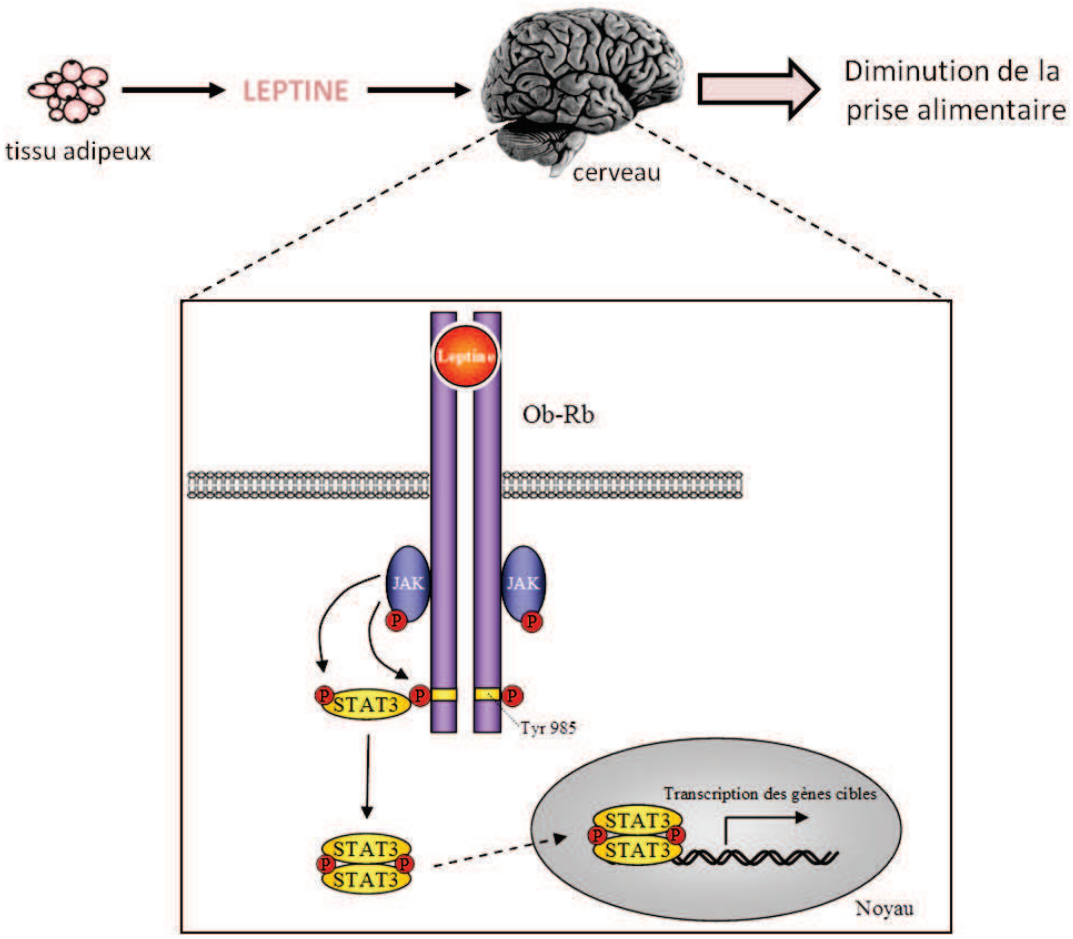


Figure 6 : Rôle de la leptine.
La leptine est un signal d'adiposité sécrétée par le tissu adipeux et agissant sur le cerveau sur des récepteurs Ob-Rb pour diminuer la prise alimentaire. Elle met en jeu la voie de signalisation JAK/STAT3.

Ensemble, ces données suggèrent que la leptine exerce son effet anorexigène via une action centrale. Cette idée est renforcée par le fait que des injections centrales mais aussi périphériques de leptine activent plusieurs noyaux hypothalamiques et bulbaires, comme en atteste l'augmentation d'expression du gène précoce c-fos admis comme étant un marqueur d'activation neuronale (Elias et al., 2000; Elmquist et al., 1997; Van Dijk et al., 1996). Ainsi les signaux d'adiposité recrutent des réseaux neuronaux qui vont freiner la prise alimentaire proportionnellement à la quantité de réserves énergétiques.

- *Les autres adipokines*

Le tissu adipeux synthétise et sécrète d'autres hormones, nommées adipokines, telles que la résistine et la vaspine. Ces hormones sont peu étudiées malgré leur capacité à agir comme signal d'adiposité. Les taux circulants de résistine sont plus élevés chez des animaux obèses que chez des animaux minces (Steppan et al., 2001). Comme l'insuline et la leptine, son injection centrale entraine une diminution de la prise alimentaire via l'activation de neurones hypothalamiques (Cifani et al., 2009; Tovar et al., 2005). Les récepteurs à la résistine n'ont pas encore été identifiés à ce jour. La vaspine entraine elle aussi un effet anorexigène lors de son injection centrale ou périphérique et sa concentration plasmatique est corrélée avec l'IMC (Klöting et al., 2011; Youn et al., 2008; Brunetti et al., 2011).

2.2. Rôle de l'hypothalamus dans l'intégration des signaux d'adiposité

Les données actuelles indiquent que l'hypothalamus est la principale structure cérébrale qui réceptionne les signaux d'adiposité véhiculés par voie humorale. Cela entraine alors l'activation d'un ensemble de neurones impliqués dans la régulation de la prise alimentaire et de la dépense énergétique. Le noyau arqué (ARC) de l'hypothalamus est à la base de ce circuit neuronal. Il contient deux populations distinctes de neurones : orexigènes et anorexigènes. Ces neurones sont dits de « premier ordre » car ils traduisent directement les signaux d'adiposité en signaux nerveux. Grâce à leurs nombreuses projections neuronales, ils transmettent ces informations aux autres noyaux hypothalamiques dans lesquels sont localisés les neurones de « second ordre ».

- *Neurones de premier ordre du noyau arqué*

L'ARC est un site de forte expression des récepteurs à l'insuline et à la leptine. Situé dans la partie médiobasale de l'hypothalamus, ce noyau étroit borde la partie ventrale du troisième ventricule. Il est situé à proximité de l'éminence médiane (EM), un organe circumventriculaire, c'est-à-dire dépourvu de BHE, ce qui favorise la pénétration des signaux circulants. Des micro-injections de leptine dans l'ARC induisent une diminution de prise alimentaire (Satoh et al., 1997), alors que la destruction de ce noyau annule l'anorexie induite par l'administration icv de leptine (Dawson et al., 1997; Tang-Christensen et al., 1999).

L'ARC contient deux populations distinctes de neurones sensibles aux signaux d'adiposité et exerçant des effets opposés sur la prise alimentaire : une population de neurones principalement situés dans la zone médiane de l'ARC qui co-expriment les neuropeptides orexigènes *neuropeptide Y* (NPY) et *agouti-related peptide* (AgRP) (Hahn et al., 1998), et une population de neurones principalement situés dans la zone latérale de l'ARC qui co-expriment les peptides anorexigènes *pro-opiomélanocortine* (POMC) et *cocaïne- and amphetamine-related transcript* (CART) (Elias et al., 1998). Les neurones NPY/AgRP et POMC/CART expriment à la fois les récepteurs à l'insuline et à la leptine (Baskin et al., 1999; Benoit et al., 2002; Cheung et al., 1997). Une injection systémique de leptine active la voie de signalisation intracellulaire STAT3 dans ces deux populations de neurones (Elias et al., 1999). Des études électrophysiologiques ont montré que la leptine entraîne une dépolarisation et une augmentation de la fréquence de décharge des neurones POMC, tandis qu'elle inhibe les neurones NPY (Cowley et al., 2001; van den Top et al., 2004). De plus, elle stimule les entrées de calcium dans les neurones POMC alors qu'elle les freine dans les neurones NPY (Wang et al., 2008). Ainsi, la fixation de leptine sur les neurones anorexigènes POMC/CART entraine leur activation alors que sa fixation sur les neurones orexigènes NPY/AgRP inhibe ces derniers. De plus, les neurones NPY/AgRP, qui synthétisent également l'acide γ-amino butyrique (GABA), envoient des collatérales d'axones inhibitrices vers les neurones POMC/CART et la leptine lève cette inhibition, renforçant l'activation des neurones anorexigènes (Cowley et al., 2001) **(Figure 7)**.

Les neuropeptides anorexigènes POMC et CART :

La POMC est un propeptide dont le clivage intracellulaire aboutit à la formation du peptide anorexigène *α-melanocyte stimulating hormone* (α-MSH). L'α-MSH est un agoniste des récepteurs aux mélanocortines et son caractère anorexigène est relayé par le récepteur de type 4 (MC4-R) qui est exprimé dans plusieurs noyaux hypothalamiques (Mountjoy et al., 1994). En effet, des injections centrales d'agonistes ou d'antagonistes mélanocortinergiques induisent respectivement une diminution ou une augmentation de la prise alimentaire (Fan et al., 1997) et les modèles génétiques murins n'exprimant pas MC4-R présentent un phénotype hyperphagique associé à une forte obésité (Huszar et al., 1997). De plus, les souris Agouti, caractérisées par une surexpression de la protéine agouti qui est un antagoniste endogène des récepteurs MC4-R, présentent un phénotype similaire à celui des souris MC4-R-/- (Lu et al., 1994).

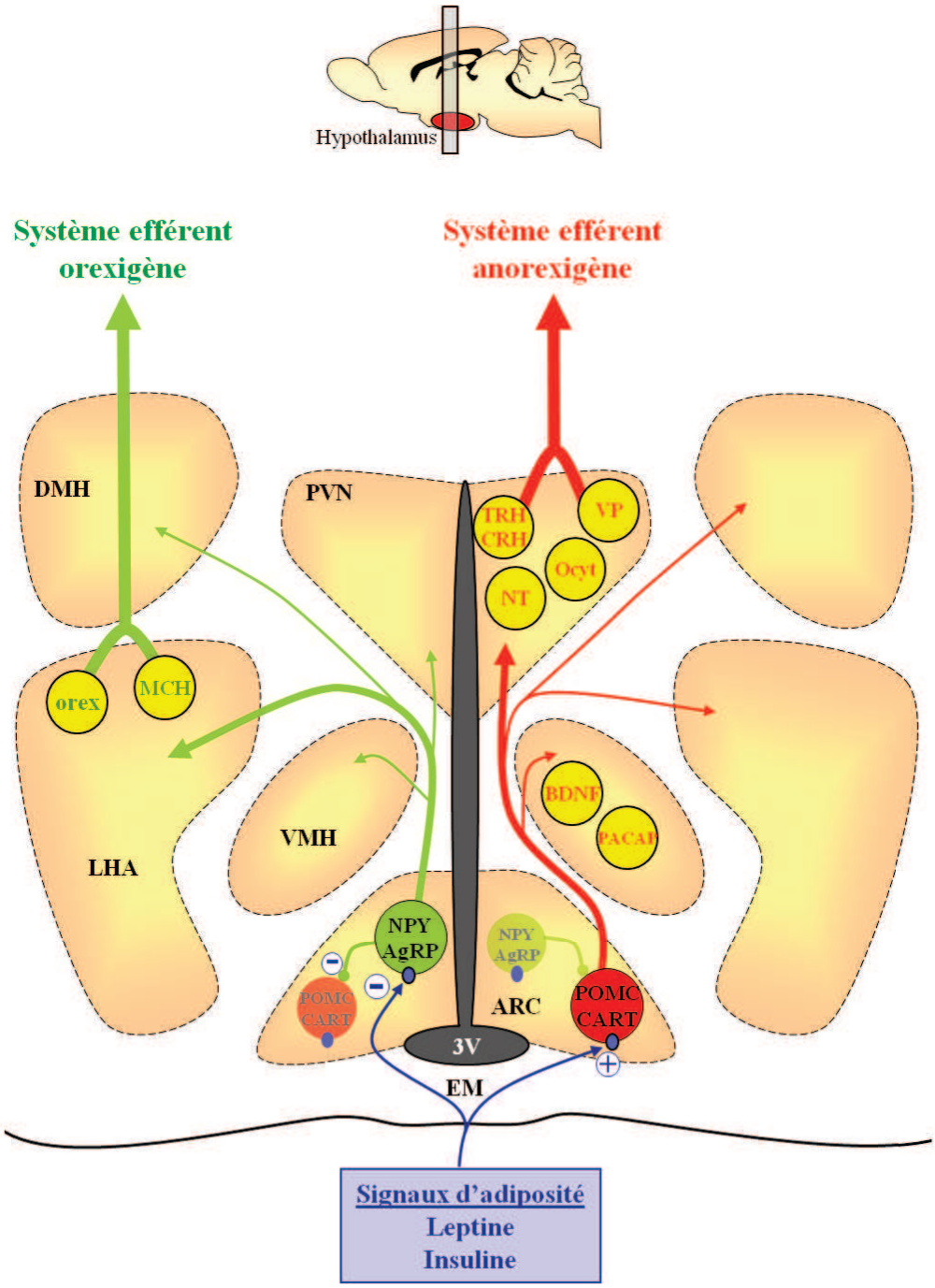


Figure 7: Implication de l'hypothalamus dans l'intégration des signaux d'adiposité.
Les signaux d'adiposité circulants activent les neurones POMC/CART de l'ARC et inhibent les neurones NPY/AgRP. Ces neurones de premier ordre projettent sur des neurones de second ordre localisés dans différents noyaux hypothalamiques et formant une voie orexigène et une voie anorexigène. Ainsi, les signaux d'adiposité stimulent le réseau hypothalamique anorexigène et inhibent le réseau orexigène, aboutissant à une diminution de prise alimentaire.

NPY: neuropeptide Y; AgRP: agouti-related peptide; POMC: pro-opiomélanocortine; CART: cocaïne- and amphetamine-related transcript; orex: orexine; MCH: melanin-concentrating hormone; TRH: thyrotropin-releasing hormone; CRH: corticotropin-releasing hormone; Ocyt: ocytocine; VP: vasopressine; NT: neurotensine; BDNF: brain-derived neurotrophic factor; PACAP: pituitary adenylate cyclase activating polypeptide; DMH: noyau dorso-médian hypothalamique; VMH: noyau ventro-médian hypothalamique; LHA: aire latérale hypothalamique; PVN: noyau paraventriculaire; ARC: noyau arqué; EM: éminence médiane; 3V: troisième ventricule.

La CART est colocalisée avec la POMC dans les neurones de l'ARC et elle exerce également un effet anorexigène puisque son injection centrale entraine une diminution de la prise alimentaire alors qu'un effet inverse est observé suite à l'injection d'anticorps anti-CART (Kristensen et al., 1998).

L'expression de POMC et CART au niveau de l'ARC varie en fonction du statut nutritionnel de l'individu : elle diminue après une privation alimentaire et augmente en présence de forts taux d'insuline et de leptine (Kim et al., 1996; Kristensen et al., 1998; Schwartz et al., 1997b). Chez les souris *ob/ob* et *db/db* fortement obèses, l'expression de POMC est réduite par rapport aux souris sauvages (Mizuno et al., 1998).

Les neuropeptides orexigènes NPY et AgRP :

Le NPY est un peptide orexigène dont l'injection centrale stimule la prise alimentaire et peut aboutir au développement de l'obésité lorsque ce traitement est chronique (Stanley et al., 1986). Il exerce cet effet orexigène via l'activation d'au moins deux types de récepteurs, les récepteurs Y1 et Y5. De manière intéressante, la délétion du gène codant pour le NPY réduit l'hyperphagie et l'obésité caractéristiques des souris *ob/ob* (Erickson et al., 1996).

L'AgRP est un antagoniste endogène des récepteurs MC4-R. Ainsi, une administration centrale d'AgRP entraine une augmentation de la prise alimentaire (Rossi et al., 1998) et des souris génétiquement modifiées surexprimant AgRP sont hyperphagiques et obèses (Ollmann et al., 1997). De même, l'injection intra-hypothalamique de vecteurs recombinants dérivés de virus adéno-associés entrainant une surexpression soit d'AgRP soit de NPY provoque une augmentation de la prise alimentaire et du poids corporel (de Backer et al., 2011; Tiesjema et al., 2007).

L'expression de NPY et AgRP au niveau de l'ARC est régulée de manière inverse à celle de POMC et CART : 1/ elle est stimulée par le jeûne et retourne à son niveau basal lorsque les animaux sont renourris (Beck et al., 1990; Hahn et al., 1998; Palou et al., 2009) ; 2/ elle est diminuée par la leptine et l'insuline (Mizuno et Mobbs, 1999; Sipols et al., 1995) ; 3/ le NPY et l'AgRP sont surexprimés chez les souris *ob/ob* (Shutter et al., 1997; Wilding et al., 1993).

Les autres neurones de premier ordre :

La délétion des récepteurs à la leptine sélectivement dans les neurones POMC ou AgRP entraine une prise de poids associée à une hyperphagie et un effet additif est observé lorsque la délétion des récepteurs est effectuée à la fois dans les neurones POMC et AgRP (Balthasar et al., 2004; Hill et al., 2010; van de Wall et al., 2008). Cependant, l'obésité engendrée par cette délétion spécifique des récepteurs à la leptine dans les neurones POMC et AgRP est moins conséquente que chez des souris *db/db* chez qui ce récepteur est absent dans tout l'organisme. Cela suggère l'existence d'autres populations de neurones de premier ordre directement sensibles à la leptine et capables de relayer une partie de ses effets. Cette hypothèse est renforcée par la présence de récepteurs à la leptine dans d'autres structures cérébrales que l'ARC, notamment le noyau paraventriculaire de l'hypothalamus (PVN), les noyaux ventro-médian et dorso-médian hypothalamiques (VMH et DMH), l'aire hypothalamique latérale (LHA) et le noyau de tractus solitaire (NTS) au niveau du tronc cérébral (Caron et al., 2010; Scott et al., 2009). De plus, des micro-injections de leptine dans des zones cérébrales éloignées de l'ARC sont capables de provoquer une diminution de prise alimentaire (Grill et al., 2002; Leinninger et al., 2009). Des études se sont tournées vers des neurones du VMH exprimant la protéine SF1 (pour steroidogenic factor 1), puisque des souris knockout pour cette protéine sont obèses (Majdic et al., 2002). SF1 est un facteur de transcription nécessaire à la mise en place des neurones du VMH. La délétion du récepteur à la leptine dans les neurones SF1 entraine une légère prise de poids (Bingham et al., 2008; Dhillon et al., 2006). Une étude récente s'est portée sur les neurones glutamatergiques et GABAergiques du SNC. La délétion des récepteurs à la leptine dans les neurones GABAergiques, grâce au système de recombinaison Cre-Lox, entraine une augmentation de la prise alimentaire et du poids corporel, alors que dans les neurones glutamatergiques cette délétion n'a pas d'effet. Les auteurs montrent qu'en l'absence de leptine, ces neurones GABAergiques exercent un tonus inhibiteur sur les neurones POMC/CART et que la leptine lève ce tonus inhibiteur (Vong et al., 2011). Nous verrons plus tard que le tronc cérébral joue également un rôle important dans l'intégration des signaux d'adiposité (Cf. : I.2.3.).

- *Neurones de second ordre*

Les neurones POMC/CART et NPY/AgRP de l'ARC envoient des projections dans d'autres noyaux hypothalamiques impliqués dans le contrôle de la prise alimentaire, notamment le

PVN, la LHA, le VMH et le DMH (Elmquist et al., 1998b). Dans le schéma actuel du contrôle de la prise alimentaire, le PVN est le centre anorexigène recevant des informations des neurones POMC/CART et la LHA est le centre orexigène recevant des informations des neurones NPY/AgRP **(Figure 7)**.

La stimulation du PVN diminue la prise alimentaire alors que sa destruction bilatérale conduit à une hyperphagie et au développement d'une obésité (King et al., 1989). En accord avec ces données, le PVN exprime plusieurs neuropeptides anorexigènes : la *corticotropin-releasing hormone* (CRH) (Glowa et al., 1992), la *thyrotropin-releasing hormone* (TRH) (Kow et Pfaff, 1991), l'ocytocine (Olson et al., 1991), la vasopressine (Langhans et al., 1991) et la neurotensine (Luttinger et al., 1982). Notons que la neurotensine est également exprimée dans plusieurs autres noyaux hypothalamiques dont l'ARC. L'expression de ces neuropeptides hypothalamiques diminue chez des animaux à jeun alors que l'injection de leptine stimule leur expression (Sahu, 2008; Tung et al., 2008; Uehara et al., 1998). De plus, l'injection icv d'α-MSH active les neurones TRH- et CRH-positifs du PVN (Sarkar et al., 2002). Ensemble, ces résultats indiquent que les neurones du PVN exprimant la CRH, la TRH, l'ocytocine, la vasopressine et la neurotensine sont des neurones de second ordre participant aux effets anorexigènes de la leptine. La présence d'un neuropeptide orexigène, la galanine, a cependant été relevée dans le PVN (Kyrkouli et al., 2006). Le VMH pourrait, avec le PVN, constituer un deuxième centre anorexigène puisque d'une part sa lésion entraine une hyperphagie et une obésité, ce qui lui avait valu le titre de « centre de la satiété » dans les années 50. D'autre part, le VMH exprime aussi des neuropeptides anorexigènes comme par exemple le *brain-derived neurotrophic factor* (BDNF) et le *pituitary adenylate cyclase activating polypeptide* (PACAP) (Kernie et al., 2000; Maekawa et al., 2006).

A l'inverse du PVN, la stimulation de la LHA entraine une augmentation de la prise alimentaire et sa lésion bilatérale entraine une anorexie et une perte de poids. La LHA est le site d'expression de neuropeptides orexigènes tels que les orexines (ou hypocrétines) et la *melanin-concentrating hormone* (MCH). L'administration centrale de MCH ou d'orexine A induit une augmentation de la prise alimentaire (Haynes et al., 1999; Qu et al., 1996) alors que des souris transgéniques présentant une délétion du gène codant pour la MCH sont anorexiques et excessivement maigres (Shimada et al., 1998). Les souris déficientes en orexine, quant à elles, ont un poids légèrement inférieur aux souris sauvages et notons qu'elles sont narcoleptiques car l'orexine est aussi impliquée dans la régulation des cycles

veille/sommeil (Fujiki et al., 2006). L'expression de la MCH et de la prépro-orexine est augmentée avec le jeûne et l'administration de leptine chez ces animaux à jeun restaure leur expression basale (Cai et al., 1999; Qu et al., 1996). Enfin, l'expression de la MCH est augmentée chez des souris *ob/ob*, indiquant là encore l'implication de la leptine dans la régulation de l'expression de ces neuropeptides orexigènes (Qu et al., 1996).

Ces neurones de second ordre sont à l'origine de deux réseaux neuronaux efférents majeurs, un réseau anorexigène par le biais des neurones du PVN et un réseau orexigène par le biais des neurones de la LHA. Ces deux réseaux efférents projettent massivement sur les systèmes viscéromoteurs et somatomoteurs du bulbe rachidien ainsi que sur le système limbique et le cortex cérébral. Ces efférences peuvent également influencer le système endocrinien via des projections sur la glande hypophysaire et l'éminence médiane (Berthoud, 2002).

2.3. Rôle du CVD dans l'intégration des signaux d'adiposité

Bien que l'hypothalamus soit le site principal d'intégration des signaux d'adiposité, quelques données suggèrent que le complexe vagal dorsal (CVD) serait également sensible à ces hormones. Le CVD est une structure située en région dorso-médiane du bulbe rachidien, au niveau du plancher du quatrième ventricule. Il est le centre intégrateur et effecteur des régulations autonomes, et est constitué de trois noyaux interconnectés **(Figure 8)** :

- l'area postrema (AP), un organe circumventriculaire favorisant l'accès des signaux circulants vers le cerveau, tout comme l'éminence médiane (EM) au niveau de l'hypothalamus ;
- le noyau du tractus solitaire (NTS) qui est le site de projection des afférences vagales et donc la cible directe des informations sensorielles issues du tractus digestif, de l'appareil respiratoire et de l'appareil cardiovasculaire ;
- le noyau dorsal moteur du nerf vague (DMNX) qui contient les corps cellulaires des prémotoneurones vagaux dont les axones constituent les fibres efférentes du nerf vague à destination des viscères thoraciques et abdominaux.

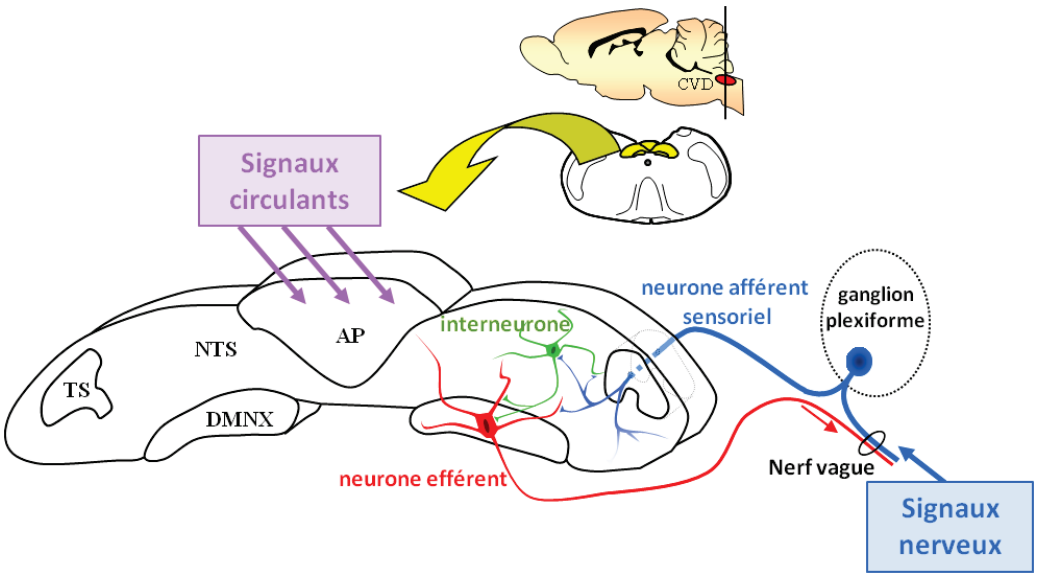


Figure 8 : Le complexe vagal dorsal.
Le CVD, localisé dans le tronc cérébral, est composé de trois noyaux interconnectés : l'AP, un organe circumventriculaire, le NTS qui reçoit les afférences du nerf vague, et le DMNX qui contient le soma des neurones efférents du nerf vague. Ainsi, le CVD intègre des signaux circulants et nerveux.

CVD: complexe vagal dorsal; AP: area postrema; NTS: noyau du tractus solitaire; DMNX: noyau dorsal moteur du nerf vague; TS: tractus solitaire.

Tout d'abord, des récepteurs à la leptine et à l'insuline ont été mis en évidence dans le CVD (Mercer et al., 1998; Unger et al., 1991). Une étude récente a montré que dans le NTS, une partie des récepteurs à la leptine se trouve sur des neurones exprimant POMC alors que les neurones NPY du NTS n'expriment pas ce récepteur (Garfield et al., 2012). L'injection périphérique de leptine aboutit à la phosphorylation de STAT3, protéine intracellulaire recrutée par le récepteur à la leptine en présence de son ligand, ainsi qu'à l'induction de c-Fos non seulement dans l'hypothalamus mais également dans le CVD, confirmant une action directe de la leptine sur le tronc cérébral (Hosoi et al., 2002; Sachot et al., 2007). De plus, l'injection de leptine dans le quatrième ventricule de rat réduit la prise alimentaire de façon significative (Grill et al., 2002) et la délétion du récepteur à la leptine uniquement dans le CVD crée une hyperphagie et une prise de poids accélérée (Hayes et al., 2010; Scott et al., 2011). Enfin, l'injection intra-NTS de vecteurs recombinants dérivés de virus adéno-associés entrainant une surexpression de POMC limite l'obésité induite par une alimentation riche en graisse, alors que, de manière surprenante, l'injection intra-ARC n'a pas d'effet (Zhang et al., 2010a). Des récepteurs à la leptine ont également été mis en évidence au niveau des afférences vagales (Buyse et al., 2001) et l'injection intra-artérielle de leptine modifie la fréquence de décharge des neurones afférents vagaux chez le rat et le chat (Gaigé et al., 2002;

Wang et al., 1997). De plus, sur des cultures primaires de neurones afférents vagaux dissociés à partir de ganglion plexiforme de rat adulte, l'application de leptine augmente la concentration en calcium intracellulaire dans environ 40% des cellules (Peters et al., 2004). Ces résultats suggèrent qu'en plus d'agir directement sur le tronc cérébral, la leptine est capable d'activer le nerf vague qui véhiculera l'information jusqu'au NTS.

Enfin, il est important de noter que l'hypothalamus et le CVD sont dotés de connexions réciproques (Cf. I.4.) et interagissent afin d'intégrer efficacement les signaux d'adiposité en provenance de la périphérie **(Figure 9)**.

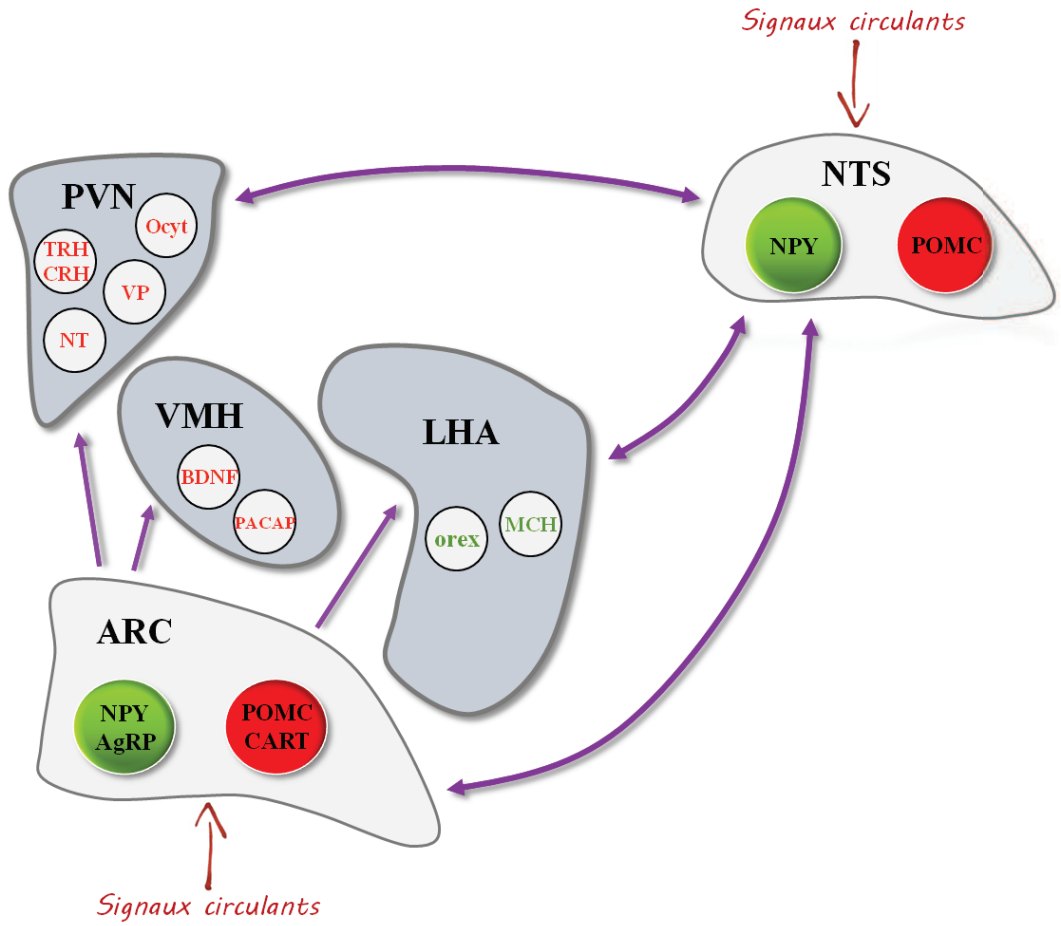


Figure 9 : Interconnections entre l'hypothalamus et le NTS.
Des noyaux hypothalamiques tels que l'ARC, le PVN et la LHA sont interconnectés avec le tronc cérébral et plus particulièrement le NTS.

NPY: neuropeptide Y; AgRP: agouti-related peptide; POMC: pro-opiomélanocortine; CART: cocaïne- and amphetamine-related transcript; orex: orexine; MCH: melanin-concentrating hormone; TRH: thyrotropin-releasing hormone; CRH: corticotropin-releasing hormone; Ocyt: ocytocine; VP: vasopressine; NT: neurotensine; BDNF: brain-derived neurotrophic factor; PACAP: pituitary adenylate cyclase activating polypeptide; VMH: noyau ventro-médian hypothalamique; LHA: aire latérale hypothalamique; PVN: noyau paraventriculaire; ARC: noyau arqué; NTS: noyau du tractus solitaire.

Ainsi, le SNC estime en permanence l'état des réserves énergétiques grâce aux signaux d'adiposité. Cette donnée permettra à l'organisme d'adapter ses apports caloriques en fonction de ses besoins. Pour cela, un mécanisme de contrôle à court terme permet de maîtriser la fréquence et la taille des repas.

3. Contrôle de la taille et de la fréquence des repas

3.1. Initiation du repas

L'initiation du repas dépend de nombreux facteurs, notamment sociaux et culturels, des habitudes alimentaires et de facteurs externes tels que les stimuli olfactifs et visuels. Cette phase implique également des signaux internes d'origine nerveuse, humorale et métabolique dont l'hypoglycémie préprandiale et la sécrétion de ghréline par le tractus gastro-intestinal.

- *L'hypoglycémie préprandiale*

Mayer fut le premier, en 1952, à corréler la prise alimentaire avec le taux de glucose circulant (Mayer, 1952). Selon lui, seules les réserves glucidiques sont capables de rendre compte de l'état énergétique de l'organisme. Sa théorie dite « glucostatique », par opposition à la théorie « lipostatique » de Kennedy, s'appuie sur le fait que le glucose est le substrat énergétique de toutes les cellules de l'organisme. Ainsi, ce serait uniquement la disponibilité du glucose sanguin circulant qui permettrait de contrôler la prise alimentaire. Bien que largement abandonnée, cette théorie fut le point de départ de nombreux travaux visant à corréler les variations de glycémie avec la prise alimentaire.

Chaque repas est précédé d'une chute transitoire de la glycémie mais si cette hypoglycémie est rapidement compensée par une injection de glucose, le repas est alors retardé de quelques heures (Campfield et al., 1985; Louis-Sylvestre et Le Magnen, 1980). C'est cependant la disponibilité cellulaire en glucose plutôt que le taux de glucose circulant qui semble être le facteur déterminant puisque l'état de faim et l'hyperglycémie peuvent coexister (cas du diabète). Ainsi, l'injection d'analogues non métabolisables du glucose tels que le 5-thioglucose (5-TG) ou le 2-désoxyglucose (2-DG) crée une glucoprivation cellulaire à l'origine d'une hyperphagie. Le SNC joue un rôle majeur dans ce phénomène puisque des

injections uniquement centrales de 5-TG suffisent à augmenter la prise alimentaire (Ritter et al., 1981).

Le cerveau, et plus particulièrement l'hypothalamus et le CVD, est équipé de neurones dits glucosenseurs ou glucodétecteurs qui sont sensibles aux variations de glucose. Ces neurones sont soit activés (*glucose excited neurons* : GE) soit inhibés (*glucose inhibited neurons* : GI) par le glucose. Les neurones GE sont localisés dans l'ARC, le PVN, le VMH, le NTS et le DMNX, et les neurones GI sont présents dans les mêmes structures à l'exception du VMH et avec en plus une forte expression dans la LHA. Ces noyaux sont interconnectés et forment un réseau capable de détecter les niveaux de glucose circulants et de mettre en place une réponse adaptée à la fois sur la prise alimentaire et sur les sécrétions hormonales (pour revue voir Marty et al., 2007).

Les glucodétecteurs impliqués dans la réponse alimentaire à une glucoprivation semblent être localisés dans le tronc cérébral et non dans l'hypothalamus. En effet, une augmentation de la prise alimentaire est observée après une injection de 5-TG dans le ventricule latéral, mais son amplitude est plus importante lorsque celui-ci est injecté dans le quatrième ventricule. Or, après occlusion de l'aqueduc de Sylvius, empêchant la circulation du liquide céphalo-rachidien (LCR) des ventricules cérébraux antérieurs vers le quatrième ventricule, seule l'injection de 5-TG dans le quatrième ventricule déclenche le repas, celle dans le ventricule latéral étant inefficace (Ritter et al., 1981). Ces résultats ont été confirmés plus récemment puisque des micro-injections de 5-TG dans le parenchyme du bulbe rachidien entraînent une forte réponse alimentaire, alors qu'elles sont quasiment inefficaces lorsqu'elles sont pratiquées dans le parenchyme de l'hypothalamus (Ritter et al., 2000).

Ainsi, l'hypoglycémie préprandiale est ressentie au niveau du SNC et plus particulièrement au niveau du tronc cérébral et contribue à l'initiation d'un repas.

- <u>*La ghréline*</u>

La ghréline est une hormone orexigène de 28 acides aminés secrétée principalement par les cellules endocrines de l'estomac. Chez le rongeur, comme chez l'homme, les taux plasmatiques de ghréline augmentent de façon transitoire avant le début du repas et retournent à un niveau basal lors de l'ingestion (Cummings et al., 2001; Tolle et al., 2002). De plus, des animaux mis à jeun présentent une augmentation du taux plasmatique de ghréline qui retourne à son niveau basal lorsqu'ils sont renourris (Tschöp et al., 2000). Le récepteur à la ghréline

(*growth hormone secretaguogue receptor* : GHS-R) est exprimé au niveau des afférences du nerf vague mais le rôle des afférences vagales dans l'action orexigène de la ghréline est actuellement sujet à controverse. En effet, alors qu'une étude montre que la vagotomie abolit l'hyperphagie déclenchée par l'injection de ghréline (Date et al., 2002), une autre étude plus récente décrit des résultats inverses chez des rats ayant subi une déafférentation subdiaphragmatique totale du nerf vague (Arnold et al., 2006). Ces auteurs concluent que les afférences vagales ne sont pas nécessaires à l'action orexigène de la ghréline et que ce peptide agit directement au niveau central. Notons que dans ces deux études le mode d'administration n'est pas le même puisque Date et al. injectent la ghréline en iv alors qu'Arnold et al. l'injectent en ip. Le récepteur GHS-R est également exprimé au niveau de l'hypothalamus et du CVD et des injections de ghréline dans le troisième ventricule ainsi que dans le quatrième ventricule augmentent la prise alimentaire (Faulconbridge et al., 2003). L'action de la ghréline sur la prise alimentaire semble être relayée par les neuropeptides orexigènes NPY et AgRP de l'ARC puisque son infusion centrale stimule l'expression de ces deux neuropeptides (Kamegai et al., 2001) et elle augmente le calcium intracellulaire des neurones de l'ARC exprimant le NPY (Kohno et al., 2003). De plus, la destruction des neurones NPY/AgRP aboutit à une perte de l'action orexigène de la ghréline (Luquet et al., 2007). Enfin, l'administration périphérique de ghréline active c-Fos dans des neurones hypothalamiques et en particulier dans des neurones de l'ARC et du DMH. Les neurones activés dans le DMH sont à proximité de fibres exprimant l'AgRP. Par contre, la ghréline ne semble pas activer les neurones du CVD (Kobelt et al., 2008). Ainsi, la ghréline a un effet opposé à celui de la leptine et de l'insuline puisqu'elle active la voie orexigène de l'hypothalamus.

En plus de son effet clairement décrit sur l'initiation des repas, la ghréline pourrait également être un signal d'adiposité mais libéré de façon inversement proportionnelle à la masse adipeuse. En effet, le taux de ghréline plasmatique est corrélé négativement avec le poids corporel chez le rat non obèse (Sugiishi et al., 2013) ainsi qu'avec l'indice de masse corporelle chez l'homme (Wadden et al., 2012).

Ainsi, à la fois la glucoprivation et l'augmentation de la sécrétion de ghréline stimulent l'initiation de l'ingestion suggérant leur rôle dans la modulation de la fréquence des repas.

3.2. Arrêt du repas

La présence d'aliments dans le tractus gastro-intestinal est détectée par les récepteurs mécano- et chémo-sensibles des afférences vagales. L'intégration de ces informations déclenche alors une sensation de satiété.

- *Signaux satiétogènes d'origine gastrique*

L'accumulation d'aliments dans l'estomac entraine une dilatation gastrique capable d'inhiber la prise alimentaire. En effet, l'occlusion du pylore empêchant le passage des aliments de l'estomac vers les intestins, à l'origine d'une augmentation du volume gastrique, entraine l'arrêt de la séquence d'ingestion (Phillips et Powley, 1996). Cet arrêt du repas par la distension gastrique résulte de l'activation de mécanorécepteurs vagaux projetant sur le bulbe rachidien. En effet, après vagotomie totale, la dilatation gastrique n'entraine plus la satiété (Phillips et Powley, 1998). De plus, la distension gastrique pratiquée à l'aide d'un ballon gonflable placé dans l'estomac entraine une augmentation de l'expression de c-Fos dans des neurones du NTS qui est le site de projection des afférences vagales (Vrang et al., 2003).

La présence d'aliments dans l'estomac entraine également la sécrétion de différents signaux de satiété agissant de façon paracrine sur des chémorécepteurs vagaux et impliqués dans la mise en place du réflexe de satiété **(Figure 10)**. Parmi ces signaux, la bombésine et ses analogues : le *gastrin-releasing peptide* (GRP) et la neuromédine B sont fortement exprimés dans l'estomac. Des administrations périphériques de ces peptides entrainent une diminution de la taille des repas et cet effet nécessite l'activation des afférences vagales (Gibbs et al., 1979; Ladenheim et Ritter, 1991). La leptine est également produite par certaines cellules de la muqueuse gastrique et sa sécrétion locale est déclenchée lors d'un repas (Sobhani et al., 2000). De plus, les afférences vagales expriment le récepteur à la leptine et STAT3 (Buyse et al., 2001). L'infusion de faibles doses de leptine dans l'artère cœliaque perfusant l'estomac entraîne une diminution rapide de la taille des repas et cet effet disparait après destruction des nerfs vagues (Peters et al., 2005). Ces données indiquent qu'au cours du repas, la leptine d'origine gastrique peut agir de façon paracrine et transitoire sur les fibres afférentes vagales et modifier leur activité afin de réguler la taille des repas.

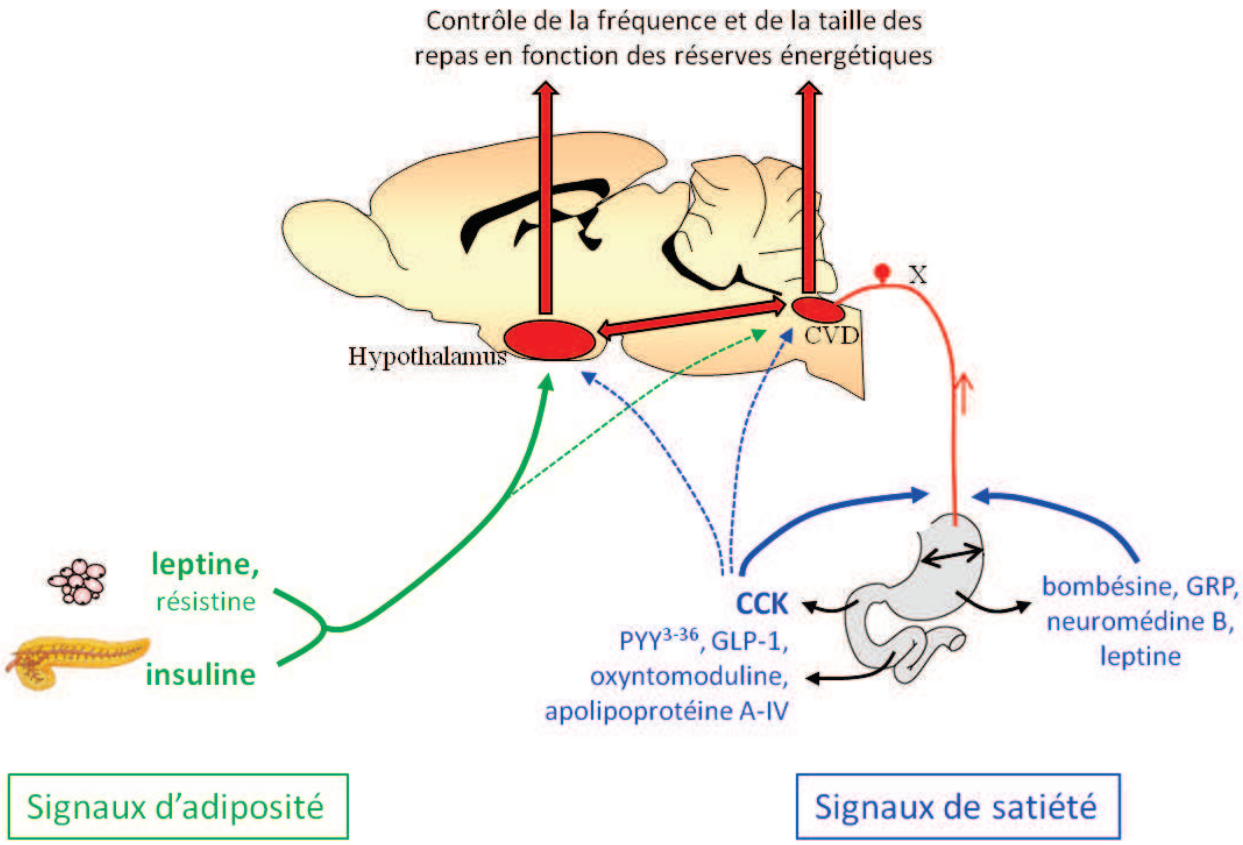


Figure 10 : Le contrôle central de la prise alimentaire.
L'hypothalamus et le CVD intègrent des signaux d'adiposité et de satiété et interagissent afin d'adapter la quantité de calories ingérées en fonction de l'état des réserves énergétiques de l'organisme.

CCK: cholécystokinine; PYY$^{3-36}$: peptide YY$^{3-36}$; GLP-1: glucagon-like peptide 1; GRP: gastrin-releasing peptide; CVD: complexe vagal dorsal; X: nerf vague.

- *Signaux satiétogènes d'origine intestinale*

La présence de macronutriments dans la lumière intestinale entraine la sécrétion de substances neuroactives par l'épithélium intestinal **(Figure 10)**. Ces substances sont capables d'inhiber la prise alimentaire via l'activation des afférences vagales. Parmi ces composés, la cholécystokinine (CCK) est le signal de satiété d'origine intestinale le plus étudié. La CCK est sécrétée par une population de cellules endocrines présentes dans la muqueuse duodénale en réponse à la présence d'aliments. Deux types de récepteurs à la CCK ont été décrits : CCK-A et CCK-B (Wank et al., 1992). Les afférences vagales expriment majoritairement le récepteur CCK-A. Des traitements périphériques de CCK réduisent la taille des repas de manière dose-dépendante alors que des traitements par des antagonistes CCK-A l'augmentent (Gibbs et al.,

1973; Moran et al., 1992). Des rats dépourvus du récepteur CCK-A (rats Otsuka Long-Evans Tokushima Fatty) présentent une obésité causée par une augmentation de la taille des repas et de leur nombre. Ainsi, la CCK agit à la fois sur le rassasiement (arrêt de la sensation de faim au cours du repas conduisant à son arrêt) et sur la satiété (absence de faim après le repas) (Marco et al., 2009). L'intégrité des afférences vagales est nécessaire à l'effet satiétogène de la CCK, suggérant une action paracrine de celle-ci sur les afférences vagales, relayée au niveau du bulbe rachidien (Moran et al., 1997). Cependant, une action directe de la CCK sur le cerveau a également été proposée puisque des récepteurs CCK-A et CCK-B sont exprimés dans plusieurs structures cérébrales et l'injection icv de CCK à faible dose diminue la prise alimentaire (Hirosue et al., 1993; Holicky et al., 2001; Mercer et Beart, 1997; Mercer et al., 2000). Les récepteurs CCK-A semblent être responsables de cet effet anorexigène car le blocage pharmacologique de ces récepteurs abolit la diminution de prise alimentaire (Hirosue et al., 1993). Ainsi, en plus d'une action paracrine sur les afférences vagales, la CCK est véhiculée dans le sang jusqu'au cerveau où elle pourrait contribuer à l'arrêt du repas.

D'autres peptides intestinaux présentent une action anorexigène tels que le glucagon-like peptide 1 (GLP-1), le peptide YY^{3-36} (PYY^{3-36}), l'oxyntomoduline et l'apolipoprotéine A-IV (Neary et Batterham, 2009). Ces composés exerceraient également leur action satiétogène par l'intermédiaire des afférences vagales (Koda et al., 2005). Cependant, l'action anorexigène du PYY^{3-36} implique également une inhibition directe des neurones orexigènes NPY/AgRP de l'ARC (Batterham et al., 2002). Récemment, des messagers lipidiques synthétisés par l'intestin en réponse à la présence d'aliments ont également été identifiés comme molécule satiétogène : l'oleoylethanolamide (OEA) et le N-acylphosphoethanolamine (NAPE) (Gillum et al., 2008; Schwartz et al., 2008).

- *Autres signaux de satiété*

D'autres hormones qui ne sont pas libérées par le tractus gastro-intestinal mais par le pancréas constituent également des signaux satiétogènes : c'est le cas du polypeptide pancréatique et de l'amyline. Le polypeptide pancréatique est sécrété post-prandialement par des cellules spécialisées des îlots de Langerhans et l'amyline est co-sécrétée avec l'insuline, également après le repas (Katsuura et al., 2002; Lutz et al., 1995b). Ces deux hormones ont un effet anorexigène lorsqu'elles sont injectées en périphérie (Asakawa et al., 2003; Batterham et al., 2003; Lutz et al., 1994).

3.3. Rôle du CVD dans l'intégration des signaux de satiété

Les premières données indiquant un rôle pivot du tronc cérébral dans l'intégration des signaux de satiété ont été obtenues par Grill et Norgren en 1978 grâce à l'utilisation d'un modèle de rat décérébré chronique. La décérébration, réalisée par transsection complète au niveau de la jonction méso-diencéphalique, permet une interruption de l'ensemble des connexions réciproques entre le mésencéphale et le cerveau antérieur. Alors que les rats décérébrés étaient incapables d'initier un repas sans qu'une nourriture liquide leur soit directement infusée dans la bouche, ils gardaient cependant la capacité à déglutir et à adapter la durée de la séquence d'ingestion à la densité énergétique de la nourriture proposée. Ainsi, les rats décérébrés se nourrissent jusqu'à atteindre un état de rassasiement et laissent alors couler l'aliment liquide hors de leur bouche (Grill et Kaplan, 1992; Grill et Norgren, 1978). Ces travaux ont ainsi permis de démontrer que le tronc cérébral, dépourvu de toute connexion avec le cerveau antérieur et notamment avec l'hypothalamus, est capable d'intégrer les signaux de satiété afin de réguler la taille des repas.

Comme nous l'avons vu, les signaux provenant du tractus gastro-intestinal exercent leur effet satiétogène en partie par l'intermédiaire du nerf vague qui envoie l'information recueillie sur le NTS. De plus, l'activation des afférences vagales par ces signaux de satiété est capable d'entraîner des modulations d'activité des neurones du CVD. Par exemple, le traitement à la CCK active des neurones du NTS, particulièrement des neurones POMC, et la mise en jeu des récepteurs MC4-R est nécessaire à l'action satiétogène de la CCK (Fan et al., 2004). De plus, l'administration d'agonistes MC3/4-R dans le quatrième ventricule réduit la taille des repas (Zheng et al., 2005). Ainsi, le système mélanocortinergique serait impliqué à la fois dans l'intégration des signaux d'adiposité et des signaux de satiété. Notons toutefois que la CCK active également des neurones hypothalamiques (Cano et al., 2003; Peter et al., 2010).

Le nerf vague n'est cependant pas impliqué dans l'effet satiétogène de l'amyline car la vagotomie subdiaphragmatique n'affecte pas la réponse alimentaire induite par l'injection périphérique de cette hormone (Lutz et al., 1995a). Une action directe sur le cerveau a alors été proposée puisque l'amyline diminue également la prise alimentaire lorsqu'elle est administrée centralement (Wielinga et al., 2010). Cette action centrale semble être relayée par l'AP qui possède un grand nombre de récepteurs à l'amyline (Sexton et al., 1994). En effet, la

diminution de prise alimentaire induite par l'administration ip d'amyline est bloquée chez des rats dont l'AP et le NTS ont été lésés (Lutz et al., 2001; Rowland et Richmond, 1999). De plus, l'injection périphérique d'amyline entraine une augmentation de l'expression de c-Fos dans l'AP, ainsi que dans le NTS et quelques structures plus antérieures : le noyau parabrachial et le noyau central de l'amygdale (Rowland et al., 1997). Cependant, la lésion de l'AP abolit l'activation neuronale dans l'ensemble de ces structures alors que la lésion du noyau barabrachial n'altère pas le niveau d'expression de c-Fos dans l'AP et le NTS (Becskei et al., 2007; Riediger et al., 2004). Ces résultats indiquent que l'amyline agit sur l'AP qui active ensuite le NTS, le noyau parabrachial et l'amygdale.

Ainsi, alors que les signaux d'adiposité permettent à l'hypothalamus d'estimer l'état des réserves énergétiques, les signaux de satiété permettent au CVD d'estimer l'apport calorique au cours d'un repas. La combinaison de ces deux phénomènes permet d'adapter la taille et la fréquence des repas en fonction des besoins énergétiques de l'organisme afin de maintenir l'homéostasie énergétique à long terme (Figure 10).

4. Réseau neuronal du comportement alimentaire

Comme nous l'avons vu, l'hypothalamus et le CVD sont sensibles aux signaux périphériques et les différents noyaux hypothalamiques sont interconnectés, tout comme les noyaux du CVD. Ces deux structures sont également connectées entre elles de manière directe : par exemples, l'ARC, le PVN et la LHA reçoivent des afférences du NTS et projettent sur celui-ci ainsi que sur le DMNX (Berthoud, 2002) **(Figure 9)**. Une partie des projections solitaro-hypothalamiques sont de nature catécholaminergique, et plus particulièrement noradrénergique (Sawchenko et Swanson, 1981). L'hypothalamus et le CVD sont également connectés de manière indirecte principalement par le biais du noyau parabrachial (PBN) qui intègre les informations issues du NTS et les transmet sur différents noyaux hypothalamiques tels que l'ARC, le PVN et la LHA (Berthoud, 2002). Ces structures sont également connectées avec la medulla ventrolatérale (VLM) et la colonne intermédiolatérale de la moelle épinière, deux structures qui, comme le CVD, assurent les composantes autonomes de la prise alimentaire. L'hypothalamus et le CVD sont également dotés de projections

réciproques avec d'autres régions du SNC assurant l'aspect émotionnel et motivationel du comportement alimentaire **(Figure 11)** :

- Le cortex cérébral, et en particulier le cortex insulaire qui contient les aires corticales sensorielles viscérales ;
- Le système limbique dont l'amygdale, le noyau accumbens, l'hippocampe et le noyau antérieur du thalamus ;
- Les noyaux du raphé et le locus coeruleus (LC) au niveau du tronc cérébral, des structures impliquées dans l'humeur et l'anxiété, ainsi que dans l'éveil.

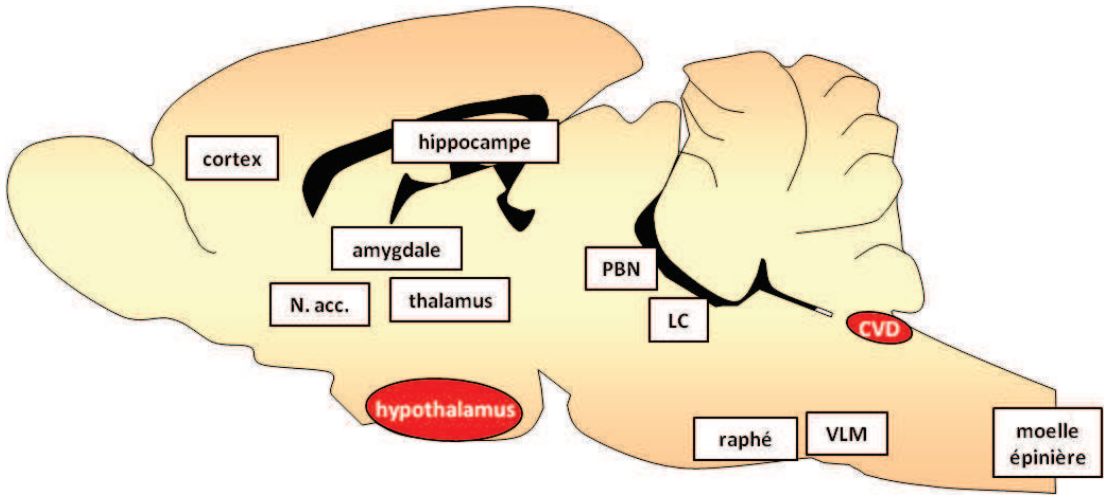


Figure 11 : Structures cérébrales impliquées dans le comportement alimentaire.
L'hypothalamus et le CVD sont anatomiquement connectés avec d'autres structures cérébrales formant un réseau responsable de la régulation du comportement alimentaire.

N.acc.: noyau accumbens; PBN: noyau parabrachial; LC: locus coeruleus; CVD: complexe vagal dorsal; VLM: medulla ventrolatérale.

Le système sérotoninergique issu des noyaux du raphé a particulièrement été étudié puisque la sérotonine (5-HT) exerce un important rôle inhibiteur sur la prise alimentaire (Hayes et Covasa, 2005). Des souris knockout pour les récepteurs à la sérotonine 5-HT2C ou 5-HT1B sont hyperphagiques et obèses (Bouwknecht et al., 2001; Heisler et al., 1998). Au niveau de l'ARC, la 5-HT augmente l'activité des neurones POMC/CART via le récepteur 5-HT2C et elle inhibe l'activité des neurones NPY/AgRP via le récepteur 5-HT1B (Heisler et al., 2006). Chez les souris 5-HT2C-/-, la réexpression de ce récepteur uniquement dans les neurones POMC suffit à rétablir une prise alimentaire et un poids normal (Xu et al., 2008). La 5-HT agit également au niveau du CVD puisqu'elle active des neurones dans le NTS qui sont pour la plupart catécholaminergiques suggérant qu'ils projettent sur l'hypothalamus (Lam et al., 2009). De plus, les récepteurs 5-HT3 du tronc cérébral participent à l'arrêt du repas. En

effet, l'administration dans le quatrième ventricule d'un antagoniste des récepteurs 5-HT3 atténue l'effet satiétogène induit par l'injection ip de CCK ou par la dilatation gastrique (Hayes et Covasa, 2006; Hayes et al., 2006). Le blocage de ces récepteurs atténue également l'hypophagie et l'activation neuronale induites par une infusion duodénale de lipides ou de glucose (Savastano et al., 2005, 2007)

5. Les cellules gliales : de nouveaux acteurs dans le contrôle central de la prise alimentaire

Longtemps considérées comme de simples cellules de soutien, les cellules gliales sont les cellules les plus nombreuses du cerveau et l'on sait aujourd'hui qu'elles contribuent largement aux fonctions neuronales. Elles participent entre autres à la survie et au guidage des neurones pendant le développement, au maintien de l'intégrité de la BHE et au maintien de l'homéostasie neuronale (Perea et al., 2009). De nombreuses études indiquent qu'elles sont également capables d'interagir avec les neurones puisqu'elles expriment une grande variété de récepteurs membranaires dont des récepteurs aux neurotransmetteurs et aux signaux circulants. L'activation de ces récepteurs entraine une augmentation du calcium intracellulaire capable de se propager aux cellules gliales avoisinantes : ce sont les « vagues calciques ». En retour, les cellules gliales libèrent des substances neuroactives appelées « gliotransmetteurs » telles que l'ATP, le glutamate et la D-sérine capables d'agir au niveau post-synaptique et de moduler l'activité neuronale (Hamilton et Attwell, 2010). Depuis quelques années, les cellules gliales apparaissent comme des acteurs directs dans la régulation de la prise alimentaire **(Cf. : annexe 1)**.

5.1. Glie et leptine

Les astrocytes du noyau arqué et de l'éminence médiane expriment le récepteur à la leptine (Cheunsuang et Morris, 2005; Hsuchou et al., 2009b), tout comme une population de cellules gliales situées entre l'AP et le NTS et baptisées vagliocytes (Dallaporta et al., 2009). Ces vagliocytes ressemblent fortement aux tanycytes de l'éminence médiane puisque tout deux expriment en plus de la « glial fibrillary acidic protein » (GFAP) des marqueurs d'immaturité tels que la nestine et la vimentine. De plus ces deux types cellulaires sont situés au niveau

d'organes circumventriculaires et semblent constituer une barrière de diffusion limitant l'accès des signaux circulants vers l'ARC et le NTS. La présence de jonctions serrées entre les tanycytes et entre les vagliocytes permet d'expliquer en partie ce phénomène (Dallaporta et al., 2009; Langlet et al., 2013b; Mullier et al., 2010; Peruzzo et al., 2000). L'application de leptine sur des cultures primaires d'astrocytes hypothalamiques entraine des vagues calciques démontrant ainsi la fonctionnalité des récepteurs à la leptine astrocytaires (Hsuchou et al., 2009a). De plus, des souris rendues obèses par une alimentation riche en graisse ainsi que les souris Agouti, qui sont naturellement obèses, présentent une augmentation de l'expression du récepteur à la leptine dans les astrocytes de l'hypothalamus permettant d'augmenter la sensibilité à la leptine (Hsuchou et al., 2009a; Pan et al., 2008). Les vagliocytes expriment la forme longue (responsable de l'effet anorexigène de la leptine) mais aussi la forme courte (responsable du transport de la leptine à travers la BHE) du récepteur à la leptine. Des modulations du statut nutritionnel font varier l'expression du récepteur de forme courte, suggérant que l'accès de la leptine au NTS est régulé par la glie (Dallaporta et al., 2009). Les cellules gliales ne sont pas sensibles uniquement à la leptine puisque la CCK est également capable de provoquer des vagues calciques dans des cultures d'astrocytes hippocampiques (Müller et al., 1997).

5.2. L'octadécaneuropeptide

Les cellules gliales expriment un peptide anorexigène, l'octadécaneuropeptide (ODN), qui n'est pas exprimé par les neurones. L'ODN est un peptide de 18 acides aminés issu du clivage d'un précurseur, le *diazepam-binding inhibitor* (DBI). C'est un ligand endogène des récepteurs aux benzodiazépines. Bien que l'ODN interagisse avec le récepteur des benzodiazépines central classique qui fait partie du complexe récepteur GABA-A, son action anorexigène est liée à sa fixation sur un récepteur couplé à une protéine G (RCPG) (do Rego et al., 2007). L'ODN est exprimé par les astrocytes protoplasmiques de diverses régions cérébrales telles que le cortex, le cervelet, l'hippocampe, ainsi que l'hypothalamus et le CVD. Il est aussi fortement exprimé par les tanycytes et les vagliocytes (Tonon et al., 1990; nos résultats non publiés) (**Figure 12A et B**). Son injection centrale réduit drastiquement la prise alimentaire ainsi que le poids corporel lorsqu'il est administré de façon chronique (de Mateos-Verchere et al., 2001). De plus, l'ODN augmente l'expression de POMC et diminue celle de NPY dans le noyau arqué (Compère et al., 2003), et une mise à jeun diminue l'expression du

DBI hypothalamique (Compère et al., 2010). Enfin, l'administration d'un antagoniste des récepteurs aux mélanocortines MC3/4-R bloque l'effet anorexigène de l'ODN (Lanfray et al., 2012). Ainsi, les cellules exprimant l'ODN et en particulier les tanycytes et vagliocytes pourraient être sensibles aux signaux circulants anorexigènes et relayer l'information sur les neurones POMC afin de réduire la prise alimentaire **(Figure 12C)**.

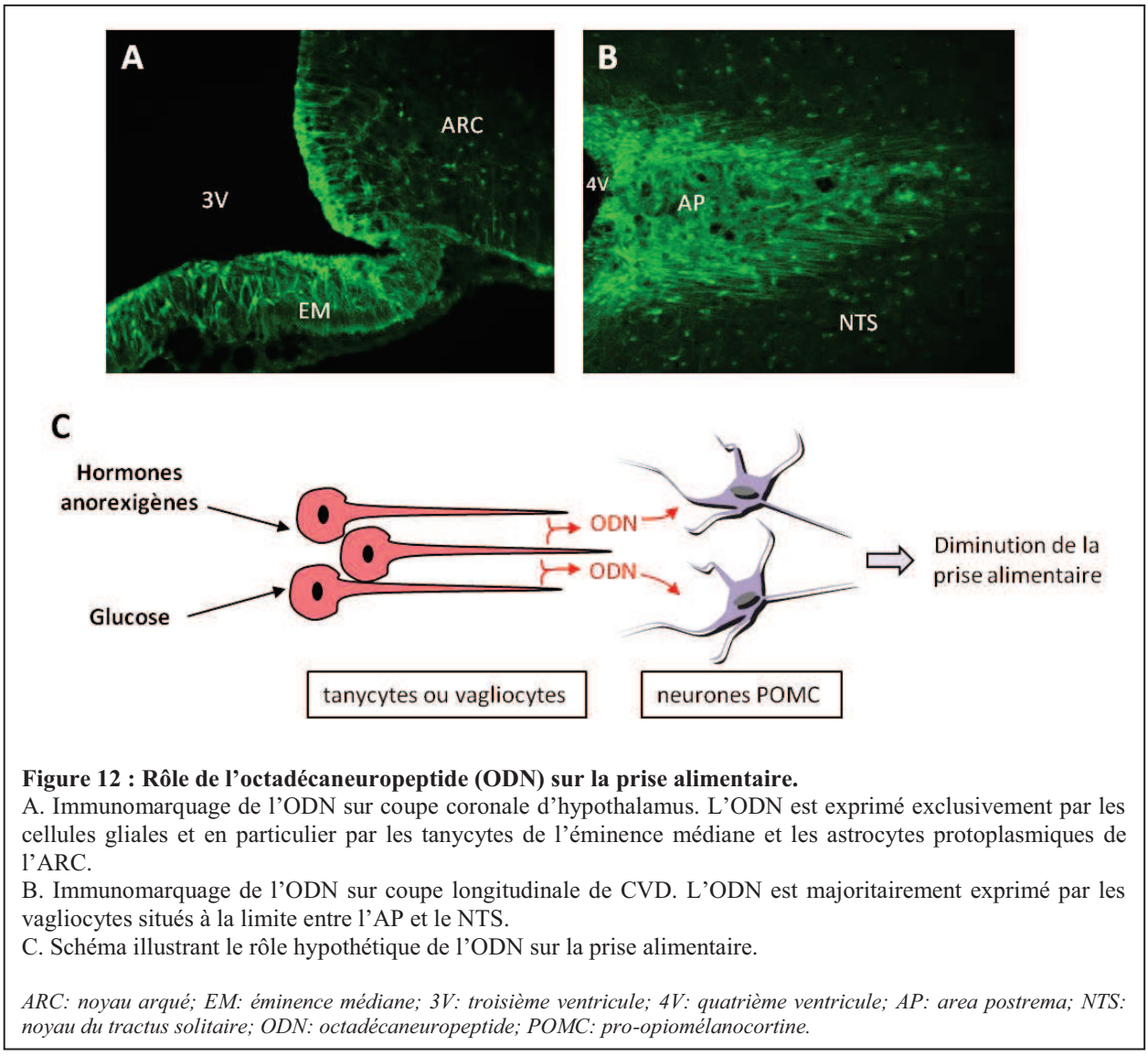


Figure 12 : Rôle de l'octadécaneuropeptide (ODN) sur la prise alimentaire.
A. Immunomarquage de l'ODN sur coupe coronale d'hypothalamus. L'ODN est exprimé exclusivement par les cellules gliales et en particulier par les tanycytes de l'éminence médiane et les astrocytes protoplasmiques de l'ARC.
B. Immunomarquage de l'ODN sur coupe longitudinale de CVD. L'ODN est majoritairement exprimé par les vagliocytes situés à la limite entre l'AP et le NTS.
C. Schéma illustrant le rôle hypothétique de l'ODN sur la prise alimentaire.

ARC: noyau arqué; EM: éminence médiane; 3V: troisième ventricule; 4V: quatrième ventricule; AP: area postrema; NTS: noyau du tractus solitaire; ODN: octadécaneuropeptide; POMC: pro-opiomélanocortine.

5.3. Glie et glucodétection

La glie et L'ODN jouent également un rôle important dans la glucodétection centrale. En effet, l'activation neuronale dans l'hypothalamus et le CVD provoquée par la glucoprivation ou à l'inverse par le glucose est bloquée en présence d'une drogue qui inhibe le métabolisme glial, la méthionine sulfoximine (Guillod-Maximin et al., 2004; Young et al., 2000).

L'application de glucose ou de 2-DG sur des tranches d'hypothalamus entraine une augmentation de calcium intracellulaire dans les tanycytes, ainsi qu'une libération d'ATP, fournissant la preuve directe que ces cellules gliales sont des senseurs de glucose (Frayling et al., 2011). Comme les neurones, les astrocytes et tanycytes expriment le transporteur du glucose GLUT-2 qui est impliqué dans les mécanismes de glucodétection centrale et, de manière intéressante, l'expression de ce transporteur GLUT-2 est régulée par le statut nutritionnel et la leptine (Fuente-Martín et al., 2012; García et al., 2003). Des souris dépourvues de GLUT-2 sont incapables de sécréter du glucagon en réponse à une glucoprivation et ne présentent pas d'activation neuronale dans le CVD suite à cette glucoprivation. La réexpression de GLUT-2 uniquement dans les cellules gliales restaure l'activation neuronale et la sécrétion de glucagon démontrant le rôle crucial des cellules gliales dans la glucodétection (Marty et al., 2005).

L'ODN semble être impliqué dans ce mécanisme de glucodétection puisque l'administration centrale de glucose augmente l'expression du DBI et l'effet anorexigène observé suite à cette injection de glucose est réduit en présence d'un antagoniste de l'ODN (Lanfray et al., 2012) **(Figure 12C)**. De plus, un traitement central à l'ODN minimise l'augmentation de la glycémie provoquée par un test de tolérance au glucose, suggérant qu'en plus de détecter les niveaux circulants de glucose, l'ODN est capable de mettre en place une réponse visant à maintenir la glycémie stable. Ces données sont en accord avec les résultats démontrant que la sécrétion d'insuline provoquée par l'injection centrale de glucose est abolie en présence de méthionine sulfoximine (Guillod-Maximin et al., 2004).

5.4. Plasticité de la barrière de diffusion par le statut nutritionnel

Comme mentionné précédemment, les tanycytes et les vagliocytes forment une barrière de diffusion limitant l'accès des signaux circulants respectivement de l'EM à l'ARC et de l'AP au NTS. Cette barrière de diffusion est cependant dotée de plasticité selon le statut nutritionnel de l'individu. Récemment, Langlet et ses collaborateurs ont montré au niveau hypothalamique une réorganisation de la barrière lors d'un jeûne de 24 heures (Langlet et al., 2013a). En effet, le jeûne stimule l'expression de la protéine *vascular endothelial growth factor A* (VEGF-A) dans les tanycytes. Le VEGF est connu pour augmenter la perméabilité vasculaire en créant des fenestrations endothéliales (Esser et al., 1998). Ainsi, lors d'un jeûne, le VEGF-A augmente la fenestration des capillaires dans l'EM et l'ARC, mais également le

nombre de jonctions serrées entre les tanycytes. Le jeûne est caractérisé par une hypoglycémie et les auteurs montrent que c'est la glucoprivation elle-même qui est à l'origine de ce phénomène. Cette réorganisation de la barrière se traduit par une amélioration de la pénétration des signaux circulants vers l'ARC et contribue ainsi à la mise en place de la réponse comportementale à la glucoprivation, c'est-à-dire l'augmentation de la prise alimentaire. Ces données indiquent que les modulations du statut nutritionnel entrainent des changements structuraux dans la barrière de diffusion gliale, modulant l'accès des signaux métaboliques vers le parenchyme cérébral. Ce phénomène semble jouer un rôle important dans le maintien de l'homéostasie énergétique.

La régulation de la prise alimentaire repose donc sur deux mécanismes :

- **l'estimation des réserves énergétiques permettant de maintenir le poids corporel à long terme,**
- **l'estimation de l'apport calorique au cours de chaque repas permettant de réguler à court terme l'ingestion.**

L'hypothalamus et le CVD sont les structures cérébrales qui assurent ces régulations en intégrant des signaux provenant de la périphérie et en mettant en place, grâce à un réseau neuronal et glial complexe, une réponse comportementale, autonome et endocrine adaptée.

II L'inflammation : un modulateur important de la balance énergétique

La présence d'une inflammation dans l'organisme entraine des troubles nutritionnels qui varient selon la durée mais surtout l'intensité de l'inflammation. Ainsi, la présence de pathogènes au sein de l'organisme (virus, bactérie…) entraine la mise en place d'une réaction inflammatoire aiguë à l'origine de plusieurs changements immuns, physiologiques et comportementaux. L'ensemble de ces changements est appelé la « réaction de phase aiguë » dont les symptômes sont la fièvre, la fatigue, la diminution des interactions sociales et une considérable diminution de la prise alimentaire. Lors de maladies chroniques (cancers, sida, insuffisance respiratoire, cardiaque, rénale, hépatique…), l'inflammation de l'organisme devient alors permanente et l'anorexie qui en résulte se prolonge, pouvant aboutir à une cachexie, c'est-à-dire un affaiblissement profond de l'organisme lié à la dénutrition. De manière surprenante, une inflammation chronique mais de bas seuil peut conduire à l'inverse à une obésité et un diabète de type 2. Les mécanismes sous-tendant ce phénomène sont à ce jour méconnus et les données de la littérature à ce sujet sont peu nombreuses. Dans ce chapitre, nous nous intéresserons tout d'abord aux mécanismes à l'origine de l'anorexie inflammatoire lors d'une inflammation de forte intensité, puis nous discuterons de l'inflammation de bas seuil dans l'obésité et le diabète de type 2.

1. La réaction de phase aiguë : bases moléculaires

1.1. Les cytokines

Le terme « cytokine » fut utilisé pour la première fois en 1974 par Stanley Cohen. Les cytokines sont les médiateurs de l'inflammation qui vont stimuler les cellules chargées du développement des défenses immunitaires lors d'une infection. Par exemple, elles stimulent la croissance et la différenciation des lymphocytes. Ce sont des polypeptides synthétisés et secrétés principalement par les macrophages au cours de la réponse de l'hôte à l'infection. Elles sont également produites au niveau du système nerveux central, majoritairement par les

cellules microgliales et les astrocytes (Dong et Benveniste, 2001; Hanisch, 2002). Les cytokines peuvent agir de façon autocrine, paracrine et endocrine. Il existe deux grands groupes de cytokines :

- les cytokines inflammatoires (interleukines : IL-1, IL-6, IL-8, IL-12, IL-17, IL-18 ; tumor necrosis factor : TNF-α, TNF-β ; interférons ; …) qui ont pour but d'initier et de coordonner la réponse inflammatoire afin de combattre efficacement l'agent pathogène,
- les cytokines anti-inflammatoires (IL-4, IL-10, …) qui ont pour but de réguler et de terminer cette réaction inflammatoire.

L'IL-1, l'IL-6 et le TNF-α sont les cytokines dont le rôle dans la réaction de phase aiguë a été le plus largement étudié.

- *L'interleukine 1*

La famille de l'interleukine-1 est constituée de l'IL-1α et de l'IL-1β, deux agonistes des récepteurs à l'IL-1, ainsi que d'un antagoniste endogène : l'*IL-1 receptor antagonist* (IL-1ra). Ces trois membres de la famille IL-1 partagent les mêmes récepteurs : l'IL-1RI et l'IL1-RII. C'est l'IL1-RI qui est responsable des effets biologiques de l'IL-1, alors que l'IL1-RII est un récepteur « leurre » c'est-à-dire que la fixation de l'IL-1 sur ce récepteur ne génère aucun signal (Sims, 2002). L'activation de l'IL1-RI recrute les voies MAPK et NFκB conduisant à la transcription de nombreux gènes impliqués dans les réponses immunitaires et inflammatoires, dont ceux codant pour les cytokines inflammatoires (Dunne et O'Neill, 2003). Dans le cerveau du rongeur, l'IL1-RI est exprimé au niveau des méninges, des plexus choroïdes, des cellules épendymaires bordant les ventricules et des cellules de la microvascularisation cérébrale (cellules endothéliales et glie périvasculaire). Il est également exprimé au niveau neuronal, dans l'hippocampe, le cervelet, le thalamus, l'amygdale, le VMH, l'ARC, l'éminence médiane, les noyaux des nerfs trijumeau, facial et hypoglosse, et l'AP (Cunningham et al., 1992; Wong et Licinio, 1994; Yabuuchi et al., 1994). L'injection centrale d'IL-1β augmente l'expression de l'IL1-RI dans plusieurs de ces structures (Ilyin et Plata-Salamán, 1996).

- *L'interleukine 6*

L'IL-6 a également la capacité d'agir au niveau central. Son récepteur, L'IL-6R qui active la voie des MAPK, est fortement exprimé dans l'hippocampe à l'état basal et plus faiblement dans le cortex, l'amygdale, le noyau d'origine de la strie terminale (BST pour *bed stria terminalis*, structure impliquée dans la réponse au stress), les organes circumventriculaires (area postrema, éminence médiane, organe vasculaire de la lame terminale, organe subfornical) et les cellules épendymaires. Lors d'une inflammation, l'expression des transcrits de l'IL-6R augmente dans ces structures et apparait dans l'hypothalamus et dans la microvascularisation cérébrale (Vallières et Rivest, 1997).

- *Le tumor necrosis factor α*

Le TNF-α peut se lier sur deux récepteurs qui recrutent des voies de signalisation intracellulaire différentes : TNF-RI qui active la voie de l'apoptose et TNF-RII qui active les voies MAPK et NFκB (Baud et Karin, 2001). A l'état basal, le récepteur TNF-RI est exprimé dans les organes circumventriculaires, les plexus choroïdes, les méninges, les cellules épendymaires bordant les ventricules et les cellules de la microvascularisation cérébrale. L'injection périphérique de TNF-α induit une augmentation de son expression dans ces régions. Le récepteur TNF-RII, quant à lui, est quasiment indétectable en condition basale sauf dans l'hippocampe. Lors d'une inflammation, son expression est induite dans les méninges, les plexus choroïdes, les cellules épendymaires et la microvascularisation cérébrale (Nadeau et Rivest, 1999).

- *Action centrale des cytokines inflammatoires*

Comme nous venons de le voir, les différents récepteurs des cytokines sont exprimés dans le cerveau. De plus, l'injection périphérique de cytokines entraine une augmentation de l'expression de c-Fos dans de nombreuses structures nerveuses (Herkenham et al., 1998; Nadeau et Rivest, 1999) ainsi que la synthèse *de novo* de cytokines inflammatoires au niveau du SNC (Churchill et al., 2006). Ainsi, les cytokines périphériques amenées par la circulation sanguine pourraient d'une part pénétrer passivement dans le cerveau au niveau des organes circumventriculaires et d'autre part se fixer aux cellules associées à la barrière hémato-

encéphalique où elles stimuleraient la synthèse de seconds messagers de l'inflammation (Konsman et al., 2004; Nadeau et Rivest, 1999; Vallières et Rivest, 1997; Vallières et al., 1997). Ces cytokines pourraient également agir par voie nerveuse via le nerf vague. En effet, l'IL-1β est capable de stimuler l'activité électrique du nerf vague provoquant ainsi une libération accrue de glutamate dans le NTS (Mascarucci et al., 1998; Niijima, 1996) et la vagotomie diminue certains symptômes centraux de la réaction de phase aiguë induits par l'IL-1β et le TNF-α tels que la fièvre (Gaykema et al., 2000; Hansen et al., 2001) et l'anorexie (Bret-Dibat et al., 1995). L'ensemble de ces données indique que les cytokines inflammatoires circulantes sont capables de modifier le fonctionnement du SNC en empruntant la voie humorale et la voie nerveuse **(Figure 13)**.

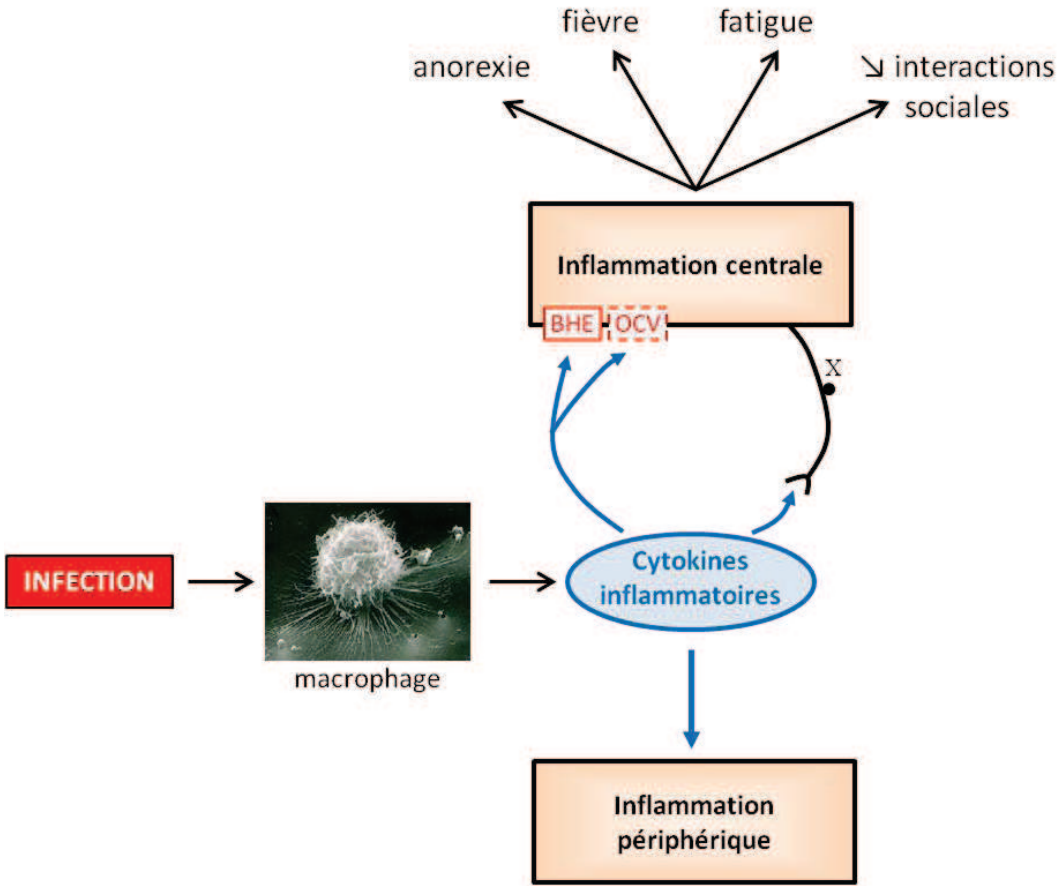


Figure 13: Implication des cytokines inflammatoires dans la réaction de phase aiguë.
Lors d'une infection, les cellules du système immunitaire sécrètent des cytokines inflammatoires à l'origine d'une inflammation périphérique et centrale. Ces cytokines sont capables d'agir sur le cerveau par deux voies: la voie nerveuse via le nerf vague et la voie humorale via les OCV et la BHE. L'inflammation centrale est responsable des symptômes caractéristiques de la réaction de phase aiguë.

BHE: barrière hémato-encéphalique; OCV: organes circumventriculaires; X: nerf vague.

L'étude des mécanismes cellulaires et moléculaires de l'inflammation se fait couramment par le lipopolysaccharide (LPS) puisque l'administration exogène de cette endotoxine entraine la synthèse de cytokines et provoque l'ensemble des symptômes de la réaction de phase aiguë.

1.2. Le lipopolysaccharide

Le LPS est un composant essentiel de la membrane externe des bactéries à Gram négatif. Il est libéré dans la circulation sanguine lorsque l'organisme est infecté par ces bactéries (exemple de bactéries à Gram négatif : Salmonella, Pseudomonas, E coli). Le LPS est transporté dans le sang grâce à une protéine de liaison, la *LPS binding protein* (LBP). Le complexe ainsi formé se fixe sur le récepteur CD14 qui interagit avec le toll-like récepteur de type 4 (TLR-4) provoquant les effets biologiques du LPS (Wright et al., 1990). Le récepteur TLR-4 a alors été identifié comme le récepteur du LPS car les souris déficientes en TLR-4 sont résistantes à cette endotoxine (Johnson et al., 1997; Poltorak et al., 1998). Les récepteurs TLR-4 sont principalement exprimés au niveau des cellules du système immunitaire inné périphérique (macrophages, neutrophiles, lymphocytes « natural killers », cellules dendritiques) mais également au niveau des cellules immunitaires du SNC (macrophages périvasculaires et microglie). Les neurones et astrocytes sont aussi capables d'exprimer TLR-4 mais plus faiblement. La présence de LPS au sein de l'organisme entraine l'activation de ces différents types cellulaires qui libèrent les cytokines inflammatoires, provoquant ainsi une augmentation de leurs concentrations plasmatiques et centrales (Janský et al., 1995; Kakizaki et al., 1999).

L'injection périphérique de LPS entraine la mise en place de la composante centrale de la réaction de phase aiguë indiquant que cette endotoxine est capable de modifier, directement ou indirectement, le fonctionnement du SNC. D'une part, l'administration périphérique de LPS induit une augmentation de l'expression des cytokines inflammatoires au niveau des organes circumventriculaires, des plexus choroïdes, des méninges et de la microvascularisation cérébrale, mais également dans le parenchyme cérébral (Quan et al., 1999; van Dam et al., 1992). De plus cette administration entraine l'activation de c-Fos dans plusieurs structures cérébrales (Dallaporta et al., 2007; Reyes et al., 2012; Sagar et al., 1995; Wan et al., 1993) **(Figure 14)** :

- structures du cerveau antérieur : amygdale, noyau d'origine de la strie terminale (BST), organe subfornical (SFO) ;

- structures hypothalamiques : aire pré-optique (APO), organe vasculaire de la lame terminale (OVLT), noyau supra-optique (SON), PVN, ARC, éminence médiane ;
- structures pontiques : PBN, LC ;
- structures bulbaires : NTS, AP, VLM.

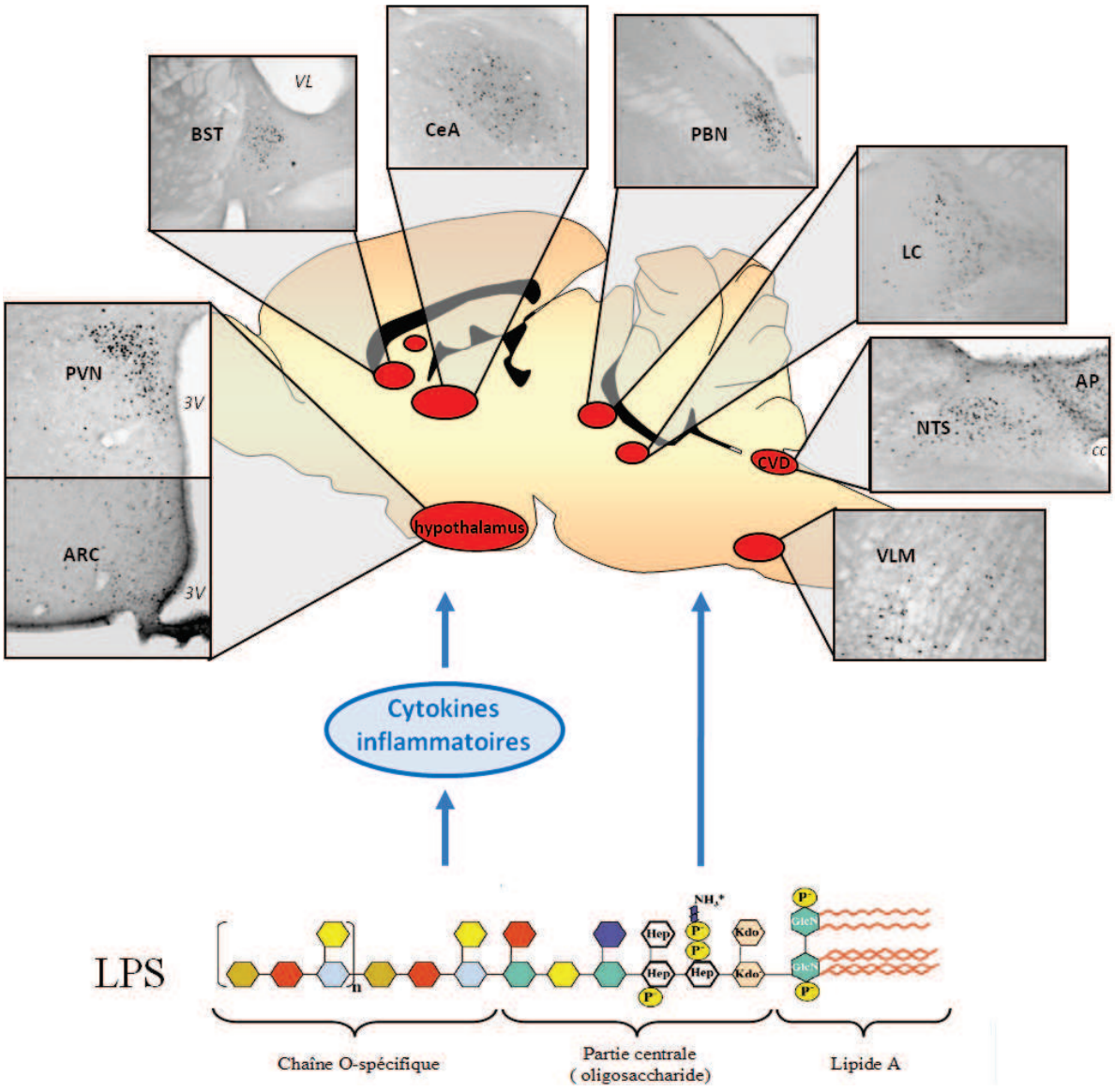


Figure 14 : Structures cérébrales activées par le LPS.
Immunohistochimie de la protéine c-Fos après administration périphérique de LPS. Le LPS active directement ou via la libération de cytokines plusieurs structures cérébrales dont l'hypothalamus et le CVD.

LPS: lipopolysaccharide; ARC: noyau arqué; PVN: noyau paraventriculaire; 3V: troisième ventricule; VL: ventricule latéral; BST: noyau d'origine de la strie terminale ; CeA: amygdale centrale, PBN: noyau parabrachial; LC: locus coeruleus; NTS: noyau du tractus solitaire; AP: area postrema; cc: canal central; VLM: medulla ventrolatérale.

Comme les cytokines, le LPS exercerait cette action centrale par deux voies : d'une part une action directe sur le cerveau, d'autre part par l'intermédiaire du nerf vague. La présence de récepteurs TLR-4 au niveau du SNC plaide en faveur d'une action directe. De plus, des injections icv de LPS provoquent les symptômes de la composante centrale de la réaction de phase aiguë (Johnson et al., 1997; Kent et al., 1992), la production de cytokines inflammatoires dans le cerveau (Gayle et al., 1998; Ilyin et al., 1998) et l'induction du gène précoce c-fos dans l'hypothalamus et le bulbe rachidien (Condé et al., 1999; Wan et al., 1993), ce qui vient appuyer l'hypothèse d'une action directe sur le SNC. Le rôle du nerf vague dans la transmission de l'information inflammatoire a également été mis en évidence car la vagotomie inhibe la fièvre induite par l'administration de LPS (Sehic et Blatteis, 1996) et abolit l'effet anorexigène de cette endotoxine (Bret-Dibat et al., 1995). Cependant ces effets n'ont pas été retrouvés dans toutes les études menées donc l'implication du nerf vague reste à ce jour controversée (Hansen et al., 2000; Konsman et al., 2000; Porter et al., 1998b). Au niveau moléculaire, la vagotomie semble inhiber l'induction des transcrits de l'IL-1β dans l'hypothalamus et l'hippocampe en réponse au LPS (Layé et al., 1995), ainsi que l'induction de c-Fos (Ge et al., 2001; Konsman et al., 2000).

De nombreuses données de la littérature indiquent que l'action inflammatoire des cytokines peut être relayée par une augmentation de la production de prostanoïdes synthétisés dans de nombreux tissus et types cellulaires lors d'une inflammation, que ce soit en périphérie ou au niveau du SNC.

1.3. Les prostanoïdes

Les prostanoïdes sont des médiateurs lipidiques agissant de manière autocrine et paracrine et dont le précurseur est l'acide arachidonique (Funk, 2001). Parmi les prostanoïdes se trouvent les prostaglandines (PG) et les thromboxanes (TX) mais le prostanoïde le plus abondant dans le corps humain est la prostaglandine E2 (PGE2). De nombreuses études ont montré que des injections de LPS ou de cytokines inflammatoires augmentent fortement la production de PGE2 au niveau du parenchyme cérébral et du LCR (Engblom et al., 2003; Moore et al., 2004) et que l'injection centrale de PGE2 induit l'activation de plusieurs structures cérébrales (Lacroix et al., 1996) ainsi que la mise en place des symptômes de la composante centrale de la réaction de phase aiguë (Levine et Morley, 1981; Milton et Wendlandt, 1971).

La PGE2 est synthétisée à partir de l'acide arachidonique par l'action de plusieurs enzymes : les cyclooxygénases (COX) et les prostaglandine E synthases (PGES) (Murakami et Kudo, 2004) **(Figure 15)**. Les COX interviennent dans les premières étapes de la synthèse de PGE2. Il existe deux COX : COX-1 qui est exprimée de manière constitutive dans de nombreux tissus, et COX-2 qui est induite par divers stimuli dont les facteurs de croissance, les hormones et les molécules inflammatoires (LPS, cytokines) (Morita, 2002; Smith et al., 2000; Tanabe et Tohnai, 2002). Les PGES, quant à elles, catalysent la dernière étape de la biosynthèse de la PGE2. Trois types de PGES sont actuellement connus : la *cytosolic PGES* (cPGES) et les *microsomal PGES-1 et -2* (mPGES-1 et -2) (Park et al., 2006). cPGES et mPGES-2 sont exprimées constitutivement (Murakami et al., 2003; Tanioka et al., 2000), alors que mPGES-1 est faiblement exprimée à l'état basal mais fortement induite par des stimuli pro-inflammatoires tels que le LPS, l'IL-1β et le TNF-α dans de nombreux tissus et types cellulaires (Catley et al., 2003; Giannico et al., 2005; Masuko-Hongo et al., 2004) **(Figure 15)**.

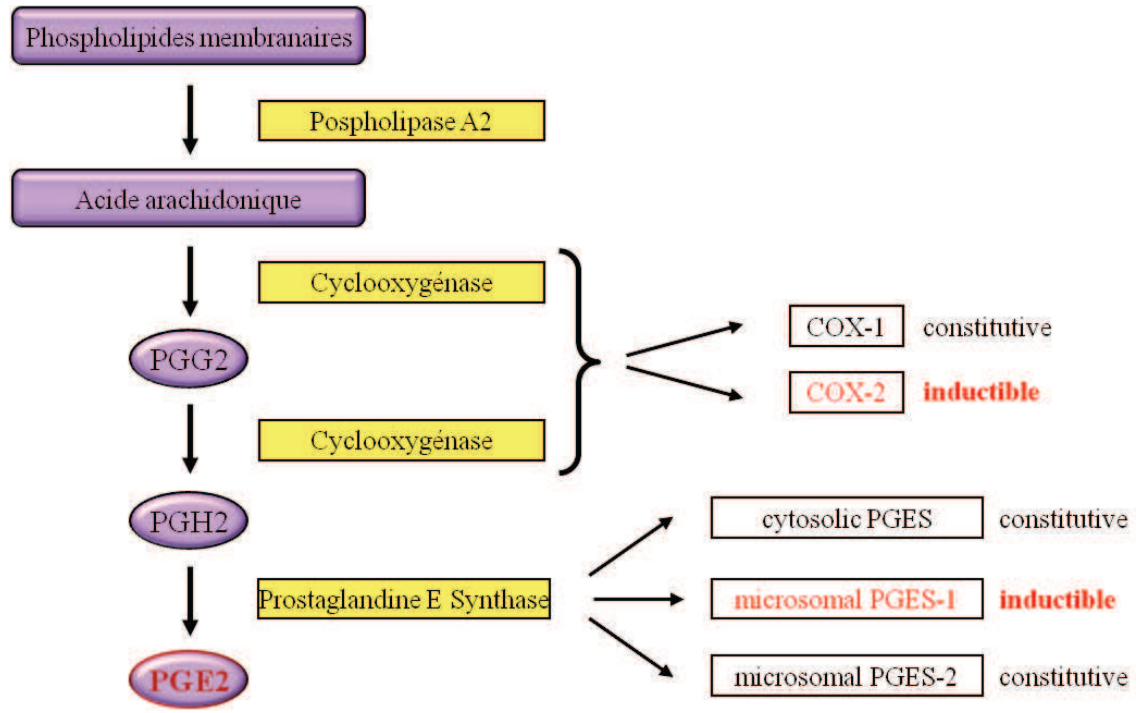


Figure 15 : Voie de synthèse de la PGE2.
La PGE2 est un médiateur de l'inflammation synthétisé à partir de l'acide arachidonique par les enzymes COX et PGES. L'activité de COX-2 et mPGES-1 est stimulée par l'inflammation aboutissant à l'augmentation de la synthèse de PGE2.

PG: prostaglandine; COX: cyclooxygénase; PGES: prostaglandine E synthase.

En condition inflammatoire, l'induction de COX-2 et de mPGES-1 serait donc responsable de l'augmentation de la production de PGE2. Cette PGE2 contribuerait à la mise en place de l'anorexie inflammatoire. Des souris dépourvues de la mPGES-1 sont d'ailleurs résistantes à l'anorexie induite par une administration périphérique d'IL-1β (Pecchi et al., 2006).

Quatre récepteurs relayant l'action pro-inflammatoire de la PGE2 ont été identifiés : les *endogene prostanoid receptor* (EP) 1 à 4. Ce sont des RCPG qui provoquent une augmentation du calcium intracellulaire via la voie phospholipase C/IP3 pour EP1 (Funk et al., 1993) et une modulation de l'activité de la voie adénylate cyclase/AMPc/PKA pour EP2-4 (Breyer et al., 2001; Katsuyama et al., 1995; Nishigaki et al., 1995). Ces récepteurs ont tous été retrouvés dans plusieurs structures cérébrales (organes circumventriculaires, hypothalamus, tronc cérébral, amygdale…) et leur expression est stimulée lors d'une inflammation (Ek et al., 2000; Zhang et Rivest, 1999).

Ainsi, lors d'une infection reproduite expérimentalement par l'injection périphérique de LPS, une augmentation de la synthèse de cytokines et de PGE2 est observée au niveau du SNC, indiquant la mise en place d'une inflammation centrale. La transmission de l'information immune de la périphérie vers le cerveau repose sur plusieurs mécanismes :

1- Le LPS peut agir directement sur les cellules du SNC qui expriment ses récepteurs, pour stimuler la synthèse de cytokines inflammatoires et de PGE2 dans le cerveau.
2- Le LPS peut agir indirectement via la synthèse de cytokines inflammatoires en périphérie, cytokines qui peuvent alors agir sur le cerveau par voie humorale ou nerveuse.

L'inflammation du SNC aboutit alors à la mise en place des symptômes de la composante centrale de la réaction de phase aiguë dont l'anorexie inflammatoire.

2. L'anorexie inflammatoire

2.1. Implication des cytokines dans l'anorexie inflammatoire

L'injection périphérique des différentes cytokines inflammatoires diminue la prise alimentaire et l'inhibition pharmacologique ou immunologique de l'action de ces cytokines atténue l'anorexie inflammatoire. Par exemple, des injections périphériques ou centrales d'IL-1ra

amoindrissent l'anorexie et la perte de poids observées après traitement au LPS (Bluthé et al., 1992; Layé et al., 2000; Swiergiel et al., 1997). De même, des injections d'anticorps dirigés contre l'IL-6 et le TNF-α atténuent l'anorexie inflammatoire induite par l'administration périphérique de LPS (Harden et al., 2006; Sharma et al., 1992). L'inhibition simultanée de plusieurs cytokines inflammatoires a un effet plus important sur l'anorexie induite par le LPS que l'inhibition d'une seule d'entre elles (Swiergiel et Dunn, 1999). En accord avec ces résultats, des souris transgéniques incapables de produire de l'IL-1β biologiquement active sont résistantes à l'anorexie induite par des traitements centraux au LPS (Yao et al., 1999). Cependant, de manière surprenante, les autres modèles génétiques ne permettent pas de corroborer ces résultats puisque des souris invalidées pour les gènes codant pour l'IL-1RI, l'IL-6 et le TNF-α présentent une anorexie comparable aux souris sauvages en réponse au LPS (Bluthé et al., 2000; Fantuzzi et al., 1996; Leon et al., 1997; Swiergiel et Dunn, 2006). Cette contradiction pourrait s'expliquer par la mise en place de mécanismes compensatoires par les autres cytokines.

2.2. Implication des médiateurs de la prise alimentaire dans l'anorexie inflammatoire

Les médiateurs de la prise alimentaire semblent relayer, au moins en partie, les effets anorexigènes des cytokines inflammatoires **(Figure 16)**.

- *Les signaux circulants*

Parallèlement à la mise en place de l'anorexie, des traitements au LPS, à l'Il-1β ou au TNF-α augmentent de manière dose-dépendante les transcrits de la leptine dans le tissu adipeux ainsi que sa concentration plasmatique (Berkowitz et al., 1998; Faggioni et al., 1998; Grunfeld et al., 1996; Sarraf et al., 1997). L'augmentation de la leptinémie par le LPS semble être relayée par l'IL-1β puisque les souris IL-1β-/- sont résistantes à l'effet hyperleptinémiant de cette endotoxine (Faggioni et al., 1998). A l'inverse, les taux circulants de ghréline sont diminués par le LPS et l'Il-1β (Asakawa et al., 2001; Hataya et al., 2003; Wang et al., 2006) et l'injection d'IL-1ra abolit l'effet inhibiteur du LPS sur la production de ghréline (Wang et al., 2006). En retour, la leptine stimule l'expression des cytokines inflammatoires dans les

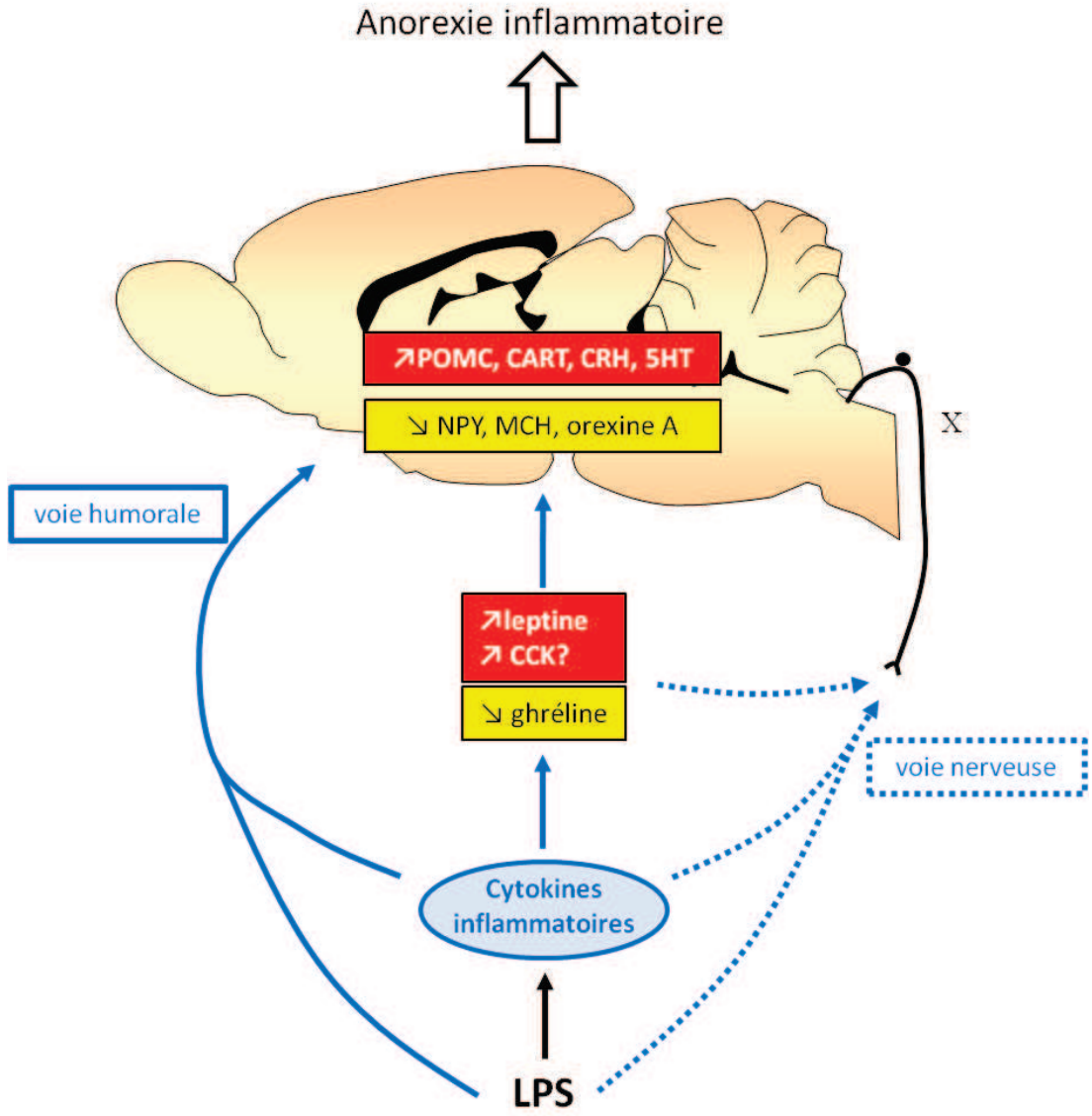


Figure 16 : Implication des médiateurs de la prise alimentaire dans l'anorexie inflammatoire.
L'inflammation stimule les hormones et neuropeptides anorexigènes alors qu'elle inhibe les hormones et neuropeptides orexigènes, aboutissant à la diminution de prise alimentaire observée durant l'inflammation.

LPS = lipopolysaccharide; CCK = cholécystokinine; NPY = neuropeptide Y; MCH = melanin-concentrating hormone; POMC = pro-opiomélanocortine; CART = cocaïne- and amphetamine-related transcript; CRH = corticotropin-releasing hormone; 5HT = sérotonine; X = nerf vague.

cellules immunitaires (Dixit et al., 2004). L'injection d'anticorps anti-leptine diminue l'anorexie observée en réponse au LPS confirmant le rôle de la leptine dans la mise en place de l'anorexie inflammatoire (Harden et al., 2006; Sachot et al., 2004). Les souris obèses *db/db* n'exprimant pas le récepteur à la leptine présentent une hypophagie atténuée en réponse au LPS (Faggioni et al., 1997). Cependant l'étude d'autres modèles génétiques d'obésité due à un déficit en signalisation leptinergique (souris *ob/ob* et rat *fa/fa*) n'a pas permis de confirmer ces résultats (Faggioni et al., 1997; Lugarini et al., 2005). La CCK semble aussi jouer un rôle dans la mise en place de l'anorexie inflammatoire puisque le blocage pharmacologique des récepteurs CCK-A atténue l'hypophagie induite par le LPS (Rorato et al., 2011).

- *Neuropeptides des neurones de premier ordre*

L'inflammation augmente l'expression de POMC et de CART dans l'hypothalamus (Sergeyev et al., 2001) alors qu'elle diminue celle de NPY (Gayle et al., 1997). Des souris transgéniques dépourvues du récepteur MC4-R sont résistantes à l'anorexie et à la perte de poids induites par l'injection de LPS (Marks et al., 2001). De même, les animaux recevant des injections centrales d'antagonistes MC4-R ne développent pas d'anorexie en réponse à l'IL-1β ou au LPS (Huang et al., 1999; Lawrence et Rothwell, 2001).

- *Neuropeptides des neurones de second ordre*

Le LPS et les cytokines inflammatoires stimulent la production de CRH dans le PVN (Turnbull et Rivier, 1999) et le LPS stimule l'expression de c-Fos dans les neurones exprimant la CRH (Rorato et al., 2011). De plus, la neutralisation de cette protéine par des anticorps anti-CRH diminue l'anorexie induite par l'IL-1β (Uehara et al., 1989). A l'inverse, dans la LHA, le LPS diminue le nombre des transcrits de la MCH (Sergeyev et al., 2001) ainsi que le nombre de neurones orexine A activés par le jeûne (Becskei et al., 2008). Ainsi, l'inflammation favorise l'action des neuropeptides anorexigènes alors qu'elle inhibe celle des neuropeptides orexigènes.

- *Sérotonine*

L'implication de la sérotonine dans l'anorexie inflammatoire aiguë a également été proposée car des traitements centraux ou périphériques aux cytokines inflammatoires ou au LPS augmentent l'activité sérotoninergique dans le noyau dorsal du raphé (Clement et al., 1997). De plus, l'injection d'antagonistes de l'autorécepteur 5HT-1A au niveau des noyaux du raphé, connus pour diminuer l'activité des neurones sérotoninergiques, atténue l'anorexie induite par des injections centrales ou périphériques de LPS ou d'IL-1β chez le rat (von Meyenburg et al., 2003a). De même l'inhibition pharmacologique du récepteur 5HT-2C amoindrit l'anorexie inflammatoire chez le rat et la souris (Asarian et al., 2007; von Meyenburg et al., 2003b).

Lors d'une infection, l'organisme libère des cytokines inflammatoires qui vont agir sur le cerveau pour mettre en place la composante centrale de la réaction de phase aiguë. Ces cytokines vont notamment activer la voie anorexigène aux dépens de la voie orexigène et ainsi provoquer l'anorexie inflammatoire (Figure 16).

3. Inflammation de bas seuil, obésité et diabète de type 2

3.1. Inflammation et diabète de type 2

De manière surprenante, de nombreuses études ont montré qu'une inflammation chronique de bas seuil était observée chez des individus obèses et diabétiques et cette inflammation participe au développement de la résistance à l'insuline, caractéristique du diabète de type 2. Une étude épidémiologique menée sur des hommes et des femmes âgés de 35 à 65 ans révèle d'ailleurs qu'un niveau élevé à la fois d'IL-1β et d'IL-6 est prédictif du diabète de type 2 : en effet, les individus présentant des taux accrus de ces deux cytokines ont des risques importants de développer un diabète dans les 3 années à venir (Spranger et al., 2003). Les cytokines inflammatoires diminuent la sensibilité à l'insuline des adipocytes, des hépatocytes et des myocytes. Les données sur l'IL-1β montrent que celle-ci diminue la capture du glucose induite par l'insuline dans les adipocytes, en altérant l'expression des transporteurs au glucose GLUT-4 (Jager et al., 2007). A l'inverse, les souris IL-1β-/- sont plus sensibles à l'insuline que les souris sauvages puisqu'elles obtiennent de meilleurs résultats au test de tolérance à

l'insuline (Stienstra et al., 2010). Enfin, l'IL-1Ra, l'antagoniste naturel des récepteurs à l'IL-1β, augmente la sensibilité des adipocytes à l'insuline *in vitro* (Stienstra et al., 2010). Chez les patients atteints de diabète de type 2, les taux endogènes d'IL-1Ra sont diminués mais un traitement avec cet antagoniste a des effets bénéfiques sur la pathologie. Cet effet ne semble cependant pas être lié à une augmentation de la sensibilité à l'insuline mais à une augmentation de sa sécrétion (Larsen et al., 2007, 2009; Marculescu et al., 2002). Ainsi, chez les personnes obèses, l'augmentation de l'IL-1β associée à une diminution de l'IL-1Ra participerait au développement du diabète de type 2.

L'IL-6 provoque également une résistance à l'insuline mais à l'inverse de l'IL-1β, elle n'agit pas sur l'expression des transporteurs GLUT-4 mais sur les récepteurs à l'insuline eux-mêmes en affectant leur activité (Kim et al., 2004; Senn et al., 2002). Le TNF-α est lui aussi plus élevé chez les individus à la fois obèses et diabétiques, et la neutralisation de cette cytokine chez des rats obèses stimule la capture du glucose en réponse à l'insuline et améliore ainsi la résistance à l'insuline (Hotamisligil et al., 1993). De plus, des souris TNF-α-/- rendues obèses par l'alimentation présentent une résistance à l'insuline moins importante que les souris sauvages (Uysal et al., 1997).

3.2. Inflammation et obésité

Chez l'homme et le rongeur, l'obésité est associée à une légère augmentation des cytokines inflammatoires dans l'organisme, notamment l'IL-1β, l'IL-6 et le TNF-α qui sont dans ce cas principalement sécrétées par le tissu adipeux (Cartier et al., 2008; Hotamisligil et al., 1993; Vozarova et al., 2001). En effet, le foyer inflammatoire semble être le tissu adipeux où des infiltrations de macrophages et de lymphocytes ont été mises en évidence (Duffaut et al., 2009; Oh et al., 2012; Weisberg et al., 2003). De plus, des patients obèses soumis à un régime durant plusieurs mois, à l'origine d'une perte de poids, voient leurs taux de TNF-α et IL-6 diminuer. Ce régime consistait à contrôler les apports alimentaires et à pratiquer de l'exercice physique. Les variations du niveau de cytokines semblent dépendre du degré d'activité physique exercé (Dandona et al., 1998; Romeo et al., 2011; Ziccardi et al., 2002). Ainsi, le niveau de cytokines inflammatoires est corrélé avec l'IMC. Mais cette inflammation chronique de bas seuil est-elle la cause ou la conséquence de l'obésité ? Aucune réponse définitive n'a été apportée à cette question mais il semblerait que lorsque la prise de poids apparait, les cellules immunitaires infiltrent le tissu adipeux et affectent sa fonction, favorisant

le développement de l'obésité (Weisberg et al., 2003). Ce serait donc l'alimentation elle-même qui initierait l'inflammation, et plus particulièrement une alimentation hypercalorique. Dans ce sens, il a été montré que le glucose est capable de stimuler la synthèse et la sécrétion d'IL-1β par différents types cellulaires tels que les cellules endothéliales, les monocytes ou encore les cellules béta (Asakawa et al., 1997; Maedler et al., 2002; Shanmugam et al., 2003). Les acides gras stimulent également la production de cytokines inflammatoires en se fixant sur les récepteurs TLR-4 (Håversen et al., 2009; Shi et al., 2006). Ainsi, une alimentation hypercalorique entrainerait d'une part un accroissement de la masse adipeuse aboutissant à une infiltration de cellules immunitaires dans ce tissu, et d'autre part une libération de cytokines inflammatoires par d'autres types cellulaires. L'ensemble de ces deux phénomènes conduirait à l'inflammation de bas seuil qui favoriserait l'obésité. Dans ce sens, une étude a montré que chez la souris, l'infusion en continu de LPS à l'aide d'une minipompe osmotique placée en sous-cutané est à l'origine d'une prise de poids similaire à celle d'animaux soumis à un régime hypercalorique. Cette prise de poids est liée à une augmentation de la masse du tissu adipeux et du foie (Cani et al., 2007).

3.3. Rôle de l'hypothalamus dans l'obésité induite par l'inflammation

C'est en 2005 qu'une inflammation centrale a été mise en évidence pour la première fois chez des animaux obèses. En effet, des rats soumis à une alimentation riche en graisse présentent une augmentation de la concentration en IL-1β, IL-6 et TNF-α dans l'hypothalamus, ainsi qu'une augmentation de PGE2 et COX-2 dans le cortex cérébral (De Souza et al., 2005; Zhang et al., 2005). Cette inflammation centrale contribue au développement de l'obésité chez ces animaux nourris avec un régime hypercalorique. En effet, l'altération de la signalisation TLR-4 spécifiquement dans les neurones protège de l'obésité, de la résistance à la leptine et de la résistance périphérique à l'insuline (Kleinridders et al., 2009). De même, des injections centrales d'anticorps dirigés contre ce récepteur TLR-4 diminue la prise alimentaire de rats rendus obèses par une alimentation hypercalorique mais cet effet n'est pas observé chez des rats nourris avec une alimentation standard (Milanski et al., 2009). En 2007, Romanatto et al. ont recréé l'inflammation centrale de bas seuil en administrant des faibles doses de TNF-α en icv. Ils ont observé chez ces animaux une résistance centrale à la leptine et à l'insuline puisque l'anorexie induite par l'injection centrale de ces hormones était moins importante que chez les animaux n'ayant pas reçu de TNF-α. Cet effet n'était pas observé pour des doses

élevées de TNF-α (Romanatto et al., 2007). Ainsi, l'inflammation chronique de bas seuil inhiberait la signalisation hypothalamique anorexigène, favorisant le développement de l'obésité (Thaler et Schwartz, 2010).

Pour résumer, l'inflammation au sein de l'organisme entraine des troubles métaboliques pouvant être opposés selon l'origine et l'intensité de celle-ci : une inflammation de forte intensité par un pathogène ou une maladie chronique entraine une anorexie et une perte de poids, alors qu'une inflammation de faible intensité entraine une obésité associée à un diabète de type 2. Ainsi, l'inflammation constitue un bon modèle d'étude des perturbations de la balance énergétique.

III Le cerveau comme cible thérapeutique de l'obésité et du diabète de type 2

Maintenant que nous avons vu comment la prise alimentaire était régulée en condition physiologique et pathologique, nous allons nous intéresser aux troubles métaboliques et plus particulièrement à leurs traitements. Comme mentionné précédemment, l'obésité et le diabète de type 2 sont des pathologies très répandues chez l'humain et constituent un grave problème de santé publique. C'est pourquoi le traitement de ces deux maladies représente un enjeu de taille. Malheureusement, à ce jour, les traitements proposés ne combinent pas efficacité et sécurité puisque d'une part, ils ne parviennent pas à remédier à l'obésité et au diabète et d'autre part, les effets secondaires sont fréquents. Dans cette partie, j'exposerai quelques traitements actuels de ces pathologies ainsi que ceux ayant été retirés du marché. Puis je décrirai des brevets récents visant à développer des agents pharmacologiques susceptibles de traiter ces deux pathologies en ciblant la neurocircuiterie dévolue à la prise alimentaire.

1. Traitements passés et présents

1.1. Drogues anti-obésité

Les premiers médicaments anti-obésité ciblaient déjà le système nerveux central puisqu'il s'agissait de dérivés amphétaminiques qui ont des propriétés coupe-faim. Ces composés mis sur le marché dans les années 50 tels que la phenmétrazine, la phentermine, la benzphétamine ou encore la phendimétrazine étaient utilisés comme traitement à court terme. Malgré leurs effets addictifs et hypertensifs, ils sont encore largement utilisés de nos jours pour lutter contre l'obésité, excepté la phenmétrazine. Des inhibiteurs de la capture de 5-HT, tels que la fenfluramine et la dexfenfluramine ont aussi longtemps été utilisés depuis les années 70. Ils étaient souvent prescrits en association avec la phentermine, et causaient une perte d'appétit (Bever et Perry, 1997). Ces drogues ont cependant été retirées du marché en 1997 puisqu'elles étaient à l'origine de valvulopathies cardiaques et d'hypertension pulmonaire (Loke et al., 2002). Actuellement, le traitement le plus communément prescrit est l'Orlistat, un inhibiteur de la lipase pancréatique commercialisé depuis 1999. Son rôle est d'empêcher l'absorption des

graisses de l'alimentation, réduisant ainsi l'apport calorique. Les graisses non digérées sont alors éliminées dans les selles. Cependant, la prise d'Orlistat entraine des diarrhées et des douleurs abdominales importantes. Une étude récente a rapporté des altérations de la vidange gastrique et des sécrétions post-prandiales telles que la CCK, le GLP-1 et le PYY^{3-36} par l'Orlistat. Ces changements augmentent l'appétit conduisant à une hyperphagie (Ellrichmann et al., 2008; Enç et al., 2009). Bien que ces médicaments contribuent à perdre du poids, les effets secondaires sont importants et leur utilisation doit être combinée avec une alimentation réduite en calories et la pratique d'une activité physique régulière.

1.2. Drogues anti-diabète

Les biguanides sont les composés utilisés en première intention dans le traitement du diabète de type 2, seuls ou en association avec d'autres anti-diabétiques oraux. Ils sont prescrits depuis la fin des années 50. Ces composés augmentent la sensibilité de l'insuline au niveau du foie, du tissu adipeux et des muscles. La phenformine a été le premier biguanide utilisé mais il fut retiré du marché à la fin des années 70 à cause de sa toxicité. La metformine est à ce jour le biguanide le plus couramment prescrit (Viollet et al., 2012). Bien que le diabète de type 2 soit caractérisé par une insulino-résistance, à des stades avancés de la maladie, un défaut de sécrétion d'insuline se développe. Une stratégie employée est alors la stimulation de sécrétion d'insuline endogène, en utilisant des sulfonylurées et des incrétines. L'insulinothérapie est également fréquemment utilisée pour traiter cette pathologie (Nadeau, 2013). Il existe deux types d'analogues de l'insuline : les analogues à action rapide qui abaissent la glycémie rapidement et transitoirement, et les analogues à action lente qui agissent avec un délai mais exercent une action hypoglycémiante durable.

Dans la suite de ce chapitre, j'exposerai quelques recherches actuelles visant à améliorer le traitement de l'obésité et du diabète de type 2. Ces deux pathologies étant très souvent associées, les études se tournent aujourd'hui vers des composés pouvant agir à la fois sur la prise alimentaire et l'homéostasie glucidique. Ces études ciblent essentiellement le système nerveux central. Bien qu'il ne s'agisse pas d'une description exhaustive des futures cibles thérapeutiques centrales de l'obésité et du diabète de type 2, je développerai ici trois voies de recherches pouvant potentiellement agir de manière combinée sur ces deux pathologies.

2. Futures cibles thérapeutiques centrales

2.1. Système mélanocortinergique

Plusieurs brevets récents relatent l'invention de nouveaux peptides dérivés de l'α-MSH pouvant agir sur les récepteurs MC3/4-R et ainsi constituer des cibles dans le traitement de l'obésité. L'efficacité de ces peptides a été testée *in vivo* chez le rat et plusieurs d'entre eux réduisent la prise alimentaire à 24 et 48 heures après leur injection ip, sous-cutanée ou orale (Lau et al., 2010 WO060901). Un autre de ces dérivés d'α-MSH, le Composé A, entraine une diminution dose-dépendante de la prise alimentaire et du poids chez des rats Zucker (Halem et Culler, 2011 WO017209). Afin d'augmenter l'activité mélanocortinergique, des brevets décrivent l'invention de peptides cycliques comme ligands des récepteurs MC3/4-R. Les peptides cycliques ont une meilleure stabilité dans les milieux biologiques car ils sont résistants aux dégradations enzymatiques naturelles. *In vivo*, ces nouveaux ligands cycliques ont montré un effet hypophagique et une diminution du poids corporel (Dong et al., 2010 US0184646 ; Shi et al., 2010 WO144344 ; Zheng, 2010 US0173834). L'utilisation de ces ligands MC3/4-R pourrait cependant s'avérer délétère car la signalisation centrale mélanocortinergique est également impliquée dans le contrôle de la pression artérielle. Chez le rongeur, une administration centrale d'α-MSH augmente la pression artérielle et la fréquence cardiaque (Greenfield et al., 2009; Ni et al., 2006). Afin de contourner ce problème, un brevet a développé des ligands mélanocortinergiques résistants à la dégradation de l'extrémité C-terminale. Ces peptides pourraient être d'un grand intérêt puisque la présence de l'extrémité C-terminale permet de minimiser les effets cardiovasculaires des mélanocortines (Gruber, 2011 WO026015).

En plus de la régulation de la prise alimentaire, le système mélanocortinergique est aussi impliqué dans l'homéostasie glucidique. En effet, des souris dépourvues de MC4-R sont d'une part hyperphagiques et obèses mais elles présentent également une hyperglycémie et une hyperinsulinémie. Le système mélanocortinergique régulerait à la fois la sécrétion d'insuline et sa sensibilité (Xu et al., 2011). Ainsi, cibler le système mélanocortinergique pourrait non seulement diminuer l'obésité mais aussi le diabète de type 2 (Lau et al., 2010 WO060901).

2.2. Le Brain-derived neurotrophic factor (BDNF)

Comme indiqué précédemment, le BDNF est un neuropeptide anorexigène qui agit en aval du système mélanocortinergique. En plus de diminuer la prise alimentaire, l'injection icv de BDNF chez des souris diabétiques améliore la sensibilité à l'insuline et diminue la glycémie (Nakagawa et al., 2000; Ono et al., 2000). Plusieurs brevets suggèrent le BDNF ou son récepteur TrkB comme traitement potentiel de l'obésité et du diabète de type 2. Un brevet propose de moduler l'expression génique du BDNF à l'aide d'un oligonucléotide antisens qui cible l'antisens naturel du BDNF. L'hybridation de ces oligonucléotides entraine une perte de fonction de l'antisens naturel du BDNF, aboutissant à une augmentation de l'expression du BDNF (Collard et al., 2010 WO093904). L'efficacité *in vivo* de cette technique sur les paramètres relatifs à l'obésité et au diabète de type 2 devra être identifiée. Le principe d'utilisation d'antisens pour traiter des pathologies a précédemment été validé par l' « Agence américaine des produits alimentaires et médicamenteux » qui a approuvé le VITRAVENE pour le traitement de la rétinite à cytomégalovirus.

Un autre brevet propose d'utiliser la neurotrophine 4/5 (NT4/5), un autre ligand endogène du récepteur TrkB pour traiter l'obésité et le diabète (Rosenthal et al., 2009 US0203610). Des injections périphériques ou centrales de NT4/5 diminuent la prise alimentaire et le poids corporel de souris normales, obèses ou diabétiques. De plus, la NT4/5 améliore la glycémie de souris diabétiques (Tsao et al., 2008). Par contre l'administration systémique de cette neurotrophine chez des primates non humains (singes rhésus et babouins) ne permet pas de corroborer ces effets (Lin et al., 2008).

2.3. La melanin-concentrating hormone (MCH)

La MCH est une hormone orexigène également connue pour favoriser la résistance à l'insuline et augmenter la glycémie (Pereira-da-Silva et al., 2005). Dans ce contexte, le développement d'antagonistes des récepteurs à la MCH constitue une piste intéressante pour le traitement de l'obésité et du diabète. Un composé, l'ALB-127158(a), est d'ailleurs en phase I d'un essai clinique. Ce composé présente une bonne affinité pour le récepteur MCH-1 et diminue significativement la prise alimentaire et le poids de souris obèses après un traitement chronique de 28 jours. D'autres brevets récents dévoilent la découverte de nouveaux antagonistes des récepteurs MCH-1 mais leur efficacité n'a pas été testée *in vivo* (Guzzo et al.,

2011 US0003737 ; Washburn et al., 2011a US0034375). Cependant, tous ces antagonistes possèdent un groupement amine pouvant entrainer des problèmes cardiovasculaires et en particulier de l'arythmie. Afin de proposer des composés sûrs, une équipe a développé des antagonistes où le groupement amine a été remplacé (Washburn et al., 2011b EP2298776). Notons que comme pour la MCH, plusieurs brevets cherchent à développer des antagonistes des récepteurs aux orexines (Branch et al., 2011 US7928124 ; Gobbi et al., 2010 US7829563 ; Knust et al., 2011 US7932269).

Ainsi, les pistes actuellement explorées pour le traitement de l'obésité et du diabète de type 2 visent la neurocircuiterie responsable de la régulation de la prise alimentaire (Cf. : annexes 2 et 3). Un nouveau peptide, la nesfatine-1, représente également un espoir thérapeutique, bien qu'il soit encore peu caractérisé à ce jour. Dans ce contexte, trois brevets proposent de cibler ce peptide pour améliorer le traitement de ces pathologies (Liu et al., 2010a US20100234274, 2010b US20100235935 ; Mori et al., 2008 US20080115231).

IV La nesfatine-1

Dans le cadre de mon travail doctoral, je me suis intéressée à un peptide identifié peu de temps avant mon arrivée dans le laboratoire : la nesfatine-1. Je me suis particulièrement attachée à rechercher l'implication des réseaux centraux exprimant ce peptide dans la régulation de l'homéostasie énergétique et les déséquilibres nutritionnels. La nesfatine-1 est à la fois un neuropeptide et une hormone aux fonctions multiples mais dont le rôle premier est la régulation de la prise alimentaire. Peu caractérisée au début de ma thèse, les données se sont progressivement accumulées permettant d'avoir une idée plus précise sur les fonctions de ce peptide. Dans ce chapitre, je ferai une synthèse relativement exhaustive des données de la littérature incluant les brevets existants sur la nesfatine-1.

1. Découverte et caractérisation de la nesfatine-1

1.1. Découverte

En 2006, Oh-I et ses collaborateurs découvrent la nesfatine-1 (Oh-I et al., 2006) en utilisant une méthode de clonage soustractif d'ADNc proche de celle utilisée en 2001 pour la découverte de la résistine dans les adipocytes (Steppan et al., 2001). La première étape dans la découverte de la nesfatine-1 fut plutôt originale puisqu'elle consista à stimuler des cellules issues d'un carcinome pulmonaire humain (lignée SQ5) par la troglitazone, un activateur de PPAR-γ (peroxisome proliferator activated receptor-γ) et à comparer les ADNc avant et après stimulation. Parmi les ADNc retenus comme étant activés par la troglitazone, les auteurs ne sélectionnèrent que ceux capables d'être adressés dans la voie de sécrétion grâce à un logiciel de prédiction de peptides signaux. Puis ils déterminèrent si les gènes activés par la troglitazone dans les cellules SQ5 étaient exprimés dans deux autres lignées : les cellules 3T3-L1 d'origine adipocytaire et les cellules HTB185 issues d'un médulloblastome, l'hypothèse étant qu'un grand nombre de peptides régulant la prise alimentaire sont exprimés à la fois dans les adipocytes et le cerveau. Au final, sur 596 ADNc activés pas la troglitazone dans les cellules SQ5, seuls 9 remplissaient les critères de sélection des auteurs, le meilleur candidat correspondant à la région 5' non traduite de la nucléobindine 2 (NUCB2) aussi appelée NEFA. Les nucléobindines sont des protéines aux fonctions multiples de par la présence de plusieurs domaines fonctionnels incluant un peptide signal, un domaine de liaison à l'ADN et

deux sites de liaison au calcium. A ce jour, deux nucléobindines ont été identifiées : NUCB1 et NUCB2 (Miura et al., 1992). En plus de pouvoir lier le calcium et les acides nucléiques, NUCB2, qui contient 396 acides aminés, peut interagir avec plusieurs protéines à l'intérieur de la cellule comme par exemple TNF-RI ou encore la necdine, une protéine régulant la croissance cellulaire (Islam et al., 2006; Taniguchi et al., 2000). Ainsi NUCB2 jouerait un rôle important dans la signalisation intracellulaire.

La deuxième étape dans la découverte de la nesfatine-1 consista à prédire les sites de clivage de la protéine NUCB2 par des proprotéines convertases à l'aide d'une technique développée par Duckert et ses collaborateurs. Cette technique consiste à identifier des couplets d'acides aminés dibasiques (lysine et arginine), sites de clivage préférentiels des proprotéines convertases (Duckert et al., 2004). Ainsi, trois peptides furent identifiés et nommés nesfatine-1 (pour *NEFA/NUCB2-encoded-satiety-* et *fat-influencing protein*), nesfatine-2 et nesfatine-3, correspondant respectivement aux acides aminés 1 à 82, 85 à 163, et 166 à 396 de la protéine NUCB2 **(Figure 17A)**.

1.2. Rôle anorexigène de la nesfatine-1

- *Etude princeps*

Oh-I et ses collaborateurs montrèrent tout d'abord qu'une injection icv (troisième ventricule) de NUCB2 chez le rat juste avant le début de la phase sombre diminuait la prise alimentaire spontanée de façon dose dépendante (Oh-I et al., 2006). A la dose de 4 pmol par rat, cet effet anorexigène durait jusqu'à 6 heures après l'injection. Ils injectèrent alors indépendamment la nesfatine-1, la nesfatine-2, ou la nesfatine-3 en icv et observèrent que seule la nesfatine-1 diminuait la prise alimentaire spontanée, également de manière dose dépendante et durant 6 heures après l'injection aux doses de 5 et 25 pmol par rat **(Figure 17B)**. Lorsque les auteurs empêchèrent le clivage de NUCB2 en nesfatine-1 par les proprotéines convertases en mutant les acides aminés Lys83-Arg84 en Ala83-Ala84, la diminution de prise alimentaire était annulée, démontrant la nécessité du clivage de NUCB2 en nesfatine-1 pour produire l'effet anorexigène. De plus, l'injection icv d'IgG Ab24 dirigés contre la nesfatine-1 bloquait les effets de la nesfatine-1 endogène et augmentait la prise alimentaire spontanée des animaux. Enfin, des injections icv chroniques de nesfatine-1 à la dose de 5 pmol/jour diminuaient d'une part la prise alimentaire journalière mais provoquaient également un retard dans la croissance

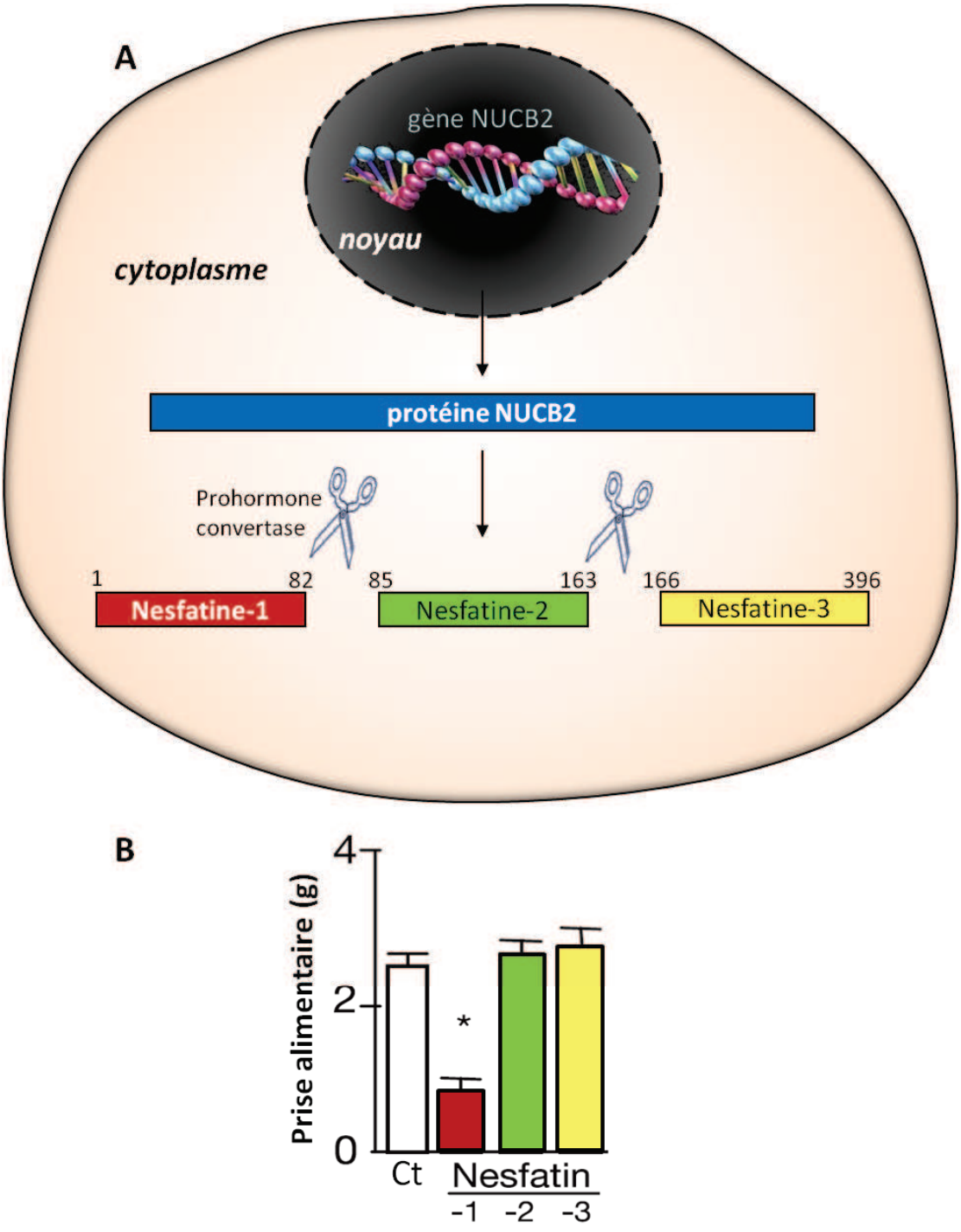


Figure 17 : caractérisation de la nesfatine-1.
A. Schéma décrivant la voie de synthèse de la nesfatine-1.
B. Mesure de la prise alimentaire (0-1h) de rats ayant reçu une injection centrale de nesfatine-1, nesfatine-2 ou nesfatine-3. *Tiré de Oh-I et al., 2006*.

pondérale. A l'inverse, l'injection icv chronique d'oligonucléotides morpholinos antisens (as-MON) qui sont des brins d'ADN synthétiques interagissant avec l'ARNm de NUCB2 afin d'empêcher sa traduction, augmentait la prise alimentaire spontanée des animaux et leur poids corporel suggérant que la nesfatine-1 exerce un tonus anorexigène. On identifia ainsi la nesfatine-1 comme un nouveau peptide anorexigène alors que la nesfatine-2 et la nesfatine-3, bien que possédant des domaines de liaison à l'ADN, n'ont pas de fonction connue à ce jour.

- *Etudes complémentaires*

Toujours chez le rat, des injections intra-hypothalamiques de nesfatine-1 (50 pmol par rat) parviennent à diminuer la prise alimentaire, en particulier lorsqu'elle est injectée dans le PVN et dans la LHA, et dans une moindre mesure dans le VMH (Chen et al., 2012b; Maejima et al., 2009). Chez la souris, une injection icv de nesfatine-1 (ventricule latéral) diminue également la prise alimentaire en affectant la taille et la fréquence des repas, augmentant ainsi l'intervalle entre les repas et le ratio de satiété (correspond au rapport entre l'intervalle et la taille des repas) (Goebel et al., 2011). Ces résultats suggèrent que la nesfatine-1 agit à la fois sur le rassasiement et la satiété. Cependant, notons que la dose nécessaire pour obtenir un effet chez la souris est largement supérieure à celle utilisée chez le rat (300 pmol pour la souris contre 5 pmol pour le rat). La nesfatine-1 agit également au niveau du tronc cérébral : une injection de nesfatine-1 dans le quatrième ventricule de rat (5 pmol) diminue la prise alimentaire spontanée dans les mêmes proportions que lorsqu'elle est injectée dans le ventricule latéral (Stengel et al., 2009a). Les mêmes résultats sont obtenus lorsque la nesfatine-1 est injectée dans la citerne cérébello-médullaire (Stengel et al., 2009a).

Par ailleurs l'effet anorexigène de la nesfatine-1 est toujours observable lorsqu'elle est injectée en ip à la dose de 0.25 nmol/g de poids corporel chez la souris, un effet n'étant pas lié à un phénomène aversif (Shimizu et al., 2009a). Cette diminution de prise alimentaire lors de l'administration périphérique de nesfatine-1 peut s'expliquer par le fait que celle-ci est capable de traverser la barrière hémato-encéphalique par un transport non saturable. En effet, quelques minutes après une injection intraveineuse de nesfatine-1 marquée radioactivement, les auteurs en retrouvent dans le parenchyme cérébral (Pan et al., 2007; Price et al., 2007). Les afférences vagales peuvent aussi contribuer au transfert du signal nesfatine-1 de la périphérie vers le cerveau puisque l'application de nesfatine-1 sur des neurones afférents vagaux isolés crée un signal calcique via un influx au travers de canaux de type N (Iwasaki et al., 2009).

Des études ont également été menées chez le poisson rouge démontrant que des injections périphériques ou centrales de nesfatine-1 produisent un effet anorexigène, indiquant que la fonction de la nesfatine-1 est conservée au cours de l'évolution, ainsi que sa structure puisque les compositions en acides aminés des nesfatine-1 de poisson rouge, de rongeur et d'humain sont proches les unes des autres (Gonzalez et al., 2010; Kerbel et Unniappan, 2012).

- *Chez l'homme*

Aucune étude clinique n'a été menée chez l'homme à ce jour. Cependant, des études génétiques ont montré qu'il existait un polymorphisme du gène NUCB2 chez l'humain. En effet, des nucléotides peuvent différer d'un individu à l'autre et quelques-unes de ces variations sont corrélées avec un phénotype obèse (Zegers et al., 2011). La présence de mutations dans le gène NUCB2 a également été reportée chez deux adolescentes souffrant d'obésité sévère : l'une âgée de 12 ans et pesant 92,4 kg possédait la mutation L125H, l'autre âgée de 14 ans et pesant 120,7 kg possédait la mutation K178X (Zegers et al., 2012). La patiente atteinte de la mutation L125H n'ayant pas souhaité coopérer d'avantage, l'impact de cette mutation sur la protéine NUCB2 et la nesfatine-1 n'a pas pu être évalué. Chez la patiente atteinte de la mutation K178X, le taux de NUCB2/nesfatine-1 dans le plasma n'était pas significativement différent des taux mesurés chez des individus sains ou obèses sans mutation du gène NUCB2. Cependant, lors de cette mesure, les auteurs n'ont pas pu différencier la protéine NUCB2 de la nesfatine-1. Ils suggèrent que la mutation pourrait entrainer un défaut de clivage de NUCB2 en nesfatine-1, ce qui reste à démontrer. Notons que cette adolescente a déclaré ne pas ressentir la satiété, et qu'aucune autre mutation n'a été mise en évidence dans les gènes de la leptine, des récepteurs aux mélanocortines de type 3 et 4 et du BDNF (Zegers et al., 2012).

1.3. Segment actif de la nesfatine-1

L'équipe ayant découvert la nesfatine-1 entreprit en 2009 de déterminer quel segment de la nesfatine-1 portait l'activité anorexigène (Shimizu et al., 2009a). Pour cela, ils synthétisèrent trois fragments de nesfatine-1 de souris : le segment N-terminal composé de 23 acides aminés, le segment central composé de 30 acides aminés et le segment C-terminal composé de 29 acides aminés. Lors de l'injection ip de chacun de ces segments chez la souris (0.25 nmol/g),

seul le segment central (acides aminés 23 à 53) était capable de diminuer la prise alimentaire **(Figure 18)**. Les auteurs le baptisèrent le M30 (pour *mid-segment* de 30 acides aminés). De plus, ils observèrent que ce M30 possédait des enchainements en acides aminés communs avec les peptides α-MSH et AgRP. Enfin, l'injection ip de M30 entrainait une activation neuronale dans le NTS et l'AP mise en évidence par immunohistochimie de la protéine c-Fos. Cette activation neuronale n'était pas retrouvée dans l'ARC suggérant l'implication du nerf vague dans le transfert de l'information de la périphérie vers le cerveau. L'injection icv du segment central de la nesfatine-1 (0,3 nmol par souris) entrainait également une anorexie qui durait plus de 24 heures et affectait la fréquence des repas mais pas leur taille. Cet diminution de la fréquence suggérait un effet sur la satiété, confirmé par une augmentation du ratio de satiété, mais pas d'effet sur le rassasiement (Stengel et al., 2012).

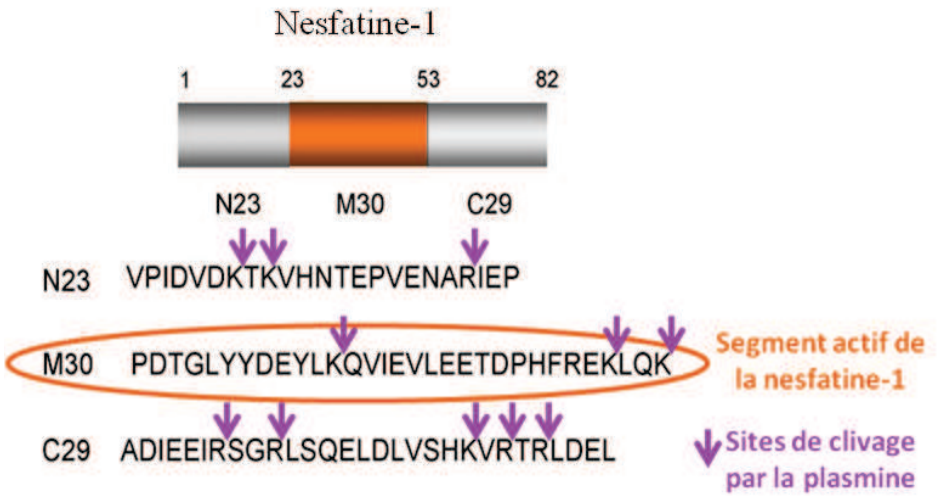


Figure 18 : Composition de la nesfatine-1 de souris.
Le segment actif de la nesfatine-1 est le segment central. Par ailleurs la nesfatine-1 peut être inactivée par la plasmine qui la clive en divers endroits. *Adapté de Shimizu et al., 2009b.*

1.4. Inactivation de la nesfatine-1

Deux brevets publiés en 2010 montrent que la nesfatine-1 est clivée en plusieurs endroits par la plasmine et digérée complètement au bout de 2 heures **(Figure 18)**. Des souris double knock out pour le récepteur à la leptine (LepR-/-) et le plasminogène (plg-/-), le précurseur de la plasmine, présentent une hyperphagie et une obésité moins importantes que des souris LepR-/- plg+/+ (Liu et al., 2010a US20100234274, 2010b US20100235935). Ainsi, lorsque

la nesfatine-1 n'est plus dégradée, son action se prolonge améliorant le phénotype obèse des souris. Aucune information n'est cependant disponible dans la littérature quant au rôle de la plasmine endogène sur l'inactivation de la nesfatine-1.

2. Expression endogène de la nesfatine-1

La nesfatine-1 est présente de manière endogène dans l'organisme. Cependant, il est important de garder en mémoire que l'anticorps utilisé classiquement en immunohistochimie pour marquer la nesfatine-1 reconnait également NUCB2.

2.1. Localisation périphérique

Rappelons tout d'abord que la découverte de la nesfatine-1 en 2006 nécessita l'utilisation de cellules de type adipocytaire. La présence de NUCB2/nesfatine-1 dans le tissu adipeux blanc a ensuite été confirmée par western blot et immunohistochimie chez la souris, le rat et l'homme (Osaki et al., 2012; Ramanjaneya et al., 2010). De plus, des cellules adipeuses murines et humaines mises en culture sont capables de sécréter la nesfatine-1 dans le milieu de culture (Ramanjaneya et al., 2010). Chez des souris privées de nourriture pendant 24 heures, l'expression des ARNm de NUCB2 et de la protéine nesfatine-1 est plus faible que chez des souris nourries *ad libitum*. A l'inverse, une alimentation riche en graisse entraine une augmentation de l'expression de la nesfatine-1 dans le tissu adipeux (Ramanjaneya et al., 2010).

En plus du tissu adipeux, la nesfatine-1 a également été retrouvée au niveau d'organes périphériques tels que l'estomac, le duodénum, le foie et le pancréas, organes qui participent aux fonctions de digestion et au maintien de l'équilibre énergétique (Mohan et Unniappan, 2012; Zhang et al., 2010b; Gonkowski et al., 2012). La présence de nesfatine-1 dans le foie reste controversée à ce jour (Osaki et al., 2012; Zhang et al., 2010b). Dans l'estomac, la nesfatine-1 est retrouvée dans les cellules de la muqueuse gastrique qui expriment également la ghréline, mais dans des vésicules de sécrétion différentes (Stengel et al., 2009b; Kerbel et Unniappan, 2012). Chez l'homme, le nombre de cellules de la muqueuse gastrique exprimant la nesfatine-1 semble être corrélé à l'indice de masse corporelle (Stengel et al., 2013). Au niveau pancréatique, elle est co-localisée avec l'insuline dans les cellules béta des îlots de Langerhans. Elle n'est cependant pas présente dans les cellules qui expriment le glucagon, la

somatostatine ou le polypeptide pancréatique (Foo et al., 2010; Gonzalez et al., 2009, 2011b) **(Figure 19)**.

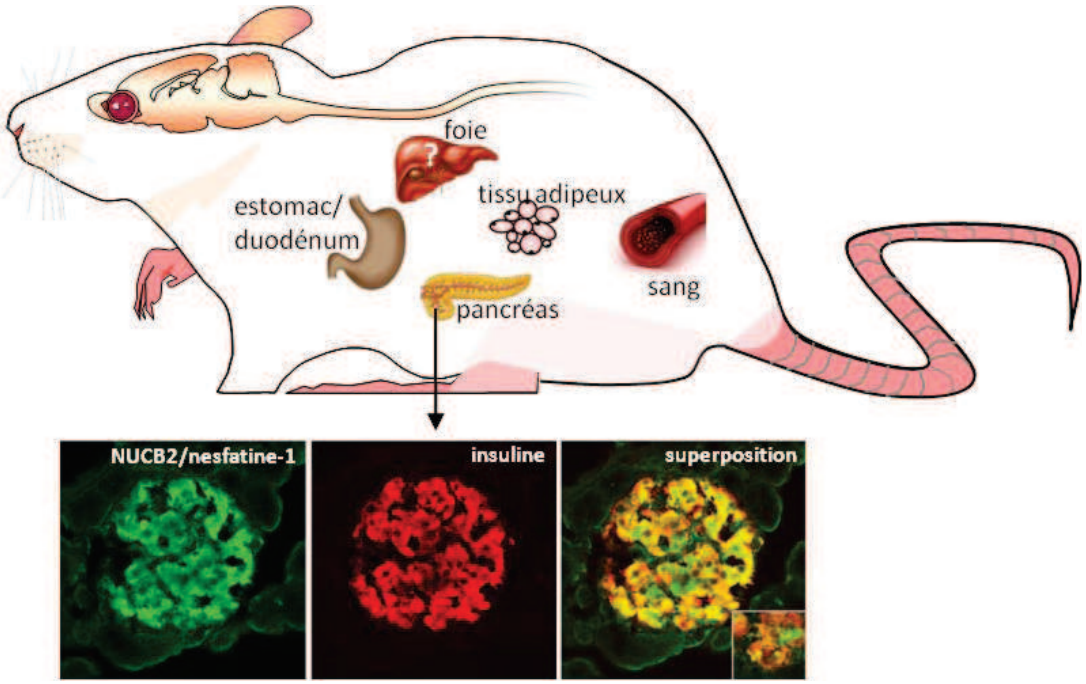


Figure 19 : Expression endogène de la nesfatine-1 au niveau périphérique.
La nesfatine-1 est exprimée dans plusieurs organes impliqués dans la régulation de l'homéostasie énergétique ainsi que dans le sang. Dans le pancréas, elle est co-localisée avec l'insuline dans les cellules béta.

Cette expression aussi bien par la muqueuse gastrique que par les cellules beta du pancréas suggère fortement que la nesfatine-1 puisse constituer un signal circulant capable d'exercer une action périphérique. La nesfatine-1 a d'ailleurs été détectée à la fois dans le sérum et le liquide céphalorachidien (LCR) de rat (Gonzalez et al., 2011a; Mohan et Unniappan, 2012; Oh-I et al., 2006; Stengel et al., 2009a) et d'humain (Aydin et al., 2011, 2009; Tan et al., 2011). Une mise à jeun de 24 heures diminue légèrement le niveau de nesfatine-1 dans le sang (Stengel et al., 2009a). De plus, les taux plasmatiques de nesfatine-1 pourraient être corrélés avec le poids corporel mais, alors qu'une équipe démontre une corrélation positive entre le taux plasmatique de nesfatine-1 et l'indice de masse corporelle chez des individus non-obèses (Ramanjaneya et al., 2010), une autre démontre une corrélation négative (Tsuchiya et al., 2010). De la même manière, la concentration plasmatique de nesfatine-1 de souris nourries avec une alimentation riche en graisse et présentant une obésité ainsi qu'une hyperglycémie est également controversée (Chaolu et al., 2011; Gonzalez et al.,

2011b). Cependant, chez l'homme, il semble que des individus obèses présentent des niveaux de nesfatine-1 accrus dans le sang et le LCR comparé à des individus non-obèses (Tan et al., 2011). A l'inverse, des femmes souffrant d'anorexie nerveuse ont moins de nesfatine-1 dans le sang que des femmes saines (Ogiso et al., 2011). Un syndrome appelé anorexie-cachexie peut se mettre en place lors d'une maladie chronique. Chez des patients cachectiques atteints d'un cancer du foie, les taux circulants de nesfatine-1 sont diminués parallèlement à la perte de poids (Cetinkaya et al., 2012). Cependant, cette régulation n'a pas été observée dans d'autres modèles de cachexie comme la mucoviscidose et l'insuffisance rénale (Cohen et al., 2013; Saldanha et al., 2012).

2.2. Localisation centrale

L'expression centrale de la nesfatine-1 est restreinte à quelques structures dont plusieurs noyaux hypothalamiques (ARC, compartiments magno- et parvocellulaires du PVN, noyau périventriculaire, LHA, SON) et le CVD (NTS et DMNX). Des neurones NUCB2/nesfatine-1 ont également été retrouvés dans des noyaux thalamiques, dans le lobe antérieur de l'hypophyse, le noyau d'Edinger-Westphal (impliqué dans le stress), le locus coeruleus, le raphé obscursus et pallidus, la medulla ventrolatérale ainsi que la colonne intermédiolatérale de la moelle épinière (Foo et al., 2008; Oh-I et al., 2006; Goebel-Stengel et al., 2011) **(Figure 20A et B)**. L'immunomarquage de NUCB2/nesfatine-1 est confiné au corps cellulaire et aux dendrites primaires. A haute résolution, il est possible de déterminer la localisation vésiculaire de la nesfatine-1, résultat confirmé en microscopie électronique à transmission montrant la présence de nesfatine-1 marquée par des particules d'or dans des vésicules de sécrétion situées autour de l'appareil de Golgi, dans des neurones du PVN (Maejima et al., 2009).

Un grand nombre d'études de colocalisation a été réalisé, en particulier au niveau de l'hypothalamus et du CVD (Brailoiu et al., 2007; Foo et al., 2008; Fort et al., 2008; Kohno et al., 2008). Dans l'ARC, la nesfatine-1 est coexprimée dans les neurones à POMC/CART, à neurotensine et à tyrosine hydroxylase (TH, enzyme de synthèse des catécholamines) mais pas dans les neurones à NPY. Dans le PVN, elle est colocalisée avec l'ocytocine, la vasopressine, la TRH, la CRH et le CART. De manière surprenante, au niveau de la LHA, on la retrouve dans des neurones à CART et MCH, qui est rappelons-le un peptide orexigène, mais pas dans les neurones à orexine. Enfin, la nesfatine-1 est également colocalisée avec l'ocytocine et la vasopressine dans le SON. On ne sait cependant pas si la nesfatine-1 est

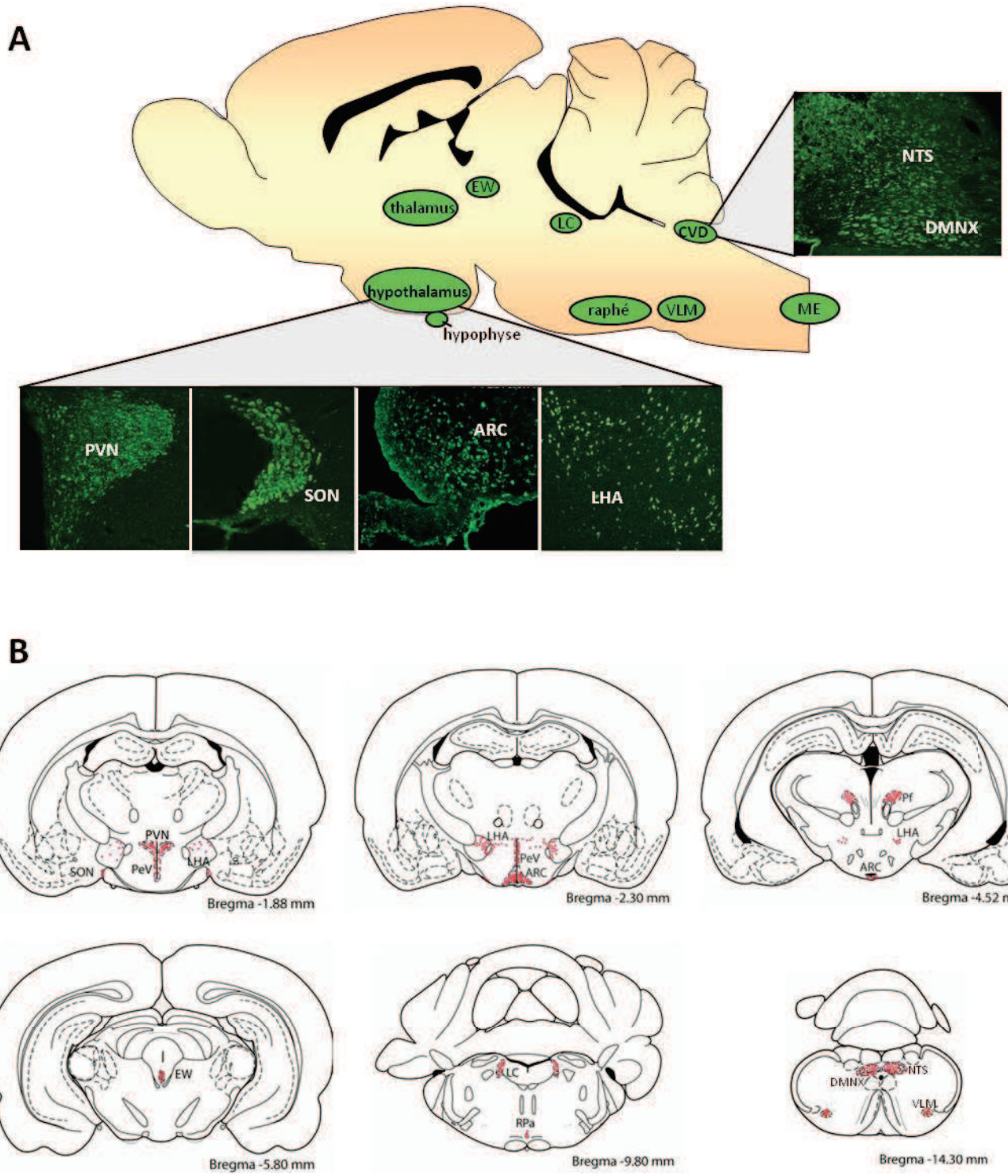


Figure 20 : Expression endogène de la nesfatine-1 au niveau central.
A. Schéma représentant les structures cérébrales possédant des neurones nesfatinergiques et immunohistochimie de NUCB2/nesfatine-1 sur coupes coronales d'hypothalamus et de CVD.
B. Localisation précise des structures exprimant la nesfatine-1.

EW: noyau d'Edinger-Westphal ; LC: locus coeruleus; CVD: complexe vagal dorsal; VLM: medulla ventrolatérale; ME: moelle épinière; PVN: noyau paraventriculaire; SON: noyau supra-optique; ARC: noyau arqué; LHA: aire latérale hypothalamique; NTS: noyau du tractus solitaire; DMNX: noyau dorsal moteur du nerf vague; PeV: noyau périventriculaire; Pf: thalamus périfornical; RPa: raphé pallidus.

située dans les mêmes vésicules de sécrétion que ces différents neuropeptides. Au niveau du tronc cérébral, la nesfatine-1 est coexprimée avec la TH et le CART dans le NTS et avec la choline acétyltransférase (ChAT, enzyme de synthèse de l'acétylcholine) dans le DMNX (Bonnet et al., 2013).

Chez le poisson rouge l'expression des transcrits de NUCB2 dans l'hypothalamus diminue avec le jeûne (Gonzalez et al., 2010). De même, dans le PVN et le SON de rat, les ARNm de NUCB2, ainsi que les concentrations en nesfatine-1 (mesurées par test ELISA) sont plus faibles lorsque les animaux sont privés de nourriture pendant 24 heures (Kohno et al., 2008; Oh-I et al., 2006). Récemment, une étude a montré une rythmicité dans l'expression de NUCB2 au cours de la journée dans le PVN de rats sains. En effet, les ARNm de NUCB2 augmentent significativement durant le début de la journée puis déclinent l'après midi pour rester bas durant la nuit. Cette augmentation des ARNm de NUCB2 en début de phase claire est corrélée à la chute rapide de prise alimentaire (Sedbazar et al., 2013). A l'inverse, chez les rats Zucker *fa/fa* souffrant d'obésité, cette rythmicité n'a pas lieu et l'absence d'augmentation de NUCB2 en début de journée semble être associée à l'hyperphagie qui subsiste durant la journée chez ces animaux. Ce trouble alimentaire est cependant partiellement corrigé par l'administration de nesfatine-1 en début de phase claire (Sedbazar et al., 2013). Chez une souche de souris spontanément obèse, hyperphagique et diabétique, les souris Tsumura Suzuki (Suzuki et al., 1999), l'expression des ARNm de NUCB2 dans l'hypothalamus est réduite par rapport à des souris non obèses et non diabétiques, ainsi que l'expression de la protéine NUCB2 elle-même (Miyata et al., 2012). Cette régulation n'est cependant pas mise en évidence chez des souris *db/db* comparé à des souris *db/+* où les taux de NUCB2 hypothalamique sont équivalents (Miyata et al., 2012). Ces résultats observés chez différents modèles murins ont été obtenus à partir d'échantillons prélevés l'après midi. De part la rythmicité dans l'expression des ARNm de NUCB2, il serait judicieux d'obtenir des valeurs à partir d'échantillons prélevés le matin, lors du pic d'expression de NUCB2.

2.3. Réponse des neurones nesfatinergiques à divers stimuli

Un grand intérêt est né dans la communauté scientifique, impliquant plusieurs équipes de recherche, autour de la sensibilité des neurones centraux exprimant la nesfatine-1 à différents effecteurs. Pour simplifier la rédaction, j'utiliserai de façon sans doute abusive le terme de neurones « nesfatinergiques » pour définir ces neurones exprimant NUCB2/nesfatine-1. Des

rats mis à jeun 48 heures puis renourris pendant 2 heures, dans le but de provoquer une satiété rapide et importante, présentent une activation de leurs neurones nesfatinergiques (attestée par la présence de la protéine c-Fos) dans le PVN et le SON, comparé à des rats nourris *ad libitum* ou des rats seulement mis à jeun. De plus, il semblerait que chez les animaux renourris, le nombre de neurones exprimant la nesfatine-1 dans le SON soit plus important (Kohno et al., 2008). Une injection périphérique de CCK-8S stimule également c-Fos dans des neurones nesfatinergiques du PVN et du NTS (Noetzel et al., 2009; Stengel et al., 2009a). Ensemble, ces données indiquent que les neurones nesfatinergiques de l'hypothalamus et du CVD pourraient être impliqués dans la mise en place du phénomène de satiété **(Figure 21)**.

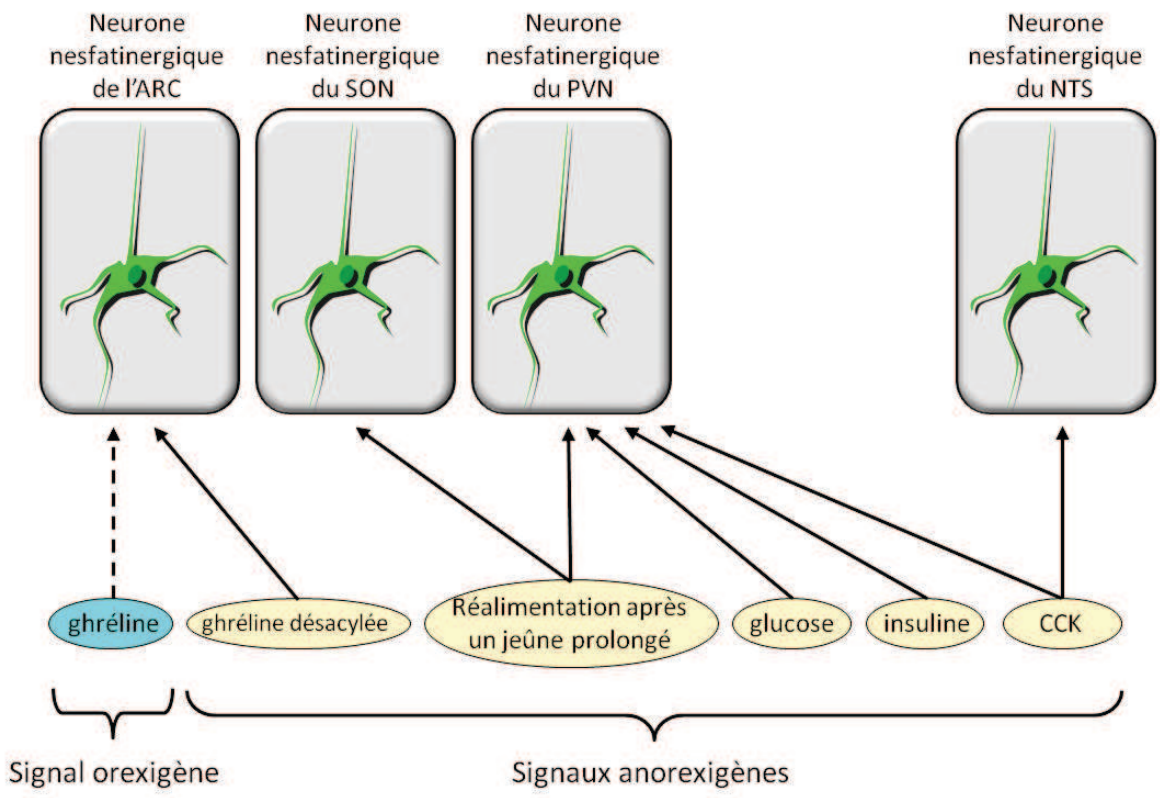


Figure 21 : Activation des neurones nesfatinergiques par divers stimuli.
Des stimuli anorexigènes, mais également orexigènes, sont capables d'activer des neurones exprimant NUCB2/nesfatine-1 dans l'hypothalamus et le NTS.

ARC: noyau arqué; SON: noyau supra-optique; PVN: noyau paraventriculaire; NTS: noyau du tractus solitaire; CCK: cholécystokinine.

La forme désacylée de la ghréline est la forme non active de la ghréline connue pour contrebalancer l'effet orexigène de celle-ci. Chez le rat, l'injection ip de ghréline active des neurones dans l'ARC dont environ 18 % expriment la nesfatine-1, alors qu'après l'injection de ghréline désacylée, 43 % des neurones activés sont nesfatinergiques (Inhoff et al., 2008). Ce résultat confirme la capacité des neurones nesfatinergiques à répondre à des signaux de type anorexigène.

Enfin, sur des neurones isolés de PVN de souris, l'application de glucose entraine une augmentation du calcium intracellulaire. Un immunomarquage de NUCB2/nesfatine-1 post-enregistrement calcique montre la présence de nesfatine-1 dans plus de 50 % des neurones sensibles au glucose. Les mêmes résultats ont été obtenus lors de l'application d'insuline (Gantulga et al., 2012). Certains neurones nesfatinergiques sont sensibles à la fois au glucose et à l'insuline, d'autres répondent uniquement à l'un des deux stimuli, et d'autres encore ne sont sensibles ni à l'un ni à l'autre. Tous ces résultats suggèrent une hétérogénéité fonctionnelle des neurones nesfatinergiques qui pourraient être sensibles à des stimuli divers et variés **(Figure 21)**.

3. Mode d'action central de la nesfatine-1

Bien que la présence de nesfatine-1 au niveau central soit clairement identifiée, son mode d'action reste incertain. Quelques études ont cependant apporté des informations permettant de mieux comprendre les mécanismes à l'origine des effets de ce peptide.

3.1. Sécrétion de la nesfatine-1

Dès la découverte de la nesfatine-1, Oh-I et ses collaborateurs avancent l'hypothèse que la nesfatine-1 est un peptide qui peut être sécrété, et appuient cette hypothèse en montrant la présence de nesfatine-1 dans le LCR de rat (Oh-I et al., 2006). De plus, l'injection icv d'anticorps neutralisant la nesfatine-1 endogène augmente la prise alimentaire. Par la suite, dans des neurones du PVN, la nesfatine-1 a été identifiée dans des vésicules de sécrétion (Maejima et al., 2009). Aucune preuve directe n'est avancée quant à la sécrétion de nesfatine-1 par les neurones, cependant elle a été démontrée en périphérie par les cellules béta pancréatiques et les adipocytes (Gonzalez et al., 2011b; Ramanjaneya et al., 2010). Toutes ces

évidences permettent tout de même de supposer que la nesfatine-1 puisse être sécrétée par les neurones.

3.2. Récepteurs de la nesfatine-1

L'utilisation de nesfatine-1 marquée radioactivement a permis de montrer qu'elle était capable de se lier spécifiquement à la surface de cellules neurales et de cellules hypothalamiques de souris (Ishida et al., 2012). L'identification du ou des récepteur(s) de la nesfatine-1 a rapidement suscité un grand intérêt. En 2007, l'équipe de Brailoiu montre que l'application de nesfatine-1 (100 nM) sur des neurones hypothalamiques mis en culture entraine une augmentation du calcium intracellulaire par entrée d'ions calcium dans les cellules (Brailoiu et al., 2007). Lorsque les cellules sont pré-traitées à la toxine pertussique, l'effet de la nesfatine-1 sur l'influx calcique est aboli, indiquant que la nesfatine-1 agit sur des récepteurs couplés aux protéines $G_{i/o}$ (RCPG). Cette entrée de calcium se fait par des canaux calciques de type L et P/Q puisque des inhibiteurs de ces canaux annulent la réponse calcique (Brailoiu et al., 2007). De plus, la nesfatine-1, de même que le M30 entrainent la phosphorylation et donc l'activation du facteur de transcription CREB (*cAMP response element binding protein*) dans une lignée de cellules neurales (Ishida et al., 2012). Cette phosphorylation est abolie en présence d'un inhibiteur de la voie MAPK. Ainsi, la nesfatine-1 se fixerait à un (ou plusieurs) RCPG, entrainant à la fois un influx calcique et l'activation de la voie MAPK, aboutissant à la phosphorylation de CREB et à la transcription de gènes **(Figure 22)**.

En 2009, un brevet étudie l'affinité de la nesfatine-1 sur un groupe de RCPG constitué de GPR3, GPR6 et GPR12, trois récepteurs hautement exprimés dans le système nerveux central et dont les ligands sont encore inconnus. Les auteurs de ce brevet montrent que GPR12 représente un bon récepteur potentiel de la nesfatine-1 (Mori et Eguchi, 2009 US0233325). De manière intéressante, des souris knock-out pour le récepteur GPR12 ont un phénotype obèse mais ne sont pas hyperphagiques, suggérant l'existence d'autres récepteurs nesfatine-1 (Bjursell et al., 2006). Pour résumer, le(s) récepteur(s) éventuel(s) de la nesfatine-1 et/ou ses sites de liaisons ne sont pas encore connus à ce jour, ce qui retarde sans doute le développement d'agents pharmacologiques agonistes et antagonistes de la nesfatine-1.

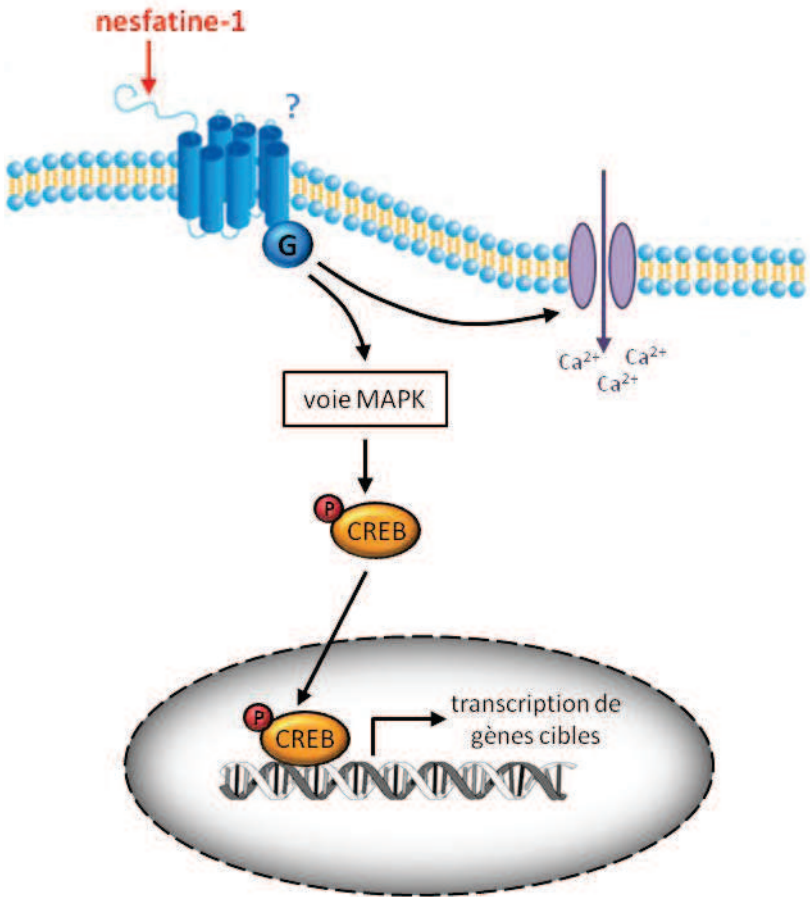


Figure 22 : Possible voie de signalisation intracellulaire de la nesfatine-1.
La nesfatine-1 agit sur un ou plusieurs RCPG encore inconnus et entraine d'une part une entrée de calcium et d'autre part l'activation du facteur de transcription CREB.

3.3. Quelques données électrophysiologiques

Des enregistrements électrophysiologiques sur tranches d'hypothalamus de rat ont montré la capacité de la nesfatine-1 à dépolariser mais aussi à hyperpolariser des neurones du PVN, de l'ARC et du NTS (Price et al., 2008a, 2008b; Mimee et al., 2012). Au niveau du PVN, ces variations de potentiel sont observables quel que soit le phénotype neuronal. En effet, elles ont été enregistrées sur des neurones à vasopressine, ocytocine, CRH, TRH et glutamate (Price et al., 2008a). Au niveau de l'ARC, les neurones à NPY sont majoritairement, mais pas exclusivement, hyperpolarisés (Price et al., 2008b). A l'inverse, dans le NTS, la nesfatine-1 exerce plutôt une action dépolarisante sur les neurones exprimant le NPY ainsi que NUCB2 (Mimee et al., 2012). Ces résultats suggèrent là-encore l'existence de plusieurs récepteurs à la nesfatine-1 aux effets différents et complexes.

3.4. Voie de signalisation de la nesfatine-1

En 2006, lors de la découverte de la nesfatine-1, Oh-I et ses collaborateurs ont observé que chez des rats Zucker obèses dépourvus de récepteurs à la leptine, l'injection icv de nesfatine-1 provoquait toujours un effet anorexigène. De plus, la neutralisation de la nesfatine-1 à l'aide d'anticorps ne bloquait pas l'anorexie induite par la leptine. Ainsi, la nesfatine-1 agirait de manière indépendante de la voie de signalisation leptinergique (Oh-I et al., 2006).

En 2009, l'équipe de Maejima entreprend alors de déterminer chez le rat la voie de signalisation nerveuse qu'emprunte la nesfatine-1 pour exercer son rôle anorexigène (Maejima et al., 2009). Ils observent tout d'abord une activation neuronale uniquement dans le PVN et le NTS lors de l'injection icv de nesfatine-1. L'activation de l'ARC et du SON par l'injection centrale de nesfatine-1 est controversée (Maejima et al., 2009; Yang et al., 2012). Beaucoup de neurones activés dans le PVN par la nesfatine-1 sont ocytocinergiques et de plus, la nesfatine-1 augmente la concentration en calcium intracellulaire de neurones isolés du PVN, neurones qui ont été identifiés *a posteriori* comme exprimant l'ocytocine, parfois même l'ocytocine et la nesfatine-1. Enfin l'incubation de tranches de PVN avec de la nesfatine-1 pendant 30 minutes aboutit à la libération d'ocytocine par les neurones du PVN. Ces résultats conduisent à l'hypothèse que la nesfatine-1 inhiberait la prise alimentaire via la stimulation du système ocytocinergique. Cette hypothèse est confirmée par le blocage des récepteurs à l'ocytocine par les antagonistes H4928 ou OVT qui abolit l'effet de la nesfatine-1 (Maejima et al., 2009; Yosten et Samson, 2010). Par ailleurs, l'activation neuronale dans le NTS a conduit les auteurs à envisager une interaction entre les neurones ocytocinergiques du PVN activés par la nesfatine-1 et le NTS. Ils montrent qu'une injection localisée de nesfatine-1 dans le PVN est capable d'augmenter l'expression de c-Fos dans le NTS et d'inhiber la prise alimentaire, inhibition qui est abolie lors d'une pré-injection de H4928 dans le quatrième ventricule (Maejima et al., 2009). Ainsi, la nesfatine-1 exerce son effet anorexigène en activant des neurones à ocytocine du PVN qui vont projeter sur le NTS **(Figure 23)**. En utilisant des souris POMC-GFP, les auteurs montrent que les terminaisons exprimant l'ocytocine contactent les neurones POMC du NTS et que l'ocytocine augmente le calcium dans ces neurones POMC. De plus, l'injection icv d'ocytocine diminue la prise alimentaire et cet effet est annulé avec un antagoniste des récepteurs MC3/4-R, le SHU9119 (Maejima et al., 2009). Dans cette étude, les auteurs montrent donc le lien entre la nesfatine-1 et les neurones

ocytocinergiques de PVN, puis entre l'ocytocine et les neurones POMC du NTS, mais pas de lien direct entre la nesfatine-1 et la voie mélanocortinergique du NTS. Oh-I et ses collaborateurs avaient précédemment établi ce lien en montrant que l'injection icv du SHU9119 abolit l'action anorexigène de la nesfatine-1 (Oh-I et al., 2006). Ils montraient également que la nesfatine-1 injectée en icv n'activaient pas les neurones exprimant les récepteurs MC3/4-R dans l'hypothalamus, ni n'affectait l'expression de POMC ou d'autres peptides alimentaires (AgRP, NPY, CRH) dans l'ARC et le PVN. Ils n'avaient cependant pas regardé au niveau du NTS. Pour conclure, la nesfatine-1 agit au niveau des neurones à ocytocine du PVN qui vont activer la voie mélanocortinergique du NTS, entrainant une diminution de prise alimentaire **(Figure 23)**. L'existence d'une boucle de régulation est envisageable puisque l'α-MSH stimule l'expression de NUCB2 (Oh-I et al., 2006) et des neurones nesfatinergiques de la LHA expriment le récepteur MC4-R (Cui et al., 2012). Il n'est pas exclu que la nesfatine-1 puisse agir directement au niveau du tronc cérébral, mais aucun mode d'action n'a été mis en évidence à ce jour. Rappelons néanmoins que l'injection de nesfatine-1 au niveau du quatrième ventricule produit un effet anorexigène et active des neurones dans le NTS et le DMNX (Xia et al., 2012). L'identification des récepteurs de la nesfatine-1 sera indispensable pour localiser les cibles neuronales de ce peptide.

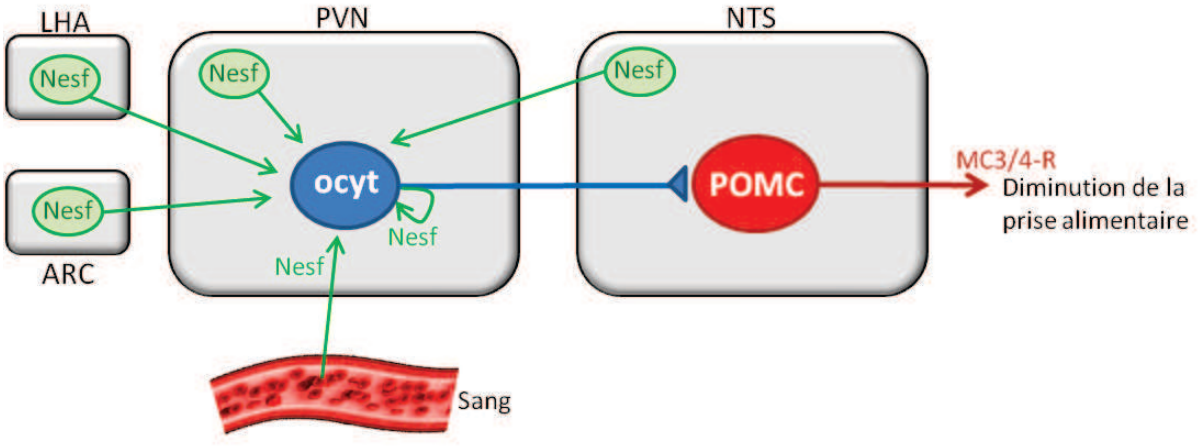


Figure 23 : Voie de signalisation de la nesfatine-1 à l'origine de l'effet anorexigène.
La nesfatine-1 agit sur des neurones ocytocinergiques du PVN qui activent des neurones POMC du NTS.

Nesf: nesfatine-1; ocyt: ocytocine, POMC: pro-opiomélanocortine; MC3/4-R: récepteurs aux mélanocortines de type 3 et 4; LHA: aire latérale hypothalamique; ARC: noyau arqué; PVN: noyau paraventriculaire; NTS: noyau du tractus solitaire.

Des études complémentaires ont mis en évidence l'interaction de la nesfatine-1 avec d'autres peptides hypothalamiques. La nesfatine-1 augmente les niveaux de CRH et TRH dans le PVN, et le blocage des voies de signalisation de ces deux facteurs atténue l'effet de la nesfatine-1 (Gotoh et al., 2013; Stengel et al., 2009a). Un inhibiteur de la formation d'histamine réduit également l'effet anorexigène de la nesfatine-1 et il en est de même chez des souris KO pour le récepteur à l'histamine de type-1. Les auteurs suggèrent que les neurones nesfatinergiques du PVN n'activeraient pas directement les neurones à histamine et que les neurones à CRH et TRH joueraient le rôle de relais (Gotoh et al., 2013). Par ailleurs, ils mettent en évidence un rétrocontrôle positif puisque plusieurs neurones nesfatinergiques du PVN expriment le récepteur à l'histamine de type-1 et l'injection centrale d'histamine augmente le taux de nesfatine-1 dans le PVN (Gotoh et al., 2013).

4. Régulation de la glycémie

La nesfatine-1 étant présente dans les cellules béta du pancréas (Gonzalez et al., 2009), la question de son implication dans la régulation de l'homéostasie glucidique s'est posée. Ainsi, en plus de son effet sur la prise alimentaire, plusieurs travaux ont démontré un lien important entre la nesfatine-1 et la glycémie.

4.1. Effets périphériques

- *In vitro*

Tout d'abord, la sécrétion de nesfatine-1 par les cellules béta pancréatiques est stimulée par la présence de glucose à forte concentration (Gonzalez et al., 2011a, 2011b). Les adipocytes ont également la capacité de sécréter la nesfatine-1 et cette sécrétion est potentialisée par l'insuline (Ramanjaneya et al., 2010). Par ailleurs, l'incubation de nesfatine-1 sur des cellules béta issues de la lignée MIN6 ou d'îlots de Langerhans isolés de souris ou de rats augmente la concentration en calcium intracellulaire des cellules béta ainsi que la sécrétion d'insuline provoquée par le glucose (Gonzalez et al., 2011a, 2011b; Nakata et al., 2011) **(Figure 24)**. En présence d'un bloqueur des canaux calciques de type L, la nitrendipine, l'entrée calcique provoquée par la nesfatine-1 est abolie ainsi que l'augmentation de la sécrétion d'insuline (Nakata et al., 2011). De manière surprenante, la nesfatine-1 stimule également la sécrétion de

glucagon par des îlots de Langerhans isolés de souris, indépendamment de la concentration en glucose (Riva et al., 2011). L'expression des ARNm de NUCB2 dans des îlots humains est corrélée positivement avec l'expression des ARNm de l'insuline, mais aussi du glucagon (Riva et al., 2011). Enfin, la nesfatine-1 stimule la capture du glucose par les cellules adipeuses en condition basale et en condition stimulée par l'insuline, mais elle la diminue dans les cellules musculaires en condition stimulée par l'insuline (Gonzalez et al., 2011a). L'ensemble de ces résultats suggèrent que la nesfatine-1 contribue au maintien de l'homéostasie glucidique de manière tissus-spécifiques et cellules-spécifiques.

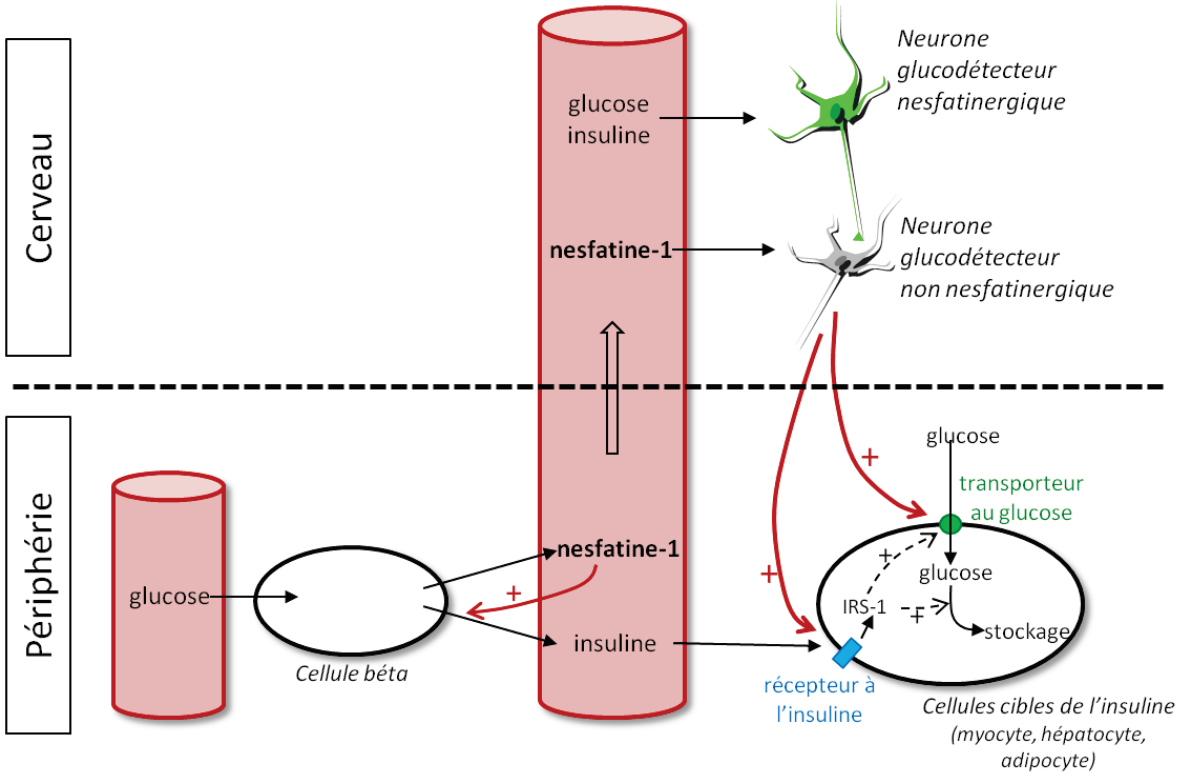


Figure 24 : Rôle anti-hyperglycémiant de la nesfatine-1.
La nesfatine-1 agit en périphérie et en central pour stimuler à la fois la sécrétion d'insuline et la sensibilité des cellules cibles à l'insuline, aboutissant à un meilleur stockage du glucose.

- *In vivo*

L'infusion périphérie continue de nesfatine-1 chez le rat augmente le niveau d'insuline circulante pendant les 30 minutes suivant un test de tolérance au glucose indiquant que, comme *in vitro*, la nesfatine-1 stimule la sécrétion d'insuline par les cellules béta en cas

d'hyperglycémie (Gonzalez et al., 2011a). Chez des souris *db/db* souffrant de diabète de type 2 caractérisé par une hyperglycémie chronique, l'injection intraveineuse (iv) de nesfatine-1 améliore la glycémie de manière dose- et temps-dépendante, alors qu'elle n'a aucun effet sur la glycémie de base de souris sauvages (Su et al., 2010). L'effet anorexigène de la nesfatine-1 n'est pas responsable de cet effet anti-hyperglycémiant puisque son injection en icv à une dose efficace sur la prise alimentaire (25 pmol) ne modifie pas la glycémie des souris *db/db*. Par contre cet effet est dépendant de l'insuline : en effet, chez des souris souffrant de diabète de type 1 (induit par la streptozotocine) et donc incapables de produire de l'insuline, la glycémie n'est pas réduite par l'injection iv de nesfatine-1. Par contre, la co-administration de nesfatine-1 et d'insuline est à l'origine d'un effet anti-hyperglycémiant plus important que celui provoqué avec l'insuline seule. La nesfatine-1 potentialise donc les effets de l'insuline (Su et al., 2010). Ainsi, la nesfatine-1 stimulerait d'une part la sécrétion d'insuline par les cellules béta pancréatiques et d'autre part la sensibilité à l'insuline.

Chez un modèle de rat diabétique de type 2, les Goto-Kakizaki, la concentration en NUCB2 dans le pancréas est inférieure à celle des rats non diabétiques (Foo et al., 2010). Il en est de même chez des humains diabétiques *versus* non diabétiques (Riva et al., 2011). Ces résultats sont en accord avec les taux de nesfatine-1 plasmatiques qui sont diminués chez des souris rendues diabétiques par une alimentation riche en graisse (Chaolu et al., 2011), et chez des humains atteints de diabète de type 2 (Li et al., 2010). La réduction du niveau de nesfatine-1 dans le sang des diabétiques de type 2 pourrait expliquer la perte de sensibilité à l'insuline. Notons que chez des femmes enceintes souffrant de diabète gestationnel, les concentrations en nesfatine-1 dans le sang mais également dans le lait maternel sont plus faibles que dans les échantillons contrôles (Aslan et al., 2012; Aydin, 2010). Dans deux autres modèles de pathologies associées à une obésité et une résistance à l'insuline, le syndrome des ovaires polykystiques et la stéatose hépatique non alcoolique (présence de graisse dans les hépatocytes), les taux sanguins de nesfatine-1 sont également diminués (Başar et al., 2012; Deniz et al., 2012).

4.2. Effets centraux

Comme mentionné précédemment, des neurones nesfatinergiques du PVN sont activés en présence de glucose ou d'insuline, attesté par une augmentation de calcium intracellulaire (Gantulga et al., 2012). On peut ainsi supposer une libération centrale de nesfatine-1 en

réponse à ces deux stimuli, ce qui amène la question de l'action centrale de la nesfatine-1 lors de variations du statut glucidique.

Des enregistrements électrophysiologiques sur tranches d'hypothalamus de rat montrent que la nesfatine-1 influence l'excitabilité des neurones glucosensibles (Chen et al., 2012b). Les neurones dits glucosensibles ou glucodétecteurs utilisent le glucose non seulement comme métabolite énergétique mais également comme signal capable de modifier leur potentiel membranaire et leur fréquence de décharge. Rappelons qu'il existe deux types de neurones glucodétecteurs : ceux excités (neurones GE) et ceux inhibés (neurones GI) par une augmentation du glucose circulant. Ces neurones glucodétecteurs intègrent des signaux hormonaux, nerveux et métaboliques et transmettent ces informations à des zones effectrices qui, en retour, induisent des réponses métaboliques et comportementales impliquées dans le contrôle de l'homéostasie énergétique (Marty et al., 2007). Des neurones GE et GI sont présents dans le PVN, l'ARC, la LHA, le NTS et le DMNX. Le VMH ne possède quasiment que des neurones GE. Au niveau du PVN, la nesfatine-1 a un effet majoritairement inhibiteur sur les neurones GE et excitateur sur les neurones GI. A l'inverse, la nesfatine-1 inhibe les neurones GI de la LHA et a peu d'effet sur les neurones GE, mais elle active les neurones GE du VMH (Chen et al., 2012b). Ces résultats contradictoires suggèrent à nouveau des rôles multiples de la nesfatine-1 selon les régions où elle agit, cependant il apparait clairement que l'activité des neurones glucodétecteurs est modulée par la nesfatine-1.

L'administration centrale de nesfatine-1 favorise la capture de glucose dans les cellules musculaires (Yang et al., 2012) alors qu'il avait été montré *in vitro* que la nesfatine-1 avait plutôt une action inhibitrice sur la capture du glucose par les myocytes (Gonzalez et al., 2011a). L'injection icv de nesfatine-1 augmente également l'activation des récepteurs à l'insuline ainsi que de leur substrat IRS-1 (Yang et al., 2012). Ainsi, l'augmentation de la sensibilité à l'insuline par la nesfatine-1 aurait une composante centrale : quand la glycémie augmente, la nesfatine-1 circulante et/ou centrale agit sur des neurones dont certains sont glucosensibles ce qui favorise en retour l'action de l'insuline **(Figure 24)**.

5. Autres fonctions de la nesfatine-1

En plus de son action sur la régulation de la prise alimentaire et de la glycémie, la nesfatine-1 exerce également une action sur la prise hydrique qui est diminuée (Yosten et al., 2012). De plus, plusieurs fonctions dont seulement certaines sont en relation directe avec la régulation de l'homéostasie énergétique ont été décrites pour la nesfatine-1. Il est donc intéressant de noter que le spectre d'action de la nesfatine-1 s'élargit et dépasse largement le cadre de la régulation de la balance énergétique **(Figure 25)**.

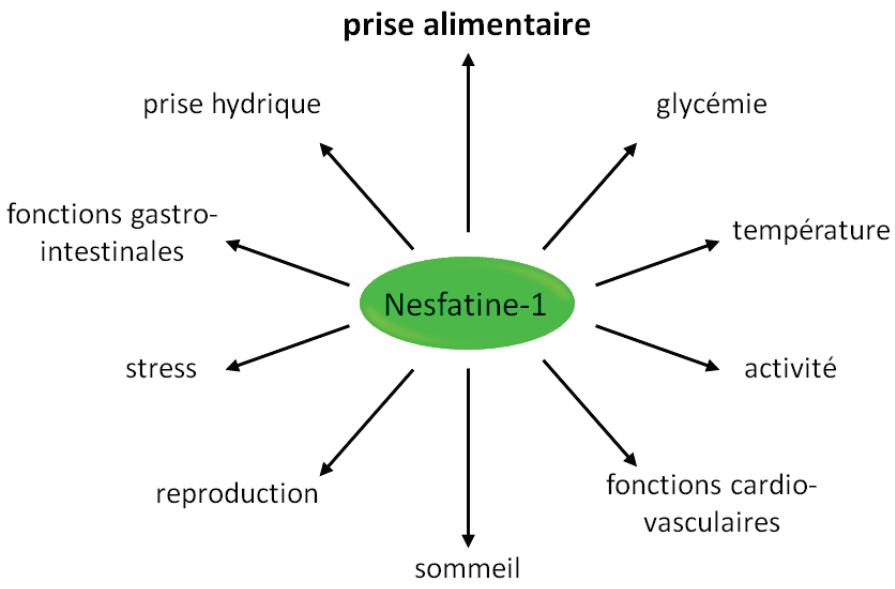


Figure 25 : Effets physiologiques de la nesfatine-1.
En plus de son action sur la prise alimentaire et sur la glycémie, la nesfatine-1 exerce d'autres effets physiologiques variés.

5.1. Effets sur la température corporelle et l'activité locomotrice

Chez le rat, l'injection icv de nesfatine-1 entraine une augmentation de la température des animaux immédiatement ou avec un court délai après administration selon la dose employée (Könczöl et al., 2012). L'administration centrale de nesfatine-1 n'affecte cependant pas l'activité des animaux. Par contre, l'infusion continue périphérique de ce peptide à l'aide d'une minipompe osmotique placée en sous-cutané augmente significativement l'activité locomotrice spontanée (Gonzalez et al., 2011a). Ainsi, ces résultats suggèrent que suite à

l'augmentation de la température et de l'activité, la nesfatine-1 augmente également la dépense énergétique.

5.2. Effets sur le tractus digestif

La nesfatine-1 injectée en icv (ventricule latéral) inhibe la vidange gastrique et la motilité gastroduodénale chez le rongeur (Atsuchi et al., 2010; Goebel-Stengel et al., 2011; Stengel et al., 2009a). Son injection dans le quatrième ventricule inhibe la sécrétion d'acide gastrique via l'activation du nerf vague (Xia et al., 2012). De plus, la chirurgie abdominale, caractérisée par une suppression du transit intestinal, active des neurones exprimant la nesfatine-1 dans plusieurs structures cérébrales incluant le SON, le locus coeruleus, le raphé pallidus, le PVN et dans une moindre mesure le NTS (Stengel et al., 2010).

5.3. Effets sur le stress

Le stress par la contention, qui est le modèle de stress aigu utilisé classiquement chez le rongeur, active des neurones nesfatinergiques dans le SON, le PVN, le noyau d'Edinger-Westphal, le locus coeruleus, le raphé pallidus, le NTS et la medulla ventrolatérale (Goebel et al., 2009; Xu et al., 2010). Dans le noyau d'Edinger-Westphal, un centre impliqué dans l'adaptation au stress, ainsi que dans le PVN, l'expression des ARNm de NUCB2 est augmentée chez les animaux soumis à la contention (Könczöl et al., 2010; Okere et al., 2010). Les auteurs n'ont pas vérifié si les transcrits de NUCB2 variaient dans les autres régions cérébrales réactives au stress. Le taux plasmatique de nesfatine-1 ne varie pas avec le stress aigu (Yoshida et al., 2010). Un stress chronique chez le rat stimule également des neurones nesfatinergiques du noyau d'Edinger-Westphal (Xu et al., 2010). Ce stress chronique est mis en place par des stress légers journaliers qui sont variables et imprédictibles (comme par exemple une baisse de la température, une privation d'eau et de nourriture, des modifications du cycle lumineux ou encore l'obligation du rat à nager dans une piscine).

Des études ont également été menées chez l'humain montrant que chez des hommes dépressifs victimes de suicide l'expression des ARNm de NUCB2 dans la partie rostro-ventrale du mésencéphale qui contient le noyau d'Edinger-Westphal est plus élevée que chez des hommes non dépressifs (Bloem et al., 2012). Chez les femmes dépressives, l'expression des ARNm de NUCB2 est à l'inverse plus faible que chez des femmes non dépressives

(Bloem et al., 2012). Les auteurs n'expliquent pas cette différence mais il faut cependant noter que le nombre d'individus dans les différents groupes est faible (n=5 pour les hommes et n=3 pour les femmes). Dans le sang, le taux de nesfatine-1 semble être corrélé positivement avec le niveau de dépression des patients (Ari et al., 2011). Pourtant, il a été montré par la suite que des individus anxieux ont moins de nesfatine-1 dans le sang que des personnes non stressées (Gunay et al., 2012). Dans ces deux études, le sexe des individus n'a pas été pris en compte.

De plus, l'injection centrale de nesfatine-1 augmente les comportements d'anxiété et de peur chez le rat : les animaux traités explorent moins leur environnement, signe d'anxiété, et ils répondent à un stimulus de peur de manière accrue par rapport à des rats non traités (sursaut plus important et temps d'immobilité après le stimulus prolongé)(Merali et al., 2008). Au niveau cellulaire et moléculaire, l'injection icv de nesfatine-1 active des neurones à CRH de l'hypothalamus et on retrouve plus d'hormone corticotrope (ACTH) et de corticostérone dans le plasma des rats traités à la nesfatine-1 que chez les contrôles, indiquant que la nesfatine-1 stimule l'axe hypothalamo-hypophysaire de la réponse au stress (Könczöl et al., 2010; Yoshida et al., 2010).

5.4. Effets sur la reproduction

Pendant la puberté de la rate (35ème jour post-natal), le nombre de neurones exprimant NUCB2 est augmenté dans le PVN, la LHA et le SON comparé à des souris âgées seulement de 20 jours (García-Galiano et al., 2010). De plus, l'injection icv de nesfatine-1 pendant la puberté provoque une augmentation des taux circulants des deux gonadotrophines : l'hormone lutéinisante (LH) et l'hormone folliculo-stimulante (FSH) qui sont libérées par l'hypophyse et contrôlent la gamétogenèse. L'injection de nesfatine-1 n'a aucun effet sur ces hormones chez la rate adulte. Afin d'inhiber la production de nesfatine-1, les auteurs ont utilisé des oligonucléotides morpholinos antisens (as-MON) empêchant la synthèse protéique de NUCB2. L'administration icv chronique de ces anti-NUCB2 MON pendant les jours 28 à 35 à l'aide d'une minipompe osmotique intradermique reliée à une canule placée dans le ventricule latéral retarde la puberté. Chez ces animaux, l'ouverture vaginale se produit plus tard, le niveau de LH circulant à la fin du traitement est réduit et la croissance des ovaires est altérée puisque ils sont plus petits. Par contre, le même traitement par les anti-NUCB2 MON chez la femelle adulte n'altère pas le pic préovulatoire de LH et FSH (García-Galiano et al., 2010). De façon surprenante, une étude menée chez le poisson rouge adulte montre que la nesfatine-1

injectée en ip diminue l'expression de la gonadolibérine (GnRH), de la LH et de la FSH dans le cerveau, ainsi que de la LH circulante (Gonzalez et al., 2012).

Des études récentes ont montré la présence de nesfatine-1 dans les testicules de rat, de souris, et d'homme (García-Galiano et al., 2012) ainsi que dans les ovaires de poisson rouge et poisson zèbre (Gonzalez et al., 2012). Dans les testicules de rat, la quantité de nesfatine-1 augmente pendant le passage de la puberté à l'âge adulte (García-Galiano et al., 2012).

5.5. Effets sur le sommeil

Au niveau de la LHA, la nesfatine-1 est coexprimée dans des neurones à MCH, neurones largement impliqués dans la régulation des états de sommeil, notamment le sommeil paradoxal. Une étude menée chez le rat démontre que des neurones nesfatinergiques de l'hypothalamus expriment c-Fos pendant le sommeil paradoxal (Jego et al., 2012). De plus, la nesfatine-1 (injectée en icv) stimule la quantité de sommeil paradoxal et la neutralisation de la nesfatine-1 par un antisérum (Ab24) ou de NUCB2 par des morpholinos antisens diminue ce sommeil paradoxal (Jego et al., 2012).

5.6. Effets cardio-vasculaires

Chez le rongeur, la nesfatine-1 injectée en icv augmente la pression artérielle sans modifier la fréquence cardiaque (Tanida et Mori, 2011; Yamawaki et al., 2012; Yosten et Samson, 2009). Cette réponse est atténuée en présence d'antagonistes adrénergiques, mélanocortinergiques et ocytocinergiques indiquant que la nesfatine-1 interagit avec les systèmes mélanocortinergique et ocytocinergique centraux pour stimuler l'activité sympathique conduisant à une augmentation de la pression de sang (Yosten et Samson, 2009, 2010). La micro-injection de nesfatine-1 dans le NTS conduit également à une augmentation de la pression sanguine (Mimee et al., 2012). En accord avec ces données, les individus souffrant de tachycardie supraventriculaire paroxystique, aussi appelée maladie de Bouveret et caractérisée par des crises de tachycardie, présentent des taux circulants de nesfatine-1 plus élevés que des individus sains (Celik et al., 2013).

Récemment il a été montré que la nesfatine-1 était exprimée dans le cœur et que son application sur une préparation de cœur isolé affecte directement la performance du myocarde en diminuant la contractilité ventriculaire (Angelone et al., 2013). Ainsi, en central, la

nesfatine-1 semble avoir un effet stimulateur sur la contraction cardiaque alors que localement l'effet est plutôt inhibiteur. Enfin, la nesfatine-1 cardiaque permettrait un meilleur rétablissement des paramètres cardiaques suite à une ischémie (Angelone et al., 2013).

5.7. La nesfatine-1 en condition pathologique

- *Inflammation*

Des données récentes de la littérature montrent que l'inflammation stimule la synthèse et la sécrétion de nesfatine-1 et que cette nesfatine-1 a un effet anti-inflammatoire. En effet, l'administration de LPS augmente les transcrits de NUCB2 dans l'estomac et les cytokines inflammatoires (TNF-α et IL-6) augmentent l'expression de la nesfatine-1 dans le tissu adipeux (Ramanjaneya et al., 2010; Stengel et al., 2011). De plus, les taux circulants de nesfatine-1 sont plus élevés lors d'une inflammation au LPS (Stengel et al., 2011). Mes travaux de thèse obtenus en 2009, qui ont été les premiers à décrire une implication de la nesfatine-1 en contexte inflammatoire, semblent accréditer cette hypothèse (voir section résultats). Toutefois, lors de lésions cérébrales causées par une hémorragie ou un traumatisme crânien, les cytokines inflammatoires sont fortement stimulées mais un traitement à la nesfatine-1 atténue cette augmentation des cytokines dans le cerveau pendant plusieurs jours (Özsavcí et al., 2011; Tang et al., 2012). Il a été suggéré que dans ces cas, l'effet anti-inflammatoire de la nesfatine-1 favoriserait la neuroprotection. Ainsi, lors d'une inflammation aiguë, la nesfatine-1 pourrait dans un premier temps contribuer à la mise en place de l'anorexie inflammatoire et dans un second temps aider à lutter contre cette inflammation grâce à ces propriétés anti-inflammatoires.

- *Epilepsie*

Les concentrations en nesfatine-1 (mesurées par ELISA) dans le sang et la salive de patients épileptiques sont beaucoup plus élevées que chez des sujets sains, que ce soit après une crise épileptique ou non (Aydin et al., 2009, 2011). Les auteurs n'expliquent pas ce phénomène mais suggèrent que la mesure de nesfatine-1 plasmatique et salivaire pourrait constituer un marqueur pour dépister l'épilepsie.

La nesfatine-1 apparait donc comme un peptide « multi-fonctions » permettant de réguler différents paramètres physiologiques. Ces effets sur l'homéostasie énergétique font de lui une cible intéressante pour le traitement de l'obésité et du diabète de type 2, deux pathologies souvent associées. Bien que son mode d'action ne soit pas clairement identifié, elle semble agir au niveau central en interaction avec les systèmes mélanocortinergique et ocytocinergique.

OBJECTIFS DE RECHERCHE

Lorsque j'ai commencé mon étude sur la nesfatine-1, en 2008, très peu de données étaient disponibles sur ce peptide puisque sa découverte ne datait que de 2006. Nous savions seulement qu'elle possédait un effet anorexigène lorsqu'elle était administrée dans le troisième ventricule, et qu'elle était exprimée dans des neurones de l'hypothalamus. Puis les données se sont rapidement accumulées sur sa localisation centrale et périphérique et son implication dans la régulation de la prise alimentaire. Cependant, les recherches menées se focalisaient essentiellement sur le phénomène de satiété. Je me suis alors intéressée à deux autres aspects intimement liés à la régulation de la prise alimentaire : la glucodétection et l'inflammation. Aucune étude sur la nesfatine-1 dans ces deux conditions n'avait été entreprise. J'ai alors suivi deux axes de recherche :

1/ les neurones nesfatinergiques de l'hypothalamus et du CVD sont ils sensibles à des variations de glycémie ? J'ai choisi comme stimulus l'hypoglycémie, bien qu'il aille à l'encontre des effets connus de la nesfatine-1. En effet, la nesfatine-1 possède des propriétés anorexigènes et anti-hyperglycémiantes.

2/ les neurones nesfatinergiques de l'hypothalamus et du CVD sont ils sensibles à une inflammation aiguë induite par une injection de LPS ?

Ces travaux menés chez le rat ont fait l'objet de deux publications que je résumerai dans la partie « résultats » de ce manuscrit.

Dans le but d'approfondir l'implication des neurones exprimant NUCB2/nesfatine-1 en contexte inflammatoire, j'ai travaillé en collaboration avec d'autres membres de mon équipe qui s'intéressaient aux effets centraux d'une toxine alimentaire : le déoxynivalénol (DON). Je leur ai alors apporté mes connaissances sur la nesfatine-1, et nous nous sommes demandés si les neurones nesfatinergiques pouvaient être activés en réponse à une intoxication aiguë au DON connue pour induire une inflammation périphérique. Nous avons testé cette hypothèse chez la souris, puis chez le porc qui est l'espèce la plus sensible à cette toxine et la plus proche de l'homme concernant les intoxications alimentaires.

Ces travaux ont fait l'objet de trois publications. Cependant, dans la suite de ce manuscrit, j'ai choisi de sélectionner et de présenter uniquement les résultats principaux qui ont permis de caractériser cette toxine, et ceux s'intéressant à la nesfatine-1. L'intégralité de ces articles sera mise en annexe.

RESULTATS

1. Activation des neurones nesfatinergiques par l'hypoglycémie

Article : Central NUCB2/nesfatin-1-expressing neurones belong to the hypothalamus-brainstem circuitry activated by hypoglycaemia.

Bonnet MS, Djelloul M, Tillement V, Tardivel C, Mounien L, Trouslard J, Troadec J-D, Dallaporta M.

Journal of Neuroendocrinology. 2013; Jan;25(1):1-13.

Dans cet article, nous avons étudié l'implication des neurones exprimant NUCB2/nesfatine-1 dans l'homéostasie glucidique. Nous avons choisi comme stimulus l'hypoglycémie, bien qu'il s'agisse d'un stimulus orexigène et non anorexigène. Ce choix s'appuie sur l'observation qu'un grand nombre de neurones catécholaminergiques du NTS exprime NUCB2/nesfatine-1 et ces neurones catécholaminergiques sont connus pour intégrer et répondre à un manque de glucose (Briski et Marshall, 2000; Ritter et al., 2006). Chez le rat, nous avons injecté de l'insuline pour provoquer une hypoglycémie ou du 2-DG pour créer une glucoprivation, puis nous avons sacrifié les animaux deux heures après l'injection, car c'est le temps nécessaire à l'activation des structures cérébrales glucosensibles. Enfin, nous avons effectué une immunohistochimie dirigée contre c-Fos et NUCB2/nesfatine-1 dans l'hypothalamus et le CVD, dans le but de mettre en évidence la sensibilité des neurones nesfatinergiques aux signaux glucoprivateurs.

Résultats :

L'injection périphérique d'insuline (10U/kg) augmente le nombre de neurones c-Fos-positifs dans l'hypothalamus et le CVD comparé aux animaux ayant reçu une injection de solution saline servant de contrôle. Parmi les régions activées, le PVN, la LHA, l'ARC, le NTS et le DMNX expriment NUCB2/nesfatine-1. Dans ces structures, une proportion non négligeable de neurones activés par l'hypoglycémie présente un phénotype nesfatinergique. En effet, le pourcentage des neurones c-Fos-positifs immunoréactifs pour NUCB2/nesfatine-1 dépasse les 20% dans tous ces noyaux et atteint même 77 ± 8% dans le DMNX.

Dans le but de s'assurer qu'il s'agissait bien de la glucoprivation centrale qui activait les neurones nesfatinergiques et non pas l'insuline elle-même, nous avons effectué le même immunomarquage après injection intrapéritonéale ou centrale de 2-DG (ip : 300mg/kg ; icv : 4mg/rat) et avons obtenu des résultats similaires.

Notre étude montre également que des neurones nesfatinergiques activés par l'hypoglycémie dans le NTS expriment la tyrosine hydroxylase (TH), et que pratiquement tous ceux activés dans le DMNX co-expriment la choline acétyltransférase (ChAT).
Enfin, en utilisant un traceur rétrograde, le Fluorogold, nous montrons que des neurones nesfatinergiques préganglionnaires du DMNX activés par l'hypoglycémie projettent sur l'estomac et le pancréas.

Conclusion :
Les neurones nesfatinergiques de l'hypothalamus et du CVD sont sensibles à une hypoglycémie ou glucoprivation. Une partie des neurones nesfatinergiques activés dans le NTS sont également catécholaminergiques puisqu'ils expriment la TH et, de manière intéressante, en plus d'être sensibles à l'hypoglycémie, ces neurones catécholaminergiques sont connus pour projeter vers l'hypothalamus (Petrov et al., 1993). C'est cependant dans le DMNX que les neurones nesfatinergiques sont les plus sollicités et nous montrons que ces neurones préganglionnaires vagaux exprimant NUCB2/nesfatine-1 et sensibles à l'hypoglycémie projettent sur l'estomac et le pancréas. Ces projections des neurones nesfatinergiques du DMNX pourraient permettre de stimuler la vidange gastrique et les sécrétions hormonales, en particulier le glucagon, afin de rétablir la glycémie de base **(Figure 26)**.

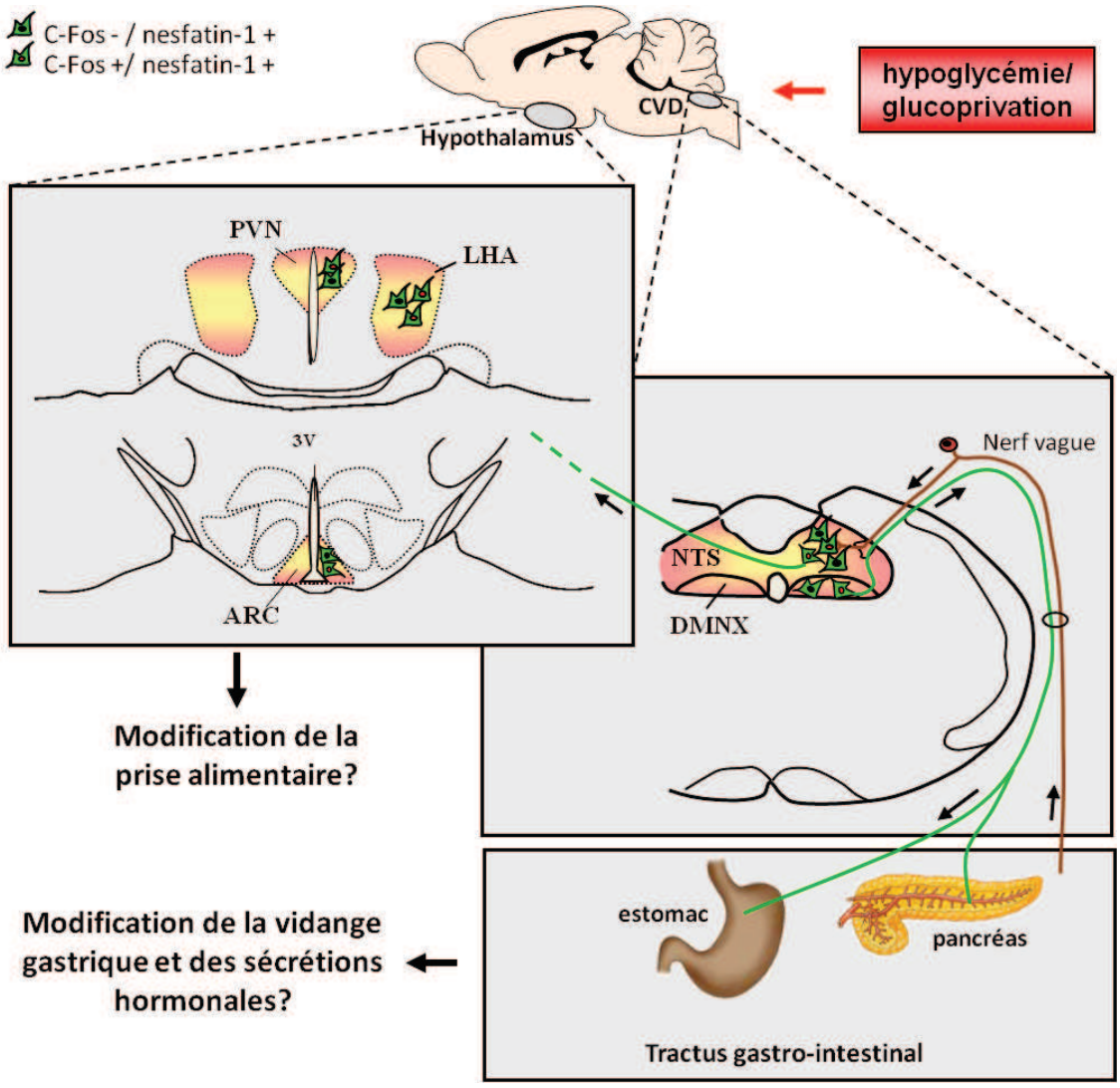


Figure 26 : schéma résumant l'implication des neurones exprimant NUCB2/nesfatine-1 dans la réponse à l'hypoglycémie.

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Journal of Neuroendocrinology

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- Burst synchronisation of oxytocin neurones



A three-dimension model of the paraventricular nucleus illustrating nesfatin-1 expression in c-Fos-activated cells

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ORIGINAL ARTICLE

Central NUCB2/Nesfatin-1-Expressing Neurones Belong to the Hypothalamic–Brainstem Circuitry Activated by Hypoglycaemia

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Nesfatin-1 is a recently identified 82 amino acid peptide shown to have an anorexigenic effect on rodents when administrered centrally and peripherally. Nesfatin-1 is expressed not only in neurones of various brain areas, including the hypothalamic and brainstem nuclei, but also in peripheral organs, such as the stomach and the pancreas. Nesfatinergic neurones were reported to participate in the regulation of satiety signals and in the responses to other stimuli, including restraint stress, abdominal surgery, and lipopolysaccharide-induced inflammation. The present study aimed to investigate whether NUCB2/nesfatin-1 expressing neurones also take part in the central signalling activated in response to hypoglycaemia and therefore are involved in central glucose sensing. Using immunolabelling methods based on the detection of the neuronal activation marker c-Fos and of nesfatin-1, we showed that peripheral injection of insulin induced a strong activation of nesfatin-1-expressing neurones in the brain vagal-regulatory nuclei, including the arcuate nucleus, paraventricular nucleus, lateral hypothalamic area, dorsal motor nucleus of the vagus (DMNX) and nucleus of the tractus solitarius. In response to intracellular glucopaenia induced by i.p. or i.c.v. 2-deoxyglucose injection, the c-Fos/nesfatin-1 colocalisations observed at the hypothalamic and brainstem levels were similar to those observed after insulin-induced hypoglycaemia. Moreover, using Fluorogold as a retrograde tracer, we showed that nesfatinergic preganglionic DMNX neurones activated by hypoglycaemia target the stomach and the pancreas. Taken together, these results suggest that a subpopulation of nesfatinergic neurones belongs to the central network activated by hypoglycaemia, and that nesfatin-1 participates in the triggering of physiological and hormonal counter-regulations observed in response to hypoglycaemia.

Key words: nesfatin-1, insulin, 2-deoxyglucose, hypoglycaemia, c-Fos

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Nesfatin-1 has recently been identified by Oh-I *et al.* (1) as a feeding behaviour regulatory molecule. Nesfatin-1, which derives from the cleavage of the precursor NUCB2, consists of an 82 amino acid peptide with a potent anorexigenic effect on rodents after both central (1) and peripheral (2) administration. Interestingly, this peptide carries out its anorexigenic effect independently of leptin signalling (1). At the central level, nesfatin-1 is expressed by neuronal populations located in different brain areas, including hypothalamic nuclei such as the paraventricular nucleus (PVN), arcuate nucleus (ARC), supraoptic nucleus and the lateral hypothalamic area (LHA), as well as at the brainstem level, including the nucleus of the tractus solitarius (NTS) and the dorsal motor nucleus of the vagus (DMNX) (3). These nesfatinergic neurones were reported to be activated in response to refeeding or i.p. injection of cholecystokinin, thereby contributing to satiety signalling (4,5). In addition to its involvement in the control of feeding behaviour, nesfatin-1 was shown to control water intake (6) and body temperature (7), to reduce gastric emptying (8), to exert cardiovascular effects (9,10) and to modulate peripheral arterial contractility (11). Moreover, nesfatin-1 expressing neurones located in both the hypothalamus and the brainstem were shown to be activated in response to other stimuli, including restraint stress (12–15), abdominal surgery (16) and lipopolysaccharide-induced inflammation (17).

Hypoglycaemia induces a counter-regulatory response that consists of a decrease of insulin secretion and an increase of glucagon and epinephrine secretion (18). These events are under the control of the central nervous system. The brain contains glucosensor neurones, which are able to detect blood glucose level. Evidence

for the presence of glucosensor neurones has been obtained by i.v. or i.c.v. injections of the glucose antimetabolites 2-deoxyglucose (2-DG) or 5-thio-glucose. The glucoprivic signal generated by these compounds induces metabolic and behavioural responses, and the activated neurones can be identified by electrophysiological recordings or detection of c-Fos-like immunoreactivity (19–21). Insulin-responsive neurones have been found in the ventromedial hypothalamus (VMH), PVN, LHA, NTS and DMNX (22). Numerous structures encompassing the medullary dorsal vagal complex (DVC) and hypothalamic nuclei (PVN, LHA), which were shown to contain a high proportion of nesfatin-1 expressing neurones (23), belong to the brain vagal-regulatory pathway activated during hypoglycaemia (22). The present study aimed to investigate whether NUCB2/nesfatin-1 expressing neurones take part in the central signalling activated in response to hypoglycaemia and therefore are involved in central glucose sensing.

Materials and methods

Animals

Adult Male Wistar rats weighing 250–300 g (Janvier, Le Genest Saint Isle, France) were housed individually in a pathogen-free facility under a 12 : 12 h light/dark cycle (lights on at 7.00 h) and controlled temperature. They were fed a standard rat diet (A04; SAFE UAR, Augy, France) and had acess to water *ad lib.* All procedures were conducted in accordance with the European Directive N886/609 and the local committees for animal use and care.

Experimental protocol

Intraperitoneal injection

For habituation, animals were handled and injected i.p. with a physiological saline solution (0.9% NaCl) daily for at least 7 days before experiment. On the day of insulin injection, rats were fasted at 09.00 h for 4 h and then i.p. injected with insulin (Actrapid, 10 U/kg, NovoNordisk, Bagsvaerd, Denmark) or saline (0.9% NaCl) at 13.00 h. Alternatively, rats received an i.p. injection of a 2-DG solution (300 mg/kg, Sigma, Saint-Quentin Fallavier, France) or saline (0.9% NaCl) at 09.00 h. Food was removed immediately after injection. Blood was taken from the tail vein and glycaemia was measured 10 min before the injection, at the injection time, and every 30 min after either the insulin or 2-DG injection. Rats were sacrificed 2 h after the injection. Before sacrifice, rats were anaesthetised with ketamine (100 mg/kg i.p; Merial, Lyon, France) and xylazine (16 mg/kg i.p; Bayer, Puteaux, France) and then transcardially perfused with a 4% paraformaldehyde (PFA)-phosphate buffered solution (0.1 M) solution. Brains were post-fixed in the 4% PFA solution for 1 h, cryoprotected in a 30% sucrose solution for 48 h and frozen in isopentane (−50 °C). The impact of insulin or 2-DG on food intake was monitored over a 4-h period on a second set of animals having received the same treatments.

Intracerebroventricular injection

A 26-gauge cannula was implanted in the lateral cerebral ventricle of the rats. Before surgery, rats were anaesthetised with ketamine (100 mg/kg i.p; Merial) and xylazine (16 mg/kg i.p; Bayer) and placed on a stereotaxic frame. Coordinates were obtained from the Paxinos and Watson rat brain atlas: 1.0 mm posterior to bregma, 2.0 mm lateral to the midline and 4.0 mm ventral to the skull surface (24). After implantation, the cannula was fixed to the skull with dental cement. After surgery, rats were accustomed to the injection procedure with physiological saline (0.9% NaCl) for 7 days. On the day of the experiment, rats received an i.c.v. injection of 2-DG (4 mg/rat, 12 μl, 2 μl/min) (25) or vehicle solution (NaCl 0.9%) at 09.00 h. Blood glucose was measured 10 min before the injection, at the time of injection and every 30 min until 90 min post-injection. Rats were sacrificed 90 min post-injection in accordance with the procedure described above. The correct cannula positioning was checked for each animal at the end of the experiment by cresyl violet staining of brain sections.

Abdominal surgery and Fluorogold injection

Rats were anaesthetised with ketamine (100 mg/kg i.p; Merial) and xylazine (16 mg/kg i.p; Bayer) and a laparotomy was performed. Five hydroxystilbamidine/FluoroGold (FG; 2%, FP-40766A; Interchim, Montluçon, France) injections were performed into the lining of the stomach (0.5 μl per injection at 0.1 μl/min) using a 26-gauge Hamilton syringe. Muscles and skin were sutured after washing the stomach with saline. In another experimental group, FG was injected into the rat pancreas. In this case, spleen and stomach were set aside, pancreas was spread on a Parafilm (Pechiney Plastic Packaging Company, Chicago, IL, USA) sheet and three or four injections were performed using a 26-gauge Hamilton syringe. FG (2 μl) was deposited on an area of the pancreas and a polymer (GC Fuji ORTHO LC, Leuven, Belgium) was placed above to embed the FG. This was repeated on several areas of the pancreas, which was then abundantly washed and replaced in the peritoneal cavity. Animals were sutured before to be rehydrated by an i.p. injection of saline (0.9% NaCl) supplemented with 5% glucose. Nine days after surgery, each animal received an i.p. injection of either insulin (10 U/kg) or saline (0.9% NaCl) solution after the habituation period as described above.

Immunohistochemistry

A double c-Fos and nesfatin-1 immunohistochemistry was performed on 40-μm coronal brain sections cut with a cryostat (CM 3050S; Leica, Rueil-Malmaison, France) and collected in 0.1 M phosphate-buffered saline (PBS). Brains were cut from −15.3 to −1 mm anterior to bregma. Coordinates for each nucleus were identified with the Paxinos and Watson rat brain atlas (24) and are given as the distance (mm) from bregma: PVN: −2 to −1.4; ARC: −3.8 to −1.8; LHA: −4.3 to −1.3; NTS rostral: −12.9 to −13.6; NTS postremal: −13.7 to −14.3 and NTS caudal: −14.3 to −15.3. The free-floating sections were incubated for 1 h in PBS containing 0.3%Triton X-100 and 3% normal goat serum (NGS). Sections were then incubated for 48 h at 4 °C in an anti c-Fos rabbit antiserum directed against amino acids 4–17 of the human protein (dilution 1 : 10 000, Ab-5; Calbiochem, San Diego, CA, USA) in PBS, 0.3% Triton X-100 and 3% NGS. After three washes in PBS, sections were treated overnight at 4 °C in PBS containing 0.3% Triton X-100, 3% NGS and biotinylated goat anti-rabbit immunoglobulin (Ig)G (dilution 1 : 400, Vector Labs, Burlingame, CA, USA). Sections were washed and incubated 10 min in a PBS solution containing 0.3% H_2O_2 for endogenous peroxidase activity quenching. Sections were washed again and treated with an avidin-biotin complex (dilution 1 : 200; ABC Vector Elite kit) for 2 h at room temperature. To visualise c-Fos protein, sections were incubated with diaminobenzidine (DAB, 0.05 ng/ml, Sigma), 0.01% H_2O_2 and 0.15% nickelous ammonium sulphate. This reaction was stopped with distilled water when the stain intensity was optimum (5 min). When animals were previously injected with FluoroGold, DAB could not be used, thus c-Fos protein labelling was performed with an anti c-Fos goat antiserum (dilution 1 : 5000, sc-52-G; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in PBS, 0.3% Triton X-100 and 5% horse serum (HS) overnight at 4 °C. Sections were washed and treated with the anti-goat IgG secondary anti-

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body conjugated with Alexa 594 (dilution 1 : 400, Vector Labs) for 2 h at room temperature. For nesfatin-1 labelling, sections were first incubated in PBS supplemented with 0.3% Triton X-100 and 10% HS for 1 h, and then overnight at 4 °C with a primary antibody raised against nesfatin-1 (dilution 1 : 10 000, H-003-22-B; Phoenix Pharmaceuticals Inc.) in PBS, 0.3% Triton X-100 and 5% HS. This anti-nesfatin-1 antibody raised against rat nesfatin-1 recognises both nesfatin-1 (26) and NUCB2, the precursor of nesfatin-1. Sections were washed in PBS and incubated for 2 h at room temperature with a secondary antibody conjugated with Alexa 488 (dilution 1 : 400; Vector Labs). Sections were finally mounted on gelatin-coated slides and coverslipped with a homemade mounting medium for fluorescence microscope preparation (Moviol, Merck-Millipore, Molsheim, France).

On some brainstem slices, tyrosine hydroxylase (TH) or acetylcholine transferase (ChAT) labelling was performed using an anti-TH mouse antibody (dilution 1 : 1000, MAB318; Millipore, Billerica, MA, USA) or an anti-ChAT goat antibody (dilution 1 : 1000, AB144; Millipore). Each labelling was revealed with an Alexa-594-conjugated secondary antibody (dilution 1 : 400).

Microscopy and image analysis

The c-Fos immunostaining in the rat brain was observed using a Nikon Eclipse E600 light microscope (Nikon, Tokyo, Japan). In selected brain nuclei, c-Fos immunostaining was further analysed by counting the positive cells on four adjacent sections. c-Fos positive nuclei count was performed on photomicrographs acquired using a ten-fold lens with a DXM 1200 Camera (Nikon) coupled to ACT-1 software. Fluorescent images acquired at 520 nm with phase contrast images allowed the visualisation of c-Fos+/nesfatin-1+ neurones. The microscope was set at a specific illumination level, as was the camera exposure time. c-Fos positive cells and c-Fos+/nesfatin-1+ neurones were then counted on these images by computer-assisted morphometry using the IMAGE J software (NIH, Bethesda, MD, USA). Images were normalised by subtracting the background, which was determined for each brain nucleus studied. For each brain structure studied, the counts performed on four distinct sections from both hemispheres were averaged to yield the number of c-Fos nuclei and c-Fos+/nesfatin-1+ cells per section. Because no consecutive sections were used for the detection of the same neuronal marker, no corrections for double counting were applied.

Construction of the three-dimensional (3D) models

The 3D models of the brainstem and the hypothalamus were performed using FREE-D, a 3D reconstruction software (27,28). First, the images of serial sections of the structure of interest were integrated into a stack, the contours of the third ventricle, fourth ventricle and area postrema were manually defined, and neurones of interest were pointed. A first stack with the c-Fos labelling sections and a second stack with the corresponding nesfatin-1-labelling sections were generated. C-fos positive neurones were marked on the first stack and these marks were transferred to the second stack of nesfatin-1 marked sections to correctly superimpose them and point the double-labelled neurones. When animals were injected with the FG, an additional stack with the FG images was generated and the same operation was performed to identify neurones expressing c-fos, nesfatin-1 and FG. Finally, consecutive sections were aligned against each other and the 3D model was generated.

Statistical analysis

Statistical analysis of data was performed with SIGMASTAT, version 3.5 (Systat Software Inc., Chicago, IL, USA) using Student's t-test. $P < 0.05$ was considered statistically significant.

Results

Intraperitoneal injections of insulin on c-Fos expression in nesfatin-1 neurones

The dose of insulin used for immunohistochemistry experiments induced a significant reduction in blood glucose level from 30 min after the injection and persisting up to 120 min (Fig. 1A). In parallel, a significant increase in food intake measured 2 and 4 h after treatment was also observed (Fig. 1B). In both the hypothalamus and the brainstem, control animals that received i.p. physiological saline solution expressed a very small number of c-fos positive cells (Table 1). By contrast, insulin-injected animals (10 U/kg) exhibited an elevated number of c-Fos positive nuclei throughout the entire DVC, including the postremal NTS (3.5 ± 0.9 versus 24.7 ± 6.2 cells per section in saline- and insulin-treated animals, respectively) and the postremal DMNX (0.8 ± 0.4 versus 15.6 ± 5.8 cells per section; Figs 1 and 2 and Table 1). More rostrally, an increase in c-Fos labelling was observed in several hypothalamic nuclei, such as the PVN (9.2 ± 2.0 versus 285.4 ± 81.8 cells per section), the ARC (3.1 ± 1.9 versus 17.7 ± 4.4 cells per section) and the LHA (10.9 ± 4.2 versus 160.5 ± 31.8 cells per section; Figs 1 and 2 and Table 1). Analysis of nesfatin-1 expression in c-Fos-activated cells revealed that a significant proportion of neurones activated by insulin treatment also expressed nesfatin-1 (Figs 1 and 2). Insulin treatment significantly increased the number of co-labelled cells in all structures studied (Figs 1 and 2 and Table 1). The percentage of c-Fos+ cells immunoreactive for nesfatin-1 reached 24 ± 6%, 38 ± 1%, 33 ± 6% and 24 ± 3%, for the postremal NTS, PVN, ARC and LHA, respectively. Remarkably, this percentage reached 77 ± 8% in the postremal DMNX (Fig. 2). Three-dimensional PVN and DVC models revealed a rather uniform rostro-caudal distribution of c-Fos+/nesfatin-1+ neurones within both structures (Fig. 1F–H).

In accordance with our previous work (17), phenotypic labelling performed at the brainstem level revealed that a subpopulation of nesfatin-1 immunoreactive neurones of the NTS was positive for TH, indicating that a fraction of nesfatin-1 neurones belongs to the A2/C2 catecholaminergic group (Fig. 3). In addition, triple-labelling performed after i.p. insulin treatment revealed the presence of c-Fos/nesfatin-1 positive neurones immunoreactive for TH (Fig. 3). Moreover, we observed that the vast majority of nesfatin-1 positive neurones located within the DMNX were positive for ChAT (Fig. 3). Almost all nesfatin-1 expressing neurones positive for c-Fos after peripheral insulin treatment were also ChAT positive (Fig. 3).

Intraperitoneal injections of 2-DG induced c-Fos expression in nesfatin-1 neurones

In response to glucopaenia induced by i.p. injection of 2-DG (300 mg/kg), an increase in blood glucose level was observed 30 min after the injection with a maximal peak reached 1 h post-injection (Fig. 4A). A significant increase in food intake was also observed 2 and 4 h after treatment (Fig. 4B). The animals that

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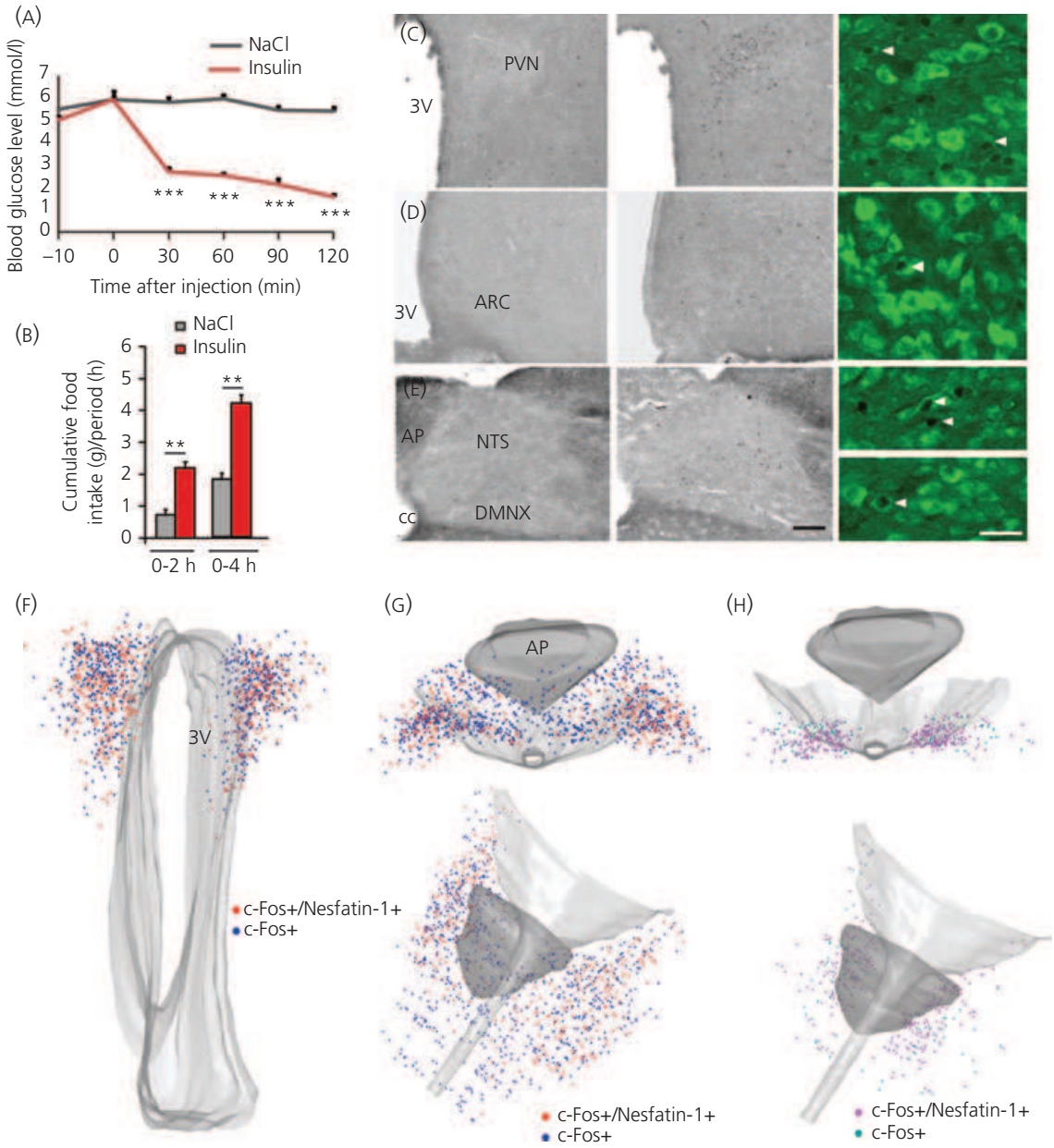


Fig. 1. Insulin-activated neurones in the hypothalamus and dorsal vagal complex (DVC) express nesfatin-1. (A) Time course changes in blood glucose level after i.p. injection of either saline solution (NaCl, n = 5) or insulin 10 U/kg (n = 4). (B) Cumulative food intake measurement after i.p. injection of saline solution (NaCl) or insulin 10 U/kg (n = 6 for each group). (C–E): c-Fos immunoreactivity and c-Fos/nesfatin-1 double-labelling in the paraventricular nucleus (PVN) (C), arcuate nucleus (ARC) (D), nucleus of the tractus solitarius (NTS) and the dorsal motor nucleus of the vagus (DMNX) (E) of rats 2 h after i.p. injection of insulin 10 U/kg. (F) The stack of the third venticle (3V) contours (grey) of serial sections and labelled neurones generate a three-dimensional (3D) model of the PVN. Blue points represent c-Fos+ neurones and red points indicate c-Fos+/nesfatin-1+ neurones. (G, H) Three-dimensional model of the brainstem representing labelled neurones of the NTS (G) and the DMNX (H) seen from the front and the top. Arrowheads in (C, D, E): c-Fos+/Nesfatin-1+ neurones. Scale bars: 200 μm (grey level images), 50 μm (fluorescence images). AP, Aera Postrema; cc, central canal. **P < 0.01; ***P < 0.001. 3V, third ventricle.

received an i.p. injection of 2-DG (300 mg/kg) exhibited an increased expression of c-Fos in the same central structures compared to that previously observed with insulin (Table 1). Double-labelling showed that a high percentage of c-Fos+ cells expressed nesfatin-1 in both hypothalamic and brainstem nuclei. At the hypothalamic level, the percentage of co-labelled cells reached 36 ± 1%, 33 ± 8% and 37 ± 1% for the PVN, ARC and LHA, respectively (Fig. 4). The greatest incidence of c-Fos/nesfatin-1 colocalisation was found in the DMNX where up to 60 ± 1% of total c-Fos+ cells also expressed nesfatin-1 (Fig. 4). Lower but still significant incidences were observed at different rostro-caudal levels of the NTS where the percentage of colocalisation reached 15 ± 1% (Fig. 4).

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Table 1. c-Fos signal and c-Fos/nesfatin-1 colocalization in brain structures of control and insulin or 2-DG (i.v. and i.c.v.) treated rats

		c-Fos+/section		c-Fos+ nesf+/section	
		Control	Treated	Control	Treated
Insulin i.p.	NTS caudal	2.8 ± 0.4	11.0 ± 3.08*	0.09 ± 0.06	2.2 ± 0.7*
	NTS postremal	3.5 ± 0.9	24.7 ± 6.2*	0.16 ± 0.08	5.6 ± 1.2**
	NTS rostral	2.6 ± 0.3	17.2 ± 4.6*	0.27 ± 0.09	2.9 ± 0.4**
	DMNX postremal	0.8 ± 0.4	15.6 ± 5.8*	0.01 ± 0.01	13.2 ± 5.3*
	DMNX rostral	0.4 ± 0.1	8.0 ± 2.1*	0.02 ± 0.02	6.0 ± 1.7*
	PVN	9.2 ± 2.0	285.4 ± 81.8*	3.0 ± 1.8	107.9 ± 30.1*
	ARC	3.1 ± 1.9	17.7 ± 4.4*	1.2 ± 0.9	5.8 ± 1.4*
	LHA	10.9 ± 4.2	160.5 ± 31.8*	2.3 ± 1.2	39.4 ± 11.8*
2-DG i.p.	NTS caudal	0.8 ± 0.1	17.2 ± 3.8*	0.0 ± 0.0	2.0 ± 0.5*
	NTS postremal	1.8 ± 0.3	47.3 ± 15.4*	0.40 ± 0.06	7.4 ± 2.7*
	NTS rostral	2.2 ± 1.0	41.7 ± 8.1**	0.4 ± 0.4	5.5 ± 0.8**
	DMNX postremal	0.9 ± 0.5	5.1 ± 1.0*	0.06 ± 0.06	3.0 ± 0.5**
	DMNX rostral	0.7 ± 0.5	4.5 ± 1.1*	0.08 ± 0.08	2.4 ± 0.7*
	PVN	2.1 ± 0.7	298.3 ± 53.9**	0.3 ± 0.1	109.2 ± 23.5**
	ARC	11.1 ± 1.9	35.2 ± 2.4***	6.5 ± 1.2	11.9 ± 3.3*
	LHA	47.1 ± 21.4	215.4 ± 29.6**	5.5 ± 3.1	78.8 ± 8.8**
2-DG i.c.v.	NTS caudal	2.6 ± 0.2	24.3 ± 3.3**	1.3 ± 0.3	6.3 ± 1.2*
	NTS postremal	7.8 ± 2.6	66.6 ± 17.2*	2.4 ± 1.2	24.3 ± 7.7*
	NTS rostral	7.3 ± 0.6	101.3 ± 14.7**	2.0 ± 0.5	37.7 ± 5.0***
	DMNX postremal	1.2 ± 0.2	12.0 ± 0.8***	0.3 ± 0.2	7.4 ± 1.1**
	DMNX rostral	0.5 ± 0.1	7.3 ± 0.7***	0.3 ± 0.2	3.9 ± 0.4***
	PVN	81.3 ± 14.9	521.6 ± 116.8**	36.4 ± 3.3	270.5 ± 49.2**
	ARC	36.1 ± 0.4	131.6 ± 21.1**	28.4 ± 1.0	76.6 ± 9.2**
	LHA	189.0 ± 2.3	339.3 ± 33.3*	50.2 ± 7.7	107.9 ± 5.6**

ARC, arcuate nucleus; 2-DG, 2-deoxyglucose; DMNX, dorsal motor nucleus of the vagus; LHA, lateral hypothalamic area; NTS, nucleus of the tractus solitarius; PVN, paraventricular nucleus. *$P < 0.05$; **$P < 0.01$; ****$P < 0.001$.

Intracerebroventricular injection of 2-DG induced c-Fos expression in nesfatin-1 neurones

When 2-DG was injected in the lateral ventricle (4 mg/rat, 12 μl), blood glucose level increased from 30 min to 90 min post-injection. At the time of sacrifice, the glycaemia value reached 8.5 ± 0.4 mM in 2-DG-treated group versus 6.8 ± 0.3 mM before treatment (Fig. 5). The central injection of 2-DG induced a cellular activation in the same hypothalamic-brainstem circuitry as previously observed after i.p. injection of insulin or 2-DG. Strong c-Fos/nesfatin-1 colocalisation was also observed in the NTS, DMNX and hypothalamic nuclei. This increase in c-Fos/nesfatin-1 positive nuclei was clearly detected in the NTS (2.4 ± 1.2 versus 24.3 ± 7.7 cells per section in saline- and 2-DG-treated animals, respectively; Fig. 5 and Table 1), postremal DMNX (0.3 ± 0.2 versus 7.4 ± 1.1 cells per section), PVN (36.4 ± 3.3 versus 270.5 ± 49.2 cells per section), ARC (28.4 ± 1.0 versus 76.6 ± 9.2 cells per section) and LHA (50.2 ± 7.7 versus 107.9 ± 5.6 cells per section). This corresponded to 54 ± 3%, 61 ± 10% and 32 ± 4% of total c-Fos cells for the PVN, ARC and LHA, respectively. At the brainstem level, 25 ± 3%, 35 ± 2% and 38 ± 1% of c-Fos positive cells colocalised nesfatin-1 in the caudal, postremal and rostral NTS, respectively. With respect to i.p. injection, a high percentage of colocalisation was observed in the DMNX (i.e. 61 ± 5% and 53 ± 3% at the postremal and rostral level of the DMNX, respectively) (Fig. 5).

Nesfatinergic neurones of the DMNX activated by hypoglycemia project to the stomach and the pancreas

A large number of preganglionic efferent neurones of the DMNX expressed c-Fos and nesfatin-1 in response to both insulin and 2-DG treatments. To identify the sites of projection of these neurones, the retrograde tracer FG was injected into either the stomach or the pancreas. Nine days after FG injection in the stomach or pancreas, FG incorporation within DMNX neurones was observed on both sides of the nucleus (Figs 6 and 7). Three-dimensional analysis revealed that, for animals injected with FG into the stomach, FG-labelled DMNX neurones were found mostly at the postremal level of the nucleus (Fig. 6). Interestingly, a strong proportion of these FG-labelled cells expressed nesfatin-1 (approximately 90%; Fig. 6). Moreover, in response to i.p. insulin, a high proportion of these FG/nesfatin-1 labelled neurones were also positive for c-Fos (Fig. 6). Three-dimensional analysis revealed that, in animals injected with FG into the pancreas, FG/nesfatin-1 labelled neurones were distributed homogeneously throughout the rostra-caudal axis of the DMNX (Fig. 7). In response to i.p. insulin injection, FG+/nesfatin

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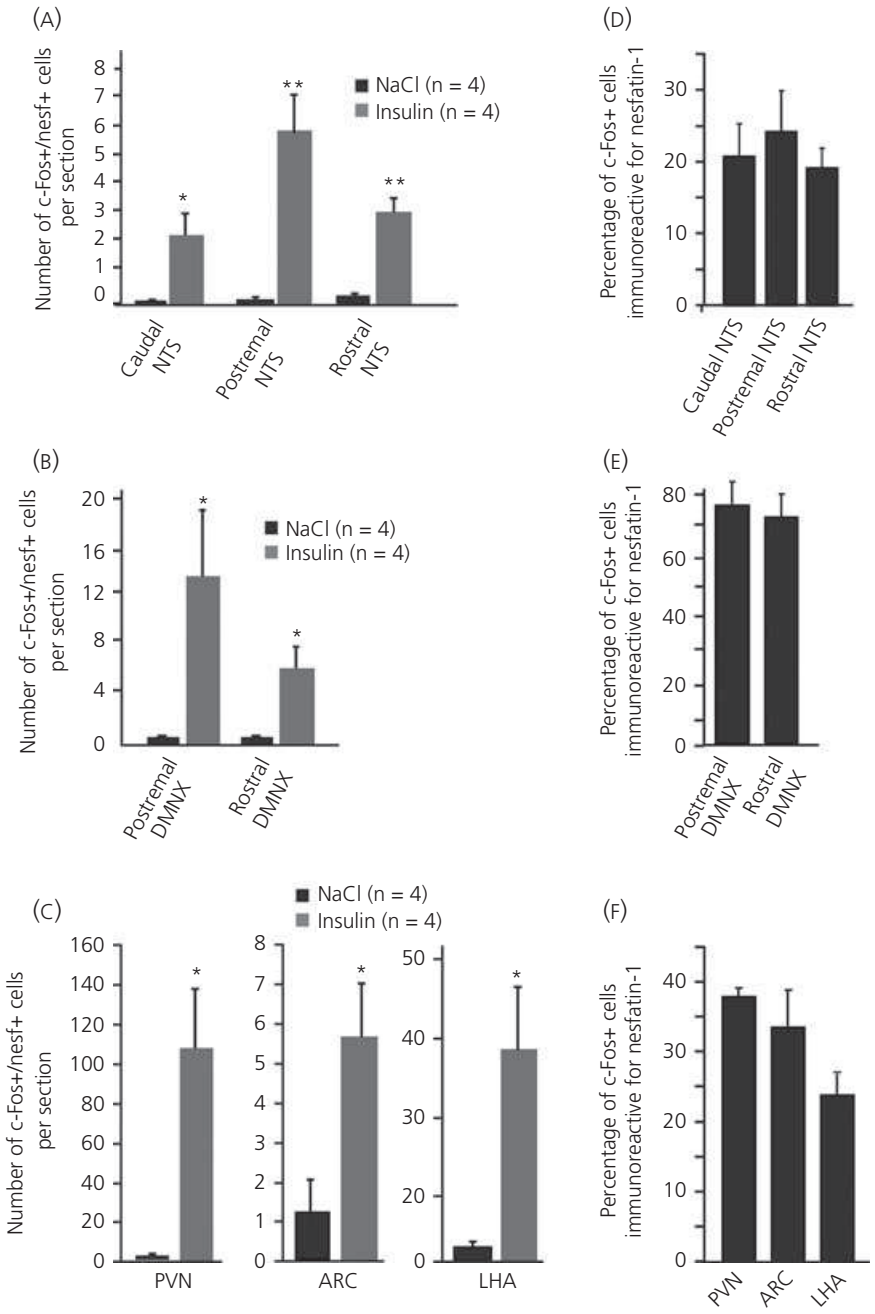


Fig. 2. Quantification of the number of c-Fos/nesfatin-1 immunoreactive cells in the nucleus of the tractus solitarius (NTS) (A), dorsal motor nucleus of the vagus (DMNX) (B), paraventricular nucleus (PVN), arcuate nucleus (ARC) and lateral hypothalamic area (LHA) (C) after i.p. injection of either saline or insulin solution (10 U/kg). Percentage of c-Fos positive cells immunoreactive for nesfatin-1 in the NTS (D), DMNX (E), PVN, ARC and LHA (F) of insulin-treated rats. *$P < 0.05$; **$P < 0.01$.

-1+/c-Fos+ were clearly visible and located mainly within the medial part of the postremal and rostral nucleus (Fig. 7).

Discussion

The responses to hypoglycaemia consist of a set of coordinated endocrine and behavioural events that provides an adaptive mechanism counterbalancing a state of negative energy balance. Under normal conditions, animals may never encounter hypoglycaemia that is able to activate these responses, although hypoglycaemia is a major complication for patients suffering from type-1 insulin-dependent diabetes mellitus, and is associated with significant physical morbidity (29). The management of hypoglycaemia remains a major limiting factor with respect to improving glycemic control. The autonomic nervous system is known to be involved in the control of these counter-regulatory responses to insulin-induced

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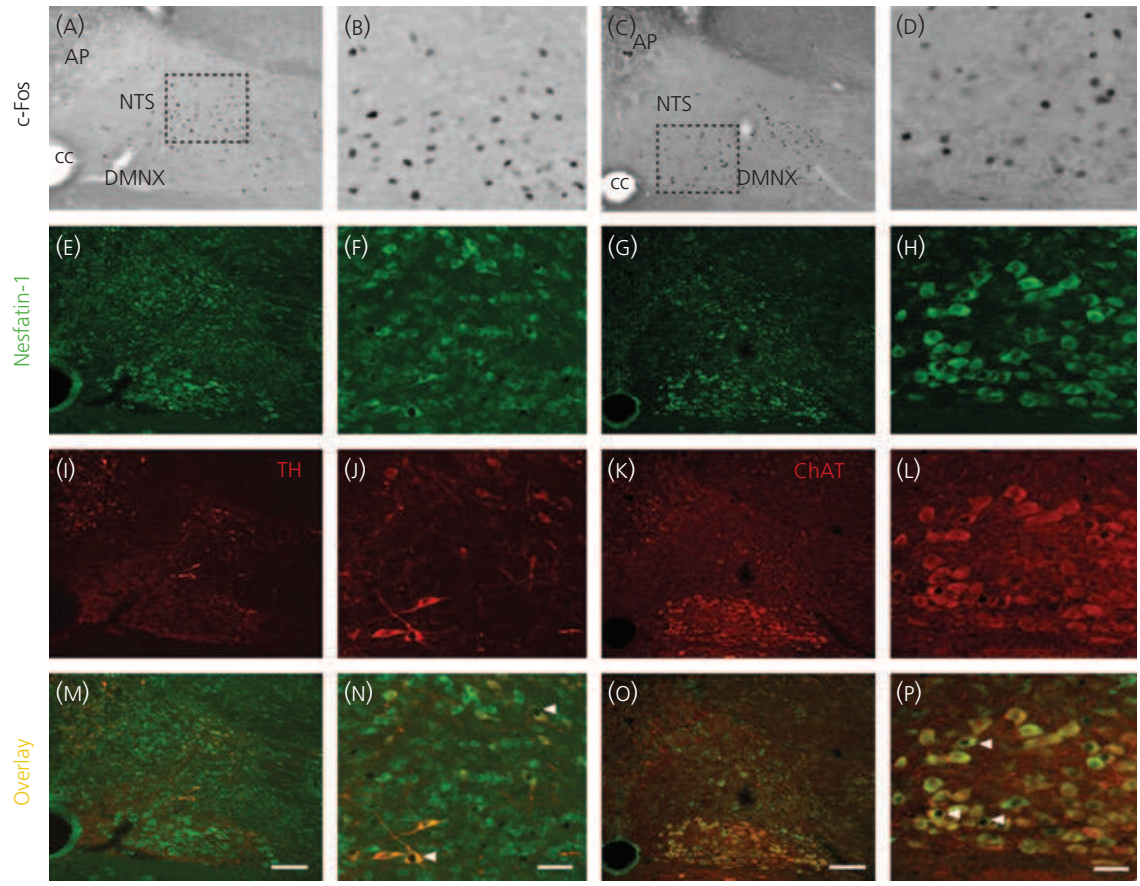


Fig. 3. Phenotype of nesfatinergic neurones activated by hypoglycaemia. c-Fos (A–D)/nesfatin-1 (E–H) labelling and tyrosine hydroxylase (TH) (I, J) or acetylcholine transferase (ChAT) (K, L) immunoreactivity in coronal sections of the dorsal vagal complex of insulin (10 U/kg i.p) treated rat. (M–P) Merged photomicrographs. Arrowheads: nesfatin-1+/c-Fos+/TH+ neurones in (N) and nesfatin-1+/c-Fos+/ChAT+ neurones in (P). Inserts in (A) and (C) indicate the origins of magnifications. Scale bars: 200 μm (A, C, E, G, I, K, M, O) and 50 μm (B, D, F, H, J, L, N, P). AP, Aera Postrema; cc, central canal. NTS, nucleus of the tractus solitarius; DMNX, dorsal motor nucleus of the vagus.

hypoglycaemia (30). The identification of signalling molecules involved in this counter-regulation is necessary to understand how our body deals with insulin-induced hypoglycaemia. In the present study, we aimed to determine whether NUCB2/nesfatin-1 expressing neurones could partake in the central neurocircuitry activated in response to acute hypoglycaemia. Accordingly, rats received a peripheral dose of insulin (10 U/kg) known to induce a significant hypoglycaemia and c-Fos expression in glucosensitive central structures, mainly the hypothalamus and the brainstem (22). Glucose-sensitive neurones are found at various brain levels and particularly in the hindbrain and hypothalamus (31). As expected, the data obtained from the present study showed that insulin induced a strong neuronal activation in the brain vagal-regulatory nuclei, including the ARC, PVN, LHA, DMNX and NTS. These data are in agreement with the well-established concept that insulin-induced hypoglycaemia is a central vagal stimulus. In all nuclei that exhibited a strong neuronal activation in response to insulin, we showed that a significant proportion of c-Fos positive neurones expressed NUCB2/nesfatin-1. The colocalisation percentage varied between 17% and 70%, with the highest value being obtained for the DMNX. These data support the hypothesis that nesfatinergic neurones are stimulated by decreasing glucose levels. Nevertheless, insulin was shown to act directly in the brain and to induce c-Fos immunoreactivity within neurones in the PVN, LHA, as well as NTS and DMNX at the brainstem level (32). To ensure that nesfatinergic neurones are stimulated in response to hypoglycaemia and subsequent intracellular glucopaenia, 2-DG, an antimetabolic drug that selectively antagonises glucose metabolism, was used. Peripheral or i.c.v. 2-DG injection was previously shown to induce c-Fos expression in the NTS, DMNX, and in the paraventricular and supraoptic hypothalamic nuclei (33), as well as counter-regulatory responses (34). In response to intracellular glucopaenia induced by i.p. or i.c.v. 2-DG injection, the c-Fos and NUCB2/nesfatin-1 colocalisation percentage observed at both the hypothalamic and brainstem levels was similar to that monitored after insulin-induced hypoglycaemia. The i.c.v. 2-DG injection induced the highest colocalisation of the two markers. Taken together, these results suggest that the nesfatinergic neurocircuitry belongs to the central network activated in response to hypoglycaemia. Accordingly, we showed that insulin produced a selective activation of a fraction of nesfatin-1 neurones that belongs to the A2/C2 catecholaminergic hindbrain group. Previous studies have demonstrated that this specific subpopulation of

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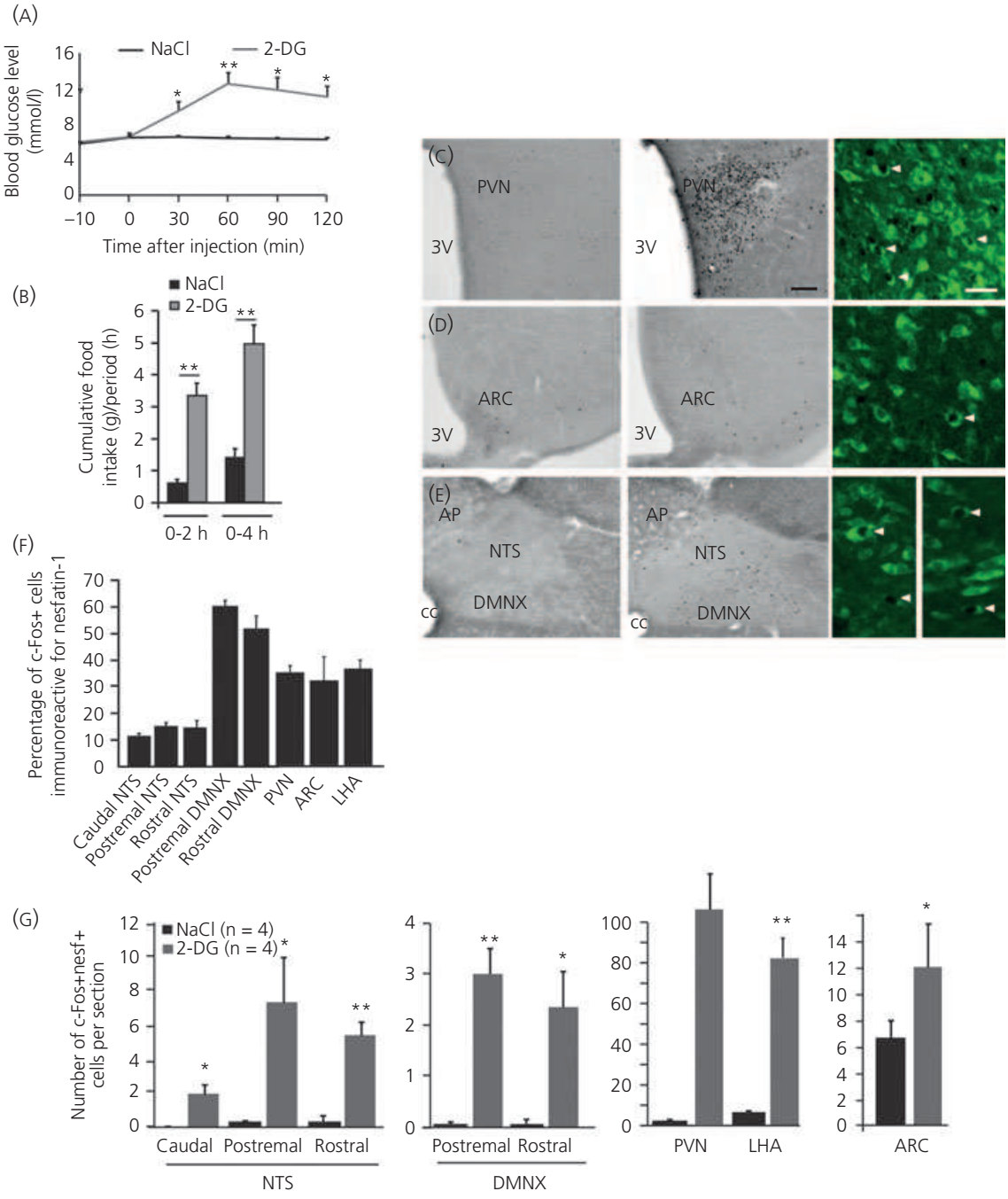


Fig. 4. Activation of nesfatinergic neurones after peripheral injection of 2-deoxyglucose (2-DG). (A) Time-course study of blood glucose levels after i.p. injection of either saline (NaCl, n = 5) or 2-DG (300 mg/kg) (n = 4). (B) Cumulative food intake measurement after i.p. injection of saline solution (NaCl) or 2-DG (n = 6 for each group). (C–E) c-Fos immunoreactivity and c-Fos/nesfatin-1 double-labelling in the paraventricular nucleus (PVN) (C), arcuate nucleus (ARC) (D), nucleus of the tractus solitarius (NTS) and dorsal motor nucleus of the vagus (DMNX) (E) of rats 2 h after i.p. injection of 2-DG (300 mg/kg). (F) Percentage of c-Fos positive cells immunoreactive for nesfatin-1 in the NTS, DMNX, PVN, ARC and lateral hypothalamic area (LHA) of 2-DG-treated rats. (G) Quantification of the number of c-Fos/nesfatin-1 immunoreactive cells in the NTS, DMNX, PVN, ARC and LHA after i.p. injection of either saline or 2-DG solution. (C–E) Scale bars: 200 μm (grey level), 50 μm (fluorescence images). AP, Aera Postrema; cc, central canal. *P < 0.05; **P < 0.01. 3V, third ventricle.

catecholamine neurones (selectively activated by hypoglycaemic stimuli) plays a significant role in feeding and hyperglycaemic responses to glucose deficit (21,35,36). It should be noted that the VMH constitutes a key structure involved in the detection of insulin-induced hypoglycaemia and in the initiation of counter-regulatory responses (37). The chemical destruction of the VMH causes a strong reduction in the hormonal counter-regulation normally observed during acute hypoglycaemia (38). Moreover, hormonal

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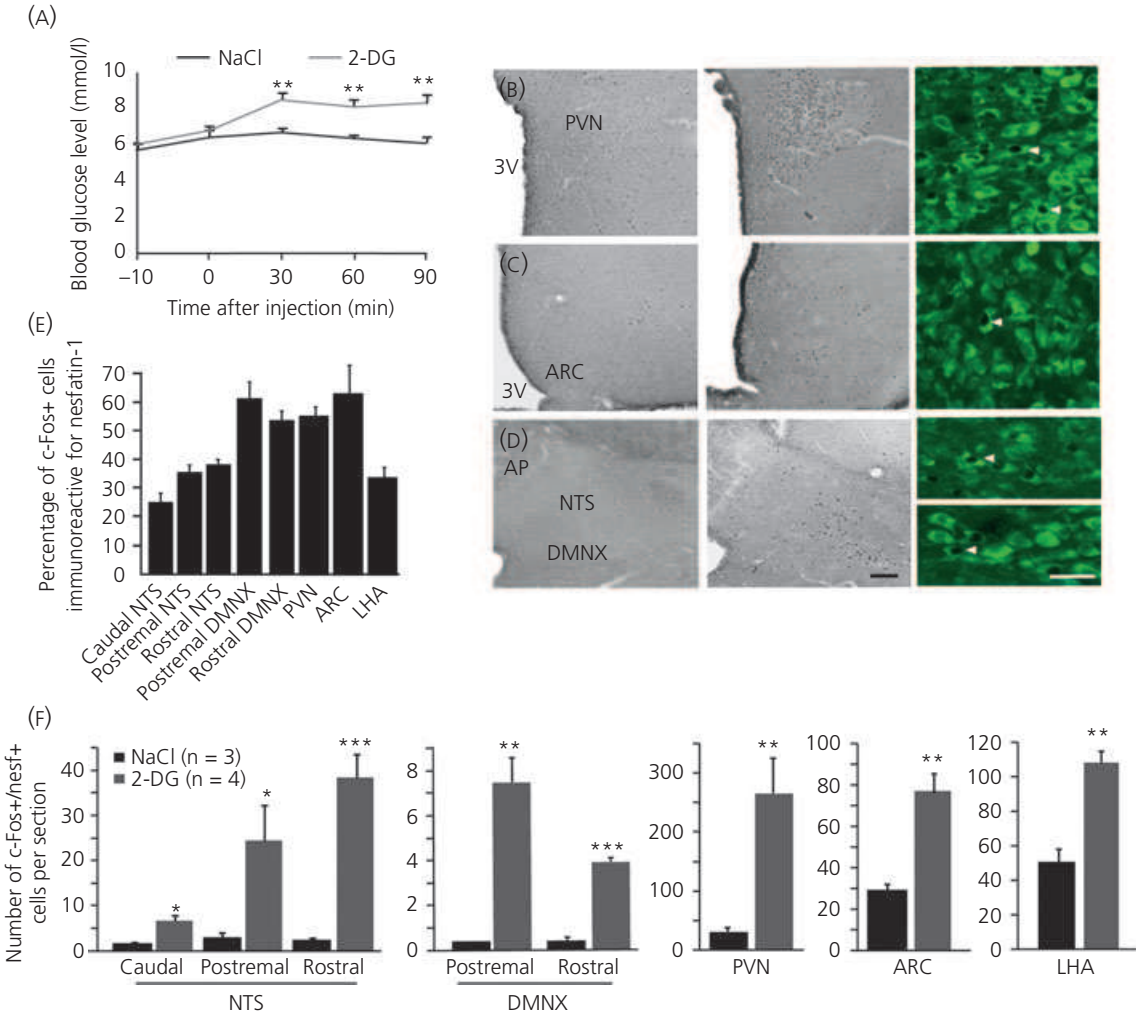


Fig. 5. Activation of nesfatinergic neurones after central injection of 2-deoxyglucose (2-DG). (A) Time-course changes in blood glucose levels after i.c.v. injection of either saline solution (NaCl, n = 3) or 2-DG (4 mg/rat) (n = 5). (B–D): c-Fos immunoreactivity and c-Fos/nesfatin-1 double-labelling in the paraventricular nucleus (PVN) (B), arcuate nucleus (ARC) (C), nucleus of the tractus solitarius (NTS) and dorsal motor nucleus of the vagus (DMNX) (D) of rats 2 h after i.c.v. injection of 2-DG (4 mg/rat). (E) Percentage of c-Fos positive cells immunoreactive for nesfatin-1 in the NTS, DMNX, PVN, ARC and lateral hypothalamic area (LHA) of 2-DG (i.c.v)-treated rats. (F) Quantification of the number of c-Fos/nesfatin-1 immunoreactive cells in the NTS, DMNX, PVN, ARC and LHA after i.c.v. injection of either saline or 2-DG solution. Scale bars: 200 μm (grey level), 50 μm (fluorescence images). AP, Aera Postrema; cc, central canal. *P < 0.05; **P < 0.01; ***P < 0.001. 3V, third ventricle.

secretion induced by systemic insulin-induced hypoglycemia is partially suppressed by the VMH perfusion with 15 mM D-glucose, and decreased by approximately 85% when the VMH is perfused with 100 mM D-glucose (39). The absence of NUCB2/nesfatin-1 expression within neurones of this nucleus (40) justifies the fact that this structure was not included in the present study despite its strong involvement in central glucosensing. The link between central nesfatin-1 network and responses to hypoglycaemia remains to be established. We are able to hypothesise that NUCB2/nesfatin-1 neurones respond to gluciprivic signals, and that locally released nesfatin-1 could also influence glucosensitive neurones excitability. Accordingly, it was recently shown that (1) nesfatin-1 neurones of the PVN are sensitive to insulin and glucose (41) and (2) nesfatin-1 modulates the excitability of glucose-excited and glucose-inhibited neurones located in the PVN, LHA and VMH (42). These assumptions are based on nesfatin-1 release from activated neurones, although evidence for the synaptic or somato-dendritic liberation of this molecule remains scarce. However, the stimulation of feeding observed after the central injection of NUCB2 neutralising antibody and the presence of nesfatin-1 in cerebrospinal fluid extracts (1) strongly suggest that this protein is an endogenous molecule released at the central level.

At the DMNX level, we report that (1) a remarkable high percentage of c-Fos and NUCB2/nesfatin-1 colocalisation (approximately 60%) occurred during hypoglycaemia; (2) the DMNX efferent neurones containing ChAT were NUCB2/nesfatin-1 positive; and (3) a substantial number of insulin hypoglycaemia-activated DMNX neurones were shown to express ChAT. These results are consistent with data

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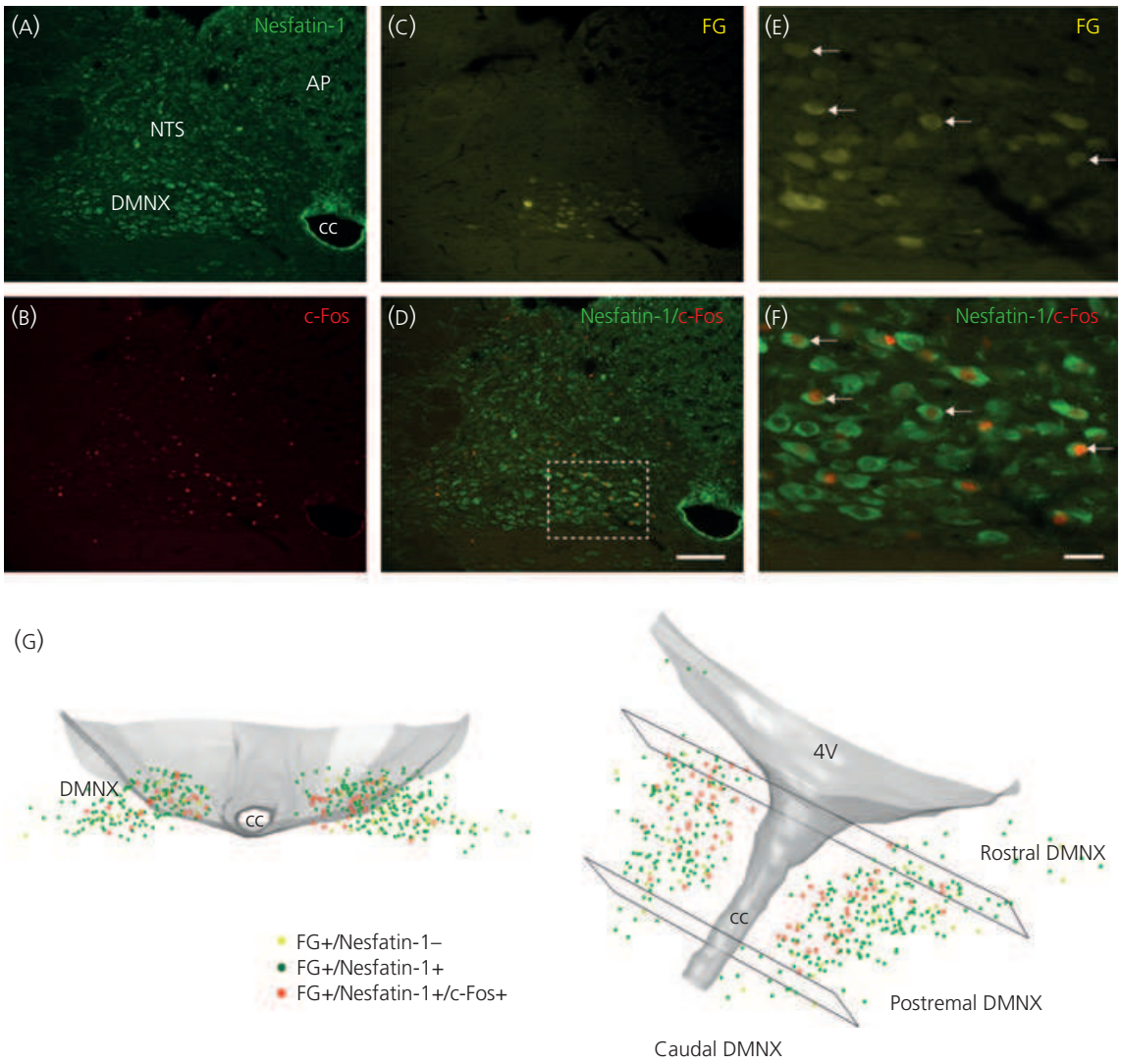


Fig. 6. Projection to the stomach of dorsal motor nucleus of the vagus (DMNX) nesfatinergic neurones activated after i.p. injection of insulin. Nesfatin-1 (A), c-Fos (B) and Fluorogold (FG) (C, E) labelling on a coronal section of the dorsal vagal complex of i.p. insulin-treated rat (10 U/kg). FG was injected into the stomach lining 9 days before sacrifice. (D, F) Merged photomicrographs for nesfatin-1 and c-Fos. (G) Location of DMNX neurones projecting to the stomach in a three-dimensional model. Grey contours, fourth ventricle (4V) and central canal (cc); yellow points, FG+/nesfatin-1- neurones; green points, FG+/nesfatin-1+ neurones; red points, FG+/nesfatin-1+/c-Fos+ neurones. Arrows: nesfatin-1+/c-Fos+/FG+ neurones, insert in (D) indicates the origin of magnification. Scale bars: 200 μm (A–D), 50 μm (E, F). AP, Aera Postrema; cc, central canal. NTS, nucleus of the tractus solitarius.

reporting that hypoglycaemia significantly induces c-Fos expression in cholinergic DMNX (22,43). Moreover, all these observations strongly support the hypothesis that nesfatinergic preganglionic neurones activated by hypoglycaemia target peripheral organs and thus intervene in the counter-regulation of hypoglycaemia. It is generally acknowledged that pancreatic islets are under powerful vagal control and that the stomach receives parasympathetic innervation from DMNX preganglionic neurones. Cell bodies of the premotoneurones that control insulin and glucagon secretion were found to occupy the medial zone of the right and left DMNX. These medial, longitudinal DMNX columns are likely to play a predominant role in the vagal control of the endocrine pancreas (44,45). Using non-transynaptic retrograde labelling, we identified NUCB2/nesfatin-1 expressing DMNX neurones activated by hypoglycaemia that project to the pancreas. Interestingly, these neurones were located in the medial part of the DMNX. This result constitutes the first demonstration that NUCB2/nesfatin-1 expressing neurones could take part in the brainstem–pancreas communication and raises the possibility that nesfatin-1 could exert regulatory functions at this level. Interestingly, it should be noted that nesfatin-1 was shown to enhance glucagon secretion from mouse islets or INS-1 (832/13) cells (46). Similarly, we also showed that some nesfatinergic neurones activated by hypoglycaemia in the DMNX project to the stomach. To date, few data are available regarding the role of nesfatin-1 in the stomach. The only observations are (1) the presence of nesfatin-1 mRNA and protein at the gastric level (26,47) and (2) the decrease

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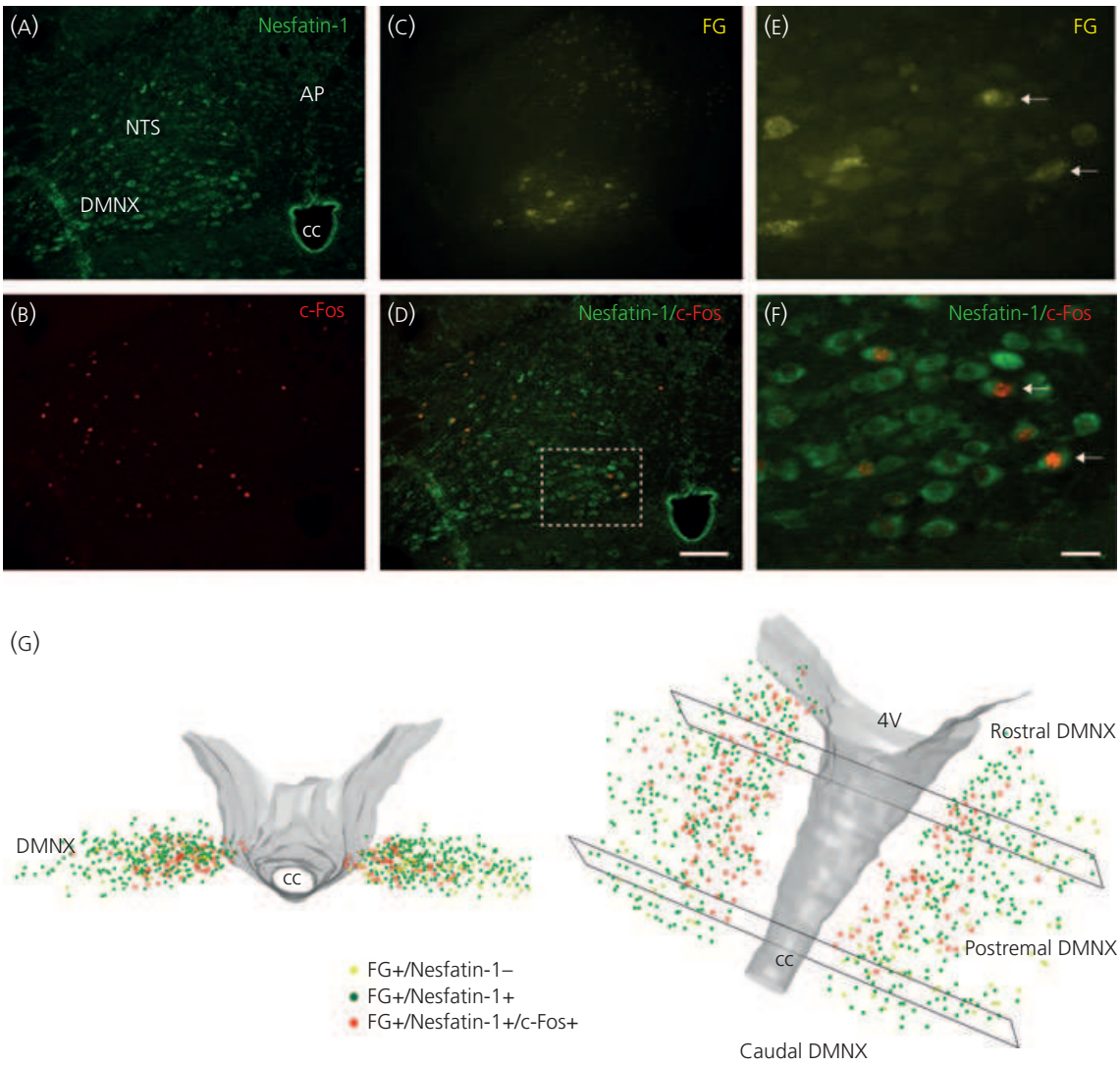


Fig. 7. Projection to the pancreas of dorsal motor nucleus of the vagus (DMNX) nesfatinergic neurones activated after i.p. injection of insulin. Nesfatin-1 (A), c-Fos (B) and Fluorogold (FG) (C, E) labelling in a coronal section of the dorsal vagal complex of insulin (i.p., 10 U/kg)-treated rat. (D, F) Merged photomicrographs for nesfatin-1 and c-Fos. (G) Location of DMNX neurones projecting to the pancreas in a three-dimensional model. Grey contours: fourth ventricle (4V) and central canal (cc); yellow points, FG+/nesfatin-1- neurones; green points, FG+/nesfatin-1+ neurones; red points, FG+/nesfatin-1+/c-Fos+ neurones. Arrows: nesfatin-1+/c-Fos+/FG+ neurones, insert in (D) indicates the origin of magnification. Scale bars: 200 μm (A–D), 50 μm (E, F). AP, Aera Postrema; cc, central canal. NTS, nucleus of the tractus solitarius.

in gastric emptying in rats and in gastroduodenal motility in mice when nesfatin-1 is injected centrally (8,48). Hence, future studies will be necessary to precisely identify the role of brainstem nesfatinergic neurones projecting to the stomach. Nevertheless, it cannot be totally excluded that, in response to hypoglycaemia, nesfatin-1 could be released via somato-dendritic release rather than a more classical axon-synapse discharge. The strong NUCB2/nesfatin-1 immunoreactivity observed on cells bodies and dendrites supports this hypothesis. In this case, nesfatin-1 may exert an autocrine and paracrine regulatory function, and thus locally modulate neurones projecting to peripheral organs.

By showing that central NUCB2/nesfatin-1 expressing neurones are activated in response to insulin-induced hypoglycaemia or 2-DG-induced glucopaenia, we provide the first evidence suggesting that nesfatinergic neurocircuitry could be involved in the counter-regulation of hypoglycaemia and the associated responses. More broadly, our results reinforce the link between nesfatin-1 and the management of glycaemia. Indeed, in addition to being present in the brain, nesfatin-1 is also found in the pancreas, where it is present in β-cells of Langerhans islets and colocalised in the same secretion vesicles as insulin (49). Moreover, a high glucose concentration (16.7 mM) was shown to stimulate nesfatin-1 release from MIN6 cells, a mouse pancreatic β-cell line, and nesfatin-1 was shown to potentiate the glucose-stimulated insulin release by isolated mouse or rat islets (50). *In vivo*, during an oral glucose tolerance test, continuous peripheral delivery of nesfatin-1 increased circulating insulin for 30 min in free-feeding rats compared to control rats (51). These data suggest that, in the presence of high

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glucose levels, peripheral nesfatin-1 is released and stimulates insulin secretion by β-cells to restore blood glucose levels (52). Our results revealing that central nesfatin-1 expressing neurones were activated by hypoglycaemia are in contrast to the data showing a peripheral anti-hyperglycaemia action of nesfatin-1. These results suggest that nesfatin-1 could act through different mechanisms at the peripheral and central levels because receptor(s) for nesfatin-1 remain(s) unknown. An action of nesfatin-1 on different signalling systems that mediate opposite effects on target cells could therefore not be excluded. Consistently, nesfatin-1 was shown to modify the excitability of neurones in the PVN and had either hyperpolarising or depolarising effects on the tested neurones (53). This multifaceted action of nesfatin-1 is also illustrated by the report of a reduction of insulin-stimulated glucose uptake in L6 myotubes by the peptide (51). Finally, although nesfatin-1 was previously shown to potentiate glucose-induced insulin release (51), Riva *et al.* (46) recently reported that nesfatin-1 could stimulate glucagon secretion. They demonstrated a positive correlation between NUCB2 mRNA and glucagon mRNA in human islets and an increase in glucagon secretion from isolated mouse islets in response to nesfatin-1. The hypoglycaemia-induced activation of efferent DMNX neurones that express NUCB2/nesfatin-1 and project to the pancreas appears to support the *in vivo* stimulating action of nesfatin-1 on glucagon release.

Recent findings suggest nesfatin-1 involvement in other important functions, such as cardivascular control, reproduction, sleep, cognition and anxiety- or stress-related responses (54). Given these very diverse physiological actions described for nesfatin-1, it could be hypothesised that nesfatin-1 released after hypoglycaemia might be involved in counter-regulation responses that differ from the control of the glycaemic state and food intake. As an example, nesfatin-1 was shown to exert cardiovascular-modulating effects (9). Central nesfatin-1 administration increases mean arterial pressure via the activation of the sympathetic nerve. Whether some nesfatin-1-neurones reported to be activated by hypoglycaemia partake in the modulation of cardiovascular function (known to intervene during insulin-induced hypoglycaemia) remains to be determined. In conclusion, our results are the first to report that a subpopulation of nesfatin-1-expressing neurones located in the hypothalamus and the brainstem belongs to the central signalling that contributes to the responses to hypoglycemia. Thus, nesfatin-1 could participate in the triggering of physiological and hormonal counter-regulations observed in response to hypoglycaemia.

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2. Activation des neurones nesfatinergiques en contexte inflammatoire

2.1. Inflammation induite par le LPS

Article : Central nesfatin-1-expressing neurons are sensitive to peripheral inflammatory stimulus.

Bonnet MS, Pecchi E, Trouslard J, Jean A, Dallaporta M, Troadec J-D.

Journal of Neuroinflammation. 2009; 6:27.

En 2009, aucune étude n'avait encore été menée sur la nesfatine-1 en condition pathologique. Nous avons donc cherché à savoir si les neurones de l'hypothalamus et du CVD exprimant ce peptide étaient sensibles à un stimulus inflammatoire. Nous avons créé une inflammation périphérique chez le rat en injectant intrapéritonéalement du LPS à la dose de 0,25 mg/kg connue pour induire une inflammation. Comme nous l'avons vu dans l'introduction, le LPS est une endotoxine bactérienne utilisée expérimentalement pour provoquer une inflammation. Elle entraine l'activation de réseaux neuronaux responsables de la mise en place des symptômes de la composante centrale de la réaction de phase aiguë. Parmi les structures cérébrales activées par le LPS, nous retrouvons le NTS ainsi que plusieurs noyaux hypothalamiques tels que l'ARC, le PVN et le SON qui expriment fortement NUCB2/nesfatine-1. Nous avons alors émis l'hypothèse que la nesfatine-1 étant anorexigène, elle pouvait relayer l'effet anorexigène du LPS. Pour vérifier cette hypothèse, nous avons sacrifié les animaux trois heures après l'injection périphérique de LPS car, à ce délai, la majorité des structures sensibles à l'inflammation est activée et les symptômes de la composante centrale de la réaction de phase aiguë sont mis en place. Comme précédemment, nous avons ensuite pratiqué une double immunohistochimie dirigée contre la protéine c-Fos et NUCB2/nesfatine-1 dans l'hypothalamus et le tronc cérébral afin de mettre en évidence une éventuelle activation des neurones exprimant NUCB2/nesfatine-1 par l'inflammation.

Résultats :

Comme attendu, l'injection de LPS entraine l'activation de l'ARC, du PVN, du SON et du NTS attesté par l'augmentation du nombre de cellules c-Fos-positives comparé à une injection de solution saline. Dans le NTS, le PVN et le SON, un nombre non négligeable de neurones activés sont nesfatinergiques. En effet, le pourcentage de neurones c-Fos-positifs

immunoréactifs pour NUCB2/nesfatine-1 s'élève à 19,7 ± 3,8% dans le NTS, 33,8 ± 2,3% dans le PVN et 51,9 ± 2,4% dans le SON. Dans l'ARC, ce pourcentage de colocalisation est beaucoup plus faible puisqu'il est inférieur à 5% (4,7 ± 0,4%) mais néanmoins significativement différent des animaux contrôles. Nous montrons également que dans le NTS, des neurones exprimant NUCB2/nesfatine-1 sont catécholaminergiques puisqu'ils co-expriment la tyrosine hydroxylase (TH), et environ 60% de ces neurones nesfatinergiques/catécholaminergiques sont activés en réponse à l'injection périphérique de LPS.

Conclusion :

Nos résultats montrent pour la première fois la mise en jeu des neurones nesfatinergiques en condition pathologique. En effet, nous mettons ici en évidence la sensibilité des neurones nesfatinergiques de l'hypothalamus et du NTS à un signal inflammatoire induit par une injection périphérique de LPS. De plus, nous montrons que des neurones nesfatinergiques du NTS activés par le LPS sont catécholaminergiques laissant supposer qu'ils projettent vers les centres supérieurs. Ainsi, ce réseau de neurones nerfatinergiques pourrait participer à la réduction de la prise alimentaire observée au cours d'une inflammation systémique. La libération centrale de nesfatine-1 et sa contribution dans la mise en place de l'anorexie inflammatoire restent à identifier.

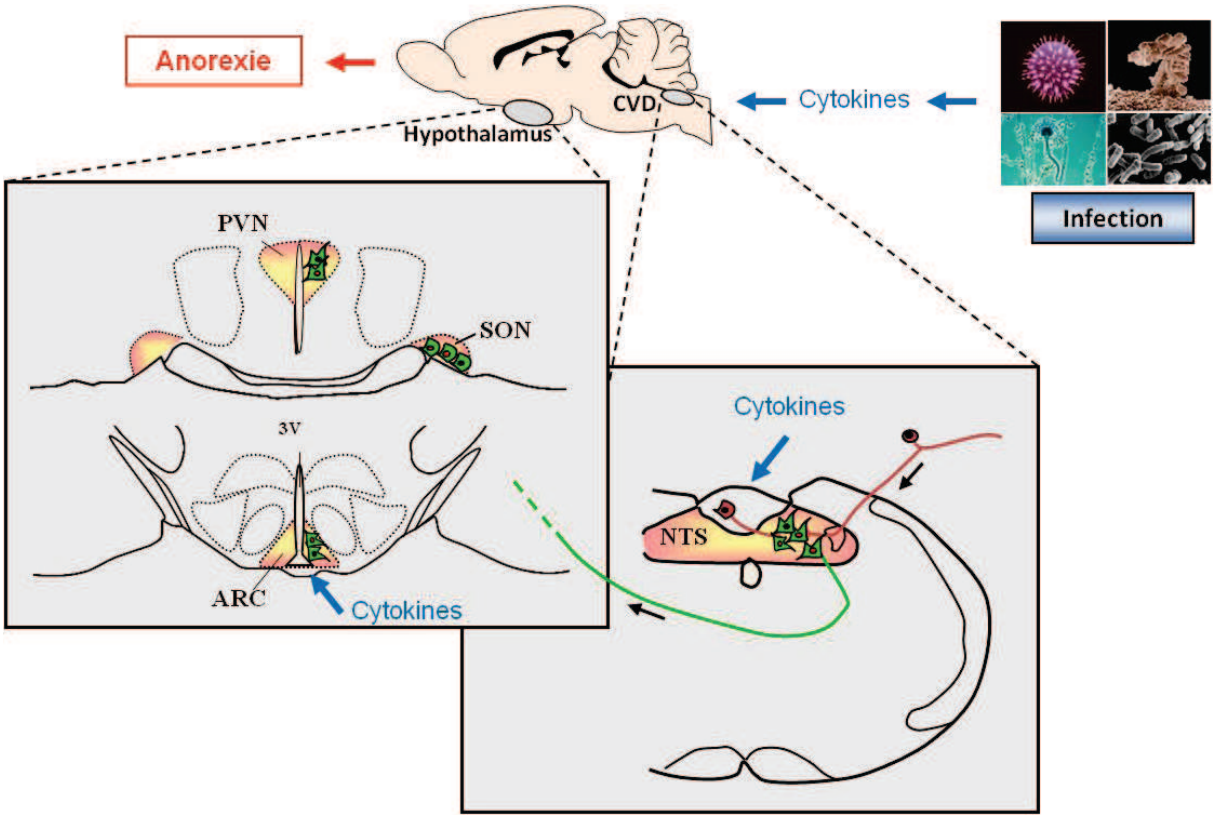


Figure 27 : schéma résumant l'implication des neurones exprimant NUCB2/nesfatine-1 dans la réponse à l'inflammation.

Journal of Neuroinflammation

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Short report

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Central nesfatin-1-expressing neurons are sensitive to peripheral inflammatory stimulus

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Abstract

Recently, a novel factor with anorexigenic properties was identified and called nesfatin-1. This protein (82 aac) is not only expressed in peripheral organs but it is also found in neurons located in specific structures including the hypothalamus and the brainstem, two sites strongly involved in food intake regulation. Here, we studied whether some of the neurons that become activated following an injection of an anorectic dose of lipopolysaccharides (LPS) exhibit a nesfatin-1 phenotype. To this end, we used double immunohistochemistry to target the expression of the immediate-early gene *c-fos* and of nesfatin-1 on coronal frozen sections of the rat brain. The number of c-Fos+/nesfatin-1+ neurons was evaluated in the immunosensitive structures reported to contain nesfatin-1 neurons; i.e. paraventricular hypothalamic nucleus (PVN), supraoptic nucleus (SON), arcuate nucleus (ARC) and nucleus of the solitary tract (NTS). LPS strongly increased the number of c-Fos+/nesfatin-1+ neurons in the PVN, SON and NTS, and to a lesser extent in the ARC. Triple labeling showed that a portion of the nesfatin-1 neurons activated in response to LPS within the NTS are catecholaminergic since they co-express tyrosine hydroxylase (TH). Our data therefore indicate that a portion of nesfatin-1 neurons of both the hypothalamus and brainstem are sensitive to peripheral inflammatory signals, and provide the first clues suggesting that centrally released nesfatin-1 may contribute to the neural mechanisms leading to endotoxaemic anorexia.

Findings

During infection and inflammation, the immune, endocrine and nervous systems closely interact to coordinate a range of physiological and behavioral changes known as the acute phase reaction. The behavioral symptoms collectively referred to as "sickness behavior" include fever, anorexia, adipsia, lethargy and reduction in social interactions [1,2]. Cytokines peripherally released act on specific central nuclei involved in feeding and homeostatic regulation, which leads to the central component of the acute phase response. The complex neuronal circuitry involved in the coordinated response to inflammation, which includes notably the nucleus of the solitary tract (NTS), area postrema (AP), ventrolateral medulla, parabrachial nucleus, paraventricular hypothalamic nucleus (PVN), supraoptic nucleus (SON), arcuate nucleus (ARC) and

central nucleus of the amygdala, has been identified mainly by quantification of the immediate-early gene *c-fos* [3,4]. The expression of the *c-fos* gene is widely considered to be a high resolution marker of neuronal activity, since a body of evidence demonstrates that Fos protein is expressed in neurons whose activity is strongly stimulated by synaptic input [5]. Used in combination with immunohistochemical determination of neuronal phenotype, this approach has led to the identification and location of neurons activated by inflammation and involved in the triggering of sickness behavior [6-8].

Recently, Oh-I and co-authors reported the identification of a novel factor with anorexigenic properties, which they called nesfatin-1 [9]. Nesfatin-1 is reported to reduce food intake both after central (3rd ventricle) and peripheral administration [9,10]. Apart from adipose tissue, from which nesfatin-1 was first isolated, this protein is also expressed by gastric mucosa [11] and pancreatic β-cells [12]. This pattern of nesfatin-1 expression, as well as the presence of nesfatin-1 within the plasma of rodents and humans, strongly suggests that this compound may act as a circulating regulatory factor. Interestingly, expression of nesfatin-1 within the brain has been reported by various groups [13-16]. Neurons expressing nesfatin-1 are found located in various areas including the brainstem (NTS, dorsal motor nucleus of the vagus: DMNX) and hypothalamic nuclei (ARC, PVN, SON).

In the present study, we investigated, by mean of a c-Fos/nesfatin-1 double immunohistochemical approach, whether a portion of those neurons activated in response to peripheral injection of an anorexic dose of lipopolysaccharides (LPS) exhibit nesfatin-1 expression. The peripheral injection of LPS is a well characterized model of inflammation, known to induce events occurring during sepsis such as the synthesis and release of inflammatory cytokines and the activation of immunosensitive structures in the brain [2].

Adult male Wistar rats weighing 250-300 g (Janvier, France) were housed individually in a pathogen-free facility at controlled temperature on a 12:12-h light/dark cycle (lights off at 18.00 h) with food (AO4, SAFE UAR, France) and water available *ad libitum*. All procedures were in accordance with the European Directive N886/609 and the local committees for animal use and care. For habituation, animals were handled and injected intraperitoneally (i.p.) with physiological saline every day for at least seven days before experiment. On the day of the experiment, animals (n = 6 for each condition) were fasted at 9.00 h and the food was withdrawn until the animal sacrifice in order to avoid activation of nesfatin-1 neurons consecutive to feeding. Four hours after the beginning of fasting, animals were injected with either saline or an LPS-containing solution (0.25 mg/kg, from *E. coli*, serotype 0127:B8, Sigma). The injection volume for all animals was 0.01 ml per gram of body weight. The rats were sacrificed three hours after injection, which corresponds to the peak of neuronal c-Fos immunoreactivity in hypothalamus and brainstem nuclei following peripheral administration of endotoxin [17]. After being deeply anaesthetized with ketamine (100 mg/kg i.p.; Merial, France) and xylazine (16 mg/kg i.p.; Bayer, France), rats were transcardially perfused with freshly depolymerized 4% PFA. The brains were removed immediately, post-fixed one hour in 4% PFA at 4°C and then cryoprotected for 24 to 48 hours in 30% sucrose at 4°C.

To identify activated nesfatin-1 neurons, double immunohistochemistry for c-Fos and nesfatin-1 was performed on brain sections. After freezing the brains in isopentane (-40°C), coronal sections (40 μm thick) were cut on a cryostat (Leica CM3050, France) and collected serially in 12-well plates containing PBS (0.1 M). Brains were cut at the brainstem level (Interaural -5.3 mm to -3.8) and at the forebrain level (Interaural +4.7 to +7.7 mm) according to the rat brain atlas from Paxinos and Watson [18]. The free-floating sections were incubated in a solution containing 0.3% H_2O_2 in PBS 0.1 M for quenching of endogenous peroxidase activity. A 1 h incubation in PBS containing 3% normal goat serum (NGS) and 0.3% Triton X-100 was performed to block non-specific binding sites. Sections were then treated for 48 hours at 4°C in PBS containing 3% NGS, 0.3% Triton X-100 and anti-c-Fos rabbit antiserum synthesized against amino acids 4-17 of human protein (1:10000, Ab-5, Calbiochem). After three washes in PBS, the sections were incubated overnight at 4°C with the biotinylated goat anti-rabbit IgG (1:400, Vector Labs). After further washes, sections were incubated with avidin-biotin complex (1:200, ABC Vector Elite kit) for 2 h at room temperature. Horseradish peroxydase activity was then visualized using a nickel-enhanced diaminobenzidine (DAB) as the chromogen to reveal a purplish-black stain of c-Fos-immunoreactive nuclei. The reaction medium contained nickelous ammonium sulphate (0.6%), DAB (0.006%) and H_2O_2 (0.02%) in PBS. The reaction was closely monitored and terminated when optimum intensity was achieved (10 min) by washing the sections in distilled water. Non-specific labeling was assessed on alternate slides that were treated identically to the above but in which the primary antibody was omitted. Sections were then incubated overnight at 4°C with a primary antibody raised against nesfatin-1 (1:10000, H-003-22-B, Phoenix Pharmaceuticals Inc.), washed in PBS, and incubated for 2 h at room temperature with a secondary antibody conjugated with Alexa 488 (1:400, Vector Labs). An additional labeling of the tyrosine hydroxylase (TH) enzyme was performed on brainstem sections. For this purpose, sections were incubated overnight at 4°C with

anti-TH antibody (1:1000, MAB318, Chemicon), and revelation was performed using an Alexa 594-conjugated secondary antibody (1/400, Vector Labs). Finally, all sections were mounted on gelatin-coated slides, air dried, and coverslipped with mounting medium for fluorescence microscope preparation (DAKO).

For the analysis of neuronal activation, images of brain sections were acquired using 10× and 20× lenses with a DXM 1200 Camera (Nikon) coupled to ACT-1 software. Fluorescent images acquired at 520 nm were merged with phase contrast images to allow visualization of c-Fos+/nesfatin-1+ neurons. For each brain structure studied, the counts performed on four distinct sections from both hemispheres were averaged to yield the number of c-Fos nuclei and c-Fos+/nesfatin-1+ cells per section. Statistical analysis of data was performed with Sigmastat (version 3.5; Systat software Inc) using Student's *t*-test. P values less than 0.05 were considered significant.

In the hypothalamus (Figure 1A-C) and brainstem (Figure 2A) of control animals injected with physiological saline solution, c-Fos-labeled perikarya were very scarce. In contrast, rats treated with LPS exhibited an increased number of c-Fos-positive nuclei in the hypothalamus and brainstem (Figures 1D-F and 2B). This increase in c-Fos immunoreactivity was clearly detected in the PVN (261.9 ± 12.5 versus 1.3 ± 0.2 c-Fos + nuclei per section in LPS and

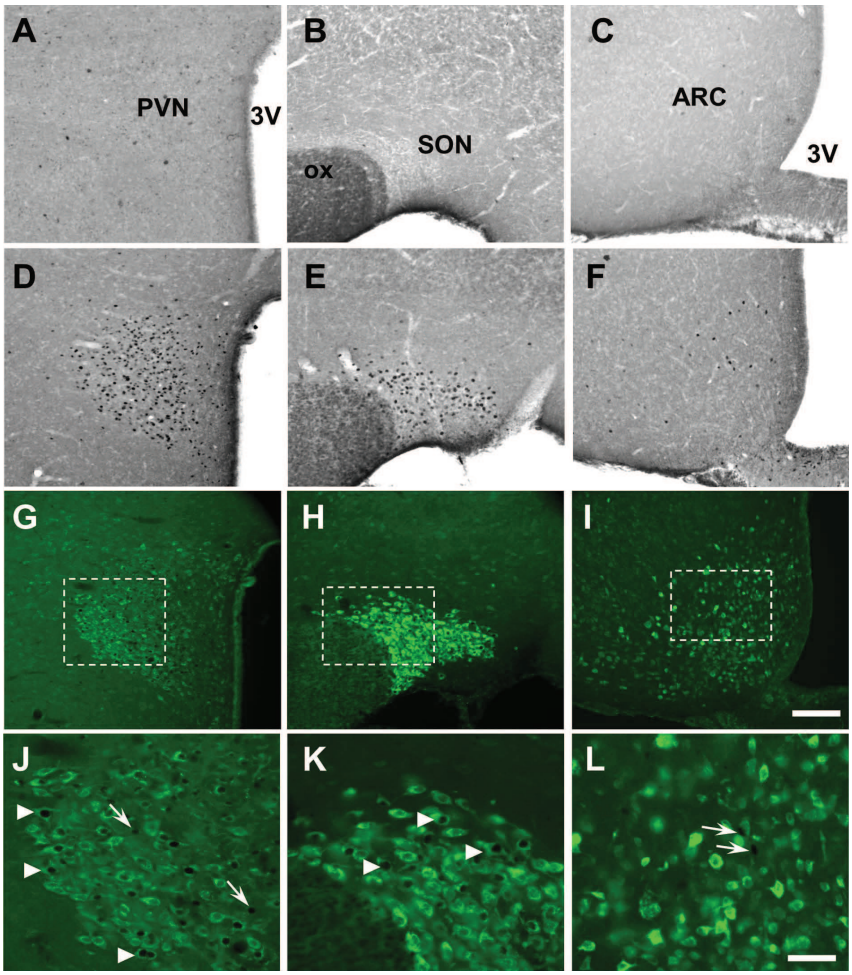


Figure 1
A-F: Representative photomicrographs of c-Fos immunoreactivity in coronal sections of the PVN, ARC and SON. Images derived from saline-injected (**A-C**) and LPS-treated (**D-F**) rats. **G-L**: Analysis of c-Fos and nesfatin-1 (green) double-labeling in coronal sections through the hypothalamus of LPS-treated rats. Dashed boxes in low magnification images (**G-I**) indicate the area where high magnification images (**J-L**) originate. Arrowheads: c-Fos+/Nesf+ neurons; arrows: c-Fos+ neurons negative for nesfatin-1. ARC arcuate nucleus; ox, optic chiasm; PVN, paraventricular nucleus of the hypothalamus; SON: supraoptic nucleus; 3V, third ventricle. Scale bars: 200 μm (A-I), 50 μm (J-L).

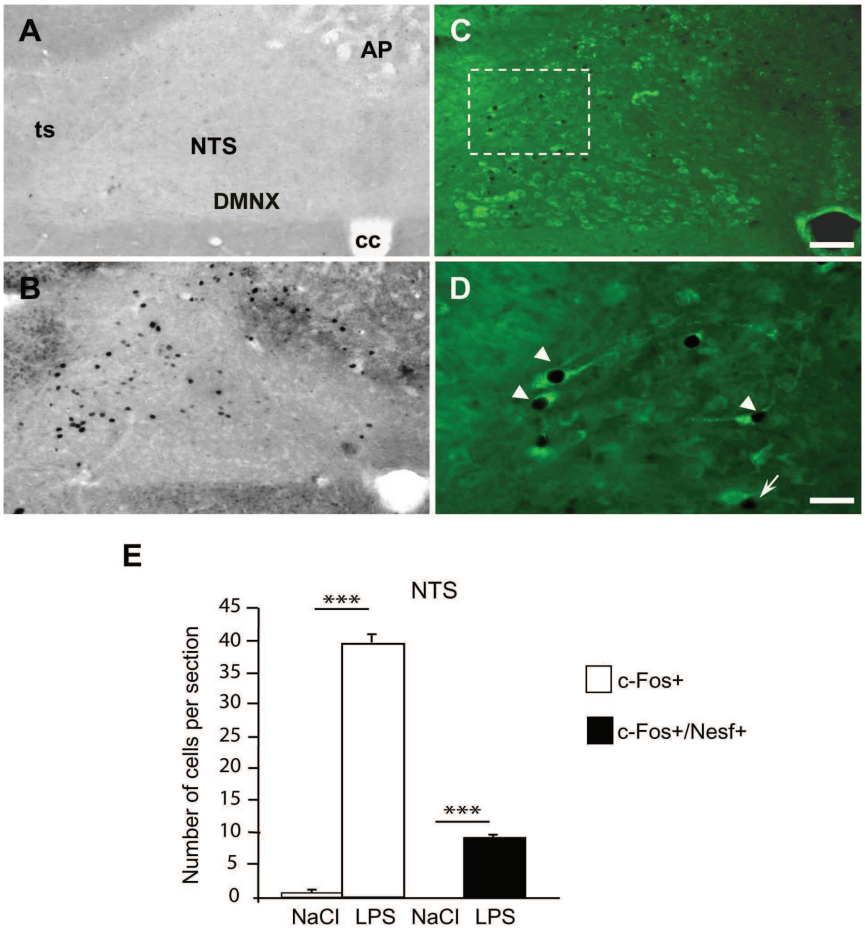


Figure 2

A-B: c-Fos immunoreactivity within the DVC. Representative coronal sections of the DVC of saline-injected (**A**) and LPS-treated (**B**) rats. **C-D**: c-Fos and nesfatin-1 (green) double-labeling in coronal sections through the DVC of LPS-treated rats. **D**: High magnification of c-Fos/nesfatin-1 positive neurons in the NTS. Dashed box in C indicates the area where high magnification image originates. Arrowheads: c-Fos+/Nesf+ neurons; arrow: c-Fos+ neuron negative for nesfatin-1. **E**: Quantification of the number of c-Fos and c-Fos/nesfatin-1 immunoreactive cells at the NTS subpostremal level of rats injected with either saline or LPS solution. Starred values are significantly different from saline-injected animals, *** $p < 0.001$. AP, area postrema; cc, central canal; DMNX, dorsal motor nucleus of the vagus; NTS, nucleus of the solitary tract; ts, tractus solitarius. Scale bars: 100 μm (A-C), 50 μm (D).

saline-treated animals respectively; Figures 1A, D and 3), the SON (69.7 ± 0.9 versus 2.0 ± 0.1 c-Fos + nuclei per section; Figures 1B, E and 3) and the ARC (22.9 ± 0.9 versus 0.5 ± 0.1 c-Fos + nuclei per section; Figures 1C, F and 3). At the brainstem level, visualisation of c-Fos immunoreactivity revealed that LPS injection induced an increase in positive perikarya mainly within the AP and the NTS (39.7 ± 1.5 versus 0.7 ± 0.2 c-Fos + nuclei per section, Figures 2A, B and 2E). Nesfatin-1-immunoreactive neurons expressing c-Fos (c-Fos+/Nesf+) were then analyzed in hypothalamic and brainstem nuclei by means of double immunohistochemistry in both experimental groups i.e saline- and LPS-treated rats. As expected, all structures studied were virtually devoid of c-Fos+/Nesf+ neurons in control condition (Figures 2E and 3). In LPS-injected animals the greatest incidence of c-Fos/Nesf colocalization was found in SON (Figures 1H, K and 3), where 51.9 ± 2.4% of total c-Fos perikarya also express nesfatin-1. Lesser, but still highly significant, incidences were observed in the PVN and NTS where the percentage of colocalization reached 33.8 ± 2.3% and 19.71 ± 3.8%, respectively (Figure 1G, J; Figures 2 C-E and 3). Interestingly, the ARC exhibited less than 5% c-Fos/Nesf colocalization, a value (4.7 ± 0.4%) which was nonetheless significantly different from control animals (Figures 1I, L and 3). It should be noted here that despite the increased

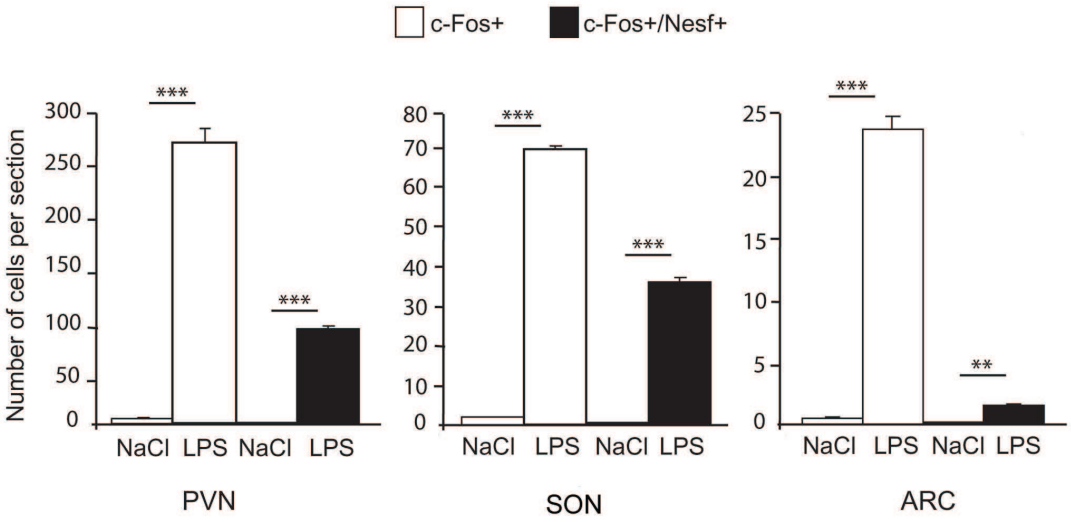


Figure 3
Quantification of the number of c-Fos and c-Fos/nesfatin-1 immunoreactive cells in the PVN, SON and ARC after an i.p. injection of either saline or LPS solution. Starred values are significantly different from saline-injected rats, ** $p < 0.01$; *** $p < 0.001$.

number of c-Fos nuclei in the AP of LPS-treated animals (Figure 2B), the absence of nesfatin-1 labeling in this area (Figure 2C) naturally prompted us to exclude this nucleus from our quantification. Finally, tyrosine hydroxylase (TH) immunohistochemistry revealed that within the NTS level some of the nesfatin-1 positive neurons were catecholaminergic in nature since they co-expressed TH (Figure 4A-C). Moreover, triple labeling for c-Fos/Nesf/TH indicated that about 60% of the Nesf+/TH+ neurons were activated during the immune challenge (Figure 4).

The present work confirms an hypothesized association between nesfatin-1 neurons and inflammation since we report here that some nesfatin-1-expressing neurons are activated during an inflammatory stimulus. These results therefore suggest that nesfatin-1 may intervene in the onset of physiological and behavioral changes occurring during acute phase reactions. The protocol used here, i.p. injection of LPS coupled to c-Fos staining, is classically employed to identify immunosensitive structures [3,4]. In the present study, animals received an i.p. injection of LPS at a concentration (0.25 mg/kg) reported to induce the symptoms of the acute phase reaction including fever [19], withdrawal [20] and anorexia [21] as well as c-Fos protein production in central nuclei [4,19]. Under our experimental conditions, the c-Fos distribution pattern observed throughout the brain of LPS-injected rats was consistent with the findings of previous studies performed to identify neurocircuitry involved in the coordinated physiological, endocrine, and behavioral response to LPS challenge [4,22].

The evidence of nesfatin-1-expressing neurons activation in the brainstem and hypothalamus emphasize the potential role played by these neurons in the transfer of information received from circulating immune factors. We observed that LPS injection substantially increased the number of co-labeled neurons in key nuclei activated during inflammation and involved in the coordinated physiological, endocrine and behavioral changes characteristic of sickness behavior. Nesfatin-1 has been reported to exert an anorexigenic activity when administered either centrally or peripherally [9,10]. Moreover, central nesfatin-1-expressing neurons located in the hypothalamus or brainstem are reported to be activated by refeeding or after cholecystokinine injection, which strongly suggests that these neurons partake in the complex central neurocircuitry involved in the termination of food intake [23,24]. The evidence that neurons expressing nesfatin-1, located within both the hypothalamus and brainstem, are activated during inflammation support the hypothesis that these neurons are involved in the reduction of food intake induced by inflammation. Our results imply that nesfatin-1, initially identified as a modulator of feeding behavior under physiological conditions, could also be involved in the modulation of feeding behavior during endotoxaemia anorexia. Such a duality of peptide-expressing neurons was first evoked for pro-opiomelanocortin and corticotrophin-releasing factor, hypothalamic neurons which both play an essential role in the control of energy homeostasis and are likely to be involved in inflammatory anorexia [25]. Finally, we cannot rule out the possibility that nesfatin-1 neurons activated by inflammatory signals con-

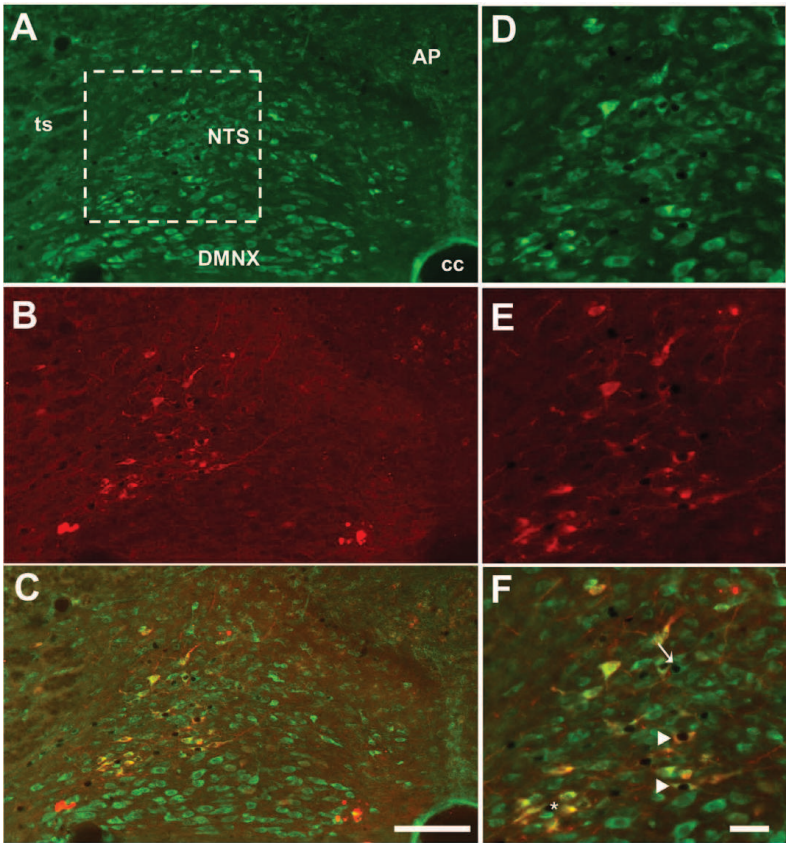


Figure 4
Representative photomicrographs of c-Fos (DAB precipitate)/nesfatin-1 (green)/TH (red) triple labeling performed on brainstem coronal sections of rats treated with LPS. Note the presence of c-Fos positive neurons co-expressing nesfatin-1 and TH (arrowheads), c-Fos+/Nesf+ neuron negative for TH (arrow) and c-Fos+/Nesf-/TH-neuron (asterisk). Dashed box in image (**A**) indicates the area where high magnification images (**D-E**) originate. AP, area postrema; cc, central canal; DMNX, dorsal motor nucleus of the vagus; NTS, nucleus of the solitary tract; ts, tractus solitarius. Scale bars: 150 μm (A-C); 40 μM (D-F).

tribute to other features of the acute-phase response. It has been proposed that the activation of neurons located in the SON contribute to a counter-hormonal response to inflammation *via* the release of neurohypophysial hormones into the circulation [26]. Interestingly, we show here that a significant fraction of LPS-activated neurons within the SON express nesfatin-1. The functional significance of such activation of nesfatin-1-expressing neurons in response to inflammation in this structure should be explored in future studies.

Double staining revealed that a large number of nesfatin-1 immunoreactive neurons of the NTS are positive for TH, indicating that a fraction of nesfatin-1 neurons belong to the A2/C2 catecholaminergic group. In accordance with the previous observation by Lacroix and Rivest [4], who reported an activation of TH-immunoreactive neurons of the NTS in response to immune challenge, triple labeling performed in the present study revealed the presence of c-Fos/nesfatin-1-positive neurons immunoreactive for TH. The activation of neurons coexpressing TH and nesfatin-1 in response to LPS might contribute to cardiovascular response to endotoxin, which consists mainly in a modification in blood pressure and heart rate [27], since catecholaminergic neurons of the NTS are involved in the regulation of cardiovascular parameters [28], and centrally injected nesfatin-1 was recently shown to affect mean arterial pressure in conscious rats [29]. The presence of c-Fos/nesfatin-1/TH triple labeled neurons in the NTS of LPS-treated rats leads also to the hypothesis that some of the nesfatin-1 neurons activated by immune challenge project to the hypothalamus. Prior research has demonstrated, for instance, the existence of catecholaminergic projections from NTS to PVN [30]. The coupling of neuronal pathway tracing studies with triple immunohisto-

chemical labelling should be performed in the future to answer this query.

In conclusion, we show for the first time that neurons of the central nesfatinergic system are sensitive to peripheral inflammatory stimulus and thus belong to the specific immunosensitive neurocircuitry activated during infection or inflammation. Given the anorexigenic properties described for nesfatin-1 and the presence of nesfatin-1-activated neurons in key nuclei of the hypothalamus and brainstem known to contribute to food intake regulation [31], one may suggest that nesfatin-1 could account for the reduction in food intake observed during endotoxaemic anorexia. This knowledge will help better understand the neural mechanisms underlying sickness behavior.

List of abbreviations

AP: area postrema; ARC: arcuate nucleus; DMNX: dorsal motor nucleus of the vagus; LPS: lipopolysaccharides; NTS: nucleus of the solitary tract; PVN: paraventricular hypothalamic nucleus; SON: supraoptic nucleus; TH: tyrosine hydroxylase.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MD, AJ, JT and JDT designed research; MSB, EP and MD performed research; MSB, MD and JDT analyzed data and MD and JDT wrote the paper.

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2.2. Inflammation induite par une mycotoxine, contaminant de l'alimentation : le Déoxynivalénol

Le déoxynivalénol (DON) est une mycotoxine de la famille des trichothécènes produite par les champignons *Fusarium*. Il contamine abondamment les céréales telles que le blé, l'orge, l'avoine, le seigle et le maïs. Le DON est très répandu dans l'alimentation des animaux de ferme ainsi que dans l'alimentation humaine. Par exemple, il a été montré que 63% des céréales pour enfants sont contaminées au DON (sur 363 céréales testées) (Lombaert et al., 2003). De plus le DON est résistant aux hautes températures (150°C) ce qui le rend stable pendant la cuisson (Bretz et al., 2006). La consommation de DON semble être à l'origine de maladies à la fois chez les animaux d'élevage et chez l'homme puisqu'elle entraine une anorexie, une perte de poids, des diarrhées, des hémorragies ainsi qu'un comportement vomitif, ce qui a valu à cette toxine d'être également appelée « vomitoxine » (Bryden, 2007; Pestka, 2010). Ainsi, la contamination des aliments par le DON crée un problème économique et un problème de santé publique **(Cf. : annexe 4)**.

L'action inflammatoire du DON en périphérie a largement été décrite (Azcona-Olivera et al., 1995; Pestka et Amuzie, 2008). Cependant, aucune donnée n'était disponible quant à l'action centrale de cette toxine. Dans ce contexte, nous avons cherché à déterminer si une intoxication aiguë au DON pouvait agir au niveau central, et plus particulièrement si les neurones exprimant NUCB2/nesfatine-1 étaient capables de relayer l'effet anorexigène du DON. Pour cela nous avons intoxiqué oralement des souris, par gavage, en fin de phase claire (1 heure avant l'extinction) afin de mieux caractériser les symptômes induits par le DON ainsi que les effets de cette toxine sur le cerveau **(Cf. : annexes 5 et 6)**. Nous avons ensuite entrepris de mettre en évidence l'action centrale du DON chez le porc qui est l'espèce la plus sensible au DON et la plus proche de l'homme concernant les intoxications alimentaires **(Cf. : annexe 7)**.

2.2.1 Inflammation centrale induite par une intoxication aiguë au DON chez la souris

Le DON étant à l'origine d'une inflammation périphérique, nous avons cherché à savoir si l'intoxication au DON causait également une inflammation centrale. L'administration orale de DON à la dose de 12,5 mg/kg provoque effectivement une augmentation de l'expression des

biomarqueurs de l'inflammation dans l'hypothalamus et le CVD, 3 heures après intoxication. Ces biomarqueurs de l'inflammation sont les cytokines IL-1β, IL-6 et TNF-α **(Figure 28A)**, et les enzymes de synthèse de la PGE2 : COX-2 et mPGES-1 **(Figure 28B)**.

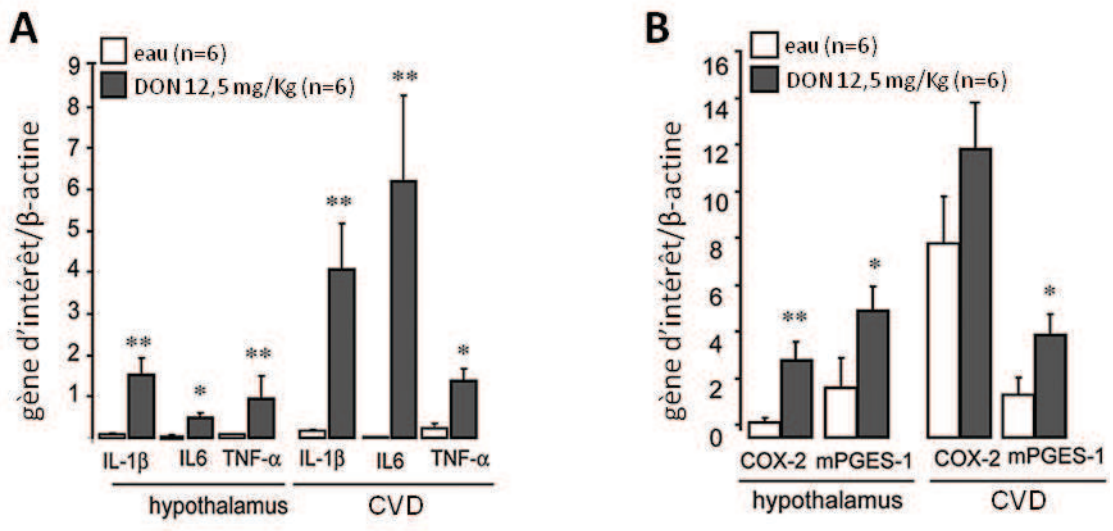


Figure 28 : L'intoxication au DON stimule l'expression centrale des biomarqueurs de l'inflammation.
Expression des transcrits de l'IL-1β, de l'IL-6, du TNF-α (A) ainsi que de COX-2 et mPGES-1 (B) dans l'hypothalamus et le CVD de souris 3 heures après une administration orale de DON (12,5 mg/kg) ou d'eau.
*p < 0,05 et **p <0,01.

2.2.2 Symptômes induits par une intoxication aiguë au DON chez la souris

- *Effets sur l'activité locomotrice et la température corporelle*

Le comportement de maladie étant caractérisé par des modifications de l'activité et de la température, nous avons réalisé des enregistrements télémétriques dans le but de mesurer ces deux paramètres suite à une administration aiguë de DON. Les souris intoxiquées oralement au DON en fin de phase claire présentent une diminution dose dépendante de l'activité locomotrice nocturne **(Figure 29A et B)**. Aux doses de 12,5 mg/kg et 25 mg/kg, l'activité locomotrice est durablement affectée puisque 3 nuits après administration, les souris traitées au DON n'ont toujours pas retrouvé un profil d'activité similaire à celui des souris traitées à l'eau **(Figure 29A)**. L'administration de DON entraine également une chute dose-dépendante de la température corporelle durant les deux premières heures suivant l'intoxication **(Figure 30A-D)**. Aux doses de 12,5 mg/kg et 25 mg/kg, la température chute de 2,5°C et cette

hypothermie se prolonge plusieurs heures avant de réaugmenter. La courbe de température reste cependant altérée plusieurs jours puisque la température corporelle des souris traitées au DON perd sa rythmicité circadienne pendant 72 heures **(Figure 30B et C)**.

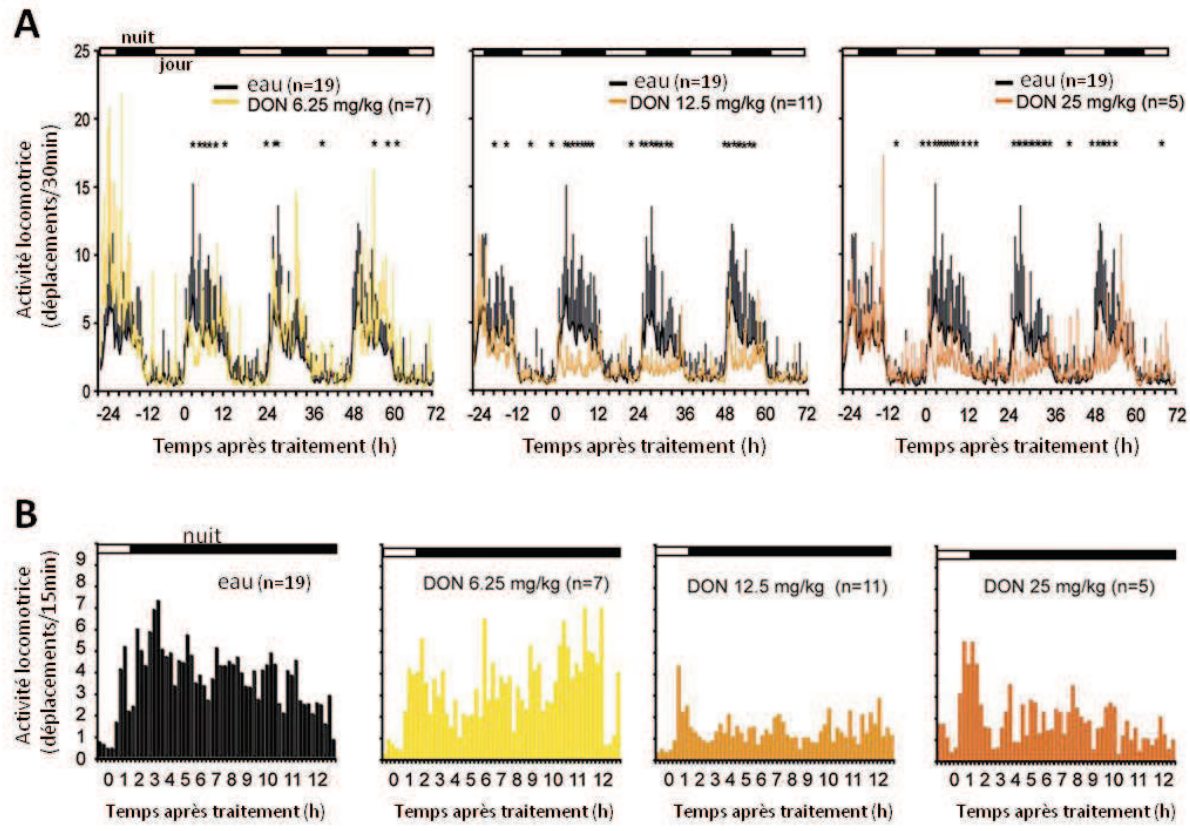


Figure 29 : L'intoxication orale au DON réduit l'activité locomotrice.
A. Enregistrement de l'activité locomotrice sur 96 heures de souris traitées au DON ou à l'eau. Les barres noires et blanches représentent les phases sombres et claires. *p < 0,05.
B. Enregistrement de l'activité locomotrice nocturne de souris traitées au DON ou à l'eau la nuit suivant l'administration de la toxine.

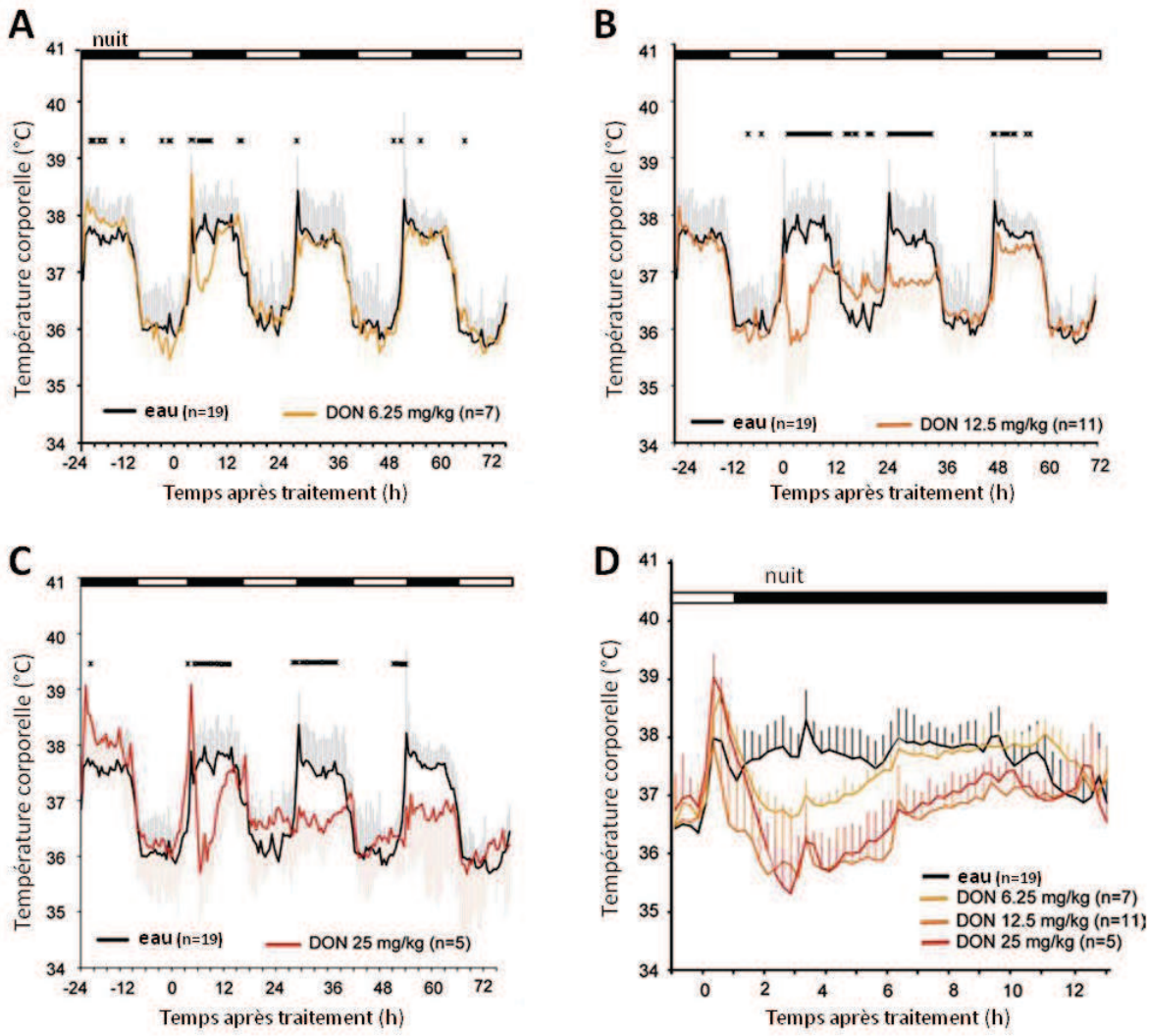


Figure 30 : L'intoxication orale au DON réduit la température corporelle.
A-C : Enregistrement de la température corporelle sur 96 heures de souris traitées au DON ou à l'eau. *p < 0,05.
D. Enregistrement de la température corporelle nocturne de souris traitées au DON ou à l'eau la nuit suivant l'administration de la toxine.

- *Effets sur la prise alimentaire*

L'intoxication orale au DON provoque une diminution dose-dépendante de la prise alimentaire qui perdure plusieurs jours pour les doses les plus élevées **(Figure 31A)**. La mesure de prise alimentaire par intervalles de temps durant les 24 premières heures suivant l'intoxication montre que le DON affecte profondément la consommation nocturne, alors que l'on observe au contraire un léger rebond dans la journée **(Figure 31B)**. Les souris traitées au DON consomment cependant plus de Kaolin pendant les premières 24 heures post-traitement que les souris ayant reçu de l'eau **(Figure 31C)**. Ce comportement connu sous le nom de

« Pica » permet d'étudier l'effet nauséeux ou vomitif d'une molécule chez les rongeurs car le kaolin est une substance non nutritive que les rongeurs ingèrent spontanément en cas de malaise gastrique (Yamamoto et al., 2002). Nous avons ensuite mesuré la prise alimentaire en continu nous permettant d'une part de confirmer que la consommation est affectée essentiellement durant la nuit et d'autre part d'analyser la microstructure des repas **(Figure 32A et B)**. Les souris ayant reçu le DON présentent une diminution de 27,9% de la fréquence des repas comparé aux souris contrôles, ainsi qu'une diminution de 42,2% de la taille des repas. De plus, l'intervalle entre les repas est augmenté de 69% et le ratio de satiété, correspondant au rapport entre l'intervalle entre les repas et la taille des repas, augmente de 40% en réponse au DON **(Figure 32B)**.

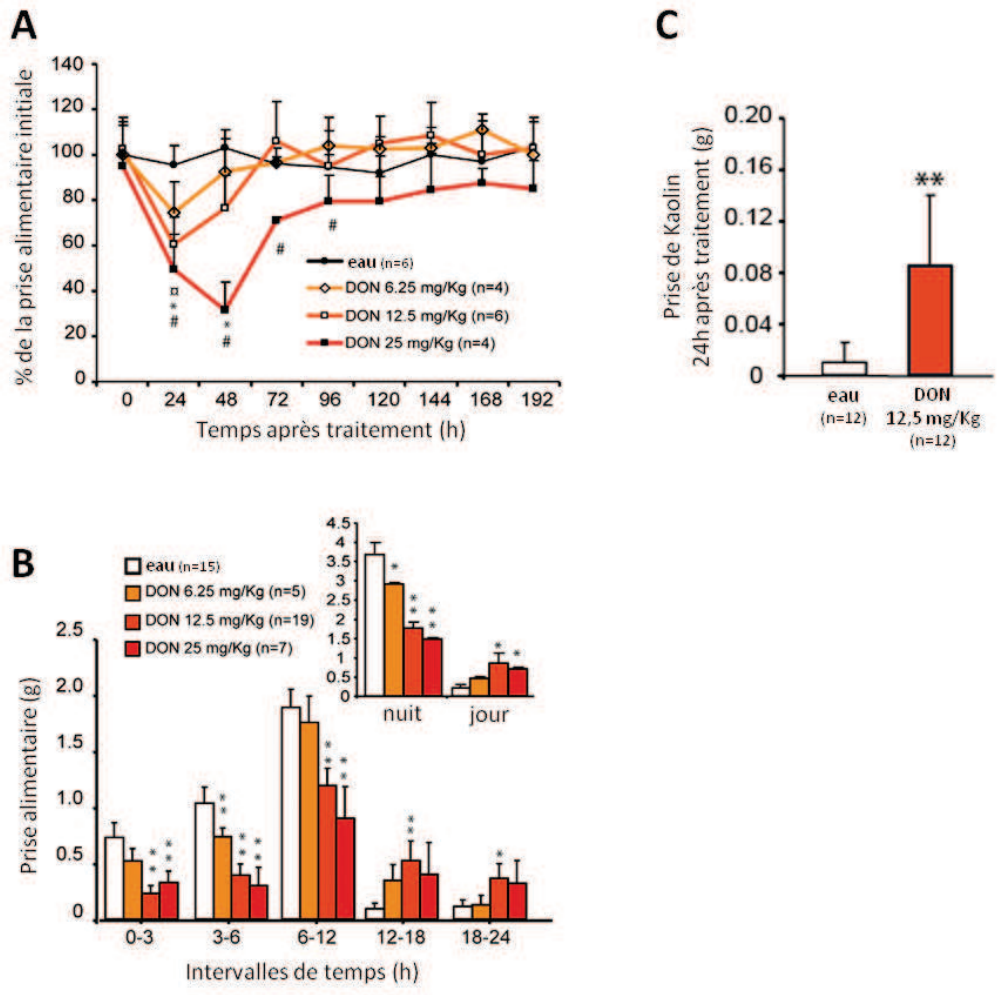


Figure 31 : L'intoxication orale au DON affecte la prise alimentaire.
A. Mesure sur plusieurs jours de la prise alimentaire journalière de souris traitées au DON ou à l'eau.
B. Prise alimentaire mesurée sur les premières 24 heures suivant l'administration de DON ou d'eau.
C. Prise de Kaolin mesurée 24 heures après traitement au DON ou à l'eau.
En A : ¤, * et # représentent $p < 0,05$ significativement différent entre les souris traitées à l'eau et celles traitées au DON respectivement aux doses de 6,25, 12,5 et 25 mg/kg. En B et C : *$p < 0,05$ et **$p < 0,01$.

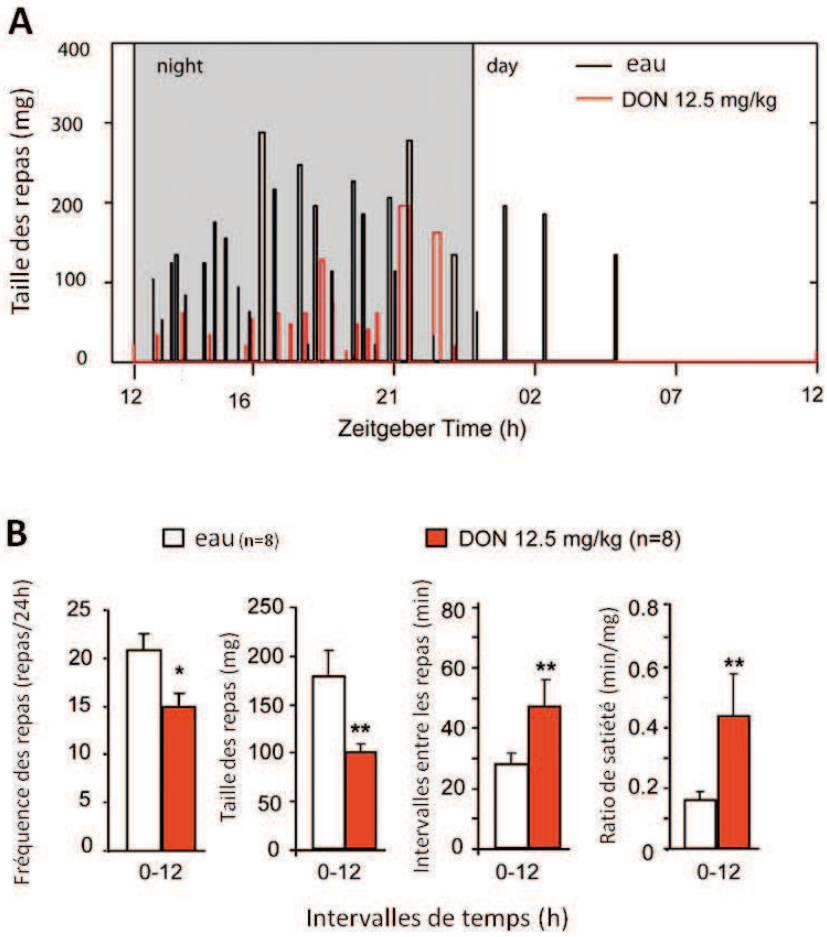


Figure 32 : Effet de l'intoxication aiguë au DON sur la microstructure des repas.
A. Mesure de la prise alimentaire en continu sur 24 heures de souris traitées au DON (12,5 mg/kg) ou à l'eau. La hauteur de chaque barre représente la taille du repas et la largeur représente sa durée.
B. Analyse de la microstructure des repas effectuée pendant la nuit suivant l'administration de DON ou d'eau.
*p < 0,05 et **p <0,01.

2.2.3 Activation neuronale induite par une intoxication aiguë au DON chez la souris

Nous avons ensuite identifié les structures cérébrales activées 3 heures après l'administration orale de DON à l'aide du marquage de la protéine c-Fos. Alors que les animaux traités à l'eau présentent un nombre très faible de cellules c-Fos-positives dans le tronc cérébral et le cerveau antérieur, ceux intoxiqués au DON présentent une forte activation neuronale dans le NTS, le locus coeruleus (LC), la partie latérale du noyau parabrachial (LPB), l'hypothalamus (PVN, SON, ARC) et le noyau central de l'amygdale (CeA). Les noyaux du Raphé, quant à eux, ne sont pas activés par l'intoxication au DON puisque aucune différence dans le nombre de neurones c-Fos-positifs n'a été observée entre les animaux traités à l'eau et ceux traités au DON **(Figure 33A et B)**. Dans, le NTS, l'ARC, le PVN et le SON, 24 à 58% des neurones

exprimant NUCB2/nesfatine-1 sont activés par le DON **(Figure 34A et B)**. De plus, en utilisant des souris exprimant la GFP dans les neurones POMC, nous avons également pu mettre en évidence que respectivement 40 et 16% des neurones POMC du NTS et de l'ARC sont activés suite à l'intoxication au DON **(Figure 35A et B)**.

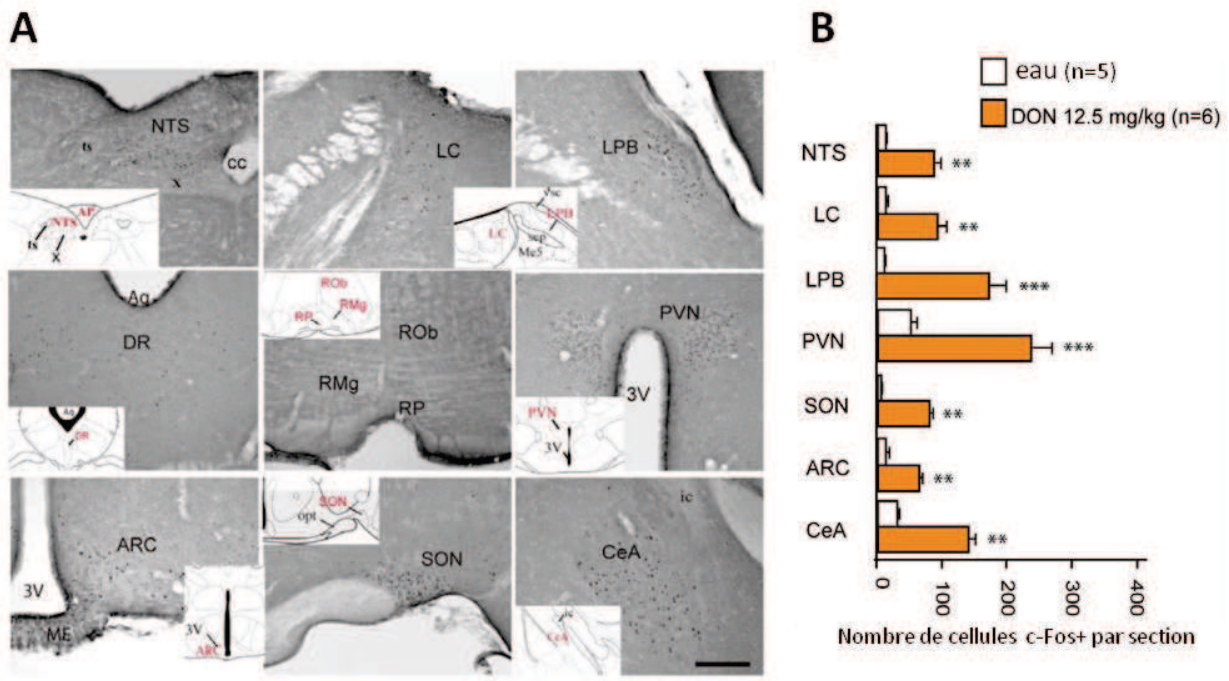


Figure 33 : Effet de l'intoxication orale au DON sur l'immunoréactivité de c-Fos.
A. Immunomarquage de c-Fos sur coupes coronales de cerveau de souris traitées au DON (12,5 mg/kg) et sacrifiées 3 heures après traitement. Barre d'échelle : 100 µm.
B. Quantification du nombre de cellules immunoréactives pour c-Fos dans le tronc cérébral, le pont et le cerveau antérieur chez des souris traitées au DON ou à l'eau. **p < 0,01 et ***p <0,001.

3V: troisième ventricule; AP: area postrema; ARC: noyau arqué; Aq: aqueduc de Sylvius; cc: canal central; CeA: amygdale centrale; DR: raphe dorsal; LPB: noyau parabrachial lateral; LC: locus coeruleus; ic: cortex insulaire; Me5: noyau trigeminal mésencephalique; NTS: noyau du tractus solitaire; opt: tractus optique; PVN: noyau paraventriculaire; RMg: raphe magnus; Rob: raphe obscursis; RP: raphe pallidus; scp: pédoncule cérébelleux supérieur; SON: noyau supraoptique; X: noyau dorsal moteur du nerf vague; ts: tractus solitaire; vsc: tractus spinocérébelleux ventral.

2.2.4 Activation des neurones exprimant la nesfatine-1 suite à une intoxication aiguë au DON chez le porc

Nous avons par la suite confirmé ce pattern d'activation neuronale chez le porc qui est l'espèce animale la plus sensible au DON parmi tous les animaux de ferme exposés à cette toxine (Trenholm et al., 1984). Comme chez la souris, des neurones du NTS, du PVN, de l'ARC et du SON activés chez le porc suite à la consommation de DON (1 mg/kg) présentent

un phénotype nesfatinergique **(Figure 36 à 39)**. C'est dans le SON que le pourcentage de colocalisation est le plus important puisque pratiquement tous les neurones c-Fos-positifs expriment NUCB2/nesfatine-1. Dans le PVN, ARC et NTS, le pourcentage de cellules c-Fos-positives immunoréactives pour NUCB2/nesfatine-1 est 40,6 ± 13,29%, 45,08 ± 6,62% et 35,72 ± 4,43% respectivement.

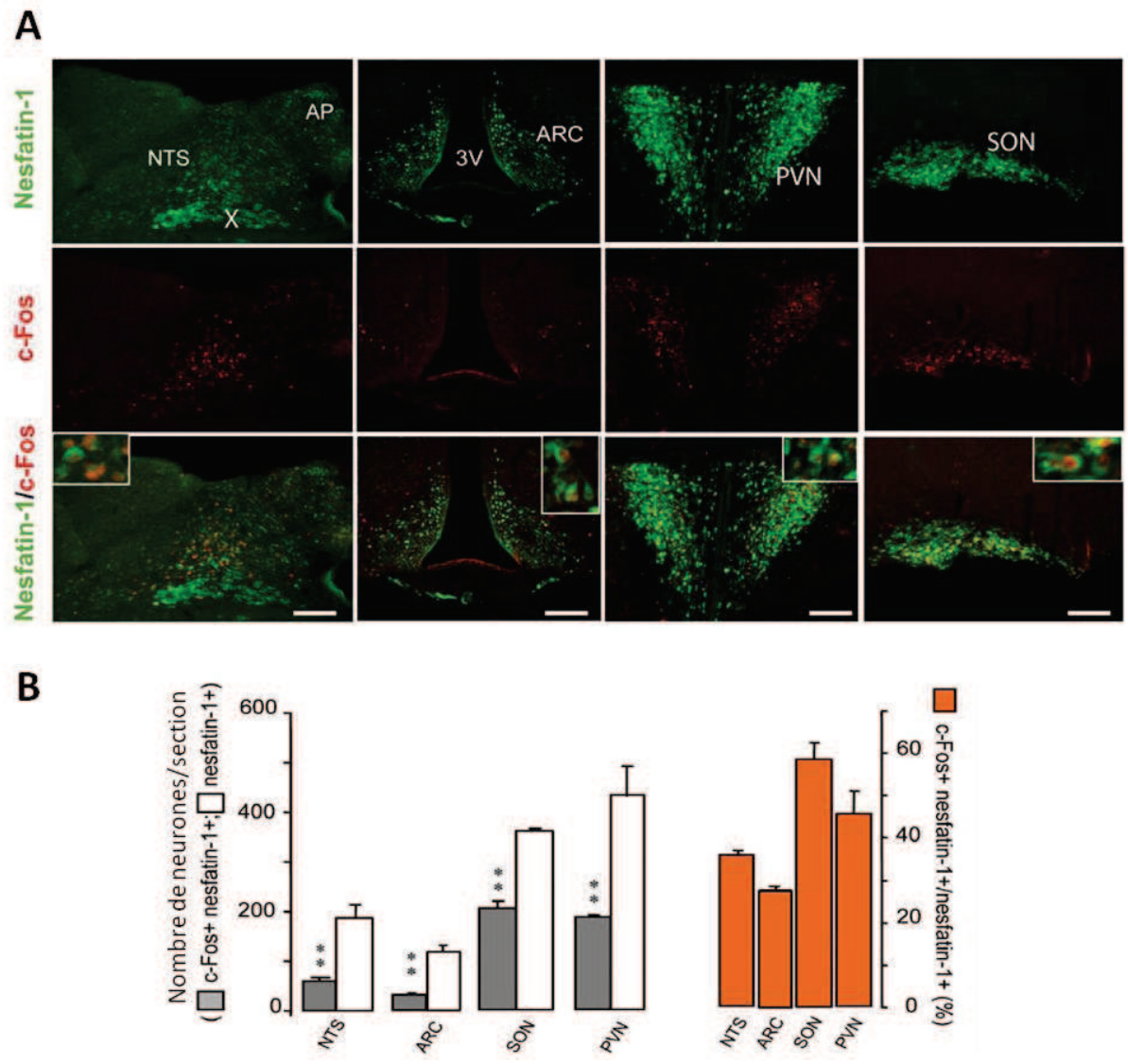


Figure 34 : Des neurones exprimant NUCB2/nesfatine-1 sont activés suite à l'intoxication au DON.
A. Double immunomarquage de NUCB2/nesfatine-1 (vert) et c-Fos (rouge) sur coupes coronales de tronc cérébral et d'hypothalamus de souris traitées oralement au DON (12,5 mg/kg) et sacrifiées 3 heures après traitement. Noter la présence de colocalisation (insert). Barre d'échelle : 200 µm.
B. Quantification du nombre de cellules immunoréactives pour NUCB2/nesfatine-1 et pour à la fois c-Fos et NUCB2/nesfatine-1. Les barres orange représentent le pourcentage de colocatisation dans chaque région. **p < 0,01 significativement différent des souris traitées à l'eau.

AP: area postrema; NTS: noyau du tractus solitaire; X: noyau dorsal moteur du nerf vague; ARC: noyau arqué; 3V: troisième ventricule; PVN: noyau paraventriculaire; SON: noyau supraoptique.

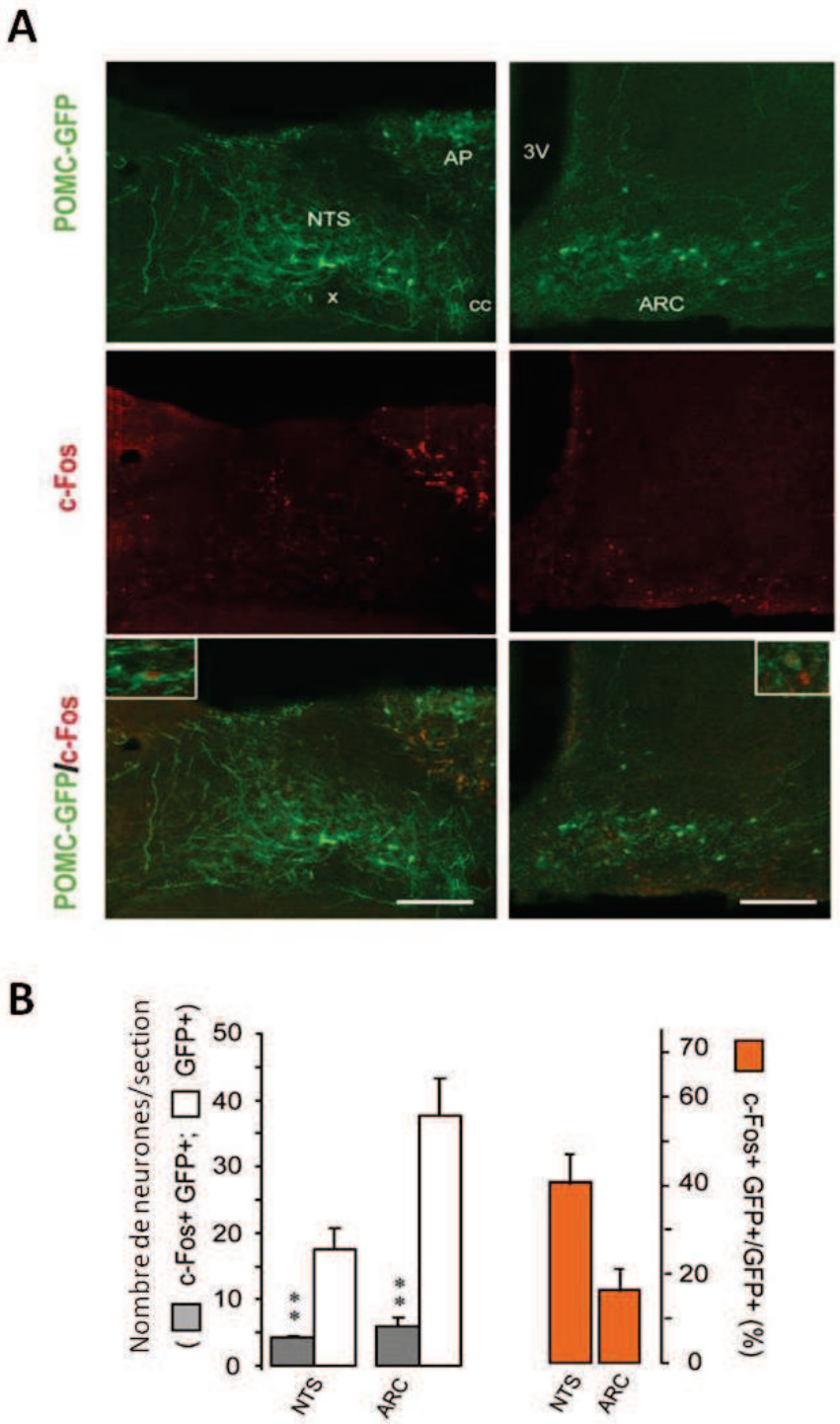


Figure 35 : Des neurones POMC sont activés suite à l'intoxication au DON.
A. Immunomarquage de c-Fos (rouge) sur coupes coronales de tronc cérébral et d'hypothalamus de souris POMC-GFP (vert) traitées oralement au DON (12,5 mg/kg) et sacrifiées 3 heures après traitement. Noter la présence de colocalisation (insert). Barre d'échelle : 150 µm.
B. Quantification du nombre de cellules immunoréactives pour la GFP et pour à la fois c-Fos et GFP. Les barres orange représentent le pourcentage de colocatisation dans chaque région. **p < 0,01 significativement différent des souris traitées à l'eau.

AP: area postrema; NTS: noyau du tractus solitaire; X: noyau dorsal moteur du nerf vague; cc: canal central ; ARC: noyau arqué; 3V: troisième ventricule.

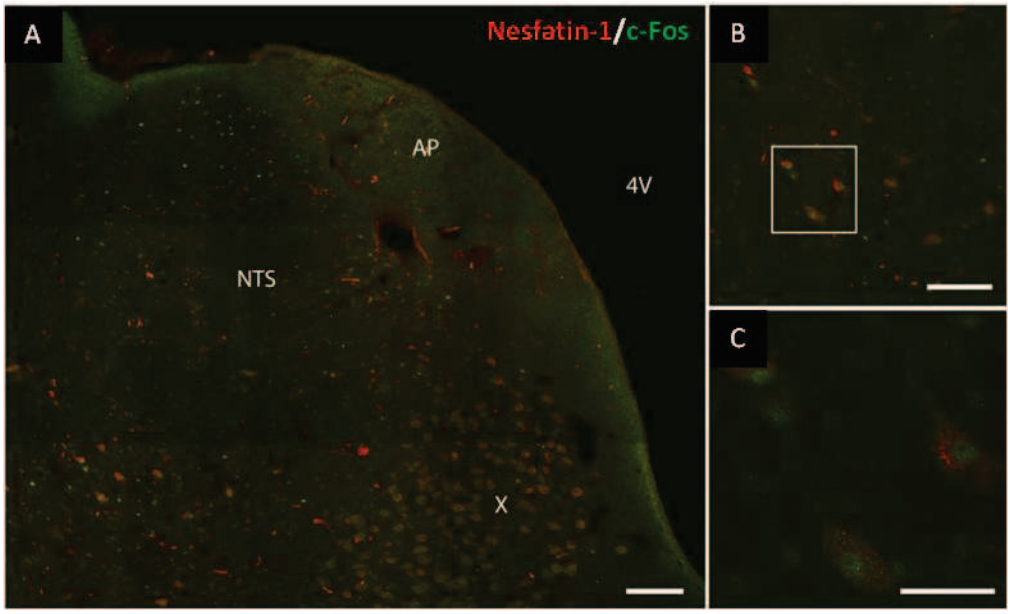


Figure 36 : Activation des neurones nesfatinergiques du NTS suite à l'intoxication au DON chez le porc.
Immunomarquage de c-Fos (vert) et NUCB2/nesfatine-1 (rouge) sur coupes coronales de tronc cérébral de porcs traités oralement au DON (1mg/kg) et sacrifiés 3 heures après traitement.
Barres d'échelles : 200 µm en A, 100 µm en B et 50 µm en C.

AP: area postrema; NTS: noyau du tractus solitaire; X: noyau dorsal moteur du nerf vague ; 4V: quatrième ventricule.

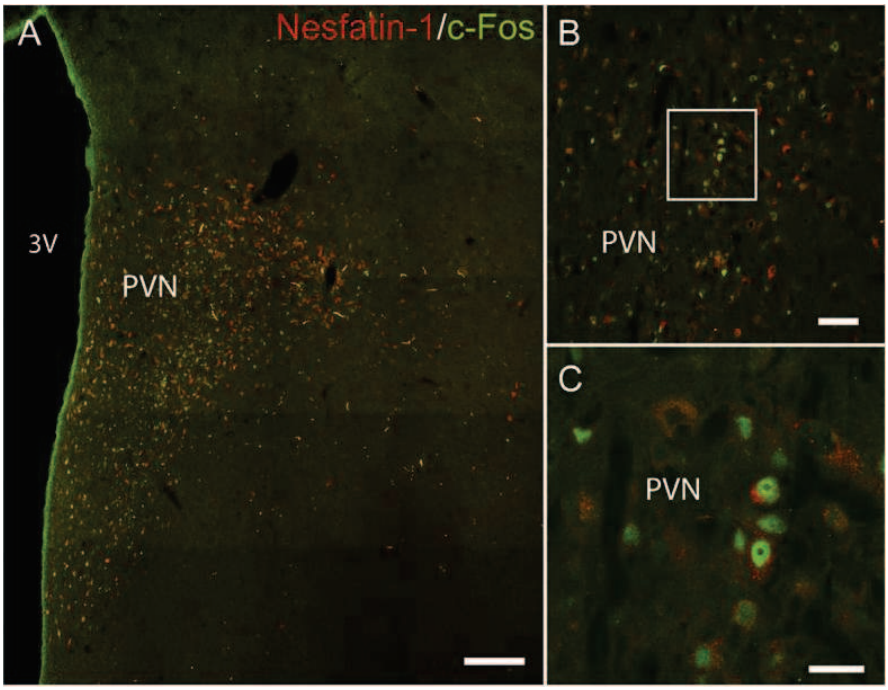


Figure 37: Activation des neurones nesfatinergiques du PVN suite à l'intoxication au DON chez le porc.
Immunomarquage de c-Fos (vert) et NUCB2/nesfatine-1 (rouge) sur coupes coronales d'hypothalamus (PVN) de porcs traités oralement au DON (1mg/kg) et sacrifiés 3 heures après traitement.
Barres d'échelles : 200 µm en A, 100 µm en B et 50 µm en C.

PVN : noyau paraventriculaire ; 3V : troisième ventricule.

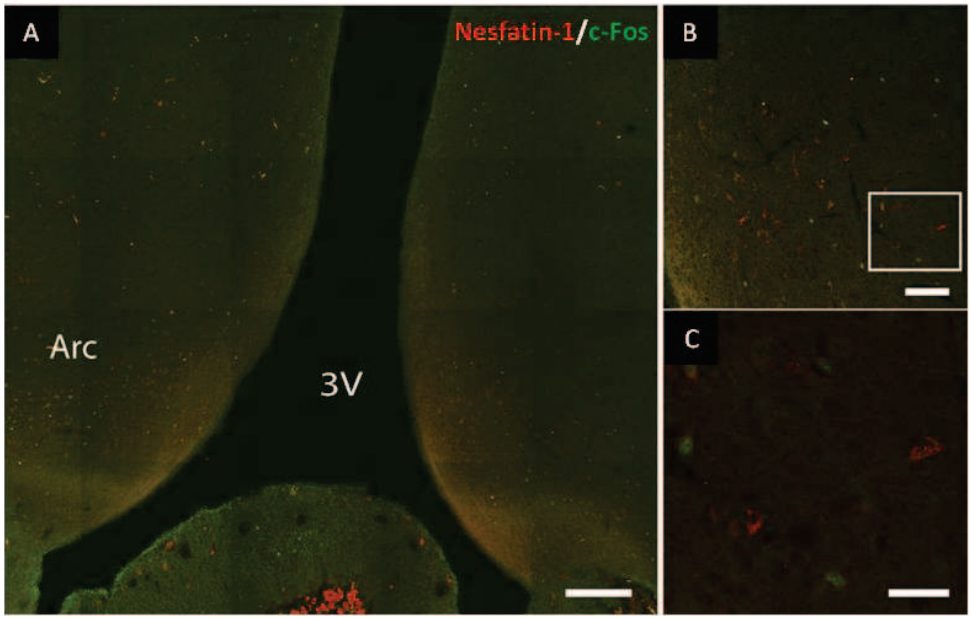


Figure 38 : Activation des neurones nesfatinergiques de l'ARC suite à l'intoxication au DON chez le porc.
Immunomarquage de c-Fos (vert) et NUCB2/nesfatine-1 (rouge) sur coupes coronales d'hypothalamus (ARC) de porcs traités oralement au DON (1mg/kg) et sacrifiés 3 heures après traitement.
Barres d'échelles : 200 µm en A, 100 µm en B et 50 µm en C.

ARC : noyau arqué ; 3V : troisième ventricule.

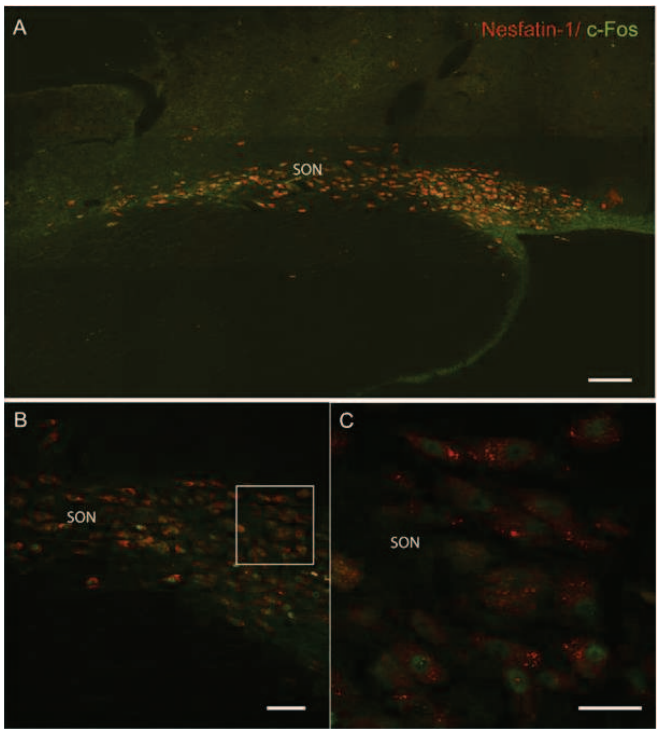


Figure 37: Activation des neurones nesfatinergiques du SON suite à l'intoxication au DON chez le porc.
Immunomarquage de c-Fos (vert) et NUCB2/nesfatine-1 (rouge) sur coupes coronales d'hypothalamus (SON) de porcs traités oralement au DON (1mg/kg) et sacrifiés 3 heures après traitement.
Barres d'échelles : 200 µm en A, 100 µm en B et 50 µm en C.

SON : noyau supraoptique.

2.2.5 Injection centrale de DON chez la souris

Afin de savoir si lors d'une intoxication alimentaire au DON celui-ci était capable d'être véhiculé dans le sang jusqu'au cerveau et d'agir directement sur les neurones, nous avons injecté le DON en icv à des souris canulées à demeure et à des faibles doses qui sont inefficaces lorsqu'elles sont administrées oralement (ventricule latéral, 2 et 20 μg/souris). Cette injection centrale de DON entraine rapidement une diminution dose-dépendante de la prise alimentaire comparé au souris contrôles traitées au NaCl **(Figure 40A)**. L'analyse de l'expression de c-Fos 1 heure après injection révèle un pattern d'activation neuronale similaire à celui observé suite à l'administration orale du DON, avec une activation des neurones anorexigènes nesfatine-1 et POMC de l'hypothalamus et du NTS **(Figure 40B)**.

Pour résumer, une intoxication au DON entraine une inflammation périphérique et centrale associée à des symptômes caractéristiques de la réaction de phase aiguë. Les neurones nesfatinergiques de l'hypothalamus et du NTS sont sensibles à l'administration orale de DON, mais également à son injection centrale, suggérant une action directe du DON sur le cerveau. Ainsi, la nesfatine-1 pourrait contribuer à la mise en place de l'anorexie induite par une intoxication au DON.

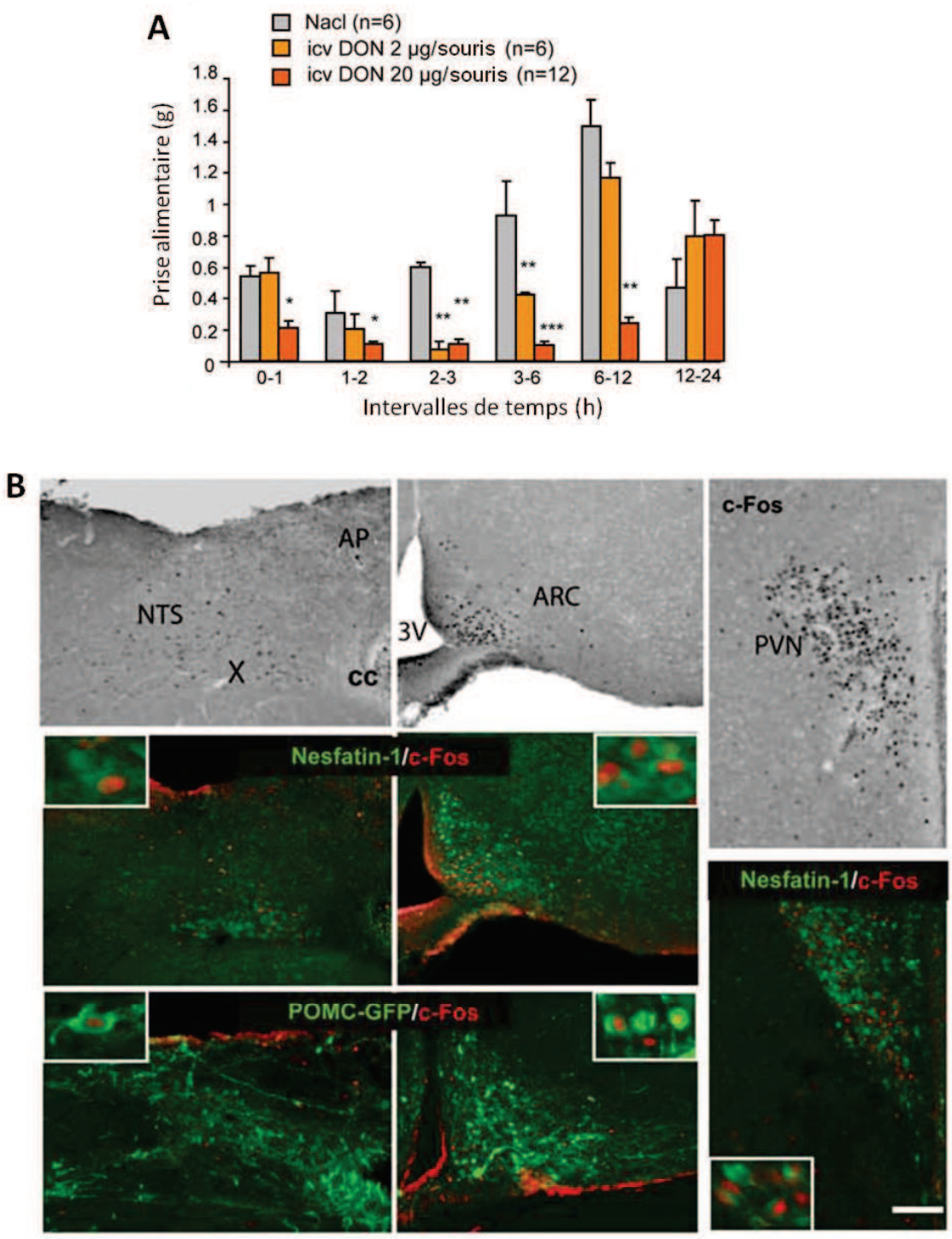


Figure 38 : Effet d'une injection icv de DON chez la souris.
A. Mesure de la prise alimentaire sur 24 heures de souris ayant reçu une injection icv (ventricule latéral) de NaCl ou de DON (2µg et 20µg/souris) en début de phase sombre. *p < 0,05 , **p < 0,01 et ***p <0,001
B. Marquage c-Fos, c-Fos/NUCB2/nesfatine-1 et c-Fos/POMC-GFP sur coupes coronales de tronc cérébral et d'hypothalamus de souris 1 heure après injection icv de DON (20µg/souris). Barre d'échelle : 100 µm.

AP: area postrema; NTS: noyau du tractus solitaire; X: noyau dorsal moteur du nerf vague; cc: canal central; ARC: noyau arqué; 3V: troisième ventricule; PVN: noyau paraventriculaire.

DISCUSSION

1. Neurones nesfatinergiques et hypoglycémie

Lors d'une hypoglycémie, l'organisme met en place une réponse métabolique et comportementale visant à rétablir la glycémie de base. Cette réponse, appelée contre-régulation, consiste notamment en une diminution de la sécrétion d'insuline, une augmentation de la sécrétion de glucagon et d'épinéphrine, ainsi qu'une stimulation de la prise alimentaire (Marty et al., 2007). Ces phénomènes sont sous le contrôle du système nerveux central. Au cours de ma thèse, nous avons cherché à savoir si les neurones exprimant NUCB2/nesfatine-1 participaient à la détection centrale de l'hypoglycémie et à la mise en place de la contre -régulation, hypothèse qui va à l'encontre des effets connus de la nesfatine-1.

1.1. La neurocircuiterie nesfatinergique est sensible à l'hypoglycémie

Nous avons démontré qu'une quantité non négligeable de neurones de l'hypothalamus et du CVD activés lors d'une hypoglycémie induite par une injection périphérique d'insuline exprime NUCB2/nesfatine-1. Cependant, l'insuline est connue pour agir directement en central et activer des structures cérébrales dont l'hypothalamus et le CVD (Carrasco et al., 2001). Pour s'assurer qu'il s'agissait bien de l'hypoglycémie et par conséquent de la glucoprivation cellulaire qui stimulait les neurones nesfatinergiques et non pas l'insuline elle-même, nous avons injecté un analogue non métabolisable du glucose, le 2-DG. L'administration de 2-DG est connue pour activer les neurones glucodétecteurs de l'hypothalamus et du CVD et mettre en place la contre-régulation (Briski, 2000; Molina et al., 1993; Ritter et al., 1998). Comme lors de l'injection d'insuline, le traitement périphérique ou central de 2-DG active une proportion non négligeable de neurones exprimant NUCB2/nesfatine-1, démontrant que les neurones nesfatinergiques de l'hypothalamus, et plus précisément du PVN, de la LHA et de l'ARC, ainsi que ceux du NTS et de DMNX sont sensibles à un déficit en glucose. En accord avec ces données, des neurones nesfatinergiques activés dans le NTS suite à l'hypoglycémie sont des neurones catécholaminergiques appartenant au groupe A2/C2 et des études ont montré que cette sous-population de neurones catécholaminergiques joue un rôle important dans les réponses hormonales et comportementales à l'hypoglycémie (Briski et Marshall, 2000; Ritter et al., 2006). Il est intéressant de noter qu'en plus d'être sensibles à l'hypoglycémie, ces neurones

catécholaminergiques sont connus pour projeter vers le cerveau antérieur et en particulier sur le PVN (Petrov et al., 1993). Ainsi, la nesfatine-1 pourrait être libérée centralement en réponse à l'hypoglycémie et contribuer à mettre en place la contre-régulation. Dans ce sens, une étude a montré que la nesfatine-1 module l'excitabilité des neurones glucodétecteurs du PVN, de la LHA et du VMH (Chen et al., 2012b). Notons que le VMH est une structure clé dans la détection de l'hypoglycémie et la mise en place de la contre-régulation. En effet, lors d'une hypoglycémie, la destruction du VMH ou la perfusion de celui-ci avec du glucose entraine une forte diminution de la réponse hormonale contre-régulatrice (Borg et al., 1994, 1997). Malgré sa forte implication dans la régulation de l'homéostasie glucidique, l'absence de neurones nesfatinergiques dans le VMH justifie le fait que nous l'ayons exclu de notre étude.

1.2. Possible implication dans la contre-régulation

Dans le DMNX, plus de 75% des neurones activés par l'hypoglycémie expriment NUCB2/nesfatine-1. De plus ces neurones nesfatinergiques sensibles à l'hypoglycémie sont des prémotoneurones vagaux puisqu'ils expriment la ChAT, suggérant qu'ils innervent des organes périphériques intervenant dans la contre-régulation. Nous montrons qu'ils projettent en effet sur l'estomac et le pancréas, probablement pour moduler la motilité et la vidange gastrique ainsi que les sécrétions hormonales. Ainsi, une libération de nesfatine-1 au niveau des ganglions intramuraux pourrait modifier les fonctions gastriques afin de provoquer un repas et stimuler la sécrétion de glucagon dans le but de rétablir la glycémie.

Nos résultats semblent en contradiction avec les effets connus de la nesfatine-1 sur la prise alimentaire et sur la glycémie puisque d'une part, l'hypoglycémie est un stimulus orexigène et non pas anorexigène, et d'autre part, une étude récente montre que les neurones nesfatinergiques du PVN sont sensibles à une augmentation de glucose (Gantulga et al., 2012). L'hypoglycémie n'est donc pas attendue comme activateur des neurones exprimant NUCB2/nesfatine-1. De plus, la nesfatine-1 améliore la glycémie de souris diabétiques en stimulant la sécrétion de l'insuline par les cellules β-pancréatiques en présence d'une forte concentration en glucose, ainsi que sa sensibilité (Gonzalez et al., 2011a; Su et al., 2010; Yang et al., 2012). Nos résultats suggèrent au contraire une action de la nesfatine-1 en réponse à l'hypoglycémie et notamment une possible stimulation de la libération de glucagon. Toutefois, les travaux de Riva et ses collaborateurs montrent que la nesfatine-1 peut stimuler

la sécrétion de glucagon par des îlots de Langerhans isolés de souris et que l'expression des ARNm de NUCB2 dans des îlots humains est corrélée positivement avec celle du glucagon (Riva et al., 2011). De plus, *in vitro*, la nesfatine-1 inhibe la capture du glucose par les cellules musculaires, résultat qui n'est cependant pas confirmé *in vivo* (Gonzalez et al., 2011a). Enfin, bien que la nesfatine-1 soit clairement identifiée comme un peptide anorexigène, il a été montré qu'elle était capable de dépolariser des neurones orexigènes exprimant le NPY dans l'ARC et le NTS (Mimee et al., 2012 ; Price et al., 2008b).

Nous montrons également que des neurones nesfatinergiques du DMNX activés par l'hypoglycémie projettent sur l'estomac. Peu de données sont disponibles quant au rôle de la nesfatine-1 sur l'estomac. Nous savons seulement qu'injectée centralement, elle diminue la vidange gastrique, la motilité gastro-duodénale et la sécrétion d'acide gastrique chez les rongeurs (Atsuchi et al., 2010; Goebel-Stengel et al., 2011; Stengel et al., 2009a; Xia et al., 2012). Cette action centrale de la nesfatine-1 ne peut cependant pas être extrapolée à nos résultats puisque nous supposons au contraire une libération de nesfatine-1 en périphérie et une action directe sur le système entérique.

Ainsi, les données de la littérature et nos résultats suggèrent la présence de plusieurs populations de neurones nesfatinergiques dans le cerveau, certains sensibles à l'hyperglycémie, d'autres à l'hypoglycémie. La nesfatine-1 semble donc exercer des effets multiples sur la régulation de la glycémie. Rappelons que le ou les récepteur(s) de la nesfatine-1 n'ont pas encore été identifiés. Il est donc possible que la nesfatine-1 agisse sur plusieurs récepteurs aux effets différents. Dans ce sens, une étude électrophysiologique a montré des effets opposés de la nesfatine-1 sur l'excitabilité des neurones du PVN avec parfois des dépolarisations et parfois des hyperpolarisations (Price et al., 2008a).

2. Neurones nesfatinergiques et inflammation

Comme nous l'avons vu dans l'introduction, lors d'une infection ou inflammation, les systèmes immunitaire, hormonal et nerveux interagissent étroitement pour mettre en place la réaction de phase aiguë visant à combattre l'inflammation. L'anorexie, qui est un des symptômes observés, résulte de l'activation de la neurocircuiterie anorexigène par les effecteurs inflammatoires. Suite à la découverte des effets anorexigènes de la nesfatine-1, nous avons recherché la sensibilité des neurones nesfatinergiques aux signaux inflammatoires en utilisant une injection périphérique de LPS chez le rat.

Nous avons utilisé une dose de LPS connue pour entrainer tous les symptômes de la réaction de phase aiguë à savoir la fièvre (Hare et al., 1995), le repli sur soi (Bret-Dibat et al., 1997) et l'anorexie (Langhans, 2007), ainsi que l'activation des structures cérébrales immunosensibles (Hare et al., 1995; Lacroix et Rivest, 1997). Nos résultats ont été obtenus dans une phase précoce de l'inflammation (3h après administration de LPS), lorsque l'anorexie est déjà installée. A ce stade, une proportion non négligeable des neurones de l'hypothalamus et du NTS activés par le LPS montre un phénotype nesfatinergique. Les neurones nesfatinergiques du DMNX ne sont quant à eux pas sensibles à l'inflammation, contrairement à l'hypoglycémie. Ces travaux dévoilent pour la première fois la sensibilité des neurones exprimant NUCB2/nesfatine-1 lors d'une inflammation et suggèrent l'implication de la nesfatine-1 dans l'anorexie inflammatoire. Alors que plusieurs études démontraient la sensibilité des neurones nesfatinergiques à des stimuli anorexigènes physiologiques tels que la CCK ou la réalimentation après un jeûne prolongé, aucune recherche n'avait encore été menée en condition inflammatoire (Kohno et al., 2008; Noetzel et al., 2009). Les neurones nesfatinergiques sont donc sollicités lors de stimuli anorexigènes physiologiques mais également pathologiques. Il est intéressant de noter que d'autres neuropeptides fortement impliqués dans la régulation physiologique de la prise alimentaire tels que POMC, CRH et 5-HT possèdent également cette caractéristique (Clement et al., 1997; Rorato et al., 2009).

De plus, nous montrons qu'une partie de ces neurones nesfatinergiques activés par l'inflammation dans le NTS exprime la TH. Or, Lacroix et Rivest ont rapporté une activation des neurones catécholaminergiques du NTS appartenant au groupe A2/C2 suite à l'administration de LPS (Lacroix et Rivest, 1997). Comme mentionné précédemment ces neurones catécholaminergiques projettent sur l'hypothalamus (Petrov et al., 1993). Ces résultats laissent penser que les neurones nesfatinergiques relaient le signal anorexigène vers l'hypothalamus. La nesfatine-1 pourrait donc être un effecteur de cette communication entre le CVD, qui est une porte d'entrée des signaux inflammatoires (Marvel et al., 2004), et les centres supérieurs. Ainsi, la nesfatine-1 pourrait être libérée centralement lors d'une inflammation et contribuer à la mise en place de l'anorexie inflammatoire. Cependant, il n'est pas exclu que les neurones nesfatinergiques activés lors de l'inflammation participent à la mise en place d'autres symptômes de la réaction de phase aiguë. Il a notamment été décrit des modifications de la fréquence cardiaque et de la pression artérielle lors d'une inflammation induite par le LPS (Xia et Krukoff, 2001). Or, des injections icv ou intra-NTS de nesfatine-1 entrainent une augmentation de la pression artérielle (Mimee et al., 2012; Yamawaki et al.,

2012; Yosten et Samson, 2009). De façon similaire, l'injection centrale de nesfatine-1 entraine une augmentation de la température corporelle ce qui suggère que ce peptide pourrait également contribuer à la mise en place de la fièvre (Könczöl et al., 2012).

A la suite de ces travaux, d'autres études sont venues étayer l'hypothèse que les neurones nesfatinergiques et la nesfatine-1 elle-même participent aux modifications physiologiques consécutives à l'inflammation. En effet, l'inflammation stimule la synthèse de nesfatine-1 en périphérie, et plus particulièrement dans le tissu adipeux et l'estomac (Ramanjaneya et al., 2010; Stengel et al., 2011). L'augmentation des taux circulants de nesfatine-1 lors d'une inflammation atteste qu'en plus de sa synthèse, sa sécrétion est également stimulée (Stengel et al., 2011). De façon contradictoire, il a également été proposé que la nesfatine-1 puisse exercer un effet anti-inflammatoire. Cette hypothèse s'appuie sur l'observation que la nesfatine-1 atténue la synthèse de cytokines inflammatoires dans le cerveau suite à des lésions cérébrales. Cet effet est observable 12 heures après la lésion et perdure plusieurs jours (Özsavcí et al., 2011; Tang et al., 2012). A la lumière de ces données, il est possible d'envisager que la nesfatine-1 puisse d'une part contribuer précocement à la mise en place de symptômes de la réaction de phase aiguë dont l'anorexie inflammatoire, mais puisse également aider à combattre cette inflammation plus tardivement grâce à ces propriétés anti-inflammatoires. Enfin, trois études ont également été menées chez des patients souffrant d'un syndrome anorexie-cachexie lié à une inflammation chronique. Les patients atteints de mucoviscidose présentent des taux circulants de nesfatine-1 plus élevés que des individus sains (Cohen et al., 2013). A l'inverse, chez des patients atteints d'un cancer du foie la concentration plasmatique en nesfatine-1 est plus faible (Cetinkaya et al., 2012). Enfin, les individus souffrant d'insuffisance rénale ne présentent pas de modification de la concentration en nesfatine-1 dans le sang (Saldanha et al., 2012). Même si les données obtenues lors d'inflammations chroniques sont contrastées, ces résultats confortent l'idée de l'intervention de la nesfatine-1 dans les processus inflammatoires. Au total, malgré de nombreuses zones d'ombres quant au rôle exact et au mode d'action de la nesfatine-1, l'ensemble des résultats obtenus permet de considérer les neurones nesfatinergiques et la nesfatine-1 comme des effecteurs de la réaction inflammatoire.

3. Neurones nesfatinergiques et intoxication au DON

Afin d'approfondir la caractérisation des neurones exprimant NUCB2/nesfatine-1 en condition pathologique, nous avons entrepris de mettre en évidence la sensibilité de ces neurones nesfatinergiques à une intoxication au DON, l'une des toxines d'origine fongique retrouvée le plus fréquemment dans l'alimentation humaine et des animaux d'élevage. Des études ont montré que des souris, rats, chiens, chats et hommes intoxiqués au DON de manière aiguë ou chronique présentent une anorexie associée à une perte de poids (Arnold et al., 1986a; Bryden, 2007; Flannery et al., 2011; Hughes et al., 1999; Robbana-Barnat et al., 1987)

3.1. Caractérisation des effets d'une intoxication au DON

Devant l'absence de données quant aux mécanismes d'action du DON, nous avons entrepris une caractérisation des effets de cette toxine. Pour réaliser cette étude, nous avons choisi comme modèle la souris. Bien que n'étant pas le modèle classiquement utilisé pour l'analyse de la toxicité au DON, plusieurs travaux ont déjà été menés chez cette espèce (Amuzie et al., 2011; Arnold et al., 1986b; Flannery et al., 2011). Nous montrons que l'injection orale de DON affecte l'activité locomotrice, la température corporelle et la prise alimentaire des souris. Ainsi l'ensemble de ces symptômes rappelle le comportement de maladie classiquement observé lors d'une infection ou inflammation (Hart, 1988). Conformément à cette hypothèse, l'intoxication de souris au DON stimule la production de cytokines (IL-1β, IL-6 et TNF-α) dans le foie, la rate, les reins, l'intestin grêle et les plaques de Peyer (constituants du tissu lymphoïde associé à l'intestin) 1 à 4 heures après l'administration de la toxine (Azcona-Olivera et al., 1995; Pestka et Amuzie, 2008). De plus, nous montrons pour la première fois une inflammation centrale avec une augmentation de l'expression de ces cytokines et des enzymes de synthèse de la PGE2 dans l'hypothalamus et le CVD à 3 heures post-intoxication.

Dans ce contexte nous nous sommes particulièrement attachés à caractériser les mécanismes à l'origine de l'anorexie. Pour cela, nous avons effectué une analyse de la microstructure des repas grâce à un système d'enregistrement continu et automatisé permettant de décrire de manière précise le comportement alimentaire des animaux. Ces enregistrements mettent en évidence une diminution à la fois de la fréquence et de la taille des

repas, associée à une augmentation de l'intervalle entre les repas et du ratio de satiété. La diminution de la fréquence des repas, qui est souvent observée lors d'administration de composés toxiques tels que le chlorure de lithium ou le LPS, suggère la présence d'un état nauséeux (Porter et al., 1998a; West et al., 1987). Nous avons testé cette hypothèse d'un état nauséeux chez la souris en utilisant le comportement de « Pica » qui correspond à la consommation d'une substance non nutritive : le Kaolin. Il est à noter que la souris, bien que pouvant ressentir des nausées, n'a pas la faculté de vomir (Yamamoto et al., 2002). Comme attendu, le DON augmente la consommation de Kaolin chez la souris. Une étude récente vient étayer nos résultats en montrant que l'administration de DON entraine une augmentation des taux de PYY^{3-36} et 5-HT dans le sang et des antagonistes des récepteurs de ces hormones atténuent l'effet nauséeux du DON (Wu et al., 2013). La diminution de la taille des repas, quant à elle, évoque un rassasiement précoce classiquement retrouvé avec des inhibiteurs physiologiques de la prise alimentaire tels que la CCK, la leptine, l'insuline et le PYY^{3-36} (Flynn et al., 1998; Hsiao et Wang, 1983; le Roux et al., 2006; VanderWeele, 1994). Cette donnée suggère que le DON agit également sur les mécanismes physiologiques mettant fin au repas. Récemment, Flannery et ces collaborateurs ont d'ailleurs montré une augmentation des taux plasmatiques des effecteurs de la prise alimentaire tels que le PYY^{3-36} et la CCK après injection orale ou ip de DON (Flannery et al., 2012). De plus, le blocage pharmacologique des récepteurs de ces deux hormones indique que l'effet anorexigène est en partie relayé par le PYY^{3-36} mais pas la CCK. Les taux de GLP-1, leptine, amyline et polypeptide pancréatique ne sont pas modifiés (Flannery et al., 2012). Ainsi, nos résultats montrent un effet complexe du DON sur la prise alimentaire avec un effet sur la satiété caractéristique d'une anorexie « pathologique » et un effet sur le rassasiement caractéristique d'une anorexie « physiologique ». L'inflammation induite par le DON n'explique donc pas totalement la diminution de la prise alimentaire observée suite à une intoxication orale au DON.

3.2. Activation neuronale en réponse à une intoxication au DON

Afin de caractériser les mécanismes sous-tendant cette anorexie, nous avons dans un premier temps décrit le pattern d'activation neuronale chez la souris puis chez le porc après intoxication au DON. Le travail réalisé sur le porc a été fait en collaboration avec l'unité TOXALIM de l'INRA de Toulouse. Le sacrifice des animaux a été réalisé 3 heures après intoxication, alors que l'anorexie et les autres symptômes sont installés. De façon intéressante,

l'activation neuronale induite par le DON est spécifique et limitée à quelques structures centrales. Ce pattern d'activation est similaire à celui observé lors d'une inflammation au LPS et suggère une réponse comportementale, autonome et endocrine (Elmquist et Saper, 1996; Lacroix et Rivest, 1997). L'activation des neurones de l'AP pourrait expliquer les nausées et l'induction de c-Fos dans le SON, le PVN, l'aire préoptique antérieure de l'hypothalamus, le LC et le NTS pourrait contribuer à la diminution de la température corporelle (Boulant, 2000; Takahashi et al., 2001). Enfin, l'anorexie induite par le DON pourrait s'expliquer par la forte induction de c-Fos dans les structures impliquées dans la régulation de la prise alimentaire telles que le NTS, l'ARC et le PVN.

De façon plus précise, nous montrons que plus d'un quart des neurones activés dans l'hypothalamus (ARC, PVN, SON) et le NTS expriment NUCB2/nesfatine-1 lors d'une intoxication au DON. Ce résultat a été obtenu aussi bien chez la souris que chez le porc. Des neurones POMC de l'ARC et du NTS sont également activés par le DON. Ces résultats laissent supposer une libération conjointe de nesfatine-1 et d'α-MSH pouvant contribuer à l'anorexie induite par le DON. De manière intéressante, nous savons que la nesfatine-1 agit en recrutant des neurones POMC ce qui pourrait faire de la voie mélanocortinergique l'effecteur principal de l'anorexie mise en place par le DON (Maejima et al., 2009). L'utilisation d'antagonistes des récepteurs MC3/4-R permettra de tester cette hypothèse. Ainsi, nos résultats montrent que le DON stimule des réseaux neuronaux dévolus à la régulation de la balance énergétique. De façon surprenante, 3 heures après intoxication, nous n'avons pas observé d'activation neuronale dans les noyaux du Raphé qui possèdent les corps cellulaires des neurones sérotoninergiques. Or, nous savons que la 5-HT exerce un important rôle anorexigène et émétique. De plus, des études ont montré une augmentation de la concentration en 5-HT dans le cerveau de rats et de porcs intoxiqués au DON (Fitzpatrick et al., 1988; Swamy et al., 2004). Chez le porc et le vison, le blocage pharmacologique des récepteurs 5-HT empêche les vomissements induits par le DON. Les auteurs n'ont cependant pas étudié l'effet de cet antagoniste sur la réponse alimentaire au DON (Prelusky et Trenholm, 1993; Wu et al., 2013). Cette divergence dans nos résultats pourrait s'expliquer par le moment où les animaux ont été sacrifiés ou par une source périphérique de 5-HT.

3.3. Le DON agit directement sur le cerveau

Bien que l'inflammation induite par le DON soit incontestable, elle n'explique pas totalement la diminution de prise alimentaire. En effet, les souris dépourvues d'IL-6, du récepteur au TNF-α ou de la mPGES-1 présentent une anorexie en réponse au DON similaire à celle des souris sauvages (Girardet et al., 2011; Pestka et Zhou, 2000, 2002). Il est donc concevable que cette toxine soit véhiculée dans le sang jusqu'au cerveau où elle pourrait modifier directement la fonction neuronale. Pour appuyer cette hypothèse, le DON a été détecté dans le LCR de porc et de mouton rapidement (moins de 2 min 30) après son administration intraveineuse (Prelusky et al., 1990). Il a également été détecté dans des extraits de cerveaux de souris exposées oralement au DON 5 min après l'intoxication (Pestka et al., 2008). Dans le cerveau, la concentration du DON est cependant beaucoup plus faible que dans les autres organes (foie, rate, cœur, reins), probablement à cause d'une faible perméabilité de la BHE à cette toxine (Pestka et al., 2008). Le DON peut cependant infiltrer le cerveau par les organes circumventriculaires (AP et EM) qui présentent justement une augmentation de l'expression de c-Fos après administration orale du DON, de même que les structures alentours (NTS et ARC).

Afin de confirmer l'action directe du DON sur le cerveau, nous l'avons injecté centralement à des doses inefficaces en périphérie mais compatibles avec les niveaux de DON détectés dans le cerveau après administration orale. L'injection icv de DON chez la souris est capable de diminuer la prise alimentaire de manière dose-dépendante, l'activité locomotrice et la température corporelle comme lors de l'administration orale. Le pattern d'expression de c-Fos après injection centrale est similaire à celui observé après l'administration périphérique de la toxine et comme précédemment, des neurones POMC et NUCB2/nesfatine-1 sont activés dans l'hypothalamus et le NTS. Ces résultats suggèrent donc que le DON est capable de cibler directement le cerveau pour modifier la prise alimentaire en agissant sur la neurocircuiterie anorexigène dont la nesfatine-1.

CONCLUSION

A ce jour, la nesfatine-1 a incontestablement rejoint la liste des neuropeptides anorexigènes. Ses fonctions et modes d'action restent cependant méconnus. Mon travail de thèse apporte des informations nouvelles sur la capacité des neurones nesfatinergiques à être recrutés dans différentes conditions. D'une part, ces neurones participent à l'arrêt du repas en condition physiologique (Kohno et al., 2008; Noetzel et al., 2009) mais nous montrons qu'ils sont également mis en jeu dans l'anorexie inflammatoire et l'anorexie induite par une intoxication alimentaire. Ils sont également impliqués dans l'homéostasie glucidique et nous montrons qu'en plus d'être sensibles au glucose (Gantulga et al., 2012), ils sont capables de détecter l'hypoglycémie, un signal orexigène, démontrant la complexité de ce peptide au point de vue fonctionnel **(Figure 39)**. Notons cependant que nous ne montrons pas directement la libération de nesfatine-1 par les neurones nesfatinergiques en réponse à ces stimuli. Bien qu'aucune preuve directe n'ait été avancée, plusieurs données de la littérature permettent de suggérer que la nesfatine-1 puisse être sécrétée par les neurones : 1/ elle est présente dans des vésicules de sécrétion dans des neurones du PVN (Maejima et al., 2009), 2/ elle a été retrouvée dans le LCR et l'injection d'anticorps neutralisant la nesfatine-1 a un effet stimulateur sur la prise alimentaire (Oh-I et al., 2006), 3/ sa libération a été démontrée en périphérie par les cellules béta du pancréas et les adipocytes (Gonzalez et al., 2011b; Ramanjaneya et al., 2010). Il sera important par la suite de démontrer cette libération neuronale de nesfatine-1.

Ainsi, le cerveau semble posséder plusieurs populations de neurones nesfatinergiques aux rôles variés. Des neurones nesfatinergiques sont d'ailleurs sensibles à des signaux très éloignés de la régulation de l'homéostasie énergétique tels que le stress et le sommeil (Goebel et al., 2009; Jego et al., 2012; Xu et al., 2010). La nesfatine-1 n'est pas le seul neuropeptide alimentaire sollicité dans d'autres fonctions physiologiques : par exemple, les neuropeptides anorexigènes CRH et vasopressine sont respectivement impliqués dans le stress et l'équilibre hydrominéral. Parmi les neuropeptides orexigènes, la MCH est également impliquée dans l'humeur et la pigmentation de la peau, et les orexines régulent l'état d'éveil. Une des explications possibles à ces effets multiples de la nesfatine-1 serait que les structures cibles des neurones nesfatinergiques recrutés puissent être différentes selon les stimuli mis en jeu. D'autre part, rappelons que les récepteurs de la nesfatine-1 n'ont pas encore été identifiés à ce jour et il est donc concevable que la nesfatine-1 puisse agir sur différents récepteurs aux effets distincts.

L'absence d'agents pharmacologiques modulant les effets de la nesfatine-1 n'a pas permis d'apporter à cette étude plus d'informations sur le rôle fonctionnel de ce nouveau peptide en réponse aux stimuli testés, mais nous montrons néanmoins pour la première fois la mise en jeu de la nesfatine-1 centrale dans l'hypoglycémie, l'inflammation et l'intoxication alimentaire. De plus, l'étude menée sur le déoxynivalénol constitue la première mise en évidence d'une action centrale de cette toxine.

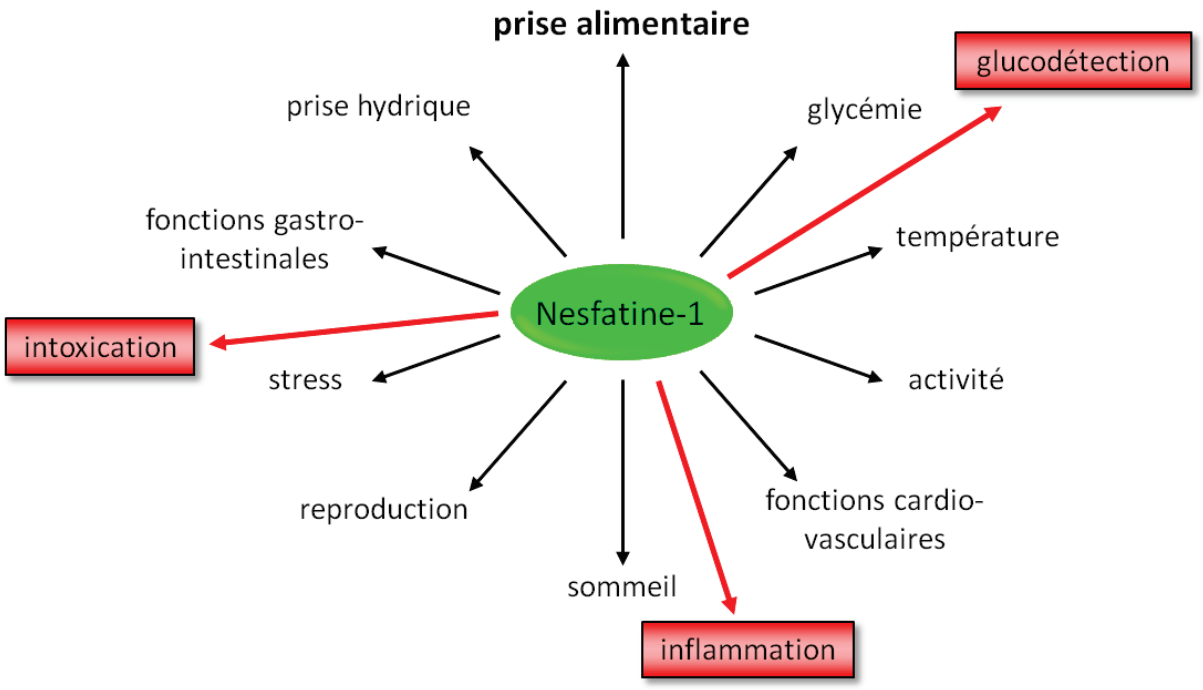


Figure 39 : Contribution de mon travail de thèse à l'avancé des connaissances sur la nesfatine-1.
La nesfatine-1 est impliquée de nombreuses fonctions physiologiques (Cf. : introduction). Mon travail de thèse permet d'ajouter à cette liste la glucodétection, l'inflammation et l'intoxication alimentaire par le DON.

PERSPECTIVES

A la lumière de mes travaux de thèse et des données de la littérature, la nesfatine-1 apparait comme un peptide mis en jeu dans la régulation de la prise alimentaire en condition physiologique et pathologique et dans le maintien de l'homéostasie glucidique. Afin d'approfondir les connaissances sur ce peptide et en particulier sur ses rôles et modes d'action en central, je propose de développer trois projets de recherche :

- la neutralisation de la nesfatine-1 endogène,
- la synthèse et l'étude de peptides dérivés de la nesfatine-1 résistants à la protéolyse,
- l'implication des cellules gliales dans la signalisation nesfatinergique.

1. Neutralisation de la nesfatine-1 endogène

Comme mentionné précédemment, les récepteurs à la nesfatine-1 n'ayant pas encore été identifiés, il n'existe pas d'agonistes ou d'antagonistes permettant de moduler ses effets. Cette absence d'agents pharmacologiques limite donc considérablement les études menées sur ce peptide. Quelques équipes de recherches ont pallié à ce problème en utilisant des oligonucléotides morpholinos antisens (as-MON) empêchant la traduction de la nesfatine-1. Par ailleurs, l'injection d'anticorps dirigés contre la nesfatine-1 pourrait neutraliser celle-ci et donc constituer un outil capable d'inhiber ses effets sans agir directement sur les récepteurs. Oh-I et ces collaborateurs avaient en effet montré que l'administration centrale d'IgG Ab24 dirigés contre la nesfatine-1 stimulait la prise alimentaire, suggérant une inhibition de la nesfatine-1 endogène (Oh-I et al., 2006). C'est cette approche que je propose d'utiliser afin d'apporter à notre étude un aspect fonctionnel.

1.1. Validation du modèle

Les anticorps anti-nesfatine-1 disponibles dans le commerce ne sont pas les mêmes que ceux utilisés par Oh-I et ses collaborateurs. La première étape consiste donc à s'assurer que ces anticorps bloquent la nesfatine-1 comme l'IgG Ab24. Pour cela nous injecterons les anticorps centralement comme l'avait fait Oh-I et ses collaborateurs et nous mesurerons la prise alimentaire. Si l'anticorps bloque effectivement la nesfatine-1, nous nous attendons à une augmentation de la prise alimentaire des animaux.

1.2. Neutralisation de la nesfatine-1 lors d'une hypoglycémie

Dans ce travail de thèse, nous avons observé que des neurones nesfatinergiques sont activés en réponse à l'hypoglycémie et nous postulons que ces neurones contribuent à mettre en place la contre-régulation en libérant de la nesfatine-1 en central mais également en périphérie, au niveau préganglionnaire. Nous proposons donc d'injecter des anticorps anti-nesfatine-1 afin de neutraliser ses effets. Nous focaliserons notre étude sur des injections centrales car celles-ci devraient nous permettre de bloquer la nesfatine-1 potentiellement libérée par les neurones nesfatinergiques. Nous pourrons ainsi appréhender le rôle de la nesfatine-1 dans la réponse centrale à l'hypoglycémie. Parallèlement nous envisageons d'injecter les anticorps en ip afin de bloquer la nesfatine-1 libérée par les efférences vagales. Toutefois, dans ces conditions, nous ne pourrons pas exclure un blocage de la nesfatine-1 libérée par d'autres organes périphériques, ce qui rendra l'interprétation des résultats plus délicate. Ainsi, afin d'évaluer l'impact du blocage de la nesfatine-1 centrale ou périphérique lors d'une hypoglycémie, différents paramètres seront analysés en présence ou en absence de ces anticorps anti-nesfatine-1 :

1/ L'évolution de la glycémie au cours du temps sera mesurée après l'injection d'insuline dans le but de comparer le temps nécessaire au retour de la glycémie de base lorsque la nesfatine-1 est neutralisée ou non.

2/ La sécrétion de glucagon et d'insuline : lors d'une hypoglycémie, la sécrétion de glucagon augmente alors que celle d'insuline est inhibée. Nous mesurerons donc les taux plasmatiques de ces deux hormones grâce à des tests ELISA après blocage de la nesfatine-1 pour savoir si elle participe à ce phénomène. Rappelons que les données de la littérature concernant le rôle de la nesfatine-1 sur les sécrétions hormonales sont contradictoires puisqu'il a été montré que celle-ci stimule à la fois la sécrétion d'insuline et de glucagon (Riva et al., 2011).

3/ La vidange gastrique : l'hypoglycémie stimule la vidange gastrique afin de favoriser la prise de nourriture. Bien que la nesfatine-1 exerce au contraire une action inhibitrice sur la vidange gastrique lorsqu'elle est injectée en central (Stengel et al., 2009a), il serait intéressant d'analyser ce paramètre au cours de l'hypoglycémie en présence d'anticorps anti-nesfatine-1. Il est notamment envisageable un rôle différentiel de la nesfatine-1 centrale et de la nesfatine-1 libérée directement sur l'estomac sur ce paramètre.

4/ La prise alimentaire pourra également être mesurée afin de voir l'influence de la nesfatine-1 sur l'ingestion au cours d'un épisode d'hypoglycémie.

1.3. Neutralisation de la nesfatine-1 lors d'une inflammation et d'une intoxication

Nous montrons également une activation des neurones nesfatinergiques lors de l'inflammation induite par le LPS et lors d'une intoxication au DON. De façon similaire à la démarche proposée pour l'étude des effets de la nesfatine-1 en réponse à l'hypoglycémie, nous envisageons d'administrer des anticorps anti-nesfatine-1 en central en contexte inflammatoire. L'objectif est d'analyser les réponses comportementales d'animaux soumis à une inflammation ou une intoxication au DON en recherchant des modifications dans les différentes composantes de la réaction de phase aiguë. L'ensemble de ces résultats permettra de confirmer ou d'infirmer notre hypothèse selon laquelle la nesfatine-1 contribuerait à l'anorexie inflammatoire ou à d'autres symptômes comme la fièvre.

2. Synthèse et étude de peptides dérivés de la Nesfatine-1 résistants à la protéolyse

Comme décrit précédemment, le diabète de type 2 est un problème majeur de santé publique à cause de sa prévalence élevée et de l'importance de ses complications cardiovasculaires et neurodégénératives. Il se caractérise par l'association d'un déficit de sécrétion d'insuline et d'une insulino-résistance. De plus, cette pathologie est souvent associée à une hyperphagie et une obésité. Bien que de nombreux traitements aident à contrôler la glycémie, ils n'agissent cependant pas de manière combinée sur la fonction de la cellule beta, la sensibilité à l'insuline et la prise alimentaire. La nesfatine-1, qui possède ces trois propriétés, semble alors être un excellent candidat pour le traitement du diabète de type 2.

Notre projet vise à synthétiser des peptides de petite taille à partir du fragment M30, le segment actif de la nesfatine-1, et de tester leur efficacité *in vitro* et *in vivo* sur la sécrétion d'insuline, le contrôle de l'homéostasie glucidique et la prise alimentaire. De plus, nous souhaitons modifier ces peptides pour leur permettre de résister à la protéolyse par la plasmine. Par cette approche, nous souhaitons identifier un ou plusieurs peptides susceptibles

de présenter des caractéristiques pharmacocinétiques et pharmacodynamiques supérieures au M30, et combinant une action insulinotropique et un effet « anti-obésité ». A terme ces composés pourraient permettre d'améliorer la glycémie des patients souffrant de diabète de type 2 et d'atténuer les symptômes aggravant la maladie tels que l'hyperphagie et la prise de poids.

2.1. Synthèse des peptides

Des peptides chevauchant et couvrant la séquence du fragment M30 seront synthétisés par voie chimique en collaboration avec le Dr Kamel Mabrouk de l'Institut de Chimie Radicalaire (équipe Chimie Radicalaire Organique et Polymères de Spécialité (CROPS), CNRS UMR 7273, Aix-Marseille Université). Une première série de peptides courts issus du M30 a déjà été synthétisée **(Figure 42)**. L'activité biologique *in vitro* et *in vivo* sur l'homéostasie glucidique et énergétique des peptides ainsi générés est actuellement en cours d'analyse. Cette activité biologique sera comparée à celle obtenue avec le fragment M30 entier et la nesfatine-1. Des analogues structuraux de ces peptides résistants à la protéolyse seront ensuite générés en remplaçant certains acides aminés par leur forme dextrogyre (Hamamoto et al., 2002). La résistance à la plasmine des peptides ainsi générés sera d'abord confirmée *in vitro* puis les peptides courts montrant une résistance à la protéolyse seront ensuite testés pour leur activité biologique *in vitro* et *in vivo*.

M30 : PDTGLYYDEYLKQVIEVLETDPHFREKLQK
M24 : YDEYLKQVIEVLETDPHFREKLQK
M19 : KQVIEVLETDPHFREKLQK
M14 : VLETDPHFREKLQK

Figure 40 : Structures primaires des peptides synthétisés dérivés de la séquence M30.

2.2. Evaluation *in vitro* de l'efficacité des nouveaux peptides issus du M30

Afin d'évaluer l'effet de ces nouveaux peptides *in vitro*, nous utiliserons une lignée de cellules béta pancréatiques : les cellules MIN6. Ces cellules sont connues pour mimer parfaitement le fonctionnement des cellules béta normales (Minami et al., 2000). Il a ainsi été

montré que les cellules MIN6 présentaient une réponse à la nesfatine-1 semblable à celle observée avec des cultures primaires de cellules béta (Gonzalez et al., 2011b) validant cette lignée comme modèle dans l'étude des effets des peptides générés dans le cadre du présent projet. Pour chaque peptide produit nous testerons :

1/ L'effet sur la sécrétion d'insuline : l'effet de doses croissantes de peptides issus du M30 sur la sécrétion d'insuline par les cellules MIN6 sera mesuré en condition basale (concentration faible de glucose) et en condition mimant l'hyperglycémie post-prandiale (concentration élevée de glucose). Pour cela, les cellules MIN6 seront cultivées dans des plaques de culture 12 puits pendant 24 h avant stimulation. Suite à l'ajout de peptides en présence de concentration basale ou élevée en glucose, la sécrétion d'insuline dans le milieu sera directement dosée à l'aide d'un test ELISA. Les effets de la nesfatine-1 et du M30 seront également testés pour comparaison avec les peptides générés et avec les résultats déjà publiés (Gonzalez et al., 2011b).

2/ L'effet sur la concentration en calcium intracellulaire : une étude récente a montré que l'effet de la nesfatine-1 sur la sécrétion d'insuline par les cellules béta passait par une élévation de la concentration en calcium intracellulaire (Nakata et al., 2011). Nous proposons donc, en parallèle des études de sécrétion d'insuline, d'étudier les effets des peptides sur la mobilisation calcique. Pour cela, les cellules MIN6 seront cultivées sur lamelles de verre afin de les observer en microscopie à fluorescence. Les cellules seront incubées avec la sonde calcique Fura-2 puis stimulées à l'aide des peptides en présence de concentrations basales ou élevées en glucose. La nesfatine-1 entière et le M30 seront également testés dans nos conditions expérimentales et leurs effets seront comparés aux résultats obtenus avec les peptides générés dans ce projet.

2.3. Evaluation *in vivo* de l'efficacité des nouveaux peptides issus du M30

Dans un modèle murin, nous évaluerons l'impact des peptides courts nouvellement synthétisés et leurs analogues structuraux protégés de la protéolyse sur les paramètres de l'homéostasie glucidique et énergétique. Ces études *in vivo* seront réalisées dans un premier temps sur des animaux sains ne présentant aucun trouble métabolique afin d'évaluer l'efficacité biologique de ces peptides. Puis les peptides les plus actifs sur ce modèle sain seront ensuite testés sur des animaux présentant des dérèglements métaboliques et glucidiques. Dans ce cas, des animaux rendus obèses et insulino-résistants par un régime

alimentaire riche en graisse et des souris diabétiques (*db/db*) seront utilisés. Nous évaluerons l'effet de ces peptides sur l'homéostasie glucidique :

1/ Test de tolérance au glucose intrapéritonéal : la capacité de normaliser rapidement l'épisode hyperglycémique induit par un bolus de glucose dépend de l'intégrité d'une réponse métabolique incluant la sécrétion d'insuline en réponse au glucose et l'action hypoglycémiante de celle-ci. Une modification de la tolérance au glucose intrapéritonéal après traitement avec les nouveaux peptides suggérera donc une action au niveau de la sécrétion d'insuline en réponse au glucose et/ou de sa sensibilité.

2/ Clamp hyperglycémique : nous évaluerons ensuite *in vivo* la sécrétion d'insuline en réponse à l'hyperglycémie à l'aide d'un clamp hyperglycémique. Pour cela, une solution de glucose sera infusée à des souris à travers un cathéter veineux fémoral afin de maintenir l'hyperglycémie (que nous contrôlerons par saignées à la queue). Un profil des taux plasmatiques d'insuline au cours du temps sera réalisé grâce à des prélèvements de sang répétés à l'aide d'un cathéter artériel.

3/ Test de tolérance à l'insuline : nous mesurerons l'étendue de l'hypoglycémie induite par une injection intrapéritonéale d'insuline après administration des nouveaux peptides dérivés du M30, ce qui nous indiquera l'effet de ces peptides sur la sensibilité à l'insuline.

L'impact des peptides sur la prise alimentaire et les dépenses énergétiques sera également quantifié :

1/ Prise alimentaire et calorimétrie indirecte : cette partie sera réalisée en collaboration avec l'entreprise Biomeostasis qui possède un système de cages individuelles permettant la mesure simultanée, non-invasive et automatisée de la prise alimentaire et de la dépense énergétique. Le système mesure en continu la prise de nourriture, la consommation d'eau et les échanges gazeux qui permettent de quantifier les dépenses énergétiques.

2/ Déglutition : des variations de la déglutition seront recherchées après injection des peptides dans le générateur central de la déglutition situé dans la partie latérale du NTS. Cette technique, développée au sein du laboratoire par Bernadette Felix, consiste à stimuler le nerf laryngé supérieur (NLS) puis à enregistrer le nombre de déglutitions provoquées par cette stimulation avant et après administration de la substance d'intérêt dans le générateur central de la déglutition (Bariohay et al., 2008; Félix et al., 2006). Des enregistrements suite à l'injection de nesfatine-1 et du peptide M14 ont déjà été effectués **(Figure 43)**.

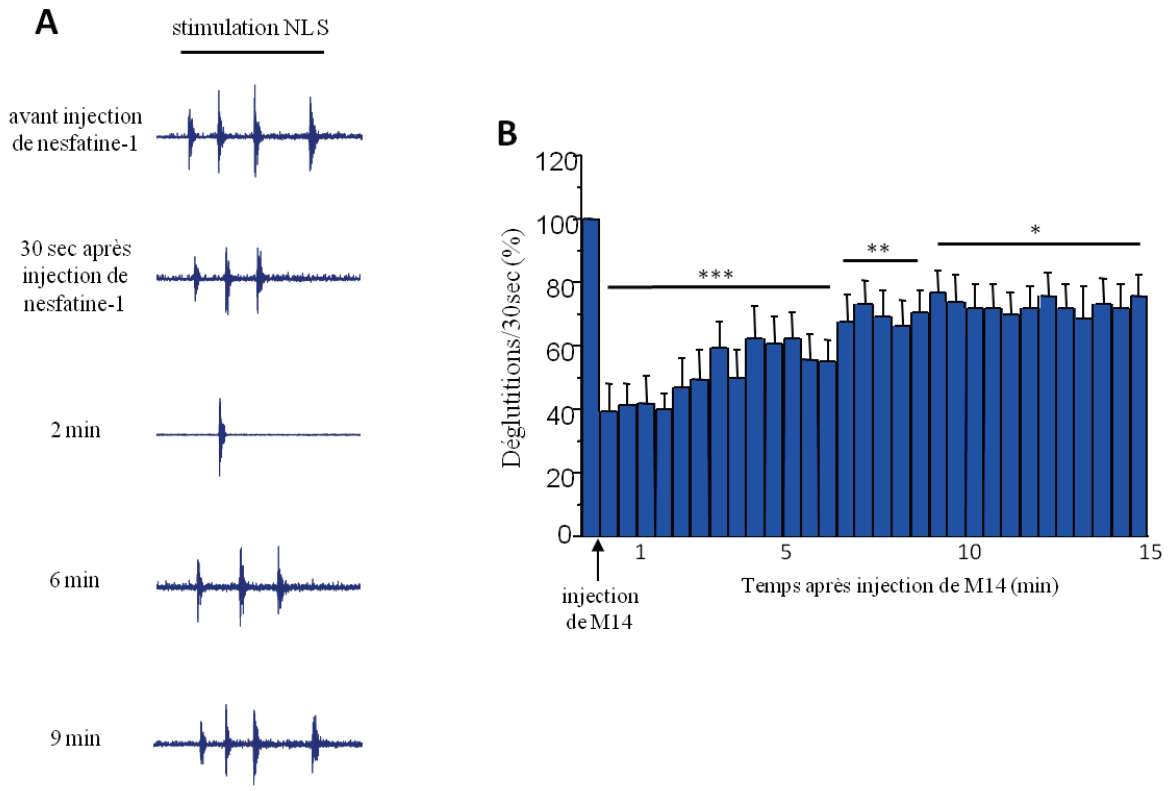


Figure 41 : Effets de la nesfatine-1 et du M14 sur la déglutition.
A. Inhibition de la déglutition chez un rat après injection de nesfatine-1 (10,4 pmol) dans le générateur central de la déglutition. Les déglutitions sont provoquées par stimulation du nerf laryngé supérieur (NLS; 20Hz, 0.14ms, 2.5V) et enregistrées à l'aide d'électrodes myographiques placées dans le muscle sublingual.
B. Moyenne des variations de la déglutition après injection de M14 (100 nmol) dans le générateur central de la déglutition (n=13). *p < 0,05; **p < 0,01 et ***p <0,001.
Résultats communiqués par Bernadette Felix et Anne Abysique.

3/ Mesure de la température corporelle et de l'activité locomotrice spontanée : l'impact des peptides sur la température corporelle et l'activité locomotrice spontanée sera quantifié à l'aide d'un système de télémétrie permettant d'enregistrer la température corporelle et l'activité locomotrice de souris évoluant librement dans leur cage. L'effet des peptides sur l'induction de symptômes caractéristiques d'un comportement de maladie et aversif sera ainsi recherché.

3. Implication des cellules gliales dans la signalisation nesfatinergique

Bien que longtemps considérées comme des cellules de soutien, les cellules gliales apparaissent aujourd'hui indispensables à la communication neuronale et au maintien de l'homéostasie dans le cerveau (Hamilton et Attwell, 2010; Perea et al., 2009). Comme décrit dans l'introduction de ce manuscrit, des données récentes de la littérature attestent que les cellules gliales participent à la régulation de la prise alimentaire. La glie semble en effet constituer un relais dans l'intégration des signaux humoraux par les neurones de l'hypothalamus et du CVD. Les cellules gliales, et en particulier les tanycytes et vagliocytes, expriment l'octadécaneuropeptide (ODN), un peptide aux propriétés anorexigènes puissantes (de Mateos-Verchere et al., 2001; do Rego et al., 2007; Tonon et al., 1990 ; nos résultats non publiés) **(Figure 40)**. Dans ce contexte, l'équipe a entrepris une collaboration avec Marie-Christine Tonon et Jérôme Leprince (laboratoire DC2N, Université de Rouen) sur la caractérisation des cellules exprimant l'ODN dans le quatrième ventricule. Le projet de recherche que je propose vise à identifier un rôle éventuel de la glie dans la signalisation nesfatinergique. Deux axes de recherches seront étudiés :

- l'activation des cellules gliales par la nesfatine-1. L'étude se focalisera sur les cellules exprimant l'ODN.
- l'activation des neurones nesfatinergiques par l'ODN.

3.1. Effets de la nesfatine-1 sur les cellules gliales du CVD et de l'hypothalamus

L'effet de la nesfatine-1 sur la glie sera tout d'abord évalué *in vitro*. Pour cela, nous effectuerons des cultures primaires de cellules gliales d'hypothalamus ou de CVD. Puis nous utiliserons plusieurs approches permettant d'évaluer l'activation des cellules gliales et la sécrétion d'ODN en réponse à la nesfatine-1:

1/ Imagerie calcique : les cellules gliales seront cultivées sur lamelles en verre et incubées avec un fluorochrome sensible à la teneur en calcium intracellulaire (Fura-2). Puis la nesfatine-1 sera perfusée sur les cellules à différentes concentrations. Les variations de calcium intracellulaire seront enregistrées à l'aide d'un microscope à imagerie dynamique.

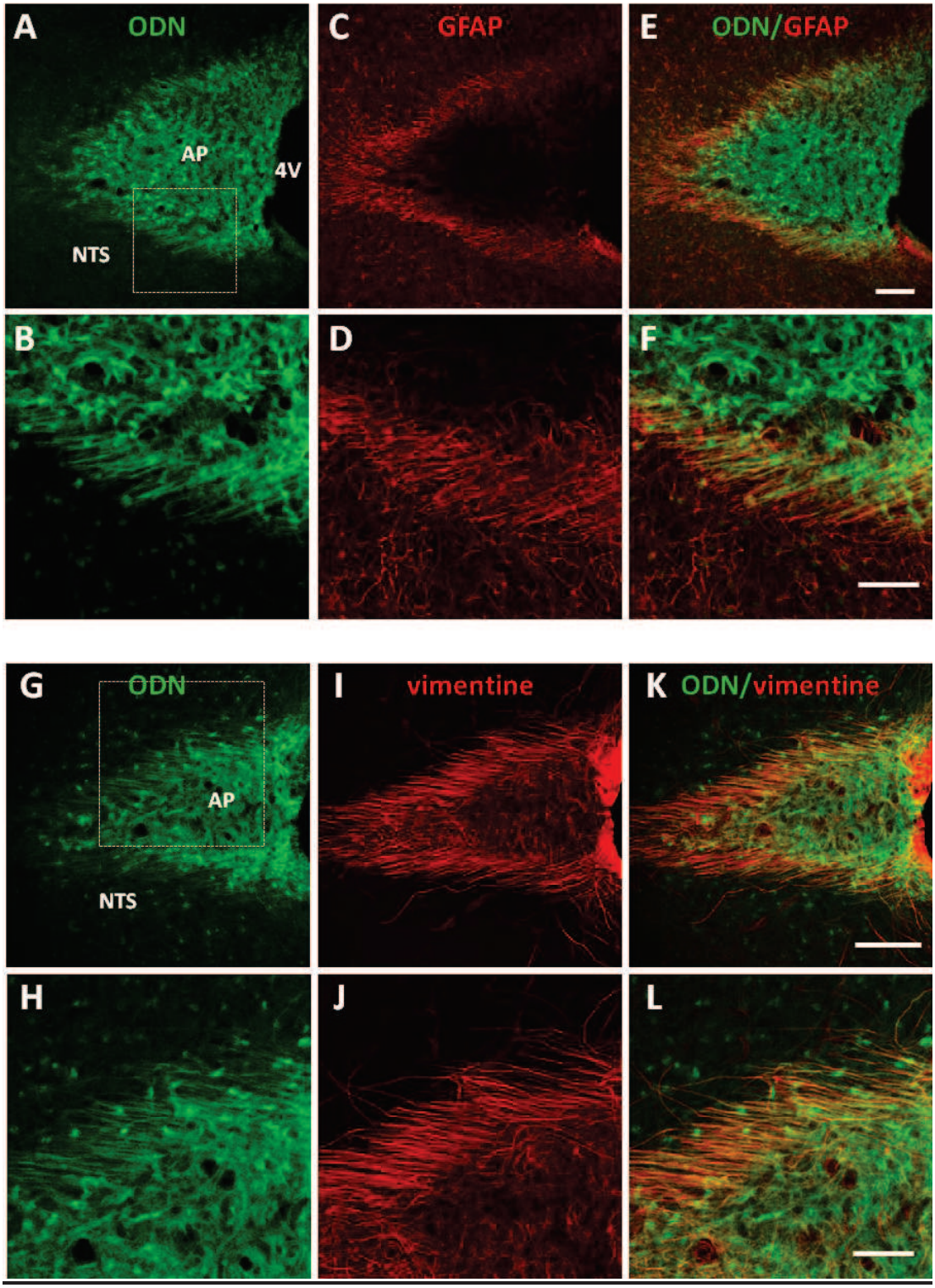


Figure 42 : Présence d'ODN dans les vagliocytes bordant l'AP.
Immunomarquage de l'ODN (A, B, G, H), de la GFAP (C, D) et de la vimentine (I, J) sur coupes longitudinales de CVD de rat. La GFAP et la vimentine sont des marqueurs des tanycytes et des vagliocytes. Noter la présence de colocalisations entre l'ODN et la GFAP (E, F) et entre l'ODN et la vimentine (K, L).
Barres d'échelles : 100 µm en E et K; 50 µm en F et L.

AP: area postrema; NTS: noyau du tractus solitaire; 4V: quatrième ventricule.

Des expériences préliminaires ont déjà été effectuées et montrent une augmentation dose-dépendante de la concentration en calcium intracellulaire des cellules en réponse à la nesfatine-1 **(Figure 41)**. Ces résultats devront être confirmés.

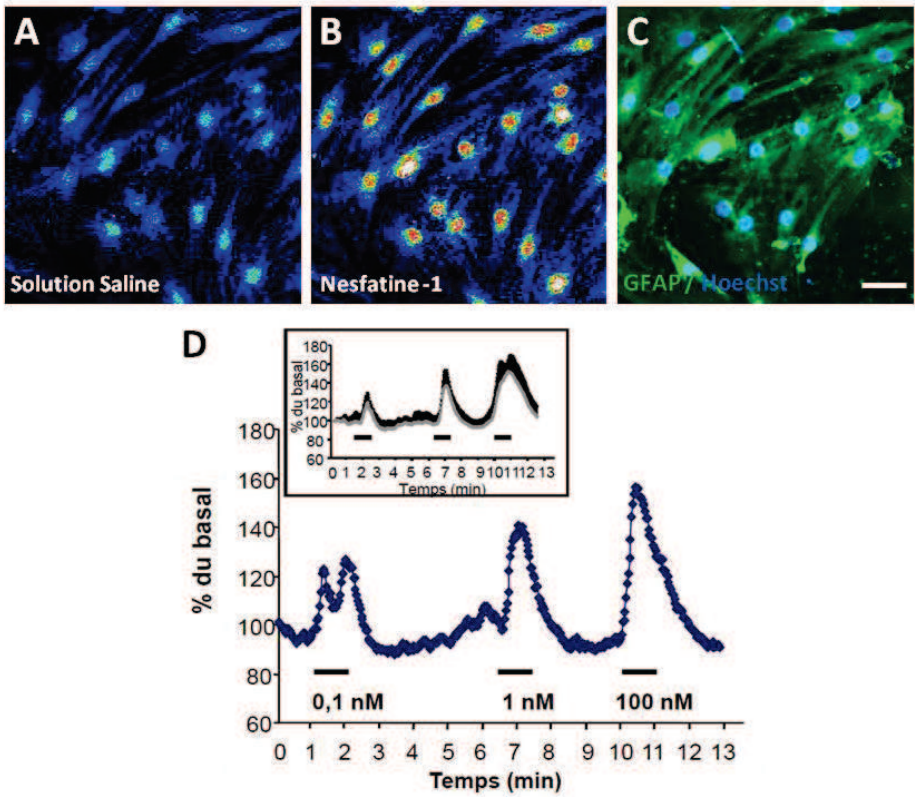


Figure 43 : Effet de la nesfatine-1 sur des cultures d'astrocytes du CVD en imagerie calcique.
A-B. Images en fausses couleurs de la fluorescence traduisant la concentration en calcium intracellulaire avant (A) et pendant (B) l'application de nesfatine-1 100 nM sur des cultures d'astrocytes du CVD.
C. Immunocytochimie de la GFAP réalisée à postériori sur les cultures sensibles à la nesfatine-1. Les noyaux sont visualisés à l'aide du Hoechst (barre d'échelle : 20 µm).
D. Variation de la fluorescence traduisant la $[Ca^{2+}]i$ dans un astrocyte en réponse à l'application de doses croissantes de nesfatine-1. Insert dans la figure D : moyenne des réponses obtenues à partir de 15 cellules.

2/ Libération d'ODN : les cellules gliales seront cultivées dans des puits de culture et incubées quelques minutes en présence de nesfatine-1. Le milieu de culture sera ensuite recueilli afin de doser la quantité d'ODN sécrété par les cellules en utilisant une technique RIA développée par l'équipe du Dr Tonon (Masmoudi et al., 2003).

Nous évaluerons ensuite l'effet de la nesfatine-1 sur les cellules gliales et l'ODN *in vivo*. Pour cela, nous testerons :

1/ L'effet de la nesfatine-1 sur l'expression du DBI, le précurseur de l'ODN. L'expression des transcrits du DBI sera quantifiée par PCR quantitative (1 à 24 heures) après

injection centrale de nesfatine-1. Cette approche aura également pu être testée *in vitro,* en particulier pour connaitre la cinétique dans les variations d'expression du DBI.

2/ L'effet du blocage de métabolisme glial par la méthionine sulfoximine sur l'anorexie induite par la nesfatine-1. La méthionine sulfoximine et la nesfatine-1 seront administrées centralement et la prise alimentaire sera mesurée à différents intervalles de temps afin de savoir si la glie constitue un relais dans la mise en place de l'anorexie induite par la nesfatine-1.

3/ L'effet du blocage des récepteurs à l'ODN sur l'anorexie induite par la nesfatine-1. Comme pour la méthionine sulfoximine, l'antagoniste des récepteurs à l'ODN et la nesfatine-1 seront injectées centralement et la prise alimentaire sera mesurée.

3.2. Effets de l'ODN sur les neurones nesfatinergiques du CVD et de l'hypothalamus

Des études récentes montrent que les tanycytes exprimant l'ODN sont sensibles à des signaux circulants anorexigènes. En réponse à ces signaux, les tanycytes pourraient libérer de l'ODN sur les neurones POMC (**Cf. : Figure 12C**). L'ODN constituerait alors un relais dans la diminution de la prise alimentaire par les signaux humoraux en activant les neurones POMC (Lanfray et al., 2012). Nous cherchons à savoir si, comme les neurones POMC, les neurones nesfatinergiques sont sensibles à l'ODN. Cette hypothèse est renforcée par la présence d'une forte colocalisation entre POMC et NUCB2/nesfatine-1 dans les neurones de l'ARC et du NTS. Nous proposons pour cette étude une approche immunohistochimique par marquage de la protéine c-Fos. Nous effectuerons ainsi deux expériences :

1/ Expression de la protéine c-Fos après injection centrale d'ODN. Nous nous intéresserons à l'activation des neurones exprimant NUCB2/nesfatine-1 en réponse à l'administration d'ODN.

2/ Effet de l'antagoniste des récepteurs à l'ODN sur l'activation des neurones nesfatinergiques induite par divers stimuli. Nous chercherons à savoir si le blocage de la signalisation à l'ODN diminue le nombre de neurones nesfatinergiques activés en réponse aux signaux de satiété comme la CCK, la réalimentation après un jeûne prolongé ou l'injection de glucose. Nous testerons également les signaux utilisés dans ce travail de thèse, à savoir l'hypoglycémie, l'inflammation et l'intoxication au DON.

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ANNEXES

Annexe 1

Revue : Glial cells of the nucleus tractus solitarius as partners of the dorsal hindbrain regulation of energy balance: a proposal for a working hypothesis.

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Review

Glial cells of the nucleus tractus solitarius as partners of the dorsal hindbrain regulation of energy balance: A proposal for a working hypothesis

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ABSTRACT

While the evidences emphasizing the role of astroglial cells in numerous aspects of information processing within the brain merges, the literature dealing with the involvement of this cell population in the signalization involved in feeding behavior and energetic homeostasis remains scarce. Nevertheless, some clues are now available indicating that glia could play a dynamic role in the regulation of energy balance, and that strengthening research effort in this field may further our understanding of the mechanisms controlling feeding behaviour. In the present review, we have summarized recent data indicating that the multifaceted glial compartment of the brainstem should be considered in future research aimed at identifying feeding-related processes operating at this level.

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1. Introduction

Glial cells constitute the numerically largest cell population in the vertebrate adult brain and have been considered for decades mainly as supporting cells. They function to maintain normal brain physiology, including survival and guidance of migrating neurons during development, formation and preservation of the blood–brain barrier (BBB), and maintenance of neuronal homeostasis (reviewed in Perea et al., 2009). More recent studies have revealed that astrocytes partake in the neuronal conversation. Through the expression of a great variety of membrane-bound receptors astrocytes generate intracellular calcium oscillations and waves in response to various stimuli, including neurotransmitters. In turn, glial activation observed during neuronal activity results in the release of neuroactive substances called "gliotransmitters" such as ATP, glutamate and D-serine. The released mediators not only induce astrocytic calcium waves (in the case of ATP) but also affect postsynaptic neurons excitability triggering either excitatory or inhibitory responses in these cells. Even if the release mechanisms (exocytotic or non-exocytotic) of this compounds remain a matter of debate (Hamilton and Attwell, 2010), gliotransmitters are believed to modulate synaptic transmission and synchronize neuronal activity (Perea et al., 2009). There is also increasing evidence suggesting a role for these cells in different forms of plasticity, including activity-dependent structural plasticity (Theodosis and Poulain, 1999) or neurogenesis (Alvarez-Buylla and Lim, 2004). In the field of ingestive behavior and control of energy balance, while glial cells have been neglected for many years, some clues are now available indicating that time has come to take the astroglial compartment into consideration when investigating the neural control of feeding behavior and its associated deregulations. In the present review, we put together recent data regarding the glial compartment of the dorsal hindbrain, its organization and possible involvement in energy homeostasis. Given its anatomic organization, comprising the close association of a circumventricular organ (CVO) the area postrema (AP), with integrative and motor nuclei the nucleus tractus solitarius (NTS) and the dorsal motor nucleus of the vagus (DMNX), respectively, the dorsal vagal complex (DVC) may constitute a relevant model to test the hypothesis of the glial cells as neuron partners in the regulation of energy balance.

2. Organization of the astroglial compartment of the adult DVC

Visualization of glial cells of the adult caudal brainstem, using glial fibrillary acidic protein (GFAP) immunohistochemistry, has revealed a striking pattern of GFAP immunoreactivity in the DVC, which distinguishes it from the surrounding brainstem nuclei, i.e., the hypoglossal nucleus, the dorsal tegmentum, and the medullary reticular nucleus (Bouzioukh et al., 2001; Pecchi et al., 2007 and Fig. 1). This simple phenotypic visualization has revealed strong heterogeneity in GFAP distribution within the DVC. The NTS and the DMNX appear heavily labelled throughout their whole rostrocaudal extent, while weak GFAP labelling is observed in the AP. In both the NTS and DMNX, most GFAP-immunoreactive cells exhibit typical features of differentiated stellate astrocytes, similar to astrocytes encountered throughout the central nervous system (CNS). Given the extensive role of astrocytes in the modulation of neurotransmission (Perea et al., 2009), such a striking abundance of stellate astrocytes in these sensory and motor nuclei raises questions about their functional significance and deserves further investigations (see paragraph 3). In addition to the classical GFAP+ astrocytes, we and others have observed a dot shaped GFAP immunoreactivity on coronal brainstem section in the midline commissural NTS and at the interface between the AP and the subpostremal NTS, i.e. the *funiculus separans* (Bouzioukh et al., 2001; Pecchi et al., 2007; Wang et al., 2008; Maolood and Meister, 2009). This labelling evokes the discrete cross-section of GFAP-positive processes extending horizontally along the rostrocaudal axis. Using GFAP immunochemistry on horizontal and sagittal brainstem sections, we have confirmed the presence of a bundle of GFAP+ processes in the midline commissural NTS and around the AP, which radiates rostrocaudally into the NTS parenchyma, and originates from cuboid shaped GFAP+ somata localized in the ependyma of the 4th ventricle (Pecchi et al., 2007). Thus, this population of GFAP-expressing cells exhibits atypical astrocyte morphology with long, thin, and unbranched processes forming a bundle at the level of the *funiculus separans*. These morphological characteristics are evocative of other sub-population of glial cells that resemble developmental radial glia and persist in a few regions of adult rodent brain. For instance, a population of glial cells termed, by Sundholm-Peters et al. (2004), "radial glia-like cells" was found within the ventral wall of adult mice lateral ventricles. These cells extend long processes from the ventricle into the surrounding parenchyma and express GFAP in addition to vimentin and nestin (Sundholm-Peters et al., 2004). Similarly, at the border of the adult ventral 3rd ventricle, specialized ependymal cells called tanycytes extend long and thin processes positive for GFAP vimentin and nestin into the hypothalamus parenchyma. These characteristics have led to the assumption that tanycytes are related to developmental radial glia (Flament-Durand and Brion, 1985). The cells we identified within the DVC appear very similar to those found in the walls of both the lateral and 3rd ventricles since they co-express vimentin and nestin (Pecchi et al., 2007 and Fig. 2A–F). In a recent paper, Maolood and Meister (2009) have reported DARPP-32 immunoreactivity, a marker classically used to identify tanycytes, in the AP and at the borderline between the AP and the NTS, reinforcing the idea of a strong homology between glial radiating cells found in the border of the 3rd and 4th ventricles. However, it should be noted that within the DVC, DAPP32 labelling has also been found in the whole AP which is devoid of GFAP radiating cells. Finally, in contrast to tanycytes whose processes terminate preferentially on perivascular spaces (for review see Wittkowski, 1998), no favoured association of GFAP+ processes and capillaries has been observed in the DVC (our unpublished data). Thus, GFAP+ cells located in the *funiculus separans* seem to share only some similarities with tanycytes located around the 3rd ventricle. However, the use of the same term of tanycytes to denote the radiating glial cells located at the AP/NTS border and the cells found around the 3rd ventricle seems to be confusing and

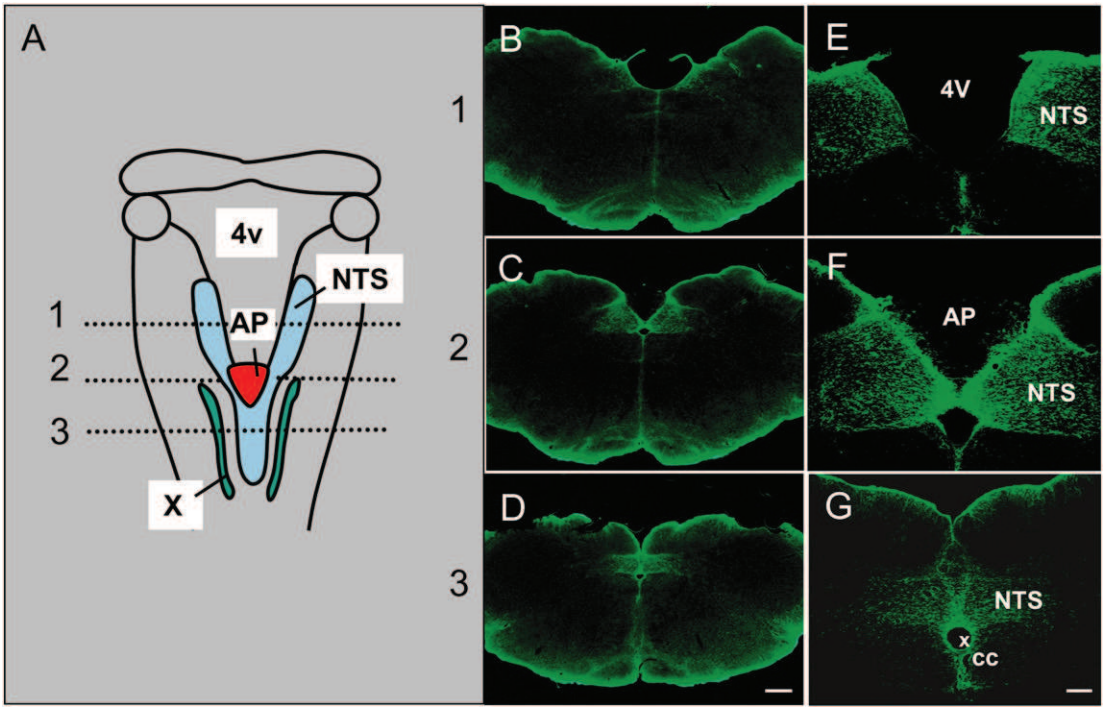


Fig. 1 – (A) Schematic representation of a DVC dorsal view illustrating the three anatomic levels of this structure shown on microphotographs B–G. GFAP immunoreactivity in the caudal brainstem of adult rat observed on coronal sections at three anatomic levels (A). GFAP immunoreactivity observed at low (B–D) or high (E–G) magnification on caudal (B and E), subpostremal (C and F), rostral (D and G) levels of the DVC reveals the striking glial compartment found in the DVC in comparison to surrounding brainstem structures. Scale bars: 700 μm in B–D and 200 μm in E–G.

somewhat improper regarding the evident differences. Hence in order to simplify future mentions of these cells and to distinguish them from 3rd ventricle tanycytes, we propose to use the term of *vagliocytes* to designate these GFAP-positive radiating cells present within the DVC at the junction with the AP.

Examination of postnatal development of the astroglial compartment in the NTS has been performed by immunoassaying of GLAST, GLT-1 and GFAP. GLAST and GLT-1 two glutamate transporters specifically expressed by glia, are considered to be relevant markers of fine glial processes devoid of GFAP (Tashiro and Kawai, 2007). Those authors have reported a strong rise in glial markers during the weeks 1–3 postnatal due to both the proliferation and differentiation of astrocytes. In the same study, 3D reconstruction has revealed that astrocytic processes are located in the vicinity of small cell bodies, suggesting that astrocytes might be able to modulate axo-somatic synaptic transmission within the NTS (Tashiro and Kawai, 2007). Regarding the atypical astrocytes, *vagliocytes*, found in the *funiculus separans*, their development has been investigated by performing immunohistochemistry for GFAP, vimentin, and nestin at P0, P10, P20 and P30 (Pecchi et al., 2007). These cells are present in the AP/NTS border and commissural NTS from birth and only little changes in the distribution and labelling density during early postnatal development were detected. The intensity of GFAP labelling increased progressively from birth to P30 while nestin labelling lessen with age and any noticeable modification in the density or distribution pattern of vimentin labelling was observed from birth to P30.

In the following paragraphs, we have reviewed the recent experimental data regarding the glial compartment of the DVC and their potential involvement in the regulation of energy balance. In the light of these results, we put forward three complementary and not exclusive hypotheses (i) the regulating function of the glial cells of the DVC in the diffusion of circulating molecules between the AP and the NTS, (ii) their dynamic role in DVC signalling and finally (iii) their contribution to the cellular dynamics found within the DVC.

3. Glial cells as a partial diffusion barrier between the AP and the NTS

The microvasculature of the AP has a less restrictive BBB than elsewhere in the CNS leading to the concept that AP, like other CVOs, constitutes an open window allowing the diffusion of circulating molecules from the blood to the NTS parenchyma. However, earlier works showing the lack of diffusion of intravenously injected tracers out of the CVOs contradict this widespread idea (Maness et al., 1998). This restricted diffusion suggests the existence of a diffusion barrier between the AP and the NTS. In this context, it has recently been shown that while intravenous injection (i.v.) of sodium fluorescein (0.3 kDa) results in the rapid labelling of both the AP and the surrounding NTS, the diffusion of the fluorescent labelled dextran (3 kDa) is restricted to the AP following its systemic injection (Willis et al., 2007). From these results, the authors concluded that 3 kDa

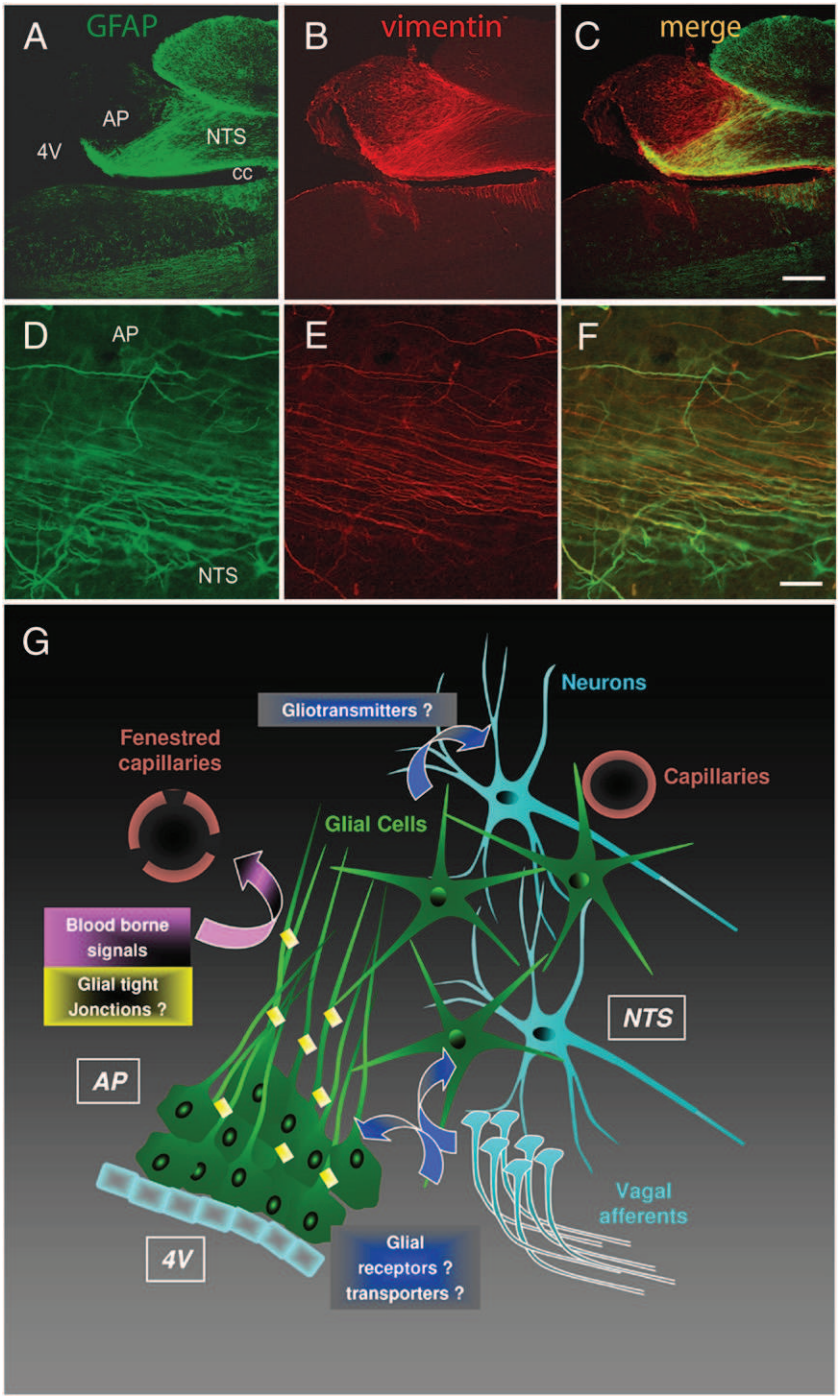


Fig. 2 – GFAP (A and D) and vimentin (B and E) immunoreactivity in the caudal brainstem of adult rat observed on sagittal sections at low (A–C) or high (D–F) magnification. GFAP and vimentin colocalize in cells located at the AP/NTS border (C–D). (G) Proposed model of astroglial compartment organization within the DVC showing the diversity of astroglial cells in this structure. Scale bars: 700 μm in A–C and 25 μm in D–F.

dextran is unable to pass through the highly GFAP-immunoreactive astrocyte boundary surrounding the AP, which forms a barrier restricting molecule movements between the AP and the NTS (Willis et al., 2007). In a similar way, we have recently observed that the fluorescent dye hydroxystilbamidine (OHSt, MW 472.53 g/mol; 0.47 kDa) is largely found confined to the AP when intravenously administered and the vast majority of the NTS cells is devoid of OHSt labelling. The dye seems to be unable to go through the border between the AP and the NTS. The elongated structure of the *vagliocytes* of the *funiculus separans*, and the way in which they radiate from the 4th ventricle to the NTS parenchyma lead to the hypothesis that they may constitute a possible obstruction to the substance diffusion from the circulation and the AP to the NTS (Fig. 2G). Similarly, it

has been proposed that tanycytes lining the median eminence (ME) and the arcuate nucleus (ARC) may constitute a barrier diffusion limiting the access to the ARC of circulating arising from the EM (Peruzzo et al., 2000; Mullier et al., 2010; Rodríguez et al., 2010). In accordance, the AP/NTS border has been recently shown to be immuno-positive for zona occludin-1 (ZO-1), a protein constitutive of tight junctions, (Wang et al., 2008; Maolood and Meister, 2009). The glial identity of ZO-1 positive cells has been established by GFAP immunostaining coupled to electronic microscopy (Wang et al., 2008). Altogether these data strongly suggest that *vagliocytes* located at the border between the AP and the NTS may limit the diffusion of circulating molecules from the AP to the adjacent structures, but could in parallel specifically transport circulating factors from the blood into the NTS parenchyma. In regulating the access of circulating feeding-related molecules to nuclei surrounding the AP, this transport system could have strong functional implications. Finally, based on the observation that molecules with a molecular weight equal or greater than 0.47 kDa are limited in their diffusion from the AP, one could estimate or speculate about the capability of diffusion outside the AP. Based on their molecular weights, neuropeptides or hormones such as angiotensin II (1 kDa), CCK (1.06 kDa), ghrelin (3.3 kDa), NPY (4.2 kDa) and its related peptide PYY 3-36 (4.0 kDa), nesfatin-1 (9.6 kDa) and leptin (16.2 kDa) but also cytokines including interleukin-1β (17.5 kDa), tumor necrosis factor-α (17.3 kDa) or interleukin-6 (21.7 kDa) are likely to be unable to reach the NTS from the AP. On the contrary, small molecules such as glucose (0.18 kDa), amino-acids or steroid hormones (testosterone 0.28 kDa; estradiol-17β 0.27 kDa) can diffuse beyond the AP through the glial barrier. Recently, we reported the *in vivo* and *in vitro* expressions of Ob-R isoforms at the AP/NTS glial boundary level, with a strong expression of the short isoforms. These isoforms, previously proposed to catalyze leptin transport, might intervene in the receptor mediated leptin transport from the AP to the NTS. This glial leptin transport envisaged here for the glial cells has been partly proposed for other structures. Astrocytes within the median eminence and adjacent ARC can take up fluorescent dyes from the blood circulation and express receptors for both leptin and neuropeptide Y, suggesting that these glial cells may have a role in the regulation of food intake (Cheunsuang and Morris, 2005; Cheunsuang et al., 2006). Astrocytes located around the subcommissural organ display an evident immunoreactivity for the Ob-R, whereas vascular components and both the ependymal and hypendymal cells of this structure, as well as glial cells lining the third ventricle and surrounding neurons, are completely negative. It has been proposed that these astrocytes may have a role in by-passing the BBB by conveying leptin from the blood and the perivascular space down to the subcommissural organ (Dall'Aglio et al., 2006). Given the importance of leptin transport within the brain in nutritional imbalance (Van Heek et al., 1997), future works should investigate the leptin transport by glia within the DVC.

4. Glial cells of the DVC as nutrients sensors

When considering a possible involvement of glial cells in energetic regulation, their modulation of neuronal signalisation operating at the brainstem level to regulate feeding behaviour and energetic homeostasis deserves to be tackled. In a pioneer study, JK Young and colleagues (2000) reported that methionine sulfoximine (MS), a drug which rather specifically impedes astroglial metabolism, significantly reduces the response of NTS neurons to the peripheral injection of the glucoprivic agent 2-deoxyglucose (2-DG; Young et al., 2000). This work was one of the first to bring forward the idea that the glial compartment of the NTS could act as nutrient sensors, and that neuroglia interactions in this structure could participate in the regulation of energy balance. Indeed, glucose transporter-2 (GLUT-2) expression in a subpopulation of astrocytes within the DVC has been described (Leloup et al., 1994). More recently, in an elegant work, Marty et al. (2005) have reported that glial re-expression of GLUT-2 within the DVC of GLUT-2 null mice restores glucagon secretion and c-Fos labelling in the brainstem in response to glucoprivation induced either by intraperitoneal or intracerebroventricular 2-DG injections. This concept of glial cells as nutrient sensors within the DVC refers to a more general view considering glial cells, particularly those located within circumventricular organs or surrounding structures, as chemoreceptors involved in the regulation of physiological functions, an idea that emerges from recent results. For instance, specific glial cells of the subfornical organ were shown to be not only sensitive to extracellular Na+ level but also the primary site of Na+ detection. Moreover, in response to extracellular Na+ increase, these glial cells signal to neighbouring GABAergic neurons via the release of lactate to modulate salt-intake behavior (Watanabe et al., 2006; Shimizu et al., 2007). Besides, astrocytes were shown to be able to sense circulating inflammatory signals, cytokines and chemokines, and the specific glial deletion of the TNF-family receptor (RANK) results into resistance to lipopolysaccharide-, TNF-α and IL-1β-induced fever (Hanada et al., 2009).

5. Glial cells of the DVC and hormonal energetic regulation

In a recent work, we have reported the glial expression of leptin receptor isoforms in the adult DVC (Dallaporta et al., 2009). In addition to the labelling of neuronal cells bodies within the NTS and DMNX as previously described (Shioda et al., 1998; Buyse et al., 2001), we observed the Ob-R labelling of structures localised at the AP/NTS interface. Using confocal analysis, we confirmed the glial identity of Ob-R positive cells corresponding to the above described *vagliocytes* (Pecchi et al., 2007). Moreover, using primary cell cultures, we found that all transcripts encoding membrane bound Ob-R isoforms were expressed by glial cells originating from the DVC including the Ob-Rb isoform which mediates leptin signalling. Interestingly, it was recently reported that hypothalamic astrocytes expressed leptin receptor (Hsuchou et al., 2009a,b) and that agouti or high- fat diet obese mice exhibited a significant increase in Ob-R expressing astrocytes in the hypothalamus compared with lean control mice, suggesting that up-regulation of astrocytic Ob-R expression may interfere with the onset of obesity (Pan et al., 2008; Hsuchou et al., 2009a). The same group has also confirmed the

functionality of glial leptin receptors by showing the induction of calcium signalling in response to the hormone application in primary hypothalamic astrocytic culture (Hsuchou et al., 2009a). In the same way, the modulation of glial Ob-R expression within the DVC during nutritional unbalance as well as the functionality of this receptor should be investigated in the future. Furthermore, the sensitivity of DVC glial cells might be extended to other feeding related molecules previously shown to induce calcium signalling in astrocytes such as CCK (Müller et al., 1997) or cytokines (Beskina et al., 2007). In this context, the recent study by Hermann and Rogers (2009) showed that a significant proportion of c-Fos activated cells in response to vagal afferent stimulation by the pro-inflammatory anorexigenic cytokine TNF-α are astrocytes. The consequences of such astroglial activation remains to be determined, but a possible modulation of the sensitivity of the NTS circuitry can be reasonably evoked. One important point that should be necessary addressed in the future is the possible release of neuroactive compounds, "gliotransmitters," by glial cells of the DVC in response to feeding related peptides and hormones or following vagal stimulation (Fig. 2G).

6. Glial cells and cellular dynamics within the DVC

Adult neurogenesis, leading to the generation of new neurons and astrocytes in equal proportions, has been shown to take place within the adult rat DVC after vagotomy using BrdU incorporation combined with phenotypic labelling (Bauer et al., 2005). Moreover, intrinsic proliferative cells have also been detected within the DVC of adult rats by means of Ki-67 immunohistochemistry (Charrier et al., 2006). The presence of neural stem cells within the DVC has been finally demonstrated by applying the *in vitro* neurosphere assay on microdissected adult DVC explants (Charrier et al., 2006). These data have shed light on the potential plasticity of the DVC, which brings novel cues about physiological adaptations of autonomic reflexes in adult mammals. In foci of neurogenesis in the adult mammalian brain such as the subventricular zone/olfactory bulb system (SVZ/OB) and the subgranular zone of the hippocampal dentate gyrus (SGZ), neural stem cell activity is strongly modulated by the tissular microenvironment and especially by astrocytes (Song et al., 2002; Alvarez-Buylla and Lim, 2004). Glial cells have been shown to favor neurogenesis in adult forebrain germinative centers owing to soluble and membrane-bound factors (Song et al., 2002; Alvarez-Buylla and Lim, 2004). In both the SVZ and SGZ of adult rodent brain, astrocyte subpopulations retain the expression of immature glial cell markers, i.e. vimentin and nestin, that characterizes the developmental radial glia supporting embryonic neurogenesis (Garcia et al., 2004; Sundholm-Peters et al., 2004). The *vagliocytes* found at the AP/NTS border fit well with these phenotypic and morphologic characteristics and furthermore proliferative cells as well as immature neurons have been found in their vicinity (Pecchi et al., 2007). This remaining proliferative capability within the adult DVC is quite fascinating and questioned about the physiological significance of such phenomenon. The possible link between this cellular proliferation and a modulation of feeding behaviour could be envisaged but remains to be established. In this context, it should be interesting to establish whether modulation of nutritional status and satiety reflex modifies the cellular proliferation and precursor's differentiation within the DVC. Alternatively and perhaps more importantly, future studies might be determine the impact of lowered or enhanced proliferation on energy-balance regulation, body weight, food intake and satiety reflex. Interestingly, the link between energy-balance and adult neurogenesis was partly establish at the hypothalamus level (Kokoeva et al., 2005; Belsham et al., 2009) where it was shown that the neurocytokine ciliary neurotrophic factor (CNTF) known to induced a long lasting body weight loss stimulates cellular proliferation and that blocking of CNTF-induced proliferation abrogates body weight loss induced by the cytokines. Some clues indicate that the hypothesis of an intervention of neurogenesis within the DVC during energy-balance adaptation could be reasonably envisaged. Indeed, ghrelin, a gastrointestinal orexigenic peptide, whose levels are elevated in anorectic patients (Tolle et al., 2003) and lowered in obese subjects (English et al., 2002), has been shown to promote neural proliferation *in vivo* and *in vitro* both in the rat NTS and DMNX (Zhang et al., 2004, 2005). Moreover, chronic immobilization stress which results in a long-term food intake inhibition and body weight reduction induced a significant decrease of DVC neurogenesis (Chigr et al., 2009).

7. Concluding remarks

In conclusion, the available data, although preliminary, raise intriguing questions regarding the function of the glial cells of the DVC. At this anatomic level, this cell type appears to be particularly abundant with multifarious functions. We believe that their contribution to various aspects of energy balance regulation, feeding-behaviour and related disorders is strongly conceivable, and certainly merits further investigations to elucidate their precise role.

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Annexe 2

Revue : An Update in the Management of Obesity: The Weight of CNS Targets.

Bariohay B, Roux JA, Bonnet MS, Dallaporta M, Troadec JD.

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An Update in the Management of Obesity: The Weight of CNS Targets

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Abstract: Obesity is one of the most important and disturbing global epidemic that affects humans, with more than 2 billion people overweight and 700 million obese predicted for 2015 by the World Health Organization. Obesity treatment represents then one of the most exciting challenges for the academic researchers and the pharmaceutical industry. But to date, this community failed to develop safe and effective treatments with a good risk/benefit profile. Indeed, most of the drugs previously used as anti-obesity agents have been withdrawn from the market for safety issues, and therapeutic options in form of a medication are currently very limited. This last decade however, new advances in our understanding of central pathways controlling food intake, body weight and energy homeostasis have led to the discovery of new molecular targets that could provide interesting options in the fight against obesity. This review aims to be an overview of the new patents exploiting the anorexigenic properties of the central catabolic pathways or aimed at blocking the orexigenic effects of the anabolic pathways, in the hope to develop new anti-obesity drugs.

Keywords: Brainstem, hypothalamus, neurocircuitry, obesity,satiety, satiation.

OBESITY A 21ST CENTURY EPIDEMIC

Obesity is defined by the World Health Organization (WHO) as a medical condition in which excess body fat has accumulated to such an extent that it may impair health. In numerous industrialized societies, obesity has become a chronic disease and one of the main health concerns over the last decades. Its prevalence has increased at a very high rate these last decades and has reached epidemic proportions worldwide. At the present time, over 1 billion adults are estimated to be overweight worldwide, 300 million clinically obese, and by 2015, the WHO predicts the alarming numbers of 2.3 billion people overweight and 700 million clinically obese [1]. Even more alarming is the rise of childhood obesity: in 2010, nearly 43 million children under the age of five were overweight and 92 million were estimated to be at risk of overweight [2]. With such a high and increasing prevalence, and considering the associated health problems, obesity has a huge economic impact on health care systems. Indeed, in the USA only, it is estimates that the global medical costs related to obesity raised up to 147 billion dollars per year in 2008 [3]. Globally, direct health care costs attributable to obesity in developed country can exceed 7% of the total health care costs [4].

At the individual level, overweight and obesity may result from an excessive caloric intake from food or beverages containing high levels of fats and/or carbohydrates and a reduced energy expenditure resulting from a more sedentary lifestyle [1]. These two factors converge toward an energy imbalance and excessive energy available, resulting in an increase in fat mass stores and body weight. It must be noted however that a few cases of obesity are primarily due to psychiatric illness, genetics or other medical reasons. Overweight and obesity can be easily estimated by the body mass index (BMI), a value calculated by dividing the mass (in kg) by the square of the height (in m). The WHO estimates that individuals with a BMI between 25 and 30 are overweight, and that individuals with a BMI equal or above 30 are obese [1]. Overweight or obesity can be further evaluated by measuring waist circumference, which is well correlated with total body fat [5]. A waist circumference above 102 cm for men and 88 cm for women increases the risk for most weight-related illnesses. The WHO considers obesity as one of the first preventable causes of death worldwide, inducing the death of at least 2.8 million of adult each year. Obesity can severely alter the quality of life and represents an important factor of premature death. The risk factor for many disorders, such as degenerative joint diseases, diabetes mellitus type 2, hypercholesterolemia, high plasmatic triglyceride levels, high blood pressure, heart diseases [6-7] and some types of cancers [8] is significantly increased by obesity and/or abdominal fat accumulation [9]. The WHO estimates that 44% of diabetes, 23% of ischemic heart diseases but also 7 to 41% of some subtypes of cancers could be attributable to overweight and obesity.

The increasing rates of overweight and obesity are mainly attributed to environmental factors and a societal shift, with a more easily accessible palatable diet such as snacks, fast foods, and soft drinks [10], and with urbanization, mechanization of transportation, computerized changes and with the increasing use of totally inactive forms of entertainment such as TV/media and videogames [11]. The principal treatment for obesity consists of diet changes in order to reduce caloric intake and with an increased physical activity in order to increase energy burning. Such a procedure has

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been shown to rapidly decrease body weight, but success rate in the long term is low, revealing the need for complementary medical treatments. Actually, one of the most effective treatments for obesity is bariatric surgery that has been shown to induce up to 25% decrease in body weight loss after 10 years [12]. However, these beneficial effects could be counterbalanced by risks of complications and a decreased quality of life of the patient several years after surgery [13]. Then, given the huge and increasing number of obese patients with increased morbidity risk, and the unmet medical need that represent anti-obesity drugs, obesity research is a very active area in the drug development field. The challenge to discover effective anti-obesity drugs with limited side effects remains entire.

CENTRAL CIRCUITRY REGULATING FOOD INTAKE AND APPETITE

As previously described, obesity results from a dysregulation of energy homeostasis, characterized by excess body weight, closely related to an abnormal fat storage generally due to an excessive amount of ingested calories. In the adult mammal, energy homeostasis is defined as the balance between energy intake provided by food and energy expenditure based on basal metabolism, muscles activity and thermoregulatory responses. The continuous but variable energy needs are covered either by directly using of newly ingested calories, or by mobilizing energy stores mainly represented by the body fat mass. In theory, energy homeostasis can be achieved by controlling the two variables of energy balance i.e. energy intake and energy expenditure. The past decade has seen an increasing recognition of the complex interplay between the central nervous system (CNS) and the activity of organs involved in energy homeostasis to maintain appropriate energy stores Fig. (**1**). To accomplish this, the brain receives continuous information about energy stores, about eaten and absorbed food, and about basal and situational energy needs by tissues. The brain in turn controls organs that have important roles in energy homeostasis and modulates nutritional behavior [14].

Without considering social factors, meal initiation is dependent on, or at least contemporary to, a feeling of "hunger". Meal termination is linked to the disappearance of

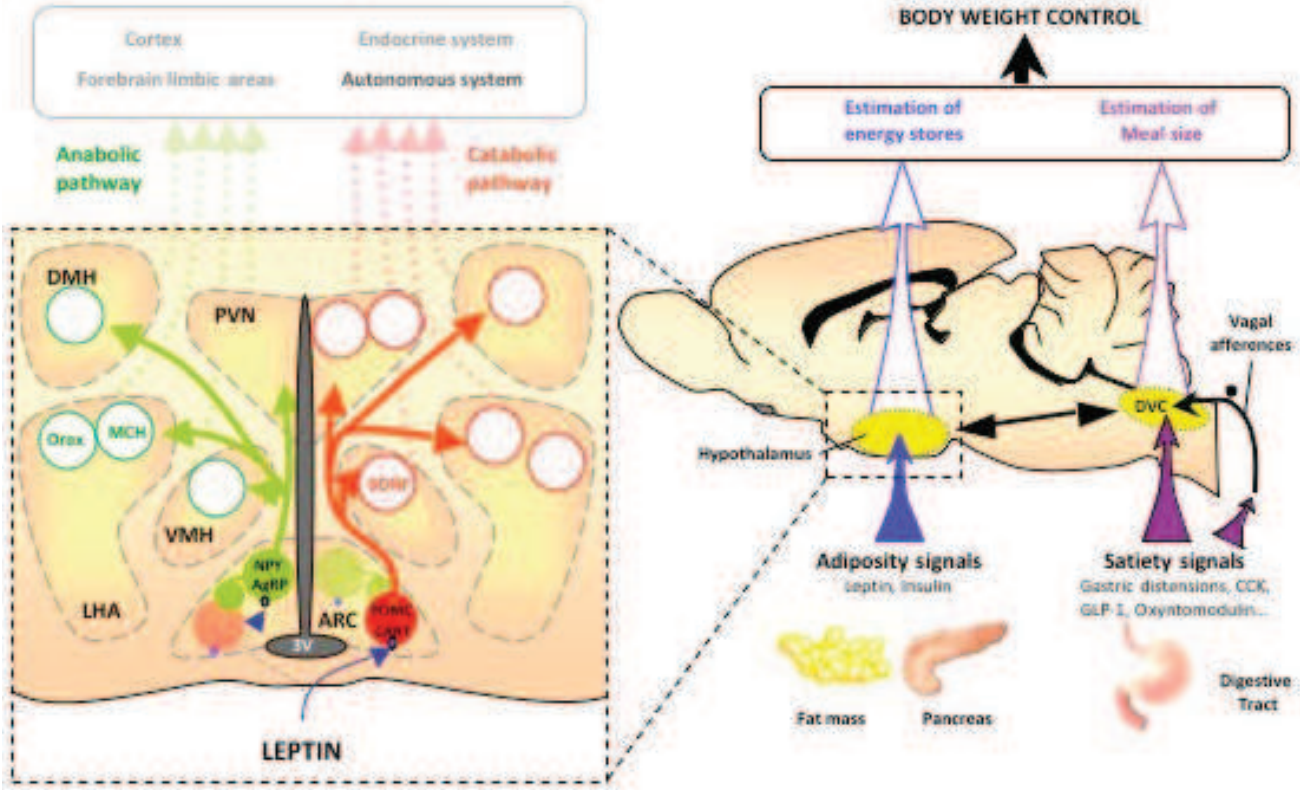


Fig. (1). Neurobiological control of food intake and body weight. body weight control implies a permanent estimate of energy stores and of the size of each meal. Satiety signals are produced when food is present in the digestive tract. They act directly at the level of the DVC of the brainstem, or indirectly through an action on vagal afferents, to inform the central nervous system about the caloric content of the meal and the digestive tract state, and to produce a feeling of satiation followed by a satiety state. Adiposity signals such as leptin, produced in proportion to the amount of body fat, first act at the level of the ARC of the hypothalamus. In the ARC, leptin receptors are expressed by two populations of "first order" neurons, one expressing the anorexigenic neuropeptides POMC and CART (in red) and the other expressing the orexigenic peptides NPY and AgRP (in green). These neurons widely project to "second-order" neurons located in the PVN, the LHA, the DMH, and the VMH. Finally, first order and second order neurons forms two parallel pathways, a catabolic pathway originating from POMC/CART neurons and an anabolic pathway originating from NPY/AgRP neurons. These pathways project in turn to the brain, in particular toward the cortex, the forebrain limbic areas, the endocrine system and the autonomous system. Finally, the integration of adiposity signals by the hypothalamus, and the permanent dialogue between this structure and the DVC, allow to adapt meal size to the size of fat stores, and to maintain the body weight.

hunger and the appearance of satiation (i.e. feelings of fullness), followed by satiety (i.e. prolongation of the meal interval until hunger) [15]. The regulation of eating behavior implies both "satiety" signals mainly produced by the digestive tract in response to food ingestion and "adiposity" signals produced in proportion to the amount of fat in the body [16]. The energy value of food ingested, which determines meal size, is estimated through a large number of satiety signals, such as gastric distension, cholecystokinin (CCK), oxyntomodulin (OXY), Glucagon-like peptide 1 (GLP-1) or pancreatic polypeptide (PP). On the other hand, the estimation of body fat stores is based on a continuous monitoring of hormonal signals such as leptin and insulin and their integration establishes a negative feedback on food intake. Satiety and adiposity signals mainly act within the hypothalamus and the caudal brainstem to control appetite and stabilize body weight [14].

The emblematic adiposity signals insulin and leptin primarily interact with specific receptors located within the hypothalamus. The hypothalamus consists of several nuclei that have been historically shown to be important in food intake. These nuclei include the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the ventromedial nucleus (VMH), the dorsomedial nucleus (DMH), and the lateral hypothalamic area (LHA). Because of a rather leaky BBB barrier at this site, the ARC is ideally located to receive body adiposity signals, as well as to integrate diverse hormonal and neural signals. Indeed, both insulin and leptin differentially were shown to affect two neuronal populations within the ARC, i.e. proopiomelanocortin/cocaine-and amphetamine related transcript (POMC/CART) and neuropeptide Y/agouti-related peptide (NPY/AgRP) neurons [14] Fig. (**1**). POMC/CART neurons relay the anorexigenic action of leptin or insulin. Briefly, in these neurons, POMC is cleaved to α-melanocyte stimulating hormones (α-MSH) which acts on melanocortin 3 and melanocortin 4 receptors (MC3R and MC4R) of neurons mainly located in the PNV to reduce appetite. In accordance, endogenous and synthetic agonists for MC3R/MC4R have been shown to cause hypophagia and weight loss in experimental animals [17]. The catabolic action of both leptin and insulin relies upon α-MSH signaling since the central administration of MC3R/MC4R antagonists was shown to block their actions in the brain. NPY/AgRP neurons projects to the same brain areas as POMC/CART neurons. AgRP acts as an antagonist on MC3/4R, an effect which thwarts the action of α-MSH secreted by POMC neurons [18, 19]. Moreover, NPY acts on Y receptors to stimulate food intake. As a result, both AgRP and NPY induce hyperphagia when acutely administered in hypothalamic structures and increase body weight when chronically infused in the same brain areas [20-22]. In agreement, injection in the hypothalamus of a recombinant adeno-associated viral vector that overexpressed either NPY [23] or AgRP [24] results in increased food intake, bodyweight and adiposity. Both insulin and leptin suppress the activity of orexigenic NPY/AgRP while they increase that of anorexigenic POMC/CART neurons in the ARC. It has been shown that NPY/AgRP neurons directly contact the POMC/CART neurons to inhibit their activity through the liberation and action of NPY and GABA [25]. Moreover, POMC/CART and NPY/AgRP neuronal populations, also called "first-order neurons", widely project to "second-order neurons" located in the PVN, the LHA, the DMH, and to a lesser extent the VMH, and schematically form two tracts often acting in parallel. In addition, these two tracts project to forebrain limbic areas involved in motivation and reward (not described here), and to areas of the hindbrain involved in satiation and satiety control [14, 26] Fig. (**1**). In summary, the POMC/CART-originating tract has an overall catabolic effect whose increased activity induces a decrease in food intake and increased energy expenditure, and may result in body fat loss. Conversely, the NPY/AgRP-originating tract is anabolic, and its increased activity causes an increase in both food intake and body fat stores.

Numerous studies have shown that the presence of food in the gastrointestinal tract induces a gastric distension and a subsequent production of many "satiety signals" which altogether promote satiation and the subsequent satiety processes Fig. (**1**). The prototypical satiety signal is the duodenal peptide cholecystokinin (CCK), which is secreted by a population of mucosal endocrine cells (I cells) in the small intestine in response to dietary lipid or protein and which acts to reduce meal size [27]. Many other satiety signals have been described to participate to the satiation and/or satiety processes such as the Glucagon-like peptide 1 (GLP-1), the Peptide YY 3-36, the Pancreatic Polypeptide (PP) and amylin. GLP-1 is generated from proglucagon and released in response to food intake by the L cells in the mucosa of the ileum and colon [28]. PYY3-36, which has a strong affinity for the Y2 receptor [29], is a member of the pancreatic polypeptide family released from the enteroendocrine cells located primarily in the ileum and colon in proportion to caloric load [30]. PP is secreted postprandially by specialized islet cells under vagal control in proportion to caloric load and acts primarily on Y4R and Y5R [31]. Finally, amylin, a peptide postprandially co-secreted with insulin by pancreatic β cells, was shown to decrease food intake [32, 33]. The integration of these satiety signals is performed at the brainstem level and especially in the Dorsal Vagal Complex (DVC). The DVC is located in the caudal brainstem and comprises three interconnected structures, namely the nucleus tractus solitarii (NTS) which is the relay of sensory inputs arising from the vagus nerve, the area postrema (AP) which constitute a circumventricular organ, and the dorsal motor nucleus of the vagus nerve (DMNV) which contains the parasympathetic preganglionic neurons. It has been shown that most satiety signals can act both in a paracrine manner on specific receptors located on vagal afferent fibers innervating the gastrointestinal tract, and in an endocrine fashion to reach the AP and the NTS.

Nevertheless, to avoid a too schematic representation of food intake behavior control, it should be mentioned that satiety signals, which mainly act via the DVC, also affect hypothalamic neuronal pathways [34]. Conversely, a growing body of data indicates that neuronal populations of the DVC also contain leptin and insulin receptors, glucose-sensing mechanisms, and neuropeptide mediators relevant to energy balance [35]. As an illustration, leptin was shown to act directly within the DVC [36, 37] and intraparenchymal injections of MC4R agonists in the DVC reduce food intake [38]. More recently it was shown that brainstem NTS neurons expressing POMC are activated by the hormone CCK,

and that activation of the brainstem neuronal MC4R is required for CCK-induced suppression of feeding [39]. In the light of these mechanisms and neuronal pathways, responsible for the homeostatic regulation of energy balance and characterized during the last decade, it was postulated that targeting these pathways and modifying the central anorexigenic/orexigenic balance may be helpful to treat obesity. In the present manuscript, we overviewed the anti-obesity drugs currently under development and the patents recently published that target central anorexigenic or orexigenic pathways, in the hope to develop new anti-obesity strategies.

PHARMACEUTICAL TREATMENT OF OBESITY, PAST AND PRESENT

To date, treatment options against obesity remain limited (see Table **1**). Orlistat (Xenical by Roche or Alli by GlaxoSmithKline), a potent gastric and pancreatic lipase inhibitor that reduces the intestinal digestion of fat, remains the most commonly used anti-obesity medication. Approved for use for about ten years, the drug dose-dependently increases fecal fat loss by 30%. Orlistat is recommended to be used with a diet that has 30% of its energy as fat. Orlistat drawback is the steatorrhea (excess fat in feces) associated with high-fat diets, which results in a low complying to treatment [40]. Moreover, Orlistat was recently reported to alter gastric emptying and to reduce the postprandial secretion of GLP-1, PYY and CCK. These changes in gastrointestinal hormone release may raise hunger feeling and increase food consumption and should therefore be considered as potential side effects when using this drug for the treatment of obesity [41]. While lifestyle changes are effective, a lot of patients struggle to comply with medical recommendations, i.e. greater physical activity and lower calories diet. The pharmaceutical industry is still searching for drugs that could be an alternative to gastric surgery and able to induce substantial long-term weight loss without excessive side effects. Historically, the early anti-obesity drugs launched on the global market were amphetamine derivatives. Phenmetrazine (Preludin), Phendimetrazine (Bontril, Adipost, Anorex-SR) and Phentermine (Adipex-P, Obenix, Oby-Trim) were used as short-term treatments and combined with exercise and diet. Despite their stimulating and addictive properties, these compounds, except for Preludin that has been withdrawn from the market, remain the number one anti-obesity therapy. 5-HT uptake inhibitors such as Dexfenfluramine (Redux) or Fenfluramine (Pondimin, Ponderax and Adifax) that increase the feeling of fullness and cause a loss of appetite [42] were also used for anti-obesity therapy. These compounds had the advantage over the amphetamine derivatives to have lower abuse potential and were used for many years for short-term (a few weeks) management of obesity. Fenfluramine and dexfenfluramine were also used in combination with phentermine, the well-known "Fen-phen" and "dexfen-phen" prescriptions. However, these drugs were removed from the market in 1997 due to the report of heart valve disease and pulmonary hypertension [43] (Table **1**). Sibutramine induces weight loss by increasing satiety through the selective inhibition of serotonin and norepinephrine neuronal reuptake. It was also shown that sibutramine might augment energy expenditure in both the basal and fed states. In addition, sibutramine treatment in animals and in humans may alter central NPY and POMC mRNA levels [44]. Sibutramine (Meridia) was approved in the USA in 1997 and in the European Union in 1999. In 2007, Sibutramine Cardiovascular OUTcomes (SCOUT) trial [45] reported a higher rate of cardiovascular disease events in the sibutramine group compared with placebo group in individuals with pre-existing cardiovascular diseases or diabetes mellitus (11.4% versus 10.0%). Sibutramine was finally withdrawn from the European and US markets in January and October 2010 respectively, due to these cardiovascular concerns. In October 2010, another promising agent, Lorquess (Arena Pharmaceutical's lorcaserin) a selective 5-HT2c receptor agonist was turned down by the US Food and Drugs Administration (FDA). Lorquess induced only minor human weight loss and preclinical studies revealed an increased risk of breast cancer and astrocytoma at low doses. Currently, a potential candidate, Tesofensine, a monoamine reuptake inhibitor, is under development by Takeda Neuroresearch. While phase II has demonstrated substantial efficacy and good tolerability with no abuse potential, phase III trial will carefully examine potential increased risk of cardiovascular diseases as previously observed for Meridia [40] (Table **2** and **3**). Since its identification, leptin has been considered as potentially helpful in the management of obesity, given its anorexigenic effect and influence on food intake and energy expenditure. However, the efficacy of exogenous leptin, even at high doses, to induce weight loss in the population of non leptin-dependent obese, has been disappointing. In diet-induced obese mouse and most obese humans, leptin levels are already elevated and tissues exhibit leptin resistance. Accordingly, exogenous leptin does not cause any weight loss [46]. Nevertheless the administration of low doses of leptin for the maintenance of weight-reduction during diet was proposed by some authors. The decreased circulating leptin level resulting from weight loss and fat mass reduction was proposed to account for the energy expenditure decline and decreased circulating T3 and T4 observed during diet. These changes seem to be reversed by 'replacement doses' of exogeneous leptin [47]. Ciliary Neurotrophic Factor (CNTF) is an endogenous neuroprotective factor (22 kD) produced by Schwann cells and astrocytes, whose expression is up-regulated by injury to these cells. CNTF was found to induce marked weight loss in non-obese patients treated with this neurotophin for amyotrophic lateral sclerosis. This result was explained by the fact that CNTF and the leptin have a related intracellular signalling mechanism i.e. Janus kinases and signal transducers and activators of transcription 3. The neurotrophin binds to the CNTF receptor and activates leptin-like intracellular signalling pathways in brain nuclei that regulate food intake and body weight. In accordance, CNTF, like leptin, is capable of causing pronounced weight loss in leptin-deficient obese mice. Interestingly, a genetically engineered variant of CNTF (rhv)CNTF called Axokine (Regeneron Pharmaceuticals, [48]) with increased potency and improved pharmacological properties causes weight loss in diet induced obese mice, suggesting that this agent may bypass the leptin resistance present in this animal model. Axokine has been tested in two phase II studies, one in overweight patients and the other in diabetic patients, and in a phase III study in overweight patients (Table **2**). Despite promising results, the benefit of the treatment was not considered as efficient

Table 1. Past and Present of Anti-obesity Drugs

Satus	Drug Name	FDA Approval Date *(or First Use)*	Type of Use	Company	Mechanism of Action	Side Effects	Ref.
Drugs Previously Used for Treatment of Obesity							
Discouraged	Thyroid hormone	*1893 (first use)*			Increase metabolic rate	Hyperthyroidism, palpitations	[40]
Withdrawn	Dinitrophenol	*1932 (first use)*			Increase metabolic rate (mitochondrial action)	Neuropathy, cardiac complications	[15, 40, 156]
Banned	desoxyephedrine (Pervitin®, Desoxyn®)	1947			Sympathomimetic drugs (DA and NA reuptake inhibitor)	Addiction, anxiety, hallucinations	[156]
Withdrawn	Phenylpropanolamine	1939				Haemmoragic strokes	[156, 157]
Withdrawn	Phenmetrazine (Preludine®)	1956	Short term	Boehringer-Ingelheim		Addiction, tachycardia, hypertension	[42]
Banned	Rainbow pills regime	1968			Combination of effects from amphetamines, thyroid hormone, diuretics, digitalis, laxatives...)	Death by arrythmia	[157]
Withdrawn	fenfluramine (Pondimin®, Ponderax®, Adifax®)	1973	Long term	Wyeth	5-HT reuptake inhibitor, Release of 5-HT	Valvulopathy, pulmonary hypertension	[42]
Withdrawn	Dexfenfluramine (Redux®)	1996	Long term	Interneuron Pharmaceutical		Valvulopathy, pulmonary hypertension	[42]
Withdrawn	Phen/Fen (mixture Phentermine/Fenfluramine)	1997	Long term		5-HT reuptake inhibitor, Release of 5-HT	Valvulopathy, pulmonary hypertension	[43]
Withdrawn	Sibutramine (Meridia®, Reductil®, Sibutral®)	1997	Long term	Abott	NA and 5-HT reuptake inhibitor	Tachycardia, hypertension, nausea	[40, 159]
Withdrawn	Rimonabant (Acomplia ®)	2006	Long term	Sanofi-Aventis	CB1-receptor antagonist	Depression, anxiety, nausea	[40]
Drugs currently used for treatment of obesity							
Current	Phentermine (Ionamin®, Adipex-P®)	1959	Short term	Medeva (Ionamin®), Gate (Adipex-P®)	DA and NA reuptake inhibitor	Addiction, tachycardia, hypertension	[35, 40, 156]
Current	Diethylpropion (Tepanil®, Tenuate®)	1959	Short term	Watson Pharms	DA and NA reuptake inhibitor	Addiction, tachycardia, hypertension	[40, 156]
Current	Phendimetrazine (Bontril®)	1959	Short term	Valeant	DA and NA reuptake inhibitor	Addiction, tachycardia, hypertension	[40]
Current	Benzphetamine (Didrex®)	1960	Short term	Pharmacia and upjohn	DA and NA reuptake inhibitor	Addiction, tachycardia, hypertension	[40]
Current	Mazindol (Sanorex®)	1973	Short term	Novartis	5-HT, DA and NA reuptake inhibitor	Hypertension, nervousness	[156, 160]
Current	Orlistat (Xenical®, Alli®)	1999	Long term	Roche (Xenical®), GSK (Alli®)	Lipase inhibitor	Steatorrhea, diarrhea	[40, 161]

Abbreviations: 5-HT: serotonin, DA: dopamine, NA: noradrenaline

enough to productively market. Two-thirds of those receiving Axokine indeed developed antibodies after 3 months that limited weight loss [49]. Regeneron Pharmaceuticals withdrew their request for FDA endorsement and Axokine development was terminated. Whether this strategy will be followed by others in the future remains an open question. Nonetheless, Novozymes Biopharma has published a patent related to the invention of a fusion protein comprising an albumin or a fragment of albumin and a fragment of active peptide that activates the CNTF receptor. These authors

Table 2. Recent Disappointments in the Development of Anti-obesity Drugs

Drug Name	Company	Satus	Mechanism of Action	Side Effects	Ref.
Lorcaserin (Lorquess®)	Arena Pharma-ceutical	Denied approval (2010)	Selective 5-HT2c receptor agonist	Breast cancer and astrocy-toma	[156], [162]
bupropion + naltrexone (Contrave®)	Orexigen	Denied approval (2011)	DA & NA reuptake inhibitor + opioid antagonist.	Tachycardia, hypertension, Nausea	[40]
phentermine + topiramate (Qnexia®)	VIVUS	Denied approval (2010)	DA and NA reuptake inhibitor + GABA agonist	Depression, Tachycardia, teratogenicity	[156]
Taranabant	Merck & Co	Development discontinued after Phase III	CB1-receptor antagonist	Depression and anxiety	[68]
Otenabant	Pfizer	Development discontinued after Phase III		Depression and anxiety	[69]
Ibipinabant	BMS/Solvay	Development discontinued after Phase II		Depression and anxiety	[70]-[73], [156]
AZD-1175	AstraZeneca	Development discontinued after Phase I			
Axokine	Regeneron	Development discontinued after Phase III	CNTF analog	Immunological response, nausea cough	[40]
CE-326597	Pfizer	Development discontinued after Phase II	CCK-1 receptor agonist		

Abbreviations: 5-HT: serotonin, DA: dopamine, NA: noradrenaline

reported that fusion of Axokine with a portion of albumin (Albunime-fused Axokine) is sufficient to prolong Axokine *in vivo* half-life by 50 fold. Albumin-fused Axokine induces a more pronounced weight loss in leptin-deficient mice when compared with Axokine ([50]; Table **3**). Two other patents related to CNTF signalisation were recently published. Signal transduction by CNTF requires its binding to CNTFR alpha and subsequent recruitment of gp130 co-receptor subunit. A first patent reported gp130 monomers with increased binding activity [51] and their potential use in medical area. The second one disclosed a method to identify and produce gp130 modulators with higher performances than native ligands [52].

One of the most promising therapeutic approach of the last decade for treating human obesity was to specifically inhibit the cannabinoid receptor type I (CB1) of the endocannabinoid system. The endocannabinoid system consists of two G-protein coupled receptors (GPCR), i.e. CB1 and CB2, of their ligands, the two major endocanna-binoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and of proteins regulating cannabinoid receptor activity and endocannabinoid tissue levels. While CB2 are mostly present on immune cells and involved in cannabinoid-mediated immune modulation, CB1 is one of the most abundant and widely distributed GPCR in the mammalian brain even though it is also expressed, at a lower extent, in some peripheral tissues such as the adipose tissue, liver, and muscles [53]. Due to its wide expression in the brain, CB1 has been involved in many functions such as memory, drug dependence, anxiety, emesis, and energy homeostasis regulation. CB1 activation has been shown to increase food intake and body weight [54], and to contribuate to fat accumulation [55]. Increasing evidences for overacting endocannabinoid system during conditions of metabolic disorders such as obesity [56] has afforded the emergence of an entirely new class of drugs selectively targeting CB1 receptors for the treatment of this pathology. The first-in-class CB1 antagonist was reported in 1994 by Rinaldi-Carmona *et al.* [57] in a study demonstrating that the orally active SR141716A, a pyrazole derivative developed by Sanofi-Aventis [58] today known as Rimonabant or Acom-plia, displays selective nanomolar affinity for the CB1 (Ki = 5.6+-0.5nM), and antagonizes classical pharmacological and behavioral effects of cannabinoid receptor agonists, e.g. tail flick, ring-immobility, and hypothermia. Afterward, various preclinical studies have shown that chronic treatments with rimonabant reduce food intake, body weight gain and improve serum lipid profile in high-fat diet induced obesity rodent models [59, 60], or in some genetic models of obesity such as ob/ob mice, db/db mice and Zucker rats [61-62]. These promising results led to engage Rimonabant in four clinical trials called Rimonabant In Obesity (RIO) whose results were published beetwen 2005 and 2008 [63-66]. All studies reported increased weight loss compared with placebo over 6–12 months, with few tolerability or safety concerns. In the RIO-Europe study, the investigators concluded that Rimonabant was generally well tolerated with mild and transient side-effects [64]. However, the individual trials showed trends to increases in depressed mood, depression, and suicidal thoughts. Furthermore, because patients with serious mental illness were excluded from the RIO programme, the estimates of the potential psychiatric side-effects of the drug are conservative. The cannabinoid (CB1) antagonist Acomplia

(Ribonabant, Sanofi-Aventis) was launched in the EU in 2006. Meta-analysis of all published randomised controlled trials to assess the safety of this new drug suggested that 20 mg per day Rimonabant increases the risk of psychiatric adverse events i.e., depressed mood disorders and anxiety [67]. The FDA asked for more detailed safety information about Rimonabant and potential risk of psychiatric disorders in larger patient numbers over the long-term before the drug could be approved. The marketing authorization for Acomplia in EU was then removed in January 2009. In parallel to the development of Rimonabant, many new small molecules acting as CB1 antagonists with diverse chemical structures have been prepared and studied for their potential anti-obesity activity. For example, Ibipinabant (SLV319; [68]; Solvay pharmaceuticals), Otenabant (CP-945,598, [69], Pfizer) or Taranabant (MK-0364; [70]; Merck & Co) have been reported to be involved in different phases of clinical trials. Despite promising preclinical efficacy studies, most companies have decided to discontinue their clinical programs either based on changing perspectives on the risk/benefit profile of the CB1 class, or due to high levels of central side effects, mainly depression and anxiety [71-73] (Table **2**). However, given the improvement of serum lipid profile induced by Rimonabant, an alternative strategy, always in the pipe, has been initiated to limit or prevent the passage of the CB1 antagonists through the blood-brain barrier (BBB) in order to prevent side effects due to large central action. The patent entitled "Cannabinoid receptor antagonist/inverse agonists useful for treating metabolic disorders, including obesity and diabetes" [74] disclosed the use of novel pyrazoles which could be linked to a P-glycoprotein, or prepared in such a manner as to create a charge within the molecule, in order not to penetrate the BBB. In the same context, the patent called "Cannabinoid receptor modulators" [75] is also related to pyrazole derivatives associated with a lowered central action on CB1 receptors that could reduce propensity to induce psychiatric and nervous system side-effects. By continuation, these inventors, from 7TM Pharma, have published experimental data on the *in vivo* properties of derivative compounds which show a better plasma/brain ratio and a potent equivalent anti-obesity effect when compared to Rimonabant [76] (Table **3**).

Therapeutic anti-diabetic approaches for enhancing incretin action were launched, which involved exenatide (Byetta), an exendin-based GLP-1 receptor agonist which resists to degradation (incretin mimetics). It has also been hypothesized that incretin mimetics could be used as a treatment against obesity in patients without diabetes [77]. GLP-1 stimulates insulin and suppresses glucagon secretion, inhibits gastric emptying, and reduces appetite and food intake. Liraglutide (NN2211, Novo Nordisk) [78], marketed under the brand name Victoza, is a long-acting GLP-1 analogue that has been developed for the treatment of type 2 diabetes. A 20-week clinical trial was performed including 564 individuals (18–65 years of age, body-mass index 30–40 kg/m^2) who received an oral administration of either Liraglutide once a day or Orlistat three times a day. In parallel to drug treatment, all individuals had a 500 kcal per day energy-deficit diet and increased their physical activity. The authors concluded from this study that Liraglutide treatment over 20 weeks is well tolerated and induces weight loss and improves certain obesity-related risk factors [79].

Alternatively, novel treatments exploring new therapeutic avenues which target the CNS are under development (Table **3**). As an illustration, Shionogi & Co, Ltd has announced positive results from a phase II study of velneperit (S-2367) a novel NPY Y5 receptor antagonist [80]. In the case of chronic administration, the hyperphagic effects of NPY are prolonged, leading to the development of an obese state [81]. Despite pharmacology with equivocal results, antagonists of the NPY Y5 receptor subtype are promising since NPY analogues with high selectivity for this receptor strongly stimulated food intake [82]. In the phase II study of velneperit, obese subjects were first submitted to low calorie diet (900-950 kcal/day) for four weeks and then received either placebo or S-2367 (400 or 1600 mg/day) once daily for a period of 12 weeks. Shionogi & Co, Ltd reports that S-2367 produced a significant continuation of weight loss following the low calorie diet. It should be noted here that another company i.e. Merck & Co has abandoned its Y-5 antagonist (MK-0557) as an anti-obesity therapy. In the same context, 7TM Pharma has established a program aimed at developing agonists targeting the Y receptors for treatment of obesity based on PYY3-36 and PP activity. This program includes peptide agonists having potent efficacy on the Y2 and/or Y4 receptor with excellent selectivity over the Y1 receptor, which activation is involved in causing cardiovascular side effects [83]. Originating from this program, Obinepitide (TM30339) was selected by 7TM Pharma as the best lead candidate for clinical development and is currently in Phase I/II. The drug candidate Obinepitide is a synthetic analogue of satiety signals PYY3-36 and PP. In this elegant approach, the properties of both of these hormones have been integrated into Obinepitide which has a dual selectivity profile, targeting the Y2 and Y4 receptor. 7TM Pharma announced that in pre-clinical studies conducted in diet-induced obese animals Obinepitide has demonstrated clear superiority in respect of long term reduction in body weight as compared for instance to the natural hormone PYY3-36, which targets the Y2 receptor only. 7TM Pharma also stated that first-in-man Phase I/II clinical study have demonstrated safety and well tolerability of the drug candidate. Importantly, in obese human subjects, once-a-day subcutaneous (s.c.) dosing of Obinepitide seems to significantly inhibit food intake up to 9 hours after dosing.

PROSPECTIVE CENTRAL THERAPEUTIC TARGETS

Notwithstanding, possible progress in obesity therapy using drugs currently under development and listed above, the need for additional, efficient and safety compounds able to reduce food intake and body weight on the long-term should be high in the close future. These new therapeutic avenues could emerge from our increased understanding of the central mechanisms regulating food intake. We have listed below a non-exhaustive list of patents and studies related to central pathways whose modulation could be helpful in the management of appetite (satiation or postmeal satiety) and as such constitute potential therapeutic avenues in the future (summarized in Table **3**).

Table 3. Anti-obesity Drugs Under Development and Recent Patents

Target	Drug Name / Type	Development Phase	Company	Ref.
NA and 5HT uptake inhibitor + cholinesterase inhibitor	THR-4109	Phase II	Theracos	
NA and DA reuptake inhibitor + GABA agonist	zonisamide + bupropion	Phase II	Orexigen	[40]
5-HT, NA and DA reuptake inhibitor	Tesofensine	Phase II	Neurosearch	[156]
5HT2C agonist	ATHX-105	Preclinical	Athersys	
oxyntomodulin signaling	OAP-189 (TKS-1225)	Phase I	Pfizer	
GLP-1 signaling	Exenatide (Byetta ®)	Phase II (obesity)	Eli Lilly / Amylin	[77], [156]
	Liraglutide (Victoza®)	Phase II (obesity)	Novo Nordisk	[78], [79], [156], [163]
NPY-Receptors	Velneperit	Phase II	Shionogi & Co	
	Obinepitide	Phase I/II	7TM Pharma	
	PYY3-36 (oral form)	Phase I	Emisphere	
CB1-receptors	Pyrazoles derivatives	Discovery / Preclinical	7TM PHARMA	[75], [76]
	pyrazoles linked to P-glycoprotein	Discovery	Jenrin Discovery	[74]
Amylin agonist	Pramlintide (Symlin®)	Phase II (obesity)	Amylin Phamaceuticals	[134], [135], [156]
	davalintide	Discovery / Preclinical	Amylin Phamaceuticals	[136]
MC4R	TTP435 (AgRP inhibitor)	Phase II	Transtechpharma	
	AP1030	Phase I	Action Pharma	
	Peptides modulating MC4R	Discovery / Preclinical	IPSEN Pharma, Novo Nordisk, Biomeasure	[85], [86], [90]
	Cyclic peptides modulating MC4R	Discovery / Preclinical	Palatin Technologies, Biomeasure	[87], [88], [89], [94]
	Hydrazine substitued piperidine	Discovery / Preclinical	Palatin Technologies	[96]
	Diamine-containing tetra-substituted piperazine	Discovery / Preclinical	Palatin Technologies	[97]
	Amine substitued piperazine	Discovery / Preclinical	Palatin Technologies	[98]
	Piperazine and keto-piperazine	Discovery / Preclinical	Palatin Technologies	[99]
	Acylated piperidine derivatives	Discovery / Preclinical	Merck	[100]
	α-MSH derivative-Fc conjugate	Discovery	Centocor Ortho Biotech Inc	[95]
BDNF/TrkB signaling	anti-TrkB, anti-BDNF or anti-NT4/5 antibodies	Discovery / Preclinical	Pfizer	[119]
	antisense oligonucleotides targeting BDNF	Discovery	Curna	[111]
	BDNF + PUFE/EFA	Discovery	*Personnal Patent (Allam et al)*	[114]
MCH-1 antagonists	ALB-127158(a)	Phase I	Albany Molecular Research	
	Non basic MCH-1 antagonists	Discovery	Bristol-Myers Squibb	[121]
	Azabicycloalkane-indole and -pyrrolo-pyridine	Discovery	Albany Molecular Research	[122]

(Table 3) Contd….

Target	Drug Name / Type	Development Phase	Company	Ref.
Nesfatin-1 signaling	Nesfatin-1 and related peptides	Discovery / Preclinical	Teijin Pharma limited	[147]
	nesfatin-1 fragments (	Discovery / Preclinical	Teijin Pharma limited	[148]
	recombinant Nesfatin-1 fragment Pladin	Discovery / Preclinical	Landing Biotech	[151]
CNTFsignaling	Albumin-fused Axokin	Discovery / Preclinical	Novozymes Biopharma	[50]
Calcitonin analogs	Calcitonin analogs	Discovery / Preclinical	Unigene Laboratories	[137]
Bone morphogenic factor signaling	BMP and BMP mimetics	Discovery / Preclinical	Joslin Diabetes Center	[140]

Abbreviations: 5-HT: Serotonin, DA: Dopamine, NA: Noradrenaline

Melanocortins and Melanocortin Receptors

As mentioned above, melanocortins and melanocortin receptors, especially MC4R, are key components of the central circuitry regulating appetite and food intake. Hence, compounds with either agonist or antagonist activity with high specificity for melanocortin receptor subtypes are actively searched for many years as attested by the large number of patents published this last decade which disclosed identification of melanocortin receptor modulators. The use of such compounds will permit to take advantage of physiological responses associated with the melanocortin receptors and accordingly helpful in the management of various diseases. More particularly, specific MC4R ligands are believed to be useful in the treatment of obesity and food intake related disorders (hyperphagia, binge eating, bulimia and compulsive eating; [84]). This research field is still lively and vigorous and numerous recently published patents disclosed the use of various compounds as modulators of melanocortin receptors. We have listed below some of these recent patents and associated inventions to illustrate the different strategies used to identify modulators of melanocortin receptors.

Some of these recent patents related to the invention of new peptides which derived from the primary sequence of the α-MSH hormone. The patent entitled "Use of melanocortins to treat dyslipidemia" ([85]; Ipsen Pharma), disclosed the use of various peptides which are MC4R ligands, to treat dyslipidemia characteristic of obesity and overweight and led to promising results. *In vivo* efficacy of a peptide called compound A on appetite was tested on obese Zucker rats. Compound A (50 or 500 nmol/kg/day) subcutaneously infused induced a dose-dependent inhibition of food intake and body weight in Zucker rats associated with a decrease in serum triglyceride, cholesterol and free fatty acid levels. In the same field, the patent named "Peptides for the treatment of obesity" by Novo Nordisk [86] related to novel peptides with improved water solubility at neutral pH. The inventors reported the conception of specific MC4R agonists with high affinity and *in vivo* efficacy. Several of the generated peptides exhibited high affinity for MC4R (Ki <1 nM) and *in vivo* efficacy studies on appetite showed that several of these compounds reduced significantly the 24 and 48h food consumption with a 1-3 mg/kg solution administered intraperitoneally, orally or subcutaneously. Numbers of other patents are directed to the invention of cyclic peptides as specific melanocortin receptor ligands. For instance, cyclic peptides disclosed in the patent ([87]; Palatin Technologies Inc.) have been found to be MC4R ligands. One of these peptides (peptide N°1) was reported to be a melanocortin receptor agonist, with an EC50 of 1nM and an intrinsic activity of 91 % on MC4R where 4-Norleucine, 7-D-phenylalanine-a-melanocyte-stimulating hormone (NDP-α-MSH), a synthetic analogue of α-MSH, is 100%. Interestingly, changes in food intake and body weight were evaluated for this new generated peptide for 24, 48 and 72 h. In these rat feeding studies, using bremelanotide, a non-specific MC4R agonist as a positive control, peptide N°1 (0.3-1 mg/kg) was reported to significantly reduce food intake and body weight over a period of 20 hours. Similarly in the US 2010/0173834 patent [88], authors also reported the invention of cyclic peptides as melanocortin receptor ligands. Four compounds (compound A, B, C and D) of this invention were tested in chronic feeding experiments in the rat. These compounds, subcutaneously injected three times a day for seven days with increasing doses (37.5, 150 and 600 nmol/kg/day) significantly reduced food intake and body weight by approximatively 20% at the higher doses. In another recent patent, Dong and collaborators [89] described a novel class of cyclic peptides modified with hydantoin moiety, which act as modulators of MCR, with a particular selectivity for MC4R. This new class bears modified amino-acid residues (unnatural or synthetic) in the N-terminal region of the peptides, these substitutions contributing to the selectivity of the compound and the binding affinity at the targeted receptor. These compounds exhibit a low nanomolar affinity with MC4R and improved pharmacokinetic properties when compared with α-MSH, in particular a prolonged half-life. *In vivo* efficacy studies have been conducted in the rat, using several compounds of this novel class, and the results indicate that these new compounds induce a significant decrease in food intake six hours after a single subcutaneous injection, and a significant decrease in food intake and body weight gain after chronic sc injections, and therefore might be useful in the prevention and treatment of obesity. Non-naturally occurring melanocortin peptidic ligands, in a linear or cyclized form, having a degradation-resistant C-terminal extension, can act as MC3R, MC4R and MC5R agonists or antagonists [90]. Interestingly, these new peptides provided in this patent have a degradation-resistant C-terminal extension consisting of one

amino acid, modified or not, a peptide mimetic, or a combination. These peptides could be of great interest in the treatment of obesity since the C-terminal extension allows to minimize or abolish the well known cardiovascular associated side effects observed with melanocortin analogues used [91,92]. Central melanocortinergic signaling is indeed involved in the blood pressure control in rodents and humans. Studies in rodents have shown that acute central administration of α-MSH increases mean arterial pressure and heart rate [93]. In humans, the prevalence of hypertension is significantly lower in MC4R-deficient subjects [92]. Although promising, the *in vivo* efficacy and safety of such compounds are unfortunately lacking up to now. As described in a patent by Palatin Technology Inc [94], cyclic peptides with MCR specific affinity could also be generated through a lactam bridge by the side chain of aspartic acid and lysine or alternatively through a disulfure bridge formed by the side chains of two cysteines. Finally, an original approach using peptidic compounds in the treatment of melanocortin-related diseases, developed in the patent entitled "Melanocortin Receptor binding conjugates" [95] by Centocor Ortho Biotech Inc., should also be mentioned here. This approach is based on the development of compounds called α-MSH derivative-Fc conjugate, wherein the conjugate contains a fragment homologue or analogue to the wild-type α-MSH and a portion of an immunoglobulin hinge region. Fourteen α-MSH derivative structures were described in this patent.

In addition to the development of new peptides, a large number of patent applications disclosed small molecules as promising melanocortin receptor modulators. Among these organic compounds, the six-membered ring piperazine, piperidine and spiroperidine are known ligands to melanocortin receptors and particularly to MC4R, and therefore possibly useful in the treatment of disorders such as obesity. Accordingly, numerous derivatives generated from these molecules have been disclosed in various patents in the last decade. However, such compounds have a rather poor affinity and specificity and their use as drug compounds is limited. A continuous effort has been made to develop new piperazine and piperine derivatives with high specificity and affinity for specific MCR, especially for MC4R, that could be used alone or in combination with other pharmacologically active agents useful for the treatment of obesity [96-100]. For instance, several piperazine and keto-piperazine (1-10 mg/kg ip,), developed in one of these recent patents, were shown to significantly reduce food intake in mice feeding experiments for a period of 20 hours [99].

BDNF/TrkB Pathway

Among central signaling pathways currently investigated as potential targets for obesity treatment, the central BDNF/TrkB signaling appears interesting. Indeed, it has been shown that central injections of BDNF induce anorexia [101-104] and that BDNF/TrkB is a downstream effector of the hypothalamic and brainstem melanocortin system in food intake control [105,106; Fig. (**1**)]. Moreover, BDNF/TrkB Human recombinant BDNF has been shown to exert a potent anti-obesity activity with reduction of leptin resistance in mice obesity model induced either by a high fat diet or a partial BDNF gene invalidation (heterozygous BDNF knock-out mice; [107,108]. Furthermore, BNDF normalized blood glucose and blood lipid levels in diabetic db/db mice [109,110]. Accordingly, several methods and compounds have been recently proposed and developed in order to modulate the BDNF/TrkB pathway activity. These processes were based either on TrkB agonists strategy or on the modulation of endogenous TrkB ligands availability. For instance, an approach aiming at modulating BDNF expression in tissues has been recently published. This method consists in the use of antisense oligonucleotides (5 to 30 nucleotides) that target natural antisense BDNF polynucleotides to modulate the expression and/or function of the neurotrophin [111]. Hybridization of these oligonucleotides on their targets can induce a loss of activity of the targeted antisense, resulting in an up-regulation of the BDNF gene expression. The patent also provided methods and pharmaceutical compositions for the use of these antisens oligonucleotides in treating diseases and disorders associated with the expression of BDNF, in particular obesity. Considering that the anorexigenic action of BDNF involves the central nervous system, a particular focus is done by the applicant for a delivery at the cerebral level. This can be performed by the means of an injection or infusion directly within the cerebrospinal fluid or within specific brain areas as described in previous patents "Methods for slowing familial ALS disease progression" [112] and "Adenovirus vectors for the transfer of foreign genes into cells of the central nervous system particularly in brain" [113]. Alternatively, intravenous injection with the co-administration of agents promoting the penetration of oligonucleotides across the BBB such as an antibody for the transferrin receptor could be performed as proposed in the same patent. However, the therapeutic utility of these antisens oligonucleotides in pathologies such as obesity and diabetes in animals and humans remains to be further evaluated *in vivo*. Nonetheless, this approach can be of interest, since the principle of using antisense oligonucleotides in treating diseases has previously been validated by the FDA, with the approval of VITRAVENE™ for the treatment of cytomegalovirus retinitis. As an illustration of strategies based on BDNF/TrkB signaling enhancement, one could cite a recent patent providing a method aimed at stabilizing and potentiating therapeutic actions of BDNF. The authors proposed to administer BDNF as an admixture of the neurotrophin conjugated with one or more essential fatty acids (EFAs) and polyunsaturated fatty acids (PUFAs) for treating BDNF related diseases such as obesity and diabetes [114]. This concept is based on the fact that both BDNF and EFAs/PUFAs are i) endogenous mediators of neuronal survival, neurotransmission and synaptic plasticity that are likely interacting in the control of these processes ii) involved in the physiopathology of obesity, diabetes and metabolic syndrome. Although interesting as a concept, such a method must be further evaluated *in vivo* on animal models. It was also proposed to use the neurotrophin 4/5 (NT4/5), another known endogenous ligand for TrkB, for obesity treatment. NT4/5 has been shown to efficiently reduce body weight and food intake in normal, obese and diabetic animals after subcutaneous or central intraparenchymal injections [115]. This led to the publication of a patent disclosing a method for treating obesity or diabetes using effective amounts of mature human or modified NT4/5 [116].

While promising, these strategies targeting the BDNF/TrkB pathway and its increased activity for the treatment of obesity and associated disorders appear now somewhat tricky. Indeed, recent works have brought puzzling results concerning the role of TrkB signaling in the regulation of energy balance. The systemic administration of NT4/5 in non-human primates (rhesus monkey and baboons) failed to show a body weight or food intake reducing effect. On the contrary, such treatment resulted in hyperphagia accompanied with weight gain. This orexigenic action of NT4/5 seemed to involve a peripheral action of the peptide since its central injection remained effective to decrease food intake in rhesus monkey [117]. This discovery led to the publication of patents which took the opposite view to previously mentioned patents. A first patent related, for instance, to a method for treating unwanted weight loss, in particular in the cases of cachexia or anorexia nervosa in primates using the peripheral administration of a TrkB agonist [118]. A second patent detailed the anti-obesity potential of TrkB signaling blockade by the means of antibodies such as anti-TrkB, anti-BDNF or anti-NT4/5 antibodies. Surprisingly, the authors showed that a peripheral treatment with an anti-BDNF antibody is as effective as a treatment with NT4/5 in reducing body weight and food intake in obese high-fat fed mice. Moreover, the authors showed that an intravenous treatment with a soluble TrkB-Fc fusion protein induced a significant decrease in body mass index in Cynomolgus monkey [119]. It seems obvious, in the light of such conflicting results, that additional information are needed before to consider the BDNF/TrkB pathway as an inevitable and serious candidate for obesity treatment.

Melanin Concentrating Hormone (MCH)

Neurons expressing MCH are abundant in several hypothalamic sites including the lateral hypothalamus and exhibit reciprocal connectivity with POMC/CART and NPY/AgRP neurons in the ARC Fig. **(1)**. Administration of MCH to rodents stimulates feeding and it is thought that blockade of specific receptor subtypes for this neuropeptide could serve to inhibit appetite. In mice, administration of an MCH-1 receptor antagonist decreased body weight through decreased feeding and increased energy output [120]. Albany Molecular Research, Inc. (AMRI) has recently performed a Phase I clinical trial for ALB-127158(a), a MCH-1 receptor antagonist, for anti-obesity therapy. Clinical candidate ALB-127158(a) possesses high affinity for the MCH-1 receptor and AMRI proclaimed significant reduction in food intake and weight body mass in a chronic 28-day feeding study in diet-induced obese mice. The identification of new MCH-1 receptor antagonists with high *in vivo* efficacy remains a challenge. Among recent patents disclosing the discovery of potential antagonists targeting MCH-1 receptor, two of the most recent are untitled "non-basic melanin concentrating hormone receptor-1 antagonists" [121] and "azabicycloalkane-indole and azabicycloalkane-pyrrolo-pyridine MCH-1 antagonists, methods of making, and use thereof" [122]. Both patents proposed the use of generated compounds as potential anti-obesity drugs but *in vivo* efficacy of developed compounds remains to be established.

Orexins

The orexins, also called hypocretins, are neuropeptides discovered in 1998 by two independent groups. Orexins A (33 aac) and B (28 aac) derive from a precursor the pro-orexin polypeptide. Orexins are produced by groups of neurons localized within the lateral hypothalamus Fig. **(1)** which project to different regions of the brain. Orexin signalling is involved in a variety of biological processes including feeding, energy homeostasis, sleep wake cycles and addiction [123]. There is an abundant literature illustrating the weight of orexins in feeding behavior and energy homeostasis. For instance, intracerebroventricular injection of orexins during the light period induces feeding behavior in rats and mice. Moreover, central administration of anti-orexin antibody or of an orexin antagonist reduces food intake, and prepro-orexin knockout mice and transgenic mice lacking orexin neurons eat less than control wild-type mice. Finally, an OX_1R-selective antagonist reduced food intake and reduce obesity in leptin-deficient *ob/ob* mice [124]. In this context, interfering with orexin signalling may constitute a promising central target to treat obesity. Several patents reporting the invention of compounds with orexin receptor antagonist activity have been released recently. A set of patents reported the invention of amine derivatives i.e. non cyclic aminoamides [125], sulfonamides [126], malonamides [127] or N-aroyl cyclic amine derivatives [128] as potential orexins receptors antagonists. Alternatively aromatic compounds such as pyrazole [129], pyrimidine [130] or pireridine [131] derivatives were also disclosed for their potential use as pharmaceuticals drugs targeting orexin receptor. Clearly there is a great hope in the pharmacological manipulation of orexin signalling illustrated by the active research in this field supported by pharmaceutical companies and academic laboratories. However, to date, no orexin antagonist compounds have come onto the market. Only Almorexant (ACT-078573), an orally active dual orexin receptor antagonist, developed by Actelion Ltd, in collaboration with GlaxoSmithKline, has completed phase III clinical trial for the treatment of insomnia [132].

Amylin and Calcitonin Peptides Family

Amylin, also known as islet amyloid polypeptide (IAPP), is a 37 amino acid peptide from the calcitonin gene related peptide family, secreted by pancreatic beta-cells and recognized to be a physiological signal of satiation. Amylin reduces food intake via a meal size effect by promoting meal-ending satiation. This effect seems to depend on a direct action in the area postrema (AP), which is a brain area rich in amylin receptors. Animal data, together with clinical data in humans, indicate that amylin is a promising candidate for the treatment of obesity [133]. In accordance, pramlintide (Symlin, Amylin Phamaceuticals), a human amylin analogue approved by the FDA for use in therapy in patients with type 1 or 2 diabetes, has been shown to increase satiety and, therefore, decrease caloric intake via a central mechanism [134]. A randomized placebo-controlled trial including obese or overweight nondiabetic subjects has examined the safety and efficacy of pramlintide given alone or in combination

with either phentermine or sibutramine. The results of this study supported the potential of pramlintide-containing combination treatments against obesity [135]. Interestingly, unlike amylin, pramlintide shows the interesting property to be non-aggregative. Another potential anti-obesity drug based on amylin-mimetic activity is davalintide (AC2307; Amylin Phamaceuticals) which binds with high affinity to amylin, calcitonin and calcitonin gene-related peptide receptors. In rodents, acutely administered davalintide has been shown to reduce feeding with a longer lasting effect when compared with amylin (23h versus 6h) [136]. Interestingly, davalintide had no effect on locomotor activity or on induction of nausea at doses that decreased food intake. Davalintide infusion induced a dose-dependent weight loss over a period of 8 weeks [136].

As a member of the calcitonin family of peptides, amylin shares some biological activities with calcitonin, adrenomedullin, and the calcitonin gene-related peptides. Calcitonin of salmon origin (sCT) is one of these potent amylin analogues. Dose comparisons with amylin have demonstrated that sCT produces a more robust decrease in food intake through similar changes in meal pattern. The robust feeding decrease induced by sCT is due to its longer duration of action, a property that is attributed to its irreversible binding to amylin receptors. Both acute and chronic administrations of the amylin receptor agonist sCT are known to suppress appetite in different species but also exert potent bone anti-resorptive actions. Therefore, its use as modulator of appetite is limited. A recent invention reported the generation of calcitonin analogues which keep significant appetite suppressive activity but which have limited action on bones [137]. These peptides were generated by modification of either salmon or eel calcitonin which sequences share significant homology. At least one amino acid was modified to decrease the action of the new peptides on bones. The appetite suppressive effect of peptides of the present invention is believed to be mediated through the amylin receptor. These peptides could then be considered as superagonists of the amylin receptor with a reduced affinity to the calcitonin receptor. *In vivo* efficacy studies on food intake were performed in Beagle dogs receiving sCT, PYY or a new generated peptide formulated in enteric coated capsules for oral delivery. The capsules also contained excipients that inhibit proteases and increase peptide absorption. The authors provided results showing that some of the tested peptides reduced food consumption to the same extent than salmon calcitonin, with a lower binding affinity for the calcitonin receptor.

Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMPs) are multifunctional growth factors belonging to the TGF-β superfamily. BMPs play a pivotal role in inducing bone, cartilage, ligament and tendon formation, but they are also pluripotent factors involved in the regulation of embryonic development and postnatal homeostasis of various organs and tissues [138]. Recently, BMP7 was shown to promote the differentiation of brown preadipocytes thus constituting a potential target for the treatment of obesity at the peripheral level [139]. Interestingly, the same group published in 2011 a patent which disclosed the use of BMP7 or agonist/peptidomimetics thereof for decreasing appetite [140]. The invention related to the methods that increase BMP7 expression, activity or signalling in the subject central nervous system. This invention is based on data showing that mice hypothalamic expression of BMP7 and Type I receptor Acvr1 (ALK2) increased 14 days after the shift from normal diet toward high fat diet. This increase was not observed in the ARC nucleus but in nuclei containing second-order neurons. Acute icv BMP7 injection resulted in a 44.4% decrease in food consumed during the 4 hours following the administration and in a 60% up-regulation of POMC mRNA expression. Moreover, adenoviral-mediated BMP7 expression was used to chronically increase BMP7 levels. In both high fat diet induced obese mice and leptin deficient (ob/ob) mice, adenoviral-mediated BMP7 expression resulted in a significant reduction of food intake and body weight.

Nesfatin-1 and Related Peptides

Nesfatin-1, identified in 2006 by Oh-I and colleagues [141], is an 82 amino-acid peptide derived from the cleavage of the precursor nucb2. The interest generated by this peptide is based on its anorexigenic action performed independently of leptin signalization after injection in both the cerebral ventricles [141] and the peritoneal cavity [142]. Nesfatin-1 has been discovered using thiazolidine diones having PPAR-γ agonist activity. Nesfatin-1 was next found expressed in neurons of various brain areas including hypothalamic nuclei (PVN, Arc) and at the brainstem level (NTS, DMNX) [143-144]. Nesfatinergic neurons located in these structures are activated in response to refeeding or intraperitoneal injection of CCK suggesting that central nesfatin-1 could participate in the meal termination mechanismS [145]. Nesfatin-1 was also reported to be expressed by the stomach and the pancreas. In the pancreas, nesfatin-1 is present in β-cells of Langerhans islets and co-localized with insulin [146]. The discovery of nesfatin-1 gave birth to a patent entitled "Novel biological substance nesfatin and its related substances and uses thereof" disclosing the method for obtaining this peptide as well as potential related peptides [147]. This patent also encompasses the use of nesfatin-1 or related peptides in the treatment, control and diagnosis of diseases associated with eating disorders and/or body weight control. The inventors also described that peptides produced from a partial sequence of nesfatin-1, i.e. nesfatin-1M30, nesfatin-1M16, nesfatin-1M14 and nesfatin-1M10, were equally potent to reduce food intake and/or body weight [148]. To date the receptor(s) of nesfatin-1 was (were) not identified although its (their) identification is (are) highly expected in order to develop pharmacological agents or nesfatin-1 analogues able to act specifically on nesfatin-1 binding sites and mimic the action of the endogenous peptide. In 2009, a patent disclosed a screening method for the study of nesfatin-1 action using a protein receptor group consisting of GPR3, GPR6 and GPR12 [149]. GPR3, GPR6, and GPR12 constitute a family of constitutively active G protein-coupled receptors (GPCRs) that share more than 50% identity and 65% similarity at the amino acid level. They are highly expressed in the CNS, and their expression in various cell lines results in constitutive stimulation of cAMP production. Based on results comparing the cAMP production in GPCR-transiently expressing HeLa cells in response to Forskolin, commonly used to raise levels of cAMP, or Nesfatin-1, the authors proposed that

among the 33 selected GPCR, GPR12 represent a potential receptor for Nesfatin-1. Interestingly, mice deficient in this receptor were found to have higher body weight, body fat mass and were dyslipidemic [150]. Nevertheless, in this study no modification of food intake was observed in the GPCR12 KO mice. All together, these results suggest the existence of alternative nefatin-1 receptors in addition to GPR12 at least to support the anorexigenic action of the peptide. More recently, pladin, a 69-amino acid peptide has been found to be significantly elevated in the plasma of plasminogen (pgl) and leptin receptor (lepr) double knock-out mice when compared to plg+/+ lepr-/- mice [151]. This peptide seems to be responsible for the anti-diabetic effect of plasminogen invalidation observed in these double knock-out mice. Plasminogen gene invalidation indeed resulted in body weight and food intake reduction in leptin (ob/ob) or leptin receptor (db/db) deficient mice, and normalized blood glucose and serum insulin in db/db mice. In accordance, intravenous injection of recombinant pladin reduced blood glucose in diabetic db/db mice. After HPLC- isolation and MALDI-TOF sequencing, this peptide was identified as a portion of NUCB2 and called pladin for plasma anti-diabetic nucb2 peptide or plasmin related anti-diabetic nucb2 peptide. Pladin was also found in the serum of wild type mice, rat, rabbit and human. Moreover, nesfatin-1 contains the entire 69 amino-acid sequence of pladin. This identification of pladin as a portion of nesfatin-1 and its action on glycemia has led to the discovery of the anti-hyperglycemic action of nesfatin-1. Intravenous injection of nesfatin-1 was reported to significantly reduce blood glucose in hyperglycemic db/db mice [152]. Finally, the inventors reported that recombinant pladin exerted potent anorexigenic action after i.p or icv injection. In the continuation of this invention, another patent originating from the same group provided a method for treating diabetes by increasing peripheral nesfatin-1 or related peptides such as pladin. The author proposed to increase peripheral nesfatin-1 by direct administration of nesfatin-1, conjugated nesfatin-1 or plasminogen inhibitors [153]. Interestingly, blood nesfatin-1 levels were reported to be lower in type 2 diabetics than in healthy subjects [154].

CURRENT AND FUTURE DEVELOPMENTS

The discovery of the "magic pill" addressing obesity remains a complex challenge in order to stem the consequences of one of the most disturbing epidemic of the early 21th century. To date, very few compound has been firmly proved to induce a substantial weight loss without serious adverse effect on the long term. These last years, some drugs launched on the market and promising anti-obesity compounds engaged in clinical phase II or III trials finally led to a disappointment due to efficacy or safety issues, and were either withdrawn or rejected by the regulatory authorities. Safety of anti-obesity compounds is indeed of great importance since safety issues for such widely marketed drugs could have huge public health consequences considering the very broad population to which these products are intended. Besides these safety issues, the past and current drugs developed show a relatively weak effectiveness in reducing body weight in humans, with generally less than 5 or 10% reduction when compared with placebo. Even if it could provide a valuable help for a lot of overweight or obese individuals when associated with dieting and increased physical activity, this low efficacy might not be sufficient to properly handle extreme cases of morbid obesity. It appears critical to increase our knowledge of the mechanisms controlling food intake and body weight in humans, in particular by the discovery of new brain signaling pathways involved in energy homeostasis. Such pathways could provide valuable pharmacological targets, and lead to the development of new generation drugs that can safely and effectively fight against overweight and obesity. Some of the new compounds under development targeting central signaling pathways seem interesting candidates. However, strategies aiming to modulate a single signaling pathway at the central level could be therapeutically limited. A multi-target approach, with the help of co-treatments, treatments altering simultaneously multiple central targets or treatments targeting peripheral as well as central targets, could provide an interesting way of research for the future. As an illustration, new anti-obesity combination therapies are currently under development. The association of naltrexone, an opiate antagonist, and bupoprion, a catecholamine re-uptake inhibitor, was proposed by Orexigen Therapeutics (Contrave). Since their discovery in the 1970s, evidence has accumulated that opioid peptides may play a role in the short-term regulation of feeding. While causing a significant reduction in short-term food intake [155], Naloxone is associated with minimal weight loss as a monotherapy, but it does have potential utility in the treatment against obesity when combined with other drugs. Similarly, also from Orexigen Therapeutics, the combinaison of bupoprion and the anti-convulsant, zonisamide, has shown promise in phase II clinical trials. To conclude, it appears from the last decade that researches in the field of nutritional behavior have identified a rich menu of potential anti-obesity targets but it is eagerly expected that these discoveries succeed in the development of effective anti-obesity drug therapies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Annexe 3

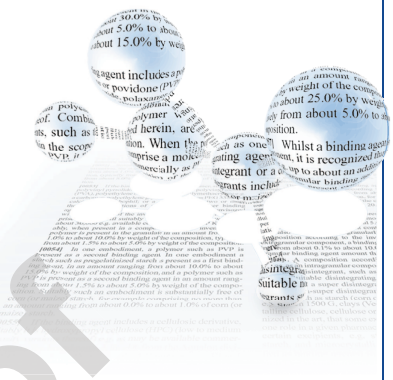
Revue : The central question of type-II diabetes.

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Pharmaceutical Patent Analyst

The central question of type 2 diabetes



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Type 2 diabetes (T2D) represents a significant global epidemic with more than 285 million people affected worldwide. Regulating glycemia in T2D patients can be partially achieved with currently available treatment, but intensive research during the last decades have led to the discovery of modified compounds or new targets that could represent great hope for safe and effective treatment in the future. Among them, targets in the CNS that are known to control feeding and body weight have been also shown to exert glucoregulatory actions, and could be a key in the development of a new generation of drugs in the field of T2D. Such drugs would be of great interest since they can be used both in the treatment of diabetes and obesity. This patent review aims to establish an overview of recent patents disclosing new therapeutic opportunities targeting peripheral, as well as central targets for the treatment of T2D.

Author Proof

According to the WHO, diabetes mellitus is described as a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both. **Type 2 diabetes** (T2D), also called noninsulin-dependent diabetes because the body fails to effectively use insulin, represents 90% of the total cases of diabetes mellitus, whereas type 1 diabetes (T1D) only represents 10%. Over recent decades, the number of people affected by T2D has increased to an alarming 285 million worldwide in 2010 [1]. Unfortunately, this rise is not slowing down and now encompasses developing countries, in particular India and China [1,2]. This exponential rise of T2D is mainly explained by changes in nutrition and by a shift towards both urbanization and a sedentary lifestyle [3]. Interestingly, a similar evolution can be observed for the cases of obesity, a pathology, which shares similar origins with T2D, underlining the very strong link between these two diseases [4]. Thus, it is estimated that 60–90% of T2D cases are related to obesity [5]. In the long term, the main complications observed in T2D are retinopathies, nephropathies and neuropathies; furthermore, diabetes is also known to increase risks for developing cardiovascular and cerebrovascular diseases. In addition, recent studies have shown that T2D also increases the risk of developing Alzheimer's disease (AD) [6]. Indeed, insulin and its receptors are present in the CNS and are involved in cognition and memorization phenomena [6]. The relationship between AD and T2D is so closed that some authors introduced the term 'type 3 diabetes' to define AD because it presents characteristics of both T1D and T2D [6]. T2D now represents a major public health problem with considerable economic consequences. As an example, in 2007, the total economic cost of diabetes represented more than US$174 billion in the USA alone (American Diabetes Association).

T2D is a chronic, progressive disease and it is important to identify people at risk before they develop diabetes. Indeed, treatment at earlier stages has been shown to prevent the progression of the disease [7]. Different tests can be used for the diagnosis of T2D and prediabetes states. The measurement of fasting plasma glucose

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Key terms

Type 2 diabetes: According to the WHO, type 2 diabetes, also called noninsulin-dependent diabetes, results from the body's ineffective use of insulin. Type 2 diabetes comprises 90% of people with diabetes around the world, and is largely the result of excess body weight and physical inactivity. It is estimated that 3.4 million people died in 2004 from consequences of high blood sugar and the WHO projects that diabetes deaths will increase by two thirds between 2008 and 2030.

Sodium-glucose co-transporters: Sodium-dependent glucose co-transporters (or sodium-glucose linked transporter) are members of a family of glucose transporter found in the intestinal mucosa (enterocytes) of the small intestine (SGLT1) and in the proximal tubule of the nephron (SGLT2 and SGLT1). At the kidney level, 90% of total glucose absorption reabsorption is facilitated by SGLT2. Thus, SGLT2 inhibitors appear as interesting candidates in order to ameliorate hyperglycemia in type 2 diabetes.

and the measurement of plasma glucose 2 h after an oral load of 75 g of glucose are commonly used. Another marker of glycemic status is HbA1C, the glycated hemoglobin, which is created by the reaction between glucose and an amino group of the protein, and the blood levels of HbA1C are correlated with average blood glucose levels. Since 2011, the use of HbA1C has been accepted by health experts and is recommended by the WHO as a diagnosis marker for diabetes mellitus [8]. A HbA1C rate of approximately 6.5% is used for the diagnosis of diabetes mellitus, as calculated from the association between HbA1C and prevalent retinopathy. This is a helpful test with great advantages as it is directly correlated with glycemia, but also provides information on the glycemic balance of the patient during the previous 8–12 weeks, and so it allows adaptation of the T2D treatment. Moreover, the measure of HbA1C avoids fasting and provides more comfort for patients compared with the 2-glucose test [2].

Physiological regulation of glycemia

■ Peripheral aspects

Insulin, secreted by pancreatic β-cells, is the most famous hormone implicated in the control of glucose homeostasis. After a meal, when blood glucose levels increase in the body, β-cells capture glucose and metabolize it resulting in an increase of ATP in cells. ATP-sensitive potassium channels localized in the plasma membrane then close and cells depolarize allowing calcium entry and insulin secretion. Insulin is conveyed to target cells localized in skeletal muscle, the main site for glucose disposal in the body, liver and adipose tissue. In these tissues, insulin receptors activate leading to the recruitment of the insulin receptor substrates (IRS). Activation of IRS-1 induces the translocation of glucose transporters, so far set aside in cytosolic vesicles, to the plasma membrane [9]. Plasma glucose is thus transported within the target cells where it will be stored as glycogen in muscle and liver or as triglycerides in adipose tissue (Figure 1). The transformation of glucose to its storage form involves the successive action of several enzymes [10]. The cytosolic expression and the activity of these enzymes are modulated according to the levels of glucose and insulin. For example, insulin stimulates glycogen synthase activation (Figure 2).

Conversely, when the organism undergoes hypoglycemia, glucagon is released by pancreatic α-cells and acts on hepatocytes and adipocytes (skeletal muscle being free from glucagon receptor) via a G-protein-coupled receptor (GPCR) to transform glycogen and provide glucose. In the liver, glucagon also stimulates gluconeogenesis. Moreover, in case of hypoglycemia, epinephrine and cortisol are secreted by adrenal glands and help to restore normoglycemia, this secretion being under the control of the CNS.

The kidney also plays an important role in glucose homeostasis. Firstly, it is capable of releasing glucose into the circulation via gluconeogenesis. Indeed, the kidney is able to release a large amount of glucose, despite its inability to produce glucose via glycogenolysis because of the lack of glycogen stores. Approximately 25% of glucose released in the circulation after an overnight fast is derived from the kidney [11]. Secondly, it is equipped with a mechanism of reabsorption of glucose from glomerular filtrate to the circulation. In healthy individuals, all filtered glucose is reabsorbed into the blood (approximately 180 g of glucose each day) and the urine is free from glucose. This renal glucose reabsorption occurs at the proximal tubule level by means of **sodium-glucose co-transporters** (SGLTs). There are six types of SGLTs, but in the kidney 90% of glucose is reabsorbed by SGLT-2 and only 10% by SGLT-1 [11] (Figure 1).

Currently, another key actor in the regulation of glucose homeostasis arouses great interest: the AMP activated protein kinase (AMPK). AMPK is an energy sensing enzyme considered as a 'fuel gauge', present in every tissue, and regulating glucose and lipid metabolism. AMPK is a heterotrimeric protein comprising a catalytic α-subunit and regulatory β- and γ-subunits activated when ATP depletion occurs in the cell, in case of hypoxia or glucose deprivation, for example. AMPK activation restores cellular ATP by both inhibiting ATP consumption processes, as well as accelerating ATP generation processes. Indeed, activation of AMPK causes inhibition of fatty acid and cholesterol synthesis in hepatocytes, muscle glycogen synthesis, gluconeogenesis in the liver and protein synthesis in many cells. Additionally, activation of AMPK stimulates glucose uptake in skeletal muscle by translocation of glucose transporter to the plasma membrane and produces ATP by glycolysis and fatty acid oxidation [12].

■ Central control of glycemia

The regulation of glycemia is also under the control of the CNS. Indeed, the presence of glucose in the oral cavity induces the activation of the parasympathetic

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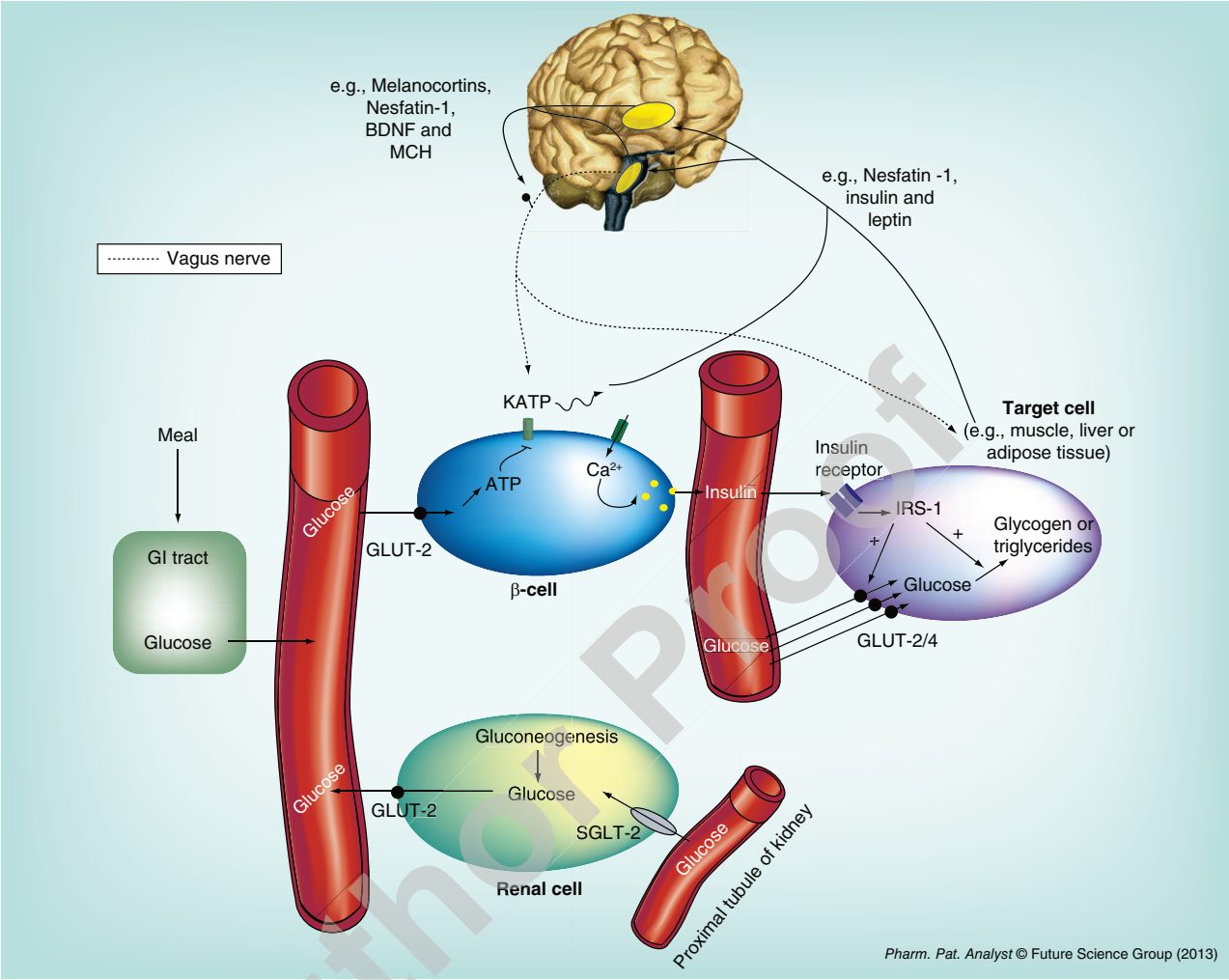


Figure 1. Global mechanisms of glycemia regulation. During a hyperglycemic episode, pancreatic β cells release insulin that act on skeletal muscle, liver, and adipose tissue to facilitate the storage of glucose. Kidneys also represent key actors in this control because of their ability to release glucose in the blood stream in a regulated manner. These mechanisms are under the control of the CNS where several hormones and neuropeptides act in order to maintain a stable glycemia.
BDNF: Brain-derived neurotrophic factor; IRS-1: Insulin receptor substrate 1; KATP: ATP-sensitive potassium channel; MCH: Melanin-concentrating hormone.

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system that leads to an anticipatory insulin release, the 'cephalic phase of insulin secretion'. To regulate glucose homeostasis the brain is equipped with glucosensor neurons, located in the hypothalamus and brainstem, which make it responsive to both hyperglycemia and hypoglycemia. These neurons are either activated (glucose-excited neurons [GE]) or inhibited (glucose-inhibited neurons [GI]) by glucose [13]. GE neurons are located in the paraventricular nucleus (PVN), arcuate nucleus (ARC) and ventromedial hypothalamic nucleus (VMH) at the hypothalamic level, and in the nucleus of the tractus solitarius (NTS) and dorsal motor nucleus of the vagus (DMNX) at the brainstem level. GI neurons, in turn, are located in the same structures except the VMH, and they are also present in the lateral hypothalamic area (LHA). These hypothalamus and brainstem nuclei are synaptically connected and form a network capable of monitoring blood glucose levels. In GE neurons, mechanisms of central glucodetection seem to be similar to those observed in pancreatic β-cells: glucose is metabolized and ATP increases resulting in plasma membrane depolarization and neurotransmitter secretion. In GI neurons, mechanisms are different: the increase of ATP leads to a stimulation of Na-K-ATPase activity and/or an opening of ATP-regulated chloride channels hyperpolarizing the plasma membrane [13].

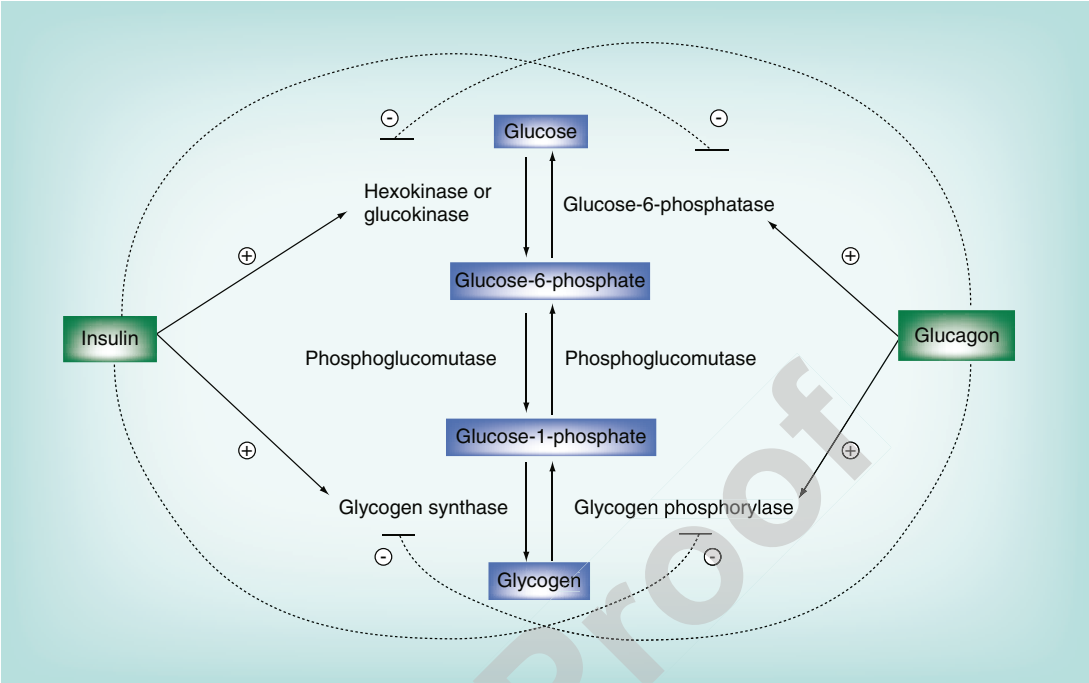


Figure 2. Conversion of glucose into glycogen. The conversion of glucose into glycogen involves several enzymes. Among them, hexokinase, glucokinase, glycogen synthase, glycogen phosphorylase and glucose-6 phosphatase show their expression and activity regulated by insulin and glucagon.

Downstream of central glucosensing neurons, β- and α-cell activity is controlled by both sympathetic and parasympathetic innervation. Insulin secretion is stimulated by parasympathetic innervation: when glucosensors neurons are activated it stimulates vagal efferents leading to an increase in insulin secretion. Parasympathetic preganglionic neurons that innervate the pancreas are located in the DMNX and can directly sense glucose, but they can also be controlled by other glucosensors neurons from other nuclei. In case of a lack of glucose, glucagon secretion is stimulated by the sympathetic and parasympathetic nervous systems and the sympathetic innervation inhibits insulin secretion and stimulates epinephrine release from adrenal glands. This mechanism is called counter-regulatory response. The VMH and the brainstem seem to be two key structures involved in this counter-regulation. Indeed, an intra-VMH injection of the glucose antimetabolite 2-deoxyglucose induces glucagon secretion [14] and, conversely, hypoglycemia-induced glucagon secretion is abolished when glucose is directly perfused into the VMH [15]. Regarding the brainstem functions, it has been shown that glucagon secretion and hyperglycemic response induced by an intraperitoneal injection of 2-deoxyglucose are preserved in decerebrate rats, suggesting that the brainstem is sufficient for the autonomic control of counter-regulation [13].

Finally, insulin itself is able to cross the blood–brain barrier to act in the brain. Insulin receptors are widely expressed in the brain, particularly in the ARC of the hypothalamus. An injection of insulin into the third cerebral ventricle inhibits food intake, but also glucose production [16]. Mice with a neuron-specific insulin receptor deletion are hyperphagic, obese and develop glucose intolerance and **insulin resistance** (IR) [17]. These data suggest that hypothalamic insulin signaling is required to control both food intake and glycemia.

Key term

Insulin resistance: Condition in which the pancreas produces insulin but muscle, fat, and liver cells do not respond properly to this hormone. As a result, higher secretion of insulin by pancreatic β-cells is needed to help glucose enter cells and to achieved normoglycemia. As for impaired glucose tolerance, insulin resistance can be observed several years before the appearance of type 2 diabetes.

■ Modulators of glycemia

In the field of modulators of glycemia and of insulin release, **incretin hormones**, namely GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are important actors of glycemic control. They are released by the intestine a few minutes after meal ingestion, more precisely by the ileum for GLP-1 and the duodenum for GIP. They are transported in the blood to pancreatic β-cells where they bind to their respective GPCR (GLP-1 receptor [GLP-1R] and GIPR) in order to stimulate insulin biosynthesis and secretion. Moreover, *in vitro*,

Future Proof

incretins stimulate β-cell proliferation and decrease β-cell apoptosis. In GLP-1R- and GIPR-knockout mice glucose-stimulated insulin secretion is decreased and animals developed glucose intolerance [18,19]. GLP-1R and GIPR are not only expressed by β-cells, but also by pancreatic α- and δ-cells, and by other organs such as GI tract, adipose tissue, kidney, lung, endothelial cells, heart and CNS. GLP-1 and GIP increase peripheral insulin sensitivity and only GLP-1 inhibits glucagon release and delays gastric emptying [20–22]. GLP-1 may also bind to vagal afferents resulting in a vagal efferent response to pancreatic β-cell and an increase in insulin secretion. Within the CNS, GLP-1R are expressed by neurons located in various nuclei regulating energy balance, including the PVN and dorsomedial nucleus of the hypothalamus, and the NTS, the area postrema and the parabrachial nucleus at the brainstem level [23]. GLP-1 may cross the blood-brain barrier but it is important to note that GLP-1 is also synthesized and secreted by neurons of the NTS and acts as a neurotransmitter locally or in sites mentioned above [23]. Both central and peripheral injection of GLP-1 reduce food intake and GLP-1 was characterized as a satiation signal [24,25]. GLP-1 and GIP are rapidly degraded by DPP-4 and metabolites are eliminated by the kidneys (half-life of 2 and 7 min for GLP-1 and GIP, respectively) [26]. The clinical use of DPP-4 inhibitors (DPP-4i) to augment incretin effect is an important area of research to the treatment of T2D.

If insulin exerts, in addition to its hypoglycemic action, a control of food intake, conversely, several hormones and neuropeptides that act at the central level are implicated both in food intake and glycemic control, such as incretin hormones, **leptin**, melanocortins, brain-derived neurotrophic factor (BDNF) or **nesfatin-1** (Figure 1). Thus, the brain could be a therapeutic target for diabetic patients also suffering from obesity.

Leptin, one of the most famous hormones regulating feeding behavior is secreted by adipose tissue and acts in the brain as a powerful anorexigenic factor. Its receptors (Ob-R) are expressed mainly in the ARC, but also in the PVN, VMH, dorsomedial nucleus of the hypothalamus, LHA and NTS [27]. Ob-R is found in other organs such as skeletal muscle, fat and the pancreas. Mice with leptin deficiency (*ob/ob* mouse) or leptin receptor deficiency (*db/db* mouse) exhibit hyperphagia and obesity, but also IR and diabetes. Leptin is able to interfere with insulin at the post-receptors intracellular pathways by modulating the activity of IRS molecules, and thus increasing insulin sensitivity. Peripheral or central administration of leptin corrects the IR and ameliorates hyperglycemia in *ob/ob* or lipodystrophic mice [28,29].

Melanocortins, and more precisely the α-melanocyte-stimulating hormone (α-MSH), a product of pro-opiomelanocortin (POMC), regulate both food intake and glucose homeostasis through different neuronal populations expressing melanocortin receptors (MC3R and MC4R). POMC neurons are located in the ARC and the NTS, and they are activated in response to anorexigenic stimuli to decrease food intake. They project to various hypothalamic and brainstem nuclei where MC3Rs and MC4Rs were found, as well as in spinal cord [30]. It is important to note that MC4R is much more widely expressed than MC3R and it is in the DMNX where MC4R is most found. MC4R-knockout mice exhibit hyperphagia, obesity, hyperinsulinemia and hyperglycemia. Reexpression of MC4Rs in cholinergic neurons attenuates hyperinsulinemia and hyperglycemia. Several authors suggest that MC4Rs expressed in parasympathetic neurons of the DMNX may regulate insulin secretion from the pancreas, whereas MC4Rs expressed by the sympathetic preganglionic neurons in the intermediolateral column may be involved in the regulation of insulin sensitivity and hepatic glucose production [31].

BDNF is a neurotrophin expressed in the central and peripheral nervous system. In addition to its proliferative role on neurons, BDNF also contributes to regulate food intake and energy homeostasis. BDNF treatment induces an anorexigenic effect and BDNF +/- mice exhibit a hyperphagic phenotype and obesity [32]. Moreover, in obese diabetic *db/db* mice, peripheral or central administration of BDNF reduces blood glucose independently of appetite alteration [33]. In young adult *db/db* mice, this decrease in blood glucose level is associated with a decrease in plasma insulin concentration, suggesting that BDNF increases insulin sensitivity [34]. BDNF is believed to exert its effect on glucose homeostasis through a central mechanism:

Key terms

Incretin hormones: Incretins consist of gastrointestinal hormones that provoke insulin release from the β cells, when glycemia is elevated (postprandial blood glucose). Incretins also decrease the rate of nutrients absorption of into the blood stream by reducing gastric emptying and may directly reduce food intake. As expected, they also inhibit glucagon release from the α-cells. The two main candidate molecules are GLP-1 and gastric inhibitory peptide (also known as glucose-dependent insulinotropic polypeptide). One of the challenge in developing GLP-1 or dependent insulinotropic polypeptide-derived drugs is that these compounds can be rapidly inactivated by the enzyme DPP-4.

Leptin: Adipocyte-secreted hormone that exert a regulatory role both in energy homeostasis and glucoregulation. Leptin primarily acts at the central level and has initially lead to great hopes in the development of anti-obesity and antidiabetic drugs, before the discovery of a central leptin-resistant state in the vast majority of obese and diabetic patients. Strategies are currently being investigated to produce leptin-derived candidates that could circle this resistance. Another strategy currently being investigated is the development of drug candidates aiming downstream effectors of leptin at the central level such as melanocortin and brain-derived neurotrophic factor/TrKB signaling pathways.

Author Proof

Key terms

Nesfatin-1: Recently discovered peptide associated with anorexigenic and antihyperglycemic effects. Nesfatin-1 can act both at the periphery and at the brain level to increase insulin secretion and sensitivity. Recent patents suggest that administration of nesfatin-1 or related peptides, or the inhibition of endogenous nesfatin-1 degradation could be therapeutic options in the treatment of both obesity and type 2 diabetes.

Glucolipotoxicity: Concept that refers to the deleterious effects of elevated glucose and fatty acid levels on β-cell resulting in decreased insulin secretion, impaired insulin gene expression and β-cell death by apoptosis. The mechanisms of glucotoxicity are, at least in part, mediated by oxidative stress while lipotoxicity probably results by an accumulation of cytosolic signals derived from the fatty acid esterification pathway. Both contribute to the progressive deterioration of glucose homeostasis observed in type 2 diabetes.

- BDNF and its receptor TrkB are widely expressed in the hypothalamus and brainstem;
- Intracerebroventricular injection of BDNF enhances insulin sensitivity in the liver of diabetic mice, while BDNF does not modify insulin signaling in primary cultured hepatocytes;
- In MC4R -/- mice, the expression of BDNF mRNA is reduced in the VMH, compared with wild-type mice suggesting a link between melanocortin signaling and BDNF [35].

A newly discovered peptide, named nesfatin-1, arouses great interest in the scientific community because of its implication in energy and glucose homeostasis. Nesfatin-1, an 82 amino-acid peptide originating from the cleavage of NUCB2, exerts an anorexigenic action after injection in both the cerebral ventricle and peritoneal cavity. Nesfatin-1 is expressed in neurons of various brain areas including hypothalamic nuclei such as PVN, ARC, LHA and in the NTS and DMNX at the brainstem level [36]. It has been reported that these nesfatinergic neurons could participate to the satiety signaling in the physiological control of food intake [37] and to the decrease in food intake observed during inflammation [38]. Interestingly, nesfatin-1 is also expressed in pancreatic β-cells, where it is co-localized with insulin in secretion vesicles. Furthermore, blood and pancreatic nesfatin-1 level are altered in diabetic subjects [39]. *In vitro* and *in vivo* studies showed that glucose-induced insulin secretion is increased by nesfatin-1 [40,41] and in hyperglycemic *db/db* mice, nesfatin-1 injected intravenously reduces blood glucose levels [42]. Finally, we recently highlighted the implication of NUCB2/nesfatin-1-expressing neurons in glucose homeostasis [43].

Physiopathology of T2D

The onset of T2D involves two key events: IR and pancreatic dysfunction. IR is a pathological condition that manifests as a reduction of insulin ability to maintain a normal glycemia [4]. The complex mechanisms hidden behind IR are still not completely understood and remain to be investigated [44,45]. However, evidence indicates that an excessive accumulation of glucose and free fatty acids (FFA) plays an important role in IR development. Indeed, '**glucolipotoxicity**' is known to impair insulin signaling in liver, adipocytes and skeletal muscle [45–47]. In addition, IR development involves many other factors increased by obesity and inactivity, such as adipokines and mediators of inflammation, which also contribute to impaired insulin signaling [4,47,48]. Nevertheless, IR cannot be solely responsible for the development of T2D considering the adaptive properties of the pancreas concerning β-cell mass and metabolism. This period, which can last several years before the apparition of the first symptoms, is called prediabetes. Two different forms of prediabetes are well documented, impaired fasting hyperglycemia (IFG) and **impaired glucose tolerance** (IGT). People affected by IFG or IGT both show IR and impairment in insulin secretion. However, a severe hepatic IR and a less severe skeletal muscle IR are observed in isolated IFG, while an opposite phenotype is observed in isolated IGT [49,50]. The second key event that contributes to the T2D development is the continued degradation of pancreatic β-cells, which eventually leads to pancreas depletion and the inability to adapt to the growing demand for insulin [4]; both insulin production and β-cell mass are decreased [4,48]. As for IR, Adrikopoulos underlined the negative impact of the excess of nutrients. On the one hand, it causes an increase in mitochondrial metabolism, which results in an increase in the production of reactive oxidative species (ROS) and contributes to β-cell dysfunction [51]. On the other hand, the overflow of nutrients leads to such an increase in insulin production that it induces a reticulum endoplasmic stress, which results in β-cell dysfunction or death. The pancreas is particularly affected by ROS production because it expresses a lower quantity of antioxidant enzymes than other organs. The crucial role of FFA in β-cell dysfunction was also highlighted by many authors [4,46,48]. It was also reported that high blood glucose level can act synergistically to liptoxicity to produce deleterious effects [4]. This process illustrates well the 'vicious circle' of T2D (Figure 3). Indeed, β-cell dysfunction induces an inadequate secretion of insulin and consequently promotes 'glucolipotoxicity', which finally appears as both a cause and a consequence of T2D. In addition to the deleterious effects of FFA accumulation, it has been suggested that the amyloid β-derived diffusible ligand, an oligomer with similar morphology and size to prions presents in degenerative diseases, is able to induce β-cell death but also impairs insulin signaling pathway in the brain. More recently, Westermark *et al.* showed that these oligomers are islet amyloid polypeptides (IAPPs), which are co-secreted with insulin [52].

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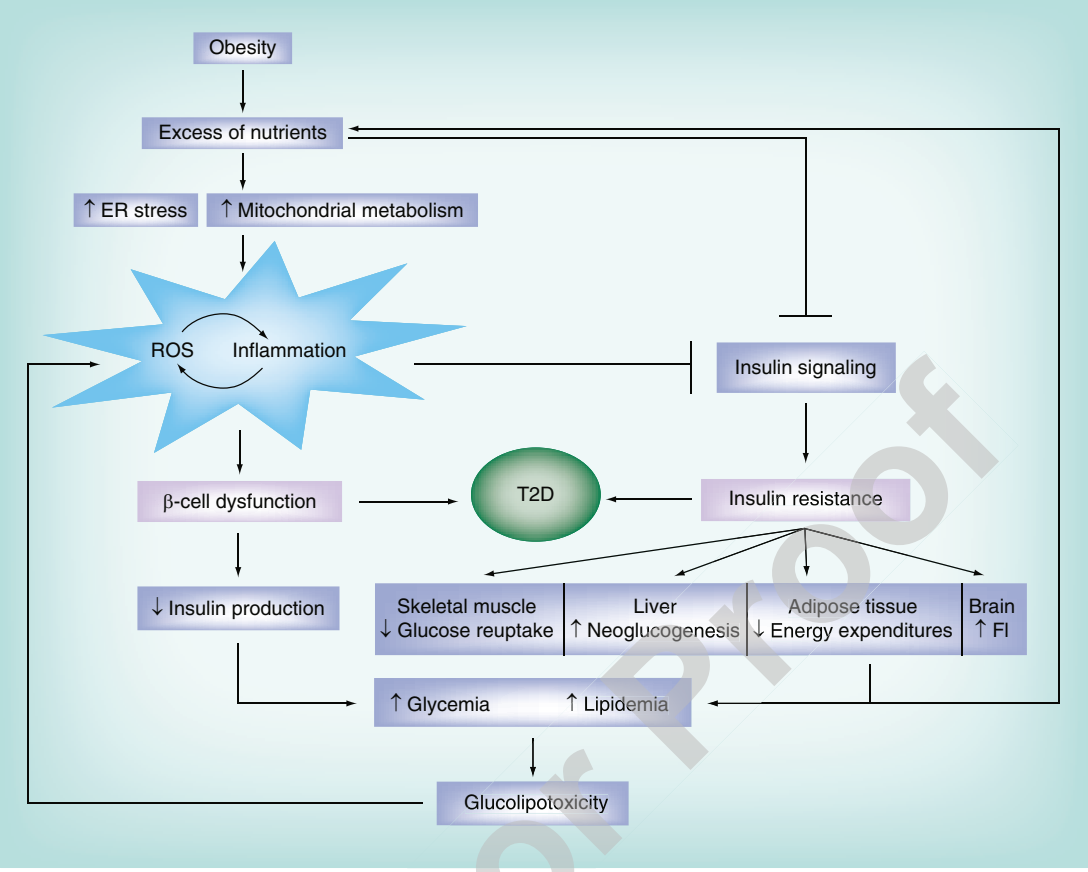


Figure 3. Role of obesity in the development of type 2 diabetes. The excess of nutrients that occurs in obesity condition impairs insulin signaling and contributes to insulin resistance in the skeletal muscle, the liver, the adipose tissue and the brain. This situation generates reactive oxidative species and a low-grade inflammation, especially by increasing the mitochondrial metabolism and the endoplasmic reticulum stress. This mechanism also exacerbates the impairment of insulin signaling and leads, in the long-term, to β-cell dysfunction or death. Both insulin resistance and β-cell dysfunction contribute to the development of type 2 diabetes, but also to the persistence of the disease by increasing glucolipotoxicity, which illustrates the vicious circle between obesity and type 2 diabetes.
ER: Endoplasmic reticulum; FI: Food intake; ROS: Reactive oxidative species; T2D: Type 2 diabetes.

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IAPPs have cytotoxic properties and contribute to increased reticulum endoplasmic stress, ROS production and β-cell death, and IAPPs aggregates are found in the pancreas of patients with T2D. In addition to ROS production, β-cells are also deteriorating by chronic inflammation characteristic of obesity, in particular due to immune cell infiltration in tissues and oxidative stress [53].

Genetic predispositions also play a crucial role in T2D development and are well known to contribute to both IR and β-cell dysfunction [2,4]. Indeed, a huge network of genetic loci was identified as risk for T2D. In addition, age, gender, smoking, hypertension, intrauterine environment, nonalcoholic liver diseases, and hepatitis virus C infection have been shown to influence its development [2,54,55].

Present pharmacological treatments & current development

To improve their quality of life, patients with T2D should adapt their lifestyle. First of all, they must pay attention to their food intake, eating a healthy diet rich in fibers, fruits and vegetables, and reducing high fat, salt and sugar meals. With this diet, patients with T2D also have to practice physical exercise. Exercise helps to control T2D by improving the body's use of insulin, burning excess body fat, helping to lose weight, lowering blood

Key term

Impaired glucose tolerance: Prediabetic state resulting in hyperglycemia, which is associated with insulin resistance. According to the WHO and the American Diabetes Association, impaired glucose tolerance is defined as a plasma glucose level of 140–199 mg/dl measured 2 h after an oral glucose tolerance test (75g of glucose). Impaired glucose tolerance can be observed several years before the appearance of type 2 diabetes and represent a major risk factor for cardiovascular pathologies.

Table 1. Current treatments for type 2 diabetes and derived drugs under clinical trials.

Target	Action	Drug name	Development phase	Company	Ref.
Insulin receptors	Fast-acting insulin analogs	Humalog®	Marketed	Eli Lilly and Company	[56]
		Novo-Rapid®	Marketed	Novo Nordisk	
		Apidra®	Marketed	Sanofi-Aventis	
	Long-lasting insulin analogs	Lantus®	Marketed	Aventis Pharmaceuticals	[56]
		Levemir®	Marketed	Novo Nordisk	
Glucagon signaling	Glucagon-receptor antagonists	Bay 27-9955	Phase II	Bayer	[57]
		MK-0893	Phase II	Merck	
		LY2409021	Phase I	Eli Lilly	
Respiratory chain complex I	Inhibitor of respiratory chain complex I	Metformine (Glucophage®)	Marketed	Merck	[64]
Low-densit lipoprotein cholesterol	Cholesterol-lowering agent	Colesevelam (Cholestagel®)	Marketed	Daiichi Sankyo	[68]
Pancreatic β-cells	Stimulate insulin release	Glimepirides (Amaryl®)	Marketed	Sanofi-Aventis	[58]
		Glipizides (Glucotrol®)	Marketed	Pfizer	
		Glyburides (DiaBeta®)	Marketed	Roche/Sanofi-Aventis	
		Repaglinide (NovoNorm®)	Marketed	Novo Nordisk	
		Nateglinide (Starlix®)	Marketed	Novartis	
PPAR-γ	Increase insulin sensitivity	Troglitazone (Rezulin®)	Withdrawn	Parke-Davis/Warner-Lambert	
		Rosiglitazone (Avantia®)	Withdrawn	GlaxoSmithKline	[70]
		Pioglitazone (Actos®)	Withdrawn (Europe)	Takeda Pharmaceutical	
		INT131 Besylate	Phase II	InteKrin Therapeutics Inc.	[71]
GLP-1 signaling	GLP-1 signaling agonists	Exenatide (Byetta®)	Marketed	Amylin Pharmaceuticals	[60]
		Liraglutide (Victoza®)	Marketed	Novo Nordisk	
		Albiglutide	Phase III	GlaxoSmithKline	
DPP-4	DPP-4 inhibitors	Sitagliptin (Januvia®)	Marketed	Merck	[60]
		Vildagliptin (Zomelis®)	Approved (Europe)	Novartis	
		Saxagliptin (Onglyza®)	Marketed	Bristol-Myers Squibb/ AstraZeneca	
		Linagliptin (Trajenta®)	Marketed	Boehringer Ingelheim and Lilly	

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Table 1. Current treatments for type 2 diabetes and derived drugs under clinical trials (cont.).

Target	Action	Drug name	Development phase	Company	Ref.
G-protein-coupled receptor	GPR 40 agonist	TAK-875	Phase III	Takeda Pharmaceutical	[72]
Sodium-glucose co-transporters	SGLT2 inhibitors	Dapagliflozin	Phase III	AstraZeneca	[73]
		Canagliflozin	Phase III	Johnson & Johnson	
		Empagliflozin	Phase III	Boehringer Ingelheim and Eli Lilly	
Glucokinase	Glucokinase activators	Piragliatin	Phase II	Hoffman-La Roche	
11β-hydroxysteroid dehydrogenase type 1	11βHSD1 inhibitors	KR-67183	Preclinical		[75]
		DIO-902	Phase II	DiObex, Inc.	
		INCB13739	Phase II	Incyte, Inc.	
		Salsalate (Disalcid®)			[76]

pressure, improving blood circulation and reducing the risk of heart disease. This lifestyle change is necessary for patients with T2D undergoing a treatment such as bariatric surgery. This type of surgery is effective in inducing diabetes remission. The principle of this surgery is to modify the GI tract (Roux-en-Y bypass) in order to decrease food intake and induce hormonal level changes improving glycemic control such as an increase of incretin secretion (e.g., GLP-1). However, this surgery is not without any risks: mortality, surgical complications in the short term such as hemorrhage, obstruction, infection, or in the long term, such as hyperinsulinemic hypoglycemia syndrome (principally caused by GLP-1 hypersecretion).

Besides the surgical option, several pharmacological options are currently available in development. In the following part of the present review, we have listed some of the current and future therapeutic options for T2D **(Tables 1 & 2)**.

■ Insulin therapy

Before biosynthetic human recombinant analogs became available, porcine insulin was chemically converted into human insulin to resolve hyperglycemia problem in T2D patients. Nowadays, insulin analogs, that is, chemically modified, act faster or slower than the native insulin. These modifications do not affect their ability to regulate hyperglycemia, but affect the rate at which the insulin is absorbed, how quickly it starts acting and how long its effects will last. There are two types of insulin analogs: the fast-acting analogs are able to induce rapid and transient hypoglyceamia response just after administration, whereas slow-lasting analogs exert a delayed and long-lasting hypoglyceamia response. Fast-acting insulin analogs are used before or just after a meal when glycemia quickly increases. Eli Lilly and Company marketed the first fast-acting insulin analog with 'lispro', trademarked as Humalog® (1966). Novo Nordisk developed 'aspart' and marketed it as Novo-Rapid® (2000). Glulisine is the newest rapid-acting insulin analog from Sanofi-Aventis, under the brand name Apidra® (2006). More recently, **long-lasting insulin analogs** have been developed in order to counter the short half-life of fast-acting insulins analogs. They are designed to replace basal insulin secretion, which is essential for the regulation of blood sugar levels between meals and during sleep. Glargine was developed by Aventis Pharmaceuticals and its brand name is Lantus® (2000). Detemir was developed by Novo Nordisk and marketed as Levemir® (2005). The use of a combination of a long-lasting and rapid-acting insulin analogs is necessary for a better regulation of glycemia. The choice of the injection depends on the patient's lifestyle. Today, insulin analogs can be infused

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Table 2. Recent patents disclosing new compounds acting on peripheral targets.

Target	Drug type	Company	Ref.
Glucagon signaling	Thiophenes as glucagon-receptor antagonists	Schering Corporation	[201]
	Pyrrolidines as glucagon-receptor antagonists	Schering Corporation	[202]
	High affinity glucagon receptor antibodies or Fab fragments	Eli Lilly	[203]
AMP-activated protein kinase signaling	Biguanide derivates	Hanall Biopharma	[208]
	Benzylparabanic acid derivative	Kyorin Pharmaceutical Co.	[209]
	Thieno [2,3-B] pyridinedione and derivates	POXEL	[210]
GLP-1 signaling	GLP-1 signaling analog with a longer half-life	Jiangsu Hansoh Pharmaceutical Group Co.	[204]
	GLP-1 signaling mimetic stable against degradation by gastrointestinal enzymes	Novo Nordisk	[205]
DPP-4	Novel substituted aminopiperidines	Merck Sharp and Dohme Corp.	[206]
	β-amino tetrahydroimidazo(1,2-A)pyrazines and tetrahydrotriazolo(4,3-A)pyrazines	Merck Sharp and Dohme Corp.	[207]
Sodium-glucose co-transporters	Compounds inhibiting SGLT2	Boehringer Ingelheim	[214]
	Benzothiophen compounds as an SGLT1/SGLT2 dual inhibitors	Astellas Pharma Inc.	[215]
Glucokinase	RO-0281675 as glucokinase activor	Hoffman-LaRoche	[211]
	PSN-GK1 as glucokinase activator	OSI/Prosidion	[212]
	RO4389620 (Piragliatin) as glucokinase activator	Hoffman-La Roche	[213]
11βHSD1	11βHSD1 inhibitors and intermediates	Boehringer Ingelheim	[216]
	1-adamantylazetidin-2-one derivate as 11βHSD1 inhibitors	Kowa Co.	[217]

using a continuous subcutaneous insulin infusion pump. Users have to check their glycemia frequently in order to know the exact quantity of insulin to inject. In the future, this system will run automatically, functioning as an 'artificial pancreas' and could detect blood glucose levels and adjust the delivery to the needs. Novo Nordisk (FlexPen®), Aventis (OptiSet®) and Eli Lilly and Company (Umuline®) commercialized a pen to inject insulin analogs, which are easy to use and to carry. Other ways to administer insulin analogs have been tested, such as oral administration and nasal administration, but without success [56].

■ Glucagon-receptor antagonists

Patients with T2D typically have fasting hyperglycemia associated with elevated rates of hepatic glucose production due to an increase in gluconeogenesis. Such patients typically have relative deficiency in their fasting and postprandial insulin-to-glucagon ratio that contributes to their hyperglycemic state. The use of glucagon receptor antagonism should improve this condition. A defect in rapid postprandial insulin secretion, as well as ineffective suppression of glucagon secretion, leads to increased glucagon levels that elevate hepatic glucose production and contribute to hyperglycemia. The concept that glucagon-receptor antagonism may be a useful approach in the therapy of diabetic patients was tested in preliminary human studies using the Bayer antagonist, Bay 27–9955, a nonpeptide compound that can be administered orally. This compound appeared safe and blocked exogenous glucagon action in short-term human studies [57]. Merck's MK-0893 and Eli Lilly's LY2409021 work by suppressing glucagon action via receptor blockade, and are currently, respectively, in Phase II and Phase I clinical trials. New patents for glucagon receptor antagonists have also been granted such as thiophenes [201] or pyrrolidines [202] by Schering Corporation. Eli Lilly and Co. have developed high affinity glucagon receptor antibodies, or Fab fragments, in order to inhibit binding of glucagon to its receptor [203], but there is no clinical data available yet. This novel class of drugs has the potential to be an effective treatment for T2D, but only if safety can be established because of a high risk of hypoglycemia associated with their use.

■ Sulfonylurea/glinides: insulin secretagog

The sulfonylureas and glinides are commonly used to stimulate insulin release from pancreatic β-cells via ATP-sensitive potassium channels. Sulfonylureas act

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during hyperglycemic states whereas glinides act without necessarily a high glycemia. The common adverse effects of these compounds are hypoglycemia and weight gain. As insulin secretagogs, the effects of sulfonylureas and glinides are dependent on β-cell integrity. When β-cell dysfunction becomes prevalent, sulfonylureas and glinides efficacy declines [58]. Different sulfonylureas are on the market such as glimepirides (brand name: Amaryl®, Sanofi-Aventis), glipizides (brand name: Glucotrol®, Pfizer), glyburides (brand name: DiaBeta®, Roche/Sanofi-Aventis) and glinides such as repaglinide (brand name: NovoNorm®, Novo Nordisk), and nateglinide (brand name: Starlix®, Novartis).

■ Incretin axis

As mentioned above, GLP-1 and GIP are strongly involved in the control of blood glucose concentration, and thus used for the treatment against T2D. Individuals with T2D show a small but significant reduction in GLP-1 plasma levels [59]. However, the GLP-1 action is still effective in these patients; this is why most pharmaceutical efforts have been focused on GLP-1R agonists. Exenatide is a synthetic version of exendin-4, a hormone found in the saliva of a lizard, the Gila monster. It is a GLP-1 agonist approved by the US FDA since April 2005 for the treatment of T2D. It belongs to the group of incretin mimetics and is manufactured by Amylin Pharmaceuticals under the brand name of Byetta®. Exenatide exerts many beneficial effects, such as an increase in insulin secretion, a suppression of pancreatic release of glucagon, a decrease in gastric emptying, and a reduction of liver fat content. It is considered as an adjunctive therapy, combined with metformin or **thiazolidinediones** (TZD). However, exenatide also induces adverse effects, principally GI disorders, such as nausea, diarrhea, vomiting.

Liraglutide, marketed under the brand name Victoza®, is a partly DPP-4-resistant GLP-1 agonist developed by Novo Nordisk and approved by the FDA in 2009–2010 for the treatment of T2D. This drug has a half-life of approximately 10–14 h. It has also an additional advantage: a stimulation of insulin secretion only when blood glucose levels are higher than normal. Consequently, it induces a negligible risk of hypoglycemia. It also lowers blood triglyceride levels. This drug has mild and transient GI adverse effects such as nausea and vomiting [60]. GLP-1 drugs are currently only available for subcutaneous administration on a daily basis, so the development of a GLP-1 drug with a longer half-life is expected in order to reduce the discomfort and inconvenience of frequent GLP-1 administration (injection weekly or biweekly instead of daily). Albiglutide, marketed by GlaxoSmithKline, has a half-life of 4 to 7 days, which is considerably longer than the other two GLP-1 analogs approved for treatment, exenatide (Byetta®) and liraglutide (Victoza®). It has not yet been determined whether albiglutide is as effective as GLP-1 drugs currently on the market and some data remains to be published in order to know the incidence of adverse effects (albiglutide is undergoing Phase III clinical trials). Endogenous GLP-1 will be degraded to inactive forms by DPP-4 quickly after the release in the GI tract (half-life: 2 min). For this reason, Jiangsu Hansoh Pharmaceutical Group Co, Ltd (Jiangsu, China) produces a GLP-1 analog with a longer half-life *in vivo* compared with native GLP-1. This analog has a longer half-life thanks to mutations increasing their resistance to DPP-4 [204] and increasing the action of GLP-1. However, these analogs still have to be administered subcutaneously. Interestingly, Novo Nordisk is developing a GLP-1 mimetic with substitutions: positions 31 and 34 W have been substituted to H and Q, respectively. These peptides and derivatives remain biologically active, but are protected against degradation by GI enzymes. This last feature appears interesting for the development of next generation GLP-1 compounds because it allows an oral administration, which is more comfortable for patients than subcutaneous treatments [205].

Another tactic to increase the action of endogenous GLP-1 is to inhibit DPP-4, the major enzyme involved in the regulation of incretin hormone. These drugs, sitagliptin (Januvia®), vildagliptin (Zomelis®), saxagliptin (Onglyza®), linagliptin (Trajenta®) increase the levels of circulating active GLP-1, enhancing glucose tolerance and insulin secretion. They can be used as a monotherapy or combined with metformin, TZD or sulfonylurea. DPP-4 inhibitors induce lower risks of hypoglycemia, no decrease in body weight (whereas it is observed with GLP-1 analogs), and are better tolerated (at the GI level). In addition, some neuropeptides, growth factors, cytokines and chemokines are also potential substrates for DPP-4. Inhibiting those enzymes could be hazardous [61]. Despite several DPP-4 inhibitors already on the market, some new patents disclose new DPP-4 inhibitors, such as the use of novel

Key terms

Long-lasting insulin analogs: Many patients with diabetes have not reached glycemic goals on currently available insulins. A need to provide more physiologic basal insulin coverage and reduce hypoglycemic risk in patients with diabetes remains. The development of long-lasting insulin analogs have shown promising safety and efficacy results. Additional studies are necessary to tailor optimal insulin delivery strategies to specific subsets of diabetes patients.

Thiazolidinediones: Also known as glitazones, thiazolidinediones are members of a class of medications used in the treatment of type 2 diabetes. They act by activating the nuclear receptors of the PPAR family, with a high specificity for PPARγ. PPARγ is predominantly found in adipose tissue, pancreatic β-cells, vascular endothelium, macrophages, and the central nervous system, and its activation leads to a reduced glycemia by increasing peripheral insulin sensitivity.

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substituted aminopiperidines by Merck Sharp and Dohme Corp. [206], β-amino tetrahydroimidazo(1,2-A)pyrazines and tetrahydrotriazolo(4,3-A)pyrazines by Merck Sharp and Dohme Corp. [207].

■ Biguanides/AMPK

Biguanides have been used as treatments against T2D since the late 1950s. They are commonly used to increase the sensitivity to insulin of the liver, fat and muscles. Phenformin was the first biguanide drug to be used but, because of toxic effects (lactic acidosis), this drug has been withdrawn from the market (in the late 1970s). Metformin, another biguanide derivate (brand name Glucophage®), is now widely used to treat hyperglycemia in individuals with T2D, because it is well tolerated with very few adverse side effects. This drug is recommended as a first-line oral therapy by the American Diabetes Association and European Association of the Study for Diabetes. The main function of metformin is to reduce hepatic glucose production and to improve peripheral sensitivity to insulin. Metformin has also been shown to enhance glucose uptake in the muscles, while reducing levels of plasma triglycerides and nonesterified fatty acids. Zhou and colleagues reported that activation of AMPK (AMP-activated protein kinase) was induced by metformin [62]. However, this theory remains controversial since, recently, Viollet and colleagues conducted studies suggesting that the respiratory chain complex I, not AMPK, was the primary target of metformin [64]. Activation of AMPK signaling pathway by metformin (or AMPK activators) has been shown to inhibit the expression of two key hepatic gluconeogenic genes PEPCK and G6Pase, which, in turn, suppresses gluconeogenesis [64]. Metformin may also act on the incretin axis. Indeed, metformin increases GLP-1 plasma levels and induces islet incretin receptor gene expression through a PPAR-α-dependent mechanism [65]. Recent evidence indicates that metformin can decrease food intake of diabetic rats [66]. This control is produced via a direct action on the CNS, maybe through a GLP-1-dependent manner. This drug can be used either as a monotherapy or combined with other treatments against T2D, such as TZD, DPP-4 inhibitors. Metformin may also be useful to prevent the transition between prediabetic state and T2D [67]. Nevertheless, more studies are needed to find out whether this drug could be used as a treatment in the prediabetic population.

Colesevelam (brand name: Cholestagel®, WelChol® in the USA) can also be combined with metformin. At the beginning of 2008, the FDA approved a new indication for Colesevelam HCl as an adjunctive therapy to improve glycemic control in adults with T2D. Colesevelam is indicated as an adjunct to diet and exercise to reduce low-density lipoprotein cholesterol in patients with primary hyperlipidemia. It is one of the bile-acid sequestrants, which, along with niacin and the statins, are three main types of cholesterol-lowering agents. This treatment causes some side effects such as constipation, skin flushing or dyspepsia [68].

AMPK activators have also proven to be beneficial for the treatment of T2D, as shown by the high efficacy of metformin in T2D treatment. Indeed, activation of the AMPK signaling pathway can lead to an increased glucose uptake into muscles, decreased gluconeogenesis in the liver, increased fatty acid oxidation in muscles and liver, decreased fatty acid synthesis in the liver and adipose tissue, and increased mitochondrial biogenesis. Patents disclosing biguanide derivates as AMPK activators have recently been published by Hanall Biopharma [208]. Kyorin Pharmaceutical have developed a benzylparabanic acid derivative, a compound with a strong AMPK activating action [209]. However, it is still an indirect way to activate AMPK pathways via other pathways or molecules (seen previously with the activation of AMPK pathway induced by metformin). Interestingly, a recent patent from POXELdisclosed promising drug candidates, the thieno [2,3-B] pyridinedione and derivatives, which act as direct activators of AMPK that could be useful for the treatment of diabetes and metabolic diseases [210].

■ Glucokinase activators

Glucokinase, an isoform of the hexokinase enzymes, serves as a glucose sensor in pancreatic islet β-cells, converts glucose to glycogen in the liver and regulates hepatic glucose production. For the past few years glucokinase has appeared to be a promising drug target for T2D and glucokinase activators (GKAs) are now being developed. In 2000, RO-0281675 (Hoffman-La-Roche; [211]) was the first GKA noted to reduce blood glucose after an oral glucose tolerance test in several rodent models of T2D. This compound also lowered basal glucose in diet-induced obese (DIO) mice, after a 2 h fasting; an accompanying rise in insulin was also noted. RO-0281675 attenuated endogenous glucose production and elevated hepatic glucose disposal [69]. Another compound, the PSN-GK1 (OSI/Prosidion; [212]) was disclosed in 2004. This agent exerts typical effects on glucokinase kinetics, that is, a stimulation of insulin secretion and hepatic glycogen synthesis without increasing glycogen stores. However, Hoffman-La Roche was the first pharmaceutical company to bring a GKA into clinical trials: RO4389620 (Piragliatin; [213]) reached Phase II clinical trials, tested as a monotherapy or in combination with metformin on volunteers and patients with T2D. This compound improves the oral glucose tolerance test in patients with T2D through

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an increase of insulin secretion and glucose uptake. RO4389620 appeared to be well-tolerated; the most common adverse event associated with this compound being headache. AstraZeneca, OSI, Eli Lilly, Merck and Novo Nordisk have been and are continuing Phase I and Phase II investigations for different GKA.

TZD

TZD, the synthetic PPAR-γ agonists are commonly used to treat T2D. They increase insulin sensitivity by acting on adipose tissue, muscle, and liver to enhance glucose uptake and decrease glucose production. They bind and activate PPAR-γ, which regulate gene expression. Troglitazone (brand name: Rezulin®, Parke-Davis/Warner- Lambert) was the first TZD approved for clinical use in the USA in 1997, but was subsequently withdrawn from the market in 2000 due to hepatotoxicity. Rosiglitazone (brand name: Avantia®, GlaxoSmithKline) and pioglitazone (brand name: Actos®, Takeda Pharmaceutical) are used as a monotherapy or in combination with sulfonylurea, metformin, or insulin. TZD have several other potential side effects such as fluid retention, body-weight gain, increased risks of heart failure and of bladder cancer [70]. Moreover, these drugs can also provoke a decrease in bone mineral density, increasing the risk of fracture. Avantia was withdrawn in 2011 because of an increased risk of heart attack and Actos has been withdrawn in 2012 in some countries (France and Germany) because of links with bladder cancer.

Recently, nonthiazolidinedione selective PPAR-γ modulators were found to be good candidates to reproduce the same effects on glucose metabolism while minimizing TZD side effects. In this context, INT131 Besylate (InteKrin Therapeutics, Inc.) is currently undergoing Phase II clinical trials [71].

GPCR & diabetes

It should be noted that GPCR 40 (GPR40) is mainly expressed in pancreatic β-cells and in intestinal cells but also at low levels in the brain. GPR40 can be activated by FFAs, especially linoleic acid, and potentiate insulin secretion in a glucose-dependent manner in β-cells, without increasing the risk of hypoglycemia. In intestinal cells, GPR40 is important for the release of incretin hormone such as GLP-1 or GIP. GRP40 may have a double effect on glucose metabolism: a direct effect on insulin secretion and an indirect one via incretin hormone. At the beginning of 2012, TAK-875, a GPR40 agonist developed by Takeda Pharmaceutical, entered Phase III clinical trials. Other GPCR receptors, also expressed in areas involved in glucose metabolism, could be good candidates for the treatment of T2D such as GPR119, GPR120, GPR41 and GPR43. Agonists for these GPRs could be used in addition to DPP-4 inhibitors [72].

Sodium-glucose cotransporters: renal target

Much evidence indicates that the kidneys may preserve a certain level of blood sugar (normoglycemia), through the mechanisms of filtration and reabsorption of glucose. In order to reduce blood glucose levels in T2D patients, some SGLT-2 inhibitors increasing urinary glucose excretion have been developed, such as dapagliflozin (AstraZeneca), canagliflozin (Johnson & Johnson) and empagliflozin (Boehringer Ingelheim and Eli Lilly and Company). Dapagliflozin, empagliflozin and canagliflozin are currently being investigated in Phase III clinical trials. For dapagliflozin, the FDA would not approve the novel diabetes drug until the drug manufacturers, Bristol-Myers Squibb and AstraZeneca, supplied more data on the drug's benefits and risks. Indeed, Dapagliflozin has been shown to increase the risk of genital, and possibly urinary, infections, and also a possible increase in the incidence of breast and bladder cancer [73]. For empagliflozin, it is one of the largest Phase III clinical trials in diabetes with over 14,500 patients planned to enroll. The program included ten multinational Phase III clinical trials, including a large safety survey on the cardiovascular outcome. Pivotal studies should have been completed in late 2012, with a market access planned in the USA and Europe by 2013. There are also some patents, such as Boehringer Ingelheim's, in which compounds are being developed to inhibit SGLT-2 in order to prevent/slow progression of/delay or treat a metabolic disorder, in particular in T2D [214]. The aim of this patent is to improve glycemic control in T2D patients and in patients with insufficient glycemic control, impaired fasting blood glucose and/or IR, and also to reduce complications associated with T2D, such as nephropathy and retinopathy. Compounds of this patent have already shown an improvement of blood glucose levels in Zucker diabetic rats. Astellas Pharma, Inc. uses benzothiophen compounds, as an SGLT-1/SGLT-2 dual inhibitory activity, which exhibit an excellent hypoglycemic action on mild hyperglycemic conditions, as well as severe hyperglycemic conditions; they also display an extremely high hypoglycemic action on mild hyperglycemic conditions compared with that of compounds that can inhibit SGLT-2 selectively. These compounds can be used for preventing and/or treating diabetes-related diseases including T1D, T2D, IR disease and obesity [215]. However, information on the long-term impact of SGLT-2 inhibitors on renal physiology is currently lacking and such effects should be investigated in the clinical trials

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■ 11β-hydroxysteroid dehydrogenase type 1 inhibitors

11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) is a NADPH-dependent enzyme, highly expressed in the liver, adipose tissue and the CNS. In these tissues, 11β-HSD1 reduces cortisone to the active hormone cortisol that, in turn, activates glucocorticoid receptors. Glucocorticoids stimulate production of glucose and inhibit β-cell insulin secretion and peripheral glucose uptake. Patients with glucocorticoid excess, for example patients with Cushing's syndrome, develop visceral obesity, IR, T2D, dyslipidemia, hypertension and increased cardiovascular mortality. A modulation of activity in the *11β-HSD1* gene can be associated with obesity and IR. Transgenic mice overexpressing 11β-HSD1 in adipose tissue faithfully replicate the metabolic syndrome (IR and glucose intolerance). Conversely, 11β-HSD1-/- mice resist the metabolic consequences of stress and high-fat feeding via insulin sensitization [74]. Emodin, a selective 11β-HSD1 inhibitor improves insulin sensitivity, lipid metabolism in DIO mice, such as the novel 11β-HSD1 inhibitor, KR-67183 [75]. Since 11β-HSD1 inhibitors appear to be a valuable target to treat T2D, numerous pharmaceutical companies develop such drugs. For instance, Boehringer Ingelheim [216] and Kowa Co. [217] disclosed 11β-HSD1 inhibitors and intermediates.

In last few years, several 11 β-HSD1 inhibitors have reached Phase IIb clinical trials, such as DIO-902 from DiObex, Inc., or INCB13739 from Incyte, Inc.

Interestingly, it has been shown that salicylates improve insulin sensitivity through a downregulation of 11β-HSD1 [76]. Salsalate (Disalcid®) is a nonsteroidal anti-inflammatory drug used to reduce pain and inflammation (class of drugs called 'salicylates'). It is also recommended as an anti-inflammatory alternative to naproxen and ibuprofen for patients who had minor stomach bleeding or stomach upset. Recent preclinical trials show that salicylates can also improve glycemic control in genetic models of obesity, including Zucker obese rats and *ob/ob* mice, as well as in nongenetic models of obesity (DIO model).

■ Anti-obesity drugs

Considering the fact that obesity is a major risk factor for T2D, effective weight-reducing drugs in obese patients is of particular relevance in the context of diabetes management. As discussed in one of our recent reviews, numerous weight-loss drugs have been withdrawn from the market and promising candidates have been abandoned during development because of serious toxic effects related to pulmonary hypertension, valvulopathies, stroke or suicidal ideation and behavior [77]. Thus, until 2012, Orlistat (Xenical® from Roche/Alli® from GSK) remained the only approved treatment for the long-term management of obesity. Among interesting candidates, lorcaserin (Arena Pharmaceutical, Lorquess®), and co-treatments with phentermine and topiramate (Vivus pharmaceuticals, Qnexia®) or with buproprion and naltrexone (Orexigen, Contrave®) were initially turned down by the FDA in 2010 (Lorquess, Qnexia) and 2011 (Contrave). However, considering the societal benefits that anti-obesity drugs could bring in a context of global epidemic, and given the lack of approved anti-obesity compounds on the market, the FDA recently announced that these drugs could get speedier US approval in order to improve the balance between innovation and safety. These recent trends in the changes in FDA policies in taking a 'fast-track' approach in the approval of anti-obesity drugs could lead sponsors to conduct smaller and faster clinical trials and could allow a rolling submission on data. In return, such approved drugs would be associated with a more restrictive labeling convention that would initially limit their use to a smaller, specific group of patients. Since these changes in the FDA policies are undergoing discussion, this lighten up on obesity drugs has already led to the approval of two new anti-obesity drugs in 2012. The Arena Pharmaceuticals' lorcaserin (Belviq®, formerly Lorquess) have been approved in June 2012 by the FDA in the light of the results of a new clinical trial, the BLOOM-DM trial (for Behavioral Modification and Lorcaserin for Obesity and Overweight patients in Diabetes Mellitus) [78]. Lorcaserin is a selective agonist of the serotonin 2C (5-HT2C) receptors and have been shown to induce greater weight loss than placebo in obese and diabetic patients, with a significant decrease in waist circumference, BMI and glycemic parameters. Improvement in the lipid profile was also noted with lorcaserin but these changes were not statistically significant. Interestingly, the treatment also induced a decrease in heart rate and blood pressure, while no significant risk of serotonin-associated valvulopathy was reported. The most commonly reported adverse effects included headache, nausea and dizziness, leading to the discontinuation of the therapy. The Vivus Pharmaceutical's phentermine/topiramate extended-release capsules (Qsymia®, formerly Qnexia) was also approved in July 2012 by the FDA for chronic weight management, in association with a reduced calories diet and increased physical activity. Its use is, however, restricted to obese adults showing an initial BMI greater than 27 kg/m² associated with hypertension, T2D or high cholesterol levels. Qsymia is a fixed-dose combination of the anorectic agent phentermine with the antiepileptic drug topiramate. Qsymia has been associated with fetal toxicity, suicidal behavior glaucoma, cognitive impairment and metabolic acidosis. At

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the cardiovascular level, Qsymia has been associated with an increased resting heart-rate but with favorable changes in blood pressure. However, considering the good magnitude of weight loss observed in the most recent clinical trials [79–81], with an average weight loss of between 5 and 11% after 1 year of treatment at the recommended doses, and given the beneficial changes in blood pressure, the FDA concluded that the benefit-to-risk ratio was positive and supported the approval of Qsymia. Even if the FDA approved Belviq® and Qsymia anti-obesity drugs, it acknowledges that more studies are required concerning the adverse effects of these drugs, and requires the manufacturers to conduct several post-approval clinical trials with a more rigorous assessment of the long-term cardiovascular effects in obese patients. In addition to these two newly approved anti-obesity drugs, Orexigen Therapeutics Inc. announced progress with the FDA on a faster path to resubmission of the Contrave (naltrexone/bupropion) New Drug Application. The FDA still wants to see data from Orexigen's cardiovascular safety trial for Contrave (the Light Study) before considering approval. However, Orexigen anticipates resubmitting the Contrave new drug application in the second half of 2012 with a potential approval in early 2013.

■ Conclusion

Nowadays, the discovery and marketing of treatments against T2D is booming. The most important challenge in these developments remains to produce safe drugs for long-term use. A 'good' treatment has to take into account both patient comfort (route of administration, frequency of administration), as well as minimizing side effects, including hypoglycemia. Concerning insulin therapy, despite advances in insulin development (fast-acting and slow-lasting analogs), there is a remaining need to provide more physiologic basal insulin coverage and to reduce hypoglycemic risk in patients with diabetes. Newer insulin analogs and routes of insulin delivery have shown promising safety and efficacy results and should improve the patient's quality of life once on the market. Paired with insulin, glucagon receptors have been considered as a prime target for a long time, but the use of glucagon antagonists remains associated with a high risk of hypoglycemia. Several pharmaceutical companies are trying to circumvent this safety problem; however, to date, the development of glucagon receptors antagonists systematically failed at the clinical stage. Besides, most of the TZDs have been withdrawn from the market due to cautionary side effects associated with their use. However, the development of non-TZD-selective PPAR-γ modulators could offer opportunities to separate the desirable from the unwanted effects. On the other hand, some pathways such as incretin axis or SGLT inhibitors appear more promising. As mentioned previously, liraglutide (Victoza®) bring an advantage compared with other treatments, since it induces a stimulation of insulin secretion only when blood glucose levels are elevated. Interestingly, a negligible risk of hypoglycemia has been associated with this compound. The development of the next generation of GLP-1 compounds created to withstand GI enzymatic degradation should allow an oral route of administration, improving the lifestyle of diabetic patients. SGLT2 inhibitors offer a novel insulin-independent approach for the control of hyperglycemia without incurring hypoglycemia. Interestingly, their efficacy in improving glycemia is not affected by the extent of IR or β-cell dysfunctions and, therefore, in principle, they could be used at any stage of the disease. Finally, current and developing anti-obesity compounds could represent an interesting way of research for T2D patients since these compounds have been proven to be effective in both decreasing body weight and in ameliorating glycemic parameters.

Focus on central therapeutic targets

The drugs currently under development and listed above could lead to great progress in the treatment of T2D. In addition, several neuropeptides and hormones acting at the brain level have been implicated both in glycemia and food intake controls. The dual mode of action of these neuropeptides could then represent a very interesting feature in the treatment of T2D since most of these patients also exhibit obesity. Thus, such therapeutic avenues could lead to a new generation of drugs addressing both T2D and obesity. We have listed below a non exhaustive list of studies and patents focused on several central signaling pathways, that is, leptin, melanocortins, BDNF/TrkB, nesfatin-1 and melanin-concentrating hormone (MCH), whose modulation could be of great interest (Table 3).

■ Leptin

The leptin and Ob-R discoveries, as well as their regulatory role in glucoregulation and energy homeostasis, have led to hopes for the use of leptin signaling as an emerging therapy for T2D. Interestingly, several studies have shown that the hypoglycemic effects of leptin were independent from its food intake and body weight lowering effects. Thus, it has been shown that intra-cerebroventricular leptin treatment, at a dose that did not modulate food intake and body weight, was able to normalize glycemia in *ob/ob* leptin-deficient mice [29]. Moreover, restricting the food intake of untreated *ob/ob* mice to the same amount of food consumed by leptin-treated *ob/ob* mice has been shown to

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Table 3. Recent patents disclosing compounds acting on CNS target for treating type 2 diabetes.

Drug name/type	Company	Ref.
Leptin receptors		
Metreleptin/pramlintide co-treatment	Amylin Pharmaceuticals, Inc.	[218,219]
Leptin-related synthetic peptides	Albany Medical College	[220,221]
D-amino acid-substituted leptin-related polypeptides	Albany Medical College	[222,223]
Small molecule CNS penetrant leptin mimetics	BioVitrum AB	[224–228]
Small leptin fragments modulating lipolysis stimulated receptor activity	Serono Genetics Institute, Inc.	[229]
Antibodies against leptin-related peptides	Albany Medical College	[230]
Melanocortin receptors		
Peptides modulating MC4R	Novo Nordisk	[231]
Cyclic peptides	Palatin Technologies	[232,233]
	Biomeasure, Inc.	[234,235]
	Grubert KA	[236]
	Palatin Technologies, Inc.	[237]
	Centocor Ortho Biotech, Inc.	[238]
Piperazine and keto-piperazine compounds	Palatin Technologies, Inc.	[242]
Diamine-containing, tetra-substituted piperazine	Palatin Technologies, Inc.	[240]
Hydrazine substituted piperidine	Palatin Technologies, Inc.	[239]
Amine substituted piperazine	Palatin Technologies, Inc.	[241]
Acylated piperidine derivatives	Merck	[243]
Pyrazolopyridine and pyrazolopyrimidine derivatives	Montreal University	[245]
Imidazolpyridine and other derivatives	Montreal University	[244]
Brain-derived neurotrophic factor/TrkB signaling		
Peptidoid compounds as brain-derived neurotrophic factor receptor agonist	Villoslada P and Messegier A	[246]
Antisense oligonucleotides	Curna	[247]
Brain-derived neurotrophic factor conjugated with fatty acids	Allam AR	[248]
Mature human or modified NT4/5	Pfizer	[249]
Anti-TrkB, anti-brain-derived neurotrophic factor or anti-NT4/5 antibodies	Pfizer	[250]
Small molecule as inhibitors of Trk receptors	IRM LLC	[251]
Nesfatin-1		
Nesfatin-1	Liu JN	[252]
Nesfatin-1 and nesfatin fragments	Teijin Pharma Ltd	[253,254]
Pladin and mutated pladin	Landing Biotech, Inc.	[255]

be insufficient to match the glucose lowering action of leptin therapy [82]. Finally, a direct glucoregulatory action of leptin has been shown in mice with lipodystrophy, in which leptin administration corrected the IR and hyperglycemia without affecting body weight [83]. Recent studies have shown that a selective re-expression of leptin receptors on POMC neurons of the ARC of obese and diabetic mice models, in which endogenous expression of the leptin receptors was prevented, was sufficient to normalize blood glucose and hepatic insulin sensitivity, while only a very subtle effect was shown on food intake and body weight [84–86]. This data indicates that leptin regulates glucose homeostasis and hepatic insulin sensitivity in mice by a direct action on POMC neurons of the ARC of the hypothalamus.

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The understanding of the action of leptin in energy and glucose homeostasis based on preclinical studies has led to great hope in emerging therapies for obesity and T2D. Clinical trials using human recombinant leptin as a potential treatment for obesity were first conducted as early as 1999. These studies revealed that while leptin treatment markedly improved obesity and endocrinological imbalance in the rare cases of obese humans in which the obesity was caused by a genetic leptin deficiency [87], it was ineffective or very poorly effective in improving obesity in the hyperleptinemic patients that represent the vast majority of obese humans [88,89]. The latter observation led to the concept of leptin resistance in the vast majority of obese subjects with hyperleptinemia. Soon after the preclinical studies conducted by Shimomura and colleagues showing the effectiveness of leptin therapy in normalizing glucose homeostasis in rodent models of lipodystrophy with hypoleptinemia [83], clinical studies confirmed these results in diabetic lipodystrophic humans showing severe hypoleptinemia (<4 ng/ml). This study showed that a long-term treatment with recombinant methionyl human leptin in these patients induced a significant improvement in IR, hyperinsulinemia, hyperglycemia and lipodystrophy [90,91]. However, a recent study failed to show any improvement in the hyperglycemia in lipodystrophic patients with moderately low levels of circulating leptin (~5 ng/ml) [92]. More recently, two clinical studies directly addressed the question of the effectiveness of leptin therapy in improving T2D in obese patients [93,94]. However, both of these studies failed to show any impact of the therapy in improving diabetes and IR.

In conclusion, it is clear that leptin therapy fails to improve metabolic imbalance in individuals who do not show severe hypoleptinemia, due to a leptin resistance in these subjects. The rate-limiting factor contributing to leptin resistance in obese humans and animals appears to be mainly related to defective leptin transport into the CNS [95–101]. Thus, ways to improve leptin resistance are currently under investigation: an effective leptin therapy for treating diabetes could be accompanied by the additional benefits observed in the hypoleptinemic nonleptin resistant patients, such as:

- An absence of hypoglycemia following leptin treatment, whereas hypoglycemia is often reached with insulin therapy;
- A decreased lipid content in adipose and extra-adipose tissues, while insulin therapy promotes lipid accumulation;
- A decreased food intake.

Failure in the clinical trials with recombinant human leptin for the vast majority of leptin-resistant patients with T2D have stimulated alternative approaches using combination therapies, leptin-related synthetic peptides analogs or mimetics, leptin receptor antagonists mimetics or leptin receptor antagonists. In this field, interesting results have been published using amylin and leptin co-treatment. Amylin is a 37 amino acid hormone secreted by pancreatic β-cells in response to nutrient ingestion [102–104]. Amylin acts on its specific receptors in the brainstem at the area postrema level, a circumventricular organ without a blood–brain barrier, and activates structures of the CNS known to regulate energy balance and glucose homeostasis. Amylin and its analog pramlintide have shown to decrease food intake and body weight in leptin-resistant animal models of obesity and in obese humans [104–109]. Interestingly, it has been shown that combination therapy using an ineffective dose of leptin with moderately effective dose of amylin induced a marked weight loss that could not be attributed to amylin alone, suggesting a synergistic action of the two treatments [105–107,110]. These promising results have been confirmed at the clinical stages with overweight and obese humans, with a synergistic effect of metreleptin and pramlintide on body weight, glycemia, insulinemia and IR [109]. These studies have led to the publication of a series of patents from Amylin Pharmaceuticals entitled 'Methods for treating obesity and obesity related diseases and disorders' [218,219]. However, while being a promising approach, Amylin Pharmaceuticals and Takeda Pharmaceutical Company announced in a press release dated March 2011 that they had *"suspended clinical activities in an ongoing Phase II study examining the safety and effectiveness of the investigational combination therapy pramlintide/metreleptin for the treatment of obesity. The clinical study was voluntarily halted to investigate a new antibody related laboratory finding with metreleptin treatment in two patients who participated in a previously completed clinical study of obesity."* These studies, however, revealed that the leptin-resistance in obese humans can be bypassed by combination therapies and could lead to new exploratory studies using leptin or leptin analogs for treating obesity and T2D.

Besides these combination therapy approaches, active research is currently ongoing into the generation of leptin-related synthetic peptides that could avoid leptin-resistance in obese animals and human. These investigations resulted from a series of publications describing the bioactivity of leptin-related synthetic peptides, in particular the LEP [111–125] fragment and its analog [D-Leu-4]-OB3 in lean and obese mice models [126–131]. Both compounds have shown to be effective in regulating food intake and body weight, and serum glucose and insulin sensitivity in *db/db* mice that are genetically deficient in functional Ob-R, suggesting an

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action independent of the activation of Ob-R [131,132]. The clinical significance of these observations may be of high importance given the leptin resistance and hyperleptinemia exhibited by the majority of obese humans. These studies have led to a series of patents from Grasso and colleagues disclosing low molecular weight synthetic peptides comprising amino acids residues of mouse and human leptin, in particular the 116–122 fragment also called OB-3 [220,221]. The invention relates methods, composition and utilization of leptin-like peptides for novel diagnostic and therapeutic applications to human obesity and obesity related dysfunctions such as T2D. The effectiveness of OB-3 and OB-3-related peptides in the treatment of *db/db* mice is shown in the patent indicating that these peptides are not acting on Ob-R. Moreover, it is thought that OB-3 can enter the CNS without assisted transport. These observations indicate that these peptides may have therapeutic applications in clinical cases where recombinant leptin have been shown to be ineffective. Grasso *et al.* further extended these patents and proposed the use of D-amino acid-substituted leptin-related polypeptides such as mouse and human [D-Leu-4]-OB3 and [D-Pro-5]-OB3 for novel therapeutic applications to human obesity and obesity related dysfunctions such as type 2 diabetes [222,223].

The development of small-molecule CNS-penetrant leptin mimetics represents another therapeutic strategy focusing on leptin system and aiming to by-pass the leptin resistance induced by limited uptake of leptin into the brain of obese patients with T2D. A series of such compounds, essentially some pyridine and piperazine derivatives, have been disclosed by BIOVITRUM AB [224–228]. These compounds have been shown to decrease food intake and body weight in wild-type c57Bl6 mice. Although interesting, such compounds must be further evaluated *in vivo* on animal models of T2D.

Besides the leptin-related synthetic peptides or small molecules therapeutic options for obesity and T2D, Yen *et al.* disclosed an invention that resulted from a focusing of the research effort on the discovery of the mechanism of leptin elimination [229]. The inventors postulated that the concentrations of leptin are increased in obese patients because of the increase in the size of adipose tissue as well as by a reduced clearance of this hormone. The resulting high level leptin causes a leptin-resistance syndrome and, thus, for the authors, the treatment of obese subjects should not be based on increasing levels of leptin, but the opposite, on normalizing leptin levels in obese patients. The invention indicates that leptin and leptin fragments generated induced a decrease in the postprandial lipemic response in *db/db* mice, indicating an action independent of the Ob-R activation. Moreover, the invention presents the discovery that leptin can increase the activity of lipolysis stimulated receptors, binds directly to these receptors and that this binding led to leptin degradation. The authors suggest that this mechanism is responsible for the decreased postprandial lipemic response in *db/db* mice following leptin treatment. The invention discloses the discovery of a 22 amino-acid region of human leptin that modulate lipolysis stimulated receptors activity *in vitro* and *in vivo* in the same way as intact leptin. The authors suggest that leptin-fragments or modified leptin-fragments could be used in disease treatment since it is active in mice at a physiologically-relevant level. The invention also features a method for treating obesity and obesity-related disorders such as diabetes with the use of a pharmaceutical composition comprising the leptin polypeptide fragments and variants of the invention or, alternatively a chimeric polynucleotide of the invention or a polynucleotide or a recombinant vector encoding a zinc finger protein of the invention.

On the same idea, a patent called 'isolated antibodies against biologically active leptin-related peptides' by Grasso *et al.* disclosed ploYclonal, monoclonal or chimeric antibodies to OB-3 and OB-3-related peptides. According to the authors, these antibodies may be used as pharmacologically active compounds [230].

■ Melanocortins

As seen above, the initial hopes in the development of a treatment for obesity and T2D by recombinant human leptin have given way to disappointment, resulting essentially from the leptin-resistant status of most of the patients. Leptin actions on energy and glucose homeostasis are mediated by downstream effectors such as melanocortin signaling at various locations of the brain. Thus, identifying the signaling pathways situated downstream of the leptin signaling and relaying its glucoregulatory actions could be a possible strategy. As previously described, leptin regulates energy and glucose homeostasis and hepatic insulin sensitivity, at least in part, by a direct action on POMC neurons of the ARC of the hypothalamus [133–135], suggesting an important role of the melanocortin system in the glucoregulation. Interestingly, it has been shown that unlike leptin, α-MSH, derived from post-translational proteolytic processing of the POMC precursor, can decrease bodyweight, weight gain, and food intake in mice with diet-induced obesity showing a leptin-resistance. Moreover, α-MSH analogs have also been shown to affect blood glucose levels in some mouse models of obesity [111]. Neurons expressing melanocortin receptors receive inputs from POMC neurons and NPY/AgRP neurons to regulate energy

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and glucose homeostasis. Among specific receptors, the melanocortin receptors 3 and 4 have been shown to be implicated in glucoregulation. For example, Fan and colleagues have shown that melanocortin agonists administered centrally decreased serum insulin concentration in hyperglycemic *ob/ob* mice, and inhibited basal insulin release as well as reduced glucose tolerance in wild-type mice [112]. Furthermore, the authors have shown that young lean MC4R-knockout mice exhibited an increased plasma insulin level and that these mice developed an IGT before the onset of detectable hyperphagia or obesity [112]. Recent evidence demonstrates that the central melanocortin system directly controls glucose and lipid metabolism independently of its actions on food intake and energy homeostasis. Notably, it has been shown that a long term intracerebroventricular (icv) infusion with MC4R antagonists increased insulin sensitivity and glucose uptake in white adipose tissue, and decreased glucose utilization in muscle and brown adipose tissue, while none of these effects are observed in control and pair-fed animals [113]. Concerning MC3R, Sutton and colleagues demonstrated that the application of a feeding restriction paradigm to MC3R-knockout mice induced hyperinsulinemia and glucose intolerance compared with wild-type controls [114]. These results indicate that melanocortin-based therapeutics specifically active in the CNS may be promising for the treatment of T2D. These observations have led to active research in identifying analogs of melanocortins pharmacologically active on MC3R and MC4R.

Recent patents disclosed the use of compounds as modulators of melanocortin receptors. Different strategies have been used to identify modulators of melanocortin receptors, with the generation of peptides, cyclic peptides and various small molecules. Some of these recent patents relate to the invention of new peptides that derive from the primary sequence of the α-MSH. In a recent patent by Novo Nordisk [231], novel peptides with improved water solubility at neutral pH are disclosed. Some of these peptides exhibit high affinity for MC4R (Ki < 1 nM) and *in vivo* efficacy. This patent also disclosed a method of treating T2D and glucose intolerance, and methods for delaying the progression from impaired glucose tolerance condition to T2D, and from noninsulin requiring T2D to insulin requiring T2D. Numerous other patents disclosed the invention of cyclic peptides acting as specific melanocortin receptor ligands. Cyclic peptides disclosed in a recent patent have been found to be MC4R ligands and are proposed as pharmacological tools to treat melanocortin receptors-mediated diseases such as obesity and T2D [232]. One of these peptides (peptide N°1) was reported to be a melanocortin receptor agonist, with an EC_{50} value of 1nM and an intrinsic activity of 91% on MC4R where 4-norleucine, 7-D-phenylalanine-α-melanocyte-stimulating hormone (NDP-α-MSH), a synthetic analog of α-MSH, is 100%. Changes in food intake and body weight were evaluated for this newly generated peptide for 24, 48 and 72 h following treatments. However, the positive actions of these peptides on glucose tolerance and insulin sensitivity remain to be demonstrated. In a second patent, others cyclic peptides acting as MC4R agonists are disclosed [233]. The biological activity of some of these compounds have been verified after an acute treatment in Sprague–Dawley rats and the peptide has been shown to decrease food intake and body weight and to increase penile erection. Similarly, in the [234] patent, authors also disclosed the invention of cyclic peptides as melanocortin receptor ligands. Four compounds of this invention were tested in long-term experiments on rats and have been shown to be pharmacologically active on feeding behavior. The authors argue that such ligand compounds activating one or more melanocortin receptors would be useful for modulating blood glucose levels. Another recent patent, from Dong and collaborators, disclosed a novel class of cyclic peptides modified with hydantoin moiety, acting as modulators of MCR, in particular for MC4R [235]. This new class bears modified amino-acid residues (unnatural or synthetic) in the N-terminal region of the peptides, conferring an increase in the selectivity of the compound and in the binding affinity at the targeted receptor. These compounds show a low nanomolar affinity with MC4R and improved pharmacokinetic properties when compared with α-MSH, in particular a prolonged half-life. The *in vivo* efficacy of several of these compounds has been demonstrated in rats, and an acute treatment has been shown to induce a decrease in food intake and body weight gain in rats. Such compounds could be useful in the prevention and treatment of obesity and related disorders such as T2D. Non-naturally occurring melanocortin peptidic ligands, in a linear or cyclized form, having a degradation-resistant C-terminal extension, can act as MC3R, MC4R and MC5R agonists or antagonists [236]. The new peptides in this patent have a degradation-resistant C-terminal extension consisting of one amino acid, modified or not, a peptide mimetic, or a combination. These peptides are of great interest in the treatment of melanocortin-related diseases since the C-terminal extension reduces the cardiovascular associated side effects observed with other melanocortin analogs [115]. However, although promising, the *in vivo* efficacy and safety of such compounds are unfortunately lacking to date. As described in a patent by Palatin Technologies, Inc., cyclic peptides with MCR specific affinity could also be generated through a lactam bridge by the side

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chain of aspartic acid and lysine, or through a disulfure bridge formed by the side chains of two cysteines [237]. Finally, a patent entitled 'melanocortin receptor binding conjugates' by Centocor Ortho Biotech, Inc. disclosed an original approach based on the development of fourteen compounds called α-MSH derivative-Fc conjugates [238]. The conjugates contain a fragment homologue or analog to the wild-type α-MSH and a portion of an immunoglobulin hinge region.

In addition to the development of new peptides, a growing number of patent applications disclosed small molecules as promising melanocortin receptor modulators. Among these compounds, the six-membered ring piperazine, piperidine and spiroperidine are known ligands to melanocortin receptors and particularly to MC4R, and are therefore possibly useful in the treatment of disorders such as obesity. This past decade, several derivatives generated from these molecules have been disclosed in numerous patents. A continuous effort has been made to develop new piperazine and piperine derivatives with high specificity and affinity for specific MCR, especially for MC4R, that could be used alone or in combination with other pharmacologically active agents useful for the treatment of diabetes and related disorders [239–245].

In conclusion, the active research in biologically active modulators of melanocortin receptors has led to an important number of potential therapeutic agents for melanocortins-related diseases. However, the effectiveness of these compounds in ameliorating glucose tolerance and insulin sensitivity still needs to be demonstrated using *in vivo* studies on physiopathological animal models of T2D.

■ BDNF

A seen above, growing evidence indicates that BDNF/TrkB signaling in the CNS plays a role in the glucose homeostasis in animal models of T2D, independent of its well known role in food intake and body weight control [35,116]. Thus, it has been shown that peripheral injections of BDNF normalize glycemia in several obese and diabetic rodent models such as DIO mice, *ob/ob*, *db/db* or Ay mice [117–120]. In *db/db* mice, BDNF hypoglycemic effects lasted for weeks, while the observed anorexigenic effects appeared transient, without any further observed effects several days after the end of the treatment [119,121]. Moreover, in the same *db/db* model, pair-feeding studies have shown that BDNF-treated mice and food-restricted mice exhibited indistinguishable body weight growth curve, while the BDNF-treated mice showed a lower glycemia than pair-fed animals [33,122,123]. These data indicate that the prolonged hypoglycemic effect of BDNF is not due simply to its actions on food intake. Moreover, a recent study has also shown that BDNF not only ameliorates hyperglycemia in diabetic *db/db* mice, but also completely prevents the progression of the disease when administered to young prediabetic mice [123]. BDNF glucoregulatory effects involve a direct action of this neurotrophin at the CNS level; intracerebroventricular injections of BDNF in *db/db* mice, at doses ineffective when injected peripherally, have also been shown to normalize glycemia [33].

The research of the mechanisms of action by which BDNF affects glycemia has led to the hypothesis that BDNF could act at the central level, resulting in an indirect enhancement of the insulin sensitivity of peripheral organs. Furthermore, hypoglycemic effects of BDNF have been associated with a simultaneous increase of plasmatic insulin levels [117,119] associated with an important increase in pancreatic insulin content. In T1D mice models, the streptozotocin (STZ)-treated mice, in which STZ induces the destruction of pancreatic β-cells and then abolishes the secretion of insulin, BDNF treatment decreased the food intake but was ineffective in reducing hyperglycemia, while co-treatment of BDNF and insulin have shown that BDNF increases the hypoglycemic action of insulin [33]. BDNF treatment has also been shown to induce an increase in the insulin transduction signals in the liver of STZ-treated mice. BDNF induced a similar increase in the insulin transduction signals in the liver, skeletal muscle and brown adipose tissue of *db/db* mice and an increased glucose utilization in the same organs [124,125]. In addition to the enhancement in insulin sensitivity, other studies suggested that BDNF can also improve hyperglycemia in diabetic mice via a decrease in hepatic production of glucose [119,136]. Interestingly, a recent study compared the antidiabetic effects of BDNF and the TZDs pioglitazone and rosiglitazone in *db/db* mice [137]. The results indicated that BDNF, pioglitazone and rosiglitazone all ameliorated hyperglycemia but that BDNF increased the pancreatic insulin content more effectively than the TZDs. Moreover, BDNF treatments induced a decrease in food intake and body weight, a positive event in T2D and obesity, while the TZDs exerted an opposite effect.

Taken together, these findings indicate that BDNF may have potential as a unique hypoglycemic agent for the treatment of diabetes at a fundamental level. Accordingly, several methods and compounds have been recently disclosed in several patents to modulate the central BDNF/TrkB signaling activity in order to treat T2D in humans.

For example, a recent patent by Villoslada and colleague disclosed the use of novel peptidoid compounds and the pharmaceutically acceptable salt and prodrugs as agonists of BDNF receptors for use as

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medication [246]. The biological activity of several of these compounds has been tested using *in vitro* and *in vivo* models. It was shown that these compounds are able to bind and activate TrkB receptors on PC12 cells cultures, this effect being competitive with BDNF. Moreover, these compounds can promote cell survival, prevent neuronal death in an *in vitro* model of Parkinson's, and reduced neuroinflammation in an *in vitro* model of neuroinflammation in a similar manner to recombinant neurotrophins. Additionally, these peptidoid compounds had a positive impact on an animal model of multiple sclerosis, a pathology in which BDNF have a potential therapeutic use. However, these compounds have not yet been tested in the context of T2D. Some of these compounds have been shown to display medium to high crossing of the blood–brain barrier by active transport, allowing the use of a peripheral method of treatment in a context where the site of action would be the CNS.

Another approach has been recently disclosed for modulating BDNF tissular expression. This method is based on the use of antisense oligonucleotides (5–30 nucleotides) targeting natural antisense BDNF polynucleotides in order to increase the expression and/or function of this neurotrophin [247]. Hybridization of these oligonucleotides on their targets induces a loss of activity of the antisense, resulting in an up-regulation of the BDNF gene expression. The patent also provided methods for the use of these antisense oligonucleotides in the treatment of diseases and disorders associated with the expression of BDNF. Considering the CNS involvement in the anorexigenic action of BDNF, a particular focus of the applicant is for a delivery at the cerebral level. The same patent also proposes an intravenous co-administration of the oligonucleotides with agents promoting their penetration across the blood–brain barrier such as anti-transferrin receptor antibodies. This approach remains to be tested in the context of obesity and T2D, but could be of interest as the use of antisense oligonucleotides has previously been validated by the FDA, with the approval of VITRAVENE™ for the treatment of cytomegalovirus retinitis. Another strategy based on BDNF/TrkB signaling enhancement has been provided in a recent patent disclosing a method stabilizing and potentiating the therapeutic actions of BDNF. The authors proposed to administer BDNF as an admixture of the neurotrophin conjugated with essential fatty acids and polyunsaturated fatty acids for treating obesity and diabetes [248]. The invention is based on the fact that both BDNF and essential fatty acids/polyunsaturated fatty acids are endogenous mediators of neuronal survival, neurotransmission and synaptic plasticity, and are likely interacting in the control of these processes, and that both are involved in the physiopathology of obesity, diabetes and metabolic syndrome. They also suggested the use of NT4/5, another known endogenous ligand for TrkB, as a treatment for obesity and diabetes. NT4/5 is known to reduce feeding and body weight and to induce long-lasting amelioration of hypertriglyceridemia and hyperglycemia in normal, obese and diabetic animals after peripheral or central intraparenchymal injections [138]. These results led to the publication of a patent revealing a method for treating obesity and diabetes using mature human or modified NT4/5 [249]. However, conflicting results have been published concerning the use of NT4/5 in nonhuman primates in the treatment of obesity, while systemic administration in these species have been shown to induce hyperphagia and weight gain, the opposite effect than observed after administration in rodents. This orexigenic action of NT4/5 in primates seems to involve a peripheral action of the peptide since its central injection remained effective in altering feeding in rhesus monkeys [139]. This discovery led to the publication of patents that took the opposite view to previously mentioned patents, and which detailed the anti-obesity and anti-diabetic potential of TrkB signaling blockade in primates by the means of antibodies, such as anti-TrkB, anti-BDNF or anti-NT4/5 antibodies, or with soluble TrkB-Fc fusion protein [250]. Another recent patent disclosed the discovery of small molecules acting as inhibitors of TrkA, TrkB or TrkC kinases, and their use in the manufacture of drugs for treating Trk-mediated disease including metabolic disease [251]. The biological activity of these compounds remains to be verified.

Thus, BDNF/TrkB signaling pathway seems to be a promising target in the treatment of T2D and obesity. However, in light of the lack of experimental data and conflicting results, it is clear that strategies targeting this pathway require additional *in vivo* studies on rodents and nonhuman primates before being considered as valuable for obesity and diabetes treatment in humans.

■ Nesfatin-1 & related peptides

As seen above, it has now been established that nesfatinergic neurons participate in responses occurring during satiety and glycemic changes. Indeed, nesfatinergic neurons are activated during a glucoprivic challenge and these activated nesfatinergic neurons could mediate the counter-regulatory response [43]. In the brain, nesfatin-1 would activate directly or indirectly POMC neurons, and thus the melanocortin pathway [140]. In the pancreas, nesfatin-1 is colocalized with insulin in β-cells, and glucose stimulates nesfatin-1 release from rat islets. In turns, nesfatin-1 stimulates glucose-dependent insulin secretion *in vitro* and *in vivo* [40]. In addition, in

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db/db mice, an intravenous injection of nesfatin-1 improves glycemia in a dose- and time-dependent manner [42]. In a recent patent entitled 'use of nesfatin-1 in the treatment for diabetes', the authors showed a reduction of nesfatin-1 level in serum and hypothalamus of *db/db* mice compared with wild-type mice [252]. These results are consistent with those obtained in humans showing that in patients with T2D the fasting plasma nesfatin-1 is reduced compared with healthy subjects and patients with T1D [39]. To date, the receptor(s) of nesfatin-1 have not been identified that hampers the development of pharmacological agents. In 2007, Brailoiu *et al*. showed that in the rat brain nesfatin-1 acts on an unidentified GPCR [141]. In 2009, a patent disclosed a screening method for the study of nesfatin-1 action using a protein receptor group consisting of GPR3, GPR6, and GPR12 [253]. GPR3, GPR6, and GPR12 constitute a family of constitutively active GPCRs highly expressed in the CNS. The authors showed that GPR12 represents a good candidate receptor of nesfatin-1. Interestingly though, GPR12 knock-out mice have an obese phenotype but no change in food intake [142], suggesting the existence of alternative nesfatin-1 receptors in addition to GPR12.

In 2008, only 2 years after the discovery of nesfatin-1, a patent proposed a method to produce nesfatin-1 and highlighted the active segment of nesfatin-1 corresponding to a short sequence at the middle of the peptide. The anti-hyperglycemic effect of nesfatin-1 was not known at this date, so the authors presented nesfatin-1 as a potential treatment for eating disorders and body weight control [254].

In 2010, two patents targeted nesfatin-1 for the treatment of diabetes: using Ob-R and plasminogen (plg) double knock-out mice, the authors showed a beneficial effect of nesfatin-1 in symptoms related to diabetes. Plasminogen is the precursor of plasmin which cleaves nesfatin-1 on several sites and inactivates it within 2 h. Compared with plg+/+ LepR-/- mice, plg-/- LepR-/- mice displayed reduced hyperphagia, obesity, hyperglycemia and hyperinsulinemia [252]. During a glucose-tolerance test the glycemic response of plg-/- LepR-/- mice was similar to wild-type mice, whereas plg+/+ LepR-/- mice failed to regulate blood glucose level. Moreover, while the hypothalamic level of the two orexigenic neuropeptideswas high in plg+/+ LepR-/- mice, it was normal in plg-/- LepR-/- mice, equal to wild-type mice. Thus, authors postulated that inhibiting the cleavage of nesfatin-1 by plasmin can prevent diabetes. They tested two plasmin inhibitors, the tranexamic acid and the aprotinin, in *db/db* mice and observed an increase of serum nesfatin-1 and a decrease of food intake and body weight when mice are treated with each inhibitor [252].

Another peptide with an anti-hyperglycemic effect was identified as a portion of NUCB2 and named pladin for plasma antidiabetic NUCB2 peptide [255]. Pladin, a 69 amino-acid peptide, was found in the serum of mice, rats, rabbits and humans, and it is of note that nesfatin-1 contains the entire 69 amino-acid sequence of pladin. As with nesfatin-1, pladin had an anorexigenic effect when injected centrally or peripherally and reduced blood glucose in diabetic *db/db* mice. The effect of pladin on blood glucose was reversed in the presence of inhibitors of PPAR-γ or AMPK, suggesting that PPAR-γ and AMPK mediates the anti-hyperglycemic effect of pladin. Moreover, pladin serum level was higher in plg-/- LepR-/- mice than in plg+/+ LepR-/- mice, indicating that pladin is also cleaved by plasmin. The authors experimented with different mutations of pladin and observed that some mutations made pladin resistant to the inactivation by plasmin. Thus, when mutated pladin was injected in *db/db* mice, blood glucose was decreased longer than with the nonmutated pladin treatment [255]. To summarize, in type 2 diabetic humans and mice, blood and hypothalamic nesfatin-1 and pladin levels were reported to be lower than in healthy subjects. The authors proposed to increase nesfatin-1and pladin levels by direct treatment with nesfatin-1, pladin or analogs displaying a resistance to cleavage by plasmin, and treatment with plasminogen inhibitors.

■ Melanin-concentrating hormone

The MCH is an orexigenic neuropeptide expressed in the LHA, which plays an important role in the regulation of feeding behavior and energy balance [143]. It was shown in rodents that icv injections of MCH induced an increase of food intake [144]. Moreover, MCH overexpression induces an increase of both body weight and insulinemia, whereas mice lacking the MCH prohormone exhibit a lean and hypophagic phenotype [145,146]. In addition to its role in obesity, Pereira-da-Silva and colleagues suggested that MCH induces IR through a mechanism independent of its orexigenic actions [147]. Indeed, a short-term icv treatment with MCH in rats did not modulate body weight and food intake, but increased basal-state glycemia, increased glucose intolerance, and decreased insulin release during a glucose-tolerance challenge and decreased insulin-induced activation of Akt. MCH-knockout mice appeared protected against aging-associated IR [148]. Pissios and colleagues hypothesized that MCH could modify metabolism by affecting appetite, but also by increasing β-cell mass and so increasing insulin secretion. Therefore, MCH appeared as a potential target to treat T2D as well as obesity [149]. Recent patents described 7-piperidinoalkyl-3,4-dihydroquinolone derivate and

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bis-pyrylpyridones as MCH receptor-1 antagonists and propose their use as potential compounds to treat obesity and/or diabetes [256,257]. However, Kowalski and MacBriar highlighted that most of the MCHR antagonists bind to their receptor thanks to a basic amine center, but this common structure promotes nonspecific interactions with other targets such as biogenic amine receptors and ion channel and especially to HERG canal in the heart [150]. Indeed, binding between ligands and HERG canal is associated with fatal arrhythmia. In order to avoid these cardiovascular problems and to produce safer compounds, Bristol Myers Squibb disclosed in two recent patents new MCHR1 antagonists where the basic amine moiety was replaced [258,259]. The authors assured that some of their compounds presented a lower affinity to nonspecific target and a satisfactory safety profile, but did not present all the necessary results to assert such a conclusion. Regarding the *in vivo* assessment, they conducted a sub-chronic treatment during 4 days in male Sprague–Dawley rats, which revealed a dose-dependent anorexic effect and a dose-response effect for some compounds. Nevertheless, the mechanism of action of these potential drugs on both obesity and diabetes still remains to be determined.

■ Conclusion

Over the past two decades, the discovery of central neuropeptides involved both in the control of energy homeostasis and glucose metabolism led to the identification of new interesting targets for the treatment of obesity and T2D. Interestingly, most of these neuropeptides have been shown to exert body weight and glucose-lowering effects by independent mechanisms. This dual mode of action appears of interest in the context of T2D since it could bring to additive effects with a direct action on glucose metabolism combined with the beneficial effects offer by a reduction of food intake and body weight. However, aiming central neuropeptides circuitries can be hampered by the inability of the drugs to cross the blood–brain barrier, as illustrated by the large disappointment provided by the ineffectiveness of recombinant leptin therapy in obese patients. Indeed, even if some efforts are currently made in the development of leptin-related synthetic mimetics, targeting central leptin signaling should not anymore be considered as a valuable therapeutic option for treating obesity and T2D. Melanocortinergic signaling has also led to strong disappointments. Considerable efforts have been made in order to develop bioactive melanocortin receptors agonists, but these compounds have been associated with adverse cardiovascular effects. Moreover, melanocortins receptors undergo considerable internalization and desensitization to agonists, rapidly reducing the potential effectiveness of these agents. Thus, the development of melanocortin agonists is not likely to represent a viable and safe approach to the development of anti-obesity and T2D therapeutics. The BDNF/TrkB signaling pathway currently appears as an interesting target since BDNF not only ameliorates hyperglycemia in animal models of T2D, but also completely prevents the progression of the disease when administered to young prediabetic mice. However, before considering BDNF as a valuable treatment for T2D in humans and to assess the efficacy and safety profiles of long-term BDNF treatments in clinical trials, additional *in vivo* studies on rodents and nonhuman primates are required. More recently, the discovery of nesfatin-1 has generated interest, this peptide acting both at the pancreas and brain levels to improve glycemic control. However, the development of nesfatin-1-derived drugs is considerably slowed since their specific receptors remain unidentified. Moreover, clinical trials remain to be conducted in order to assess the potential of nesfatin-1 for the treatment of T2D in human. In conclusion, central pathways involved in the control of energy homeostasis have been seen as the 'holy grail' as targets for the treatment of obesity and T2D, particularly by offering the opportunity to address the two pathologies that are closely linked by additive mechanisms. However, a lot of efforts remain in order to develop effective and safe drugs targeting the central signaling pathways and that could be use with a comfortable route of administration.

Future perspective

T2D represents an increasing epidemic worldwide with significant negative consequences on the quality of life of patients. While current glucose-lowering therapies include an important number of drugs among various therapeutic classes (biguanides, sulfonylurea, glinides, TZDs, insulin, GLP-1 mimetics and DPP-4 inhibitors), T2D treatment remains a tricky challenge. With current treatments, a large number of diabetic patients fail to achieve normal values in fasting glycemia and HbA1c levels, highlighting the need for new therapeutics in this field. Potential new peripheral targets have been identified over the last few years and new compounds are in development in order to normalize blood glucose in T2D patients. Among them, incretin-based therapies and SGLT-2 targeting drugs currently under development appears of particular interest because of their original modes of action. Besides, research in the field of glucoregulation and nutritional behavior at the CNS level helped to identify central signaling pathway as targets that represent an emerging strategy. Compounds targeting these pathways could bring beneficial features, such

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as slow and long-lasting effects on basal glycemia, reduced hypoglycemic events and reductions in food intake and body weight. The latter is of particular importance since such central acting drugs could be good candidates to simultaneously address T2D and obesity, two pathologies closely linked. However, the most important challenges in these developments remain to produce safe drugs for a long-term use, with the ability to freely access their action site, that is, the brain. Thus, to date, leptin and melanocortin signaling pathways have led to strong disappointments and do not represent anymore key targets in the treatment of T2D. BDNF and nesfatin-1 could bring to new therapeutics avenues, but their development is currently at the preclinical level and a long period of time will be needed before drugs targeting these pathways eventually enter the market. A last point to take into account is that central mechanisms responsible for the control of energy and glucose homeostasis involve redundant signaling pathways. Thus, drugs altering one of these signaling pathways could be ineffective due to compensation mechanisms. A multitarget approach with a combination therapy altering simultaneously several central targets or targeting peripheral as well as central targets could provide an interesting way of research for the future.

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Executive summary

Background

- The introduction of this review provides a short description of epidemiologic data related to diabetes as well as the accepted parameters and tests for the diagnosis of type 2 diabetes (T2D).

Physiological regulation of glycemia

- This section describes the physiological mechanisms involved in the regulation of glycemia, including the peripheral and central aspects of this regulation as well as the involvement of modulators of glycemia and insulin release.

Physiopathology of T2D

- This section provides the physiopathological basis for T2D, including the mechanisms responsible for glucose intolerance, insulin resistance and β-cells dysfunction.

Present pharmacological treatments and current development

- In this section, we reviewed the approved classes of hypoglycemic agents used as treatments in T2D as well as recent patent development for compounds acting peripherally. This section covers the following classes of agents: insulin, glucagon-receptor antagonists, sulfonylurea/glinides, incretins, biguanides, glucokinase activators, thiazolidinediones, G-protein-coupled receptor agonists, sodium-glucose co-transporters and 11β-hydroxysteroid dehydrogenase type 1(11β-HSD1) inhibitors. Since obesity is a major risk factor for T2D, we also made a focus on the latest approved weight-reducing drugs.

Focus on central therapeutic targets

- This section provides a review of recent patents and new developments of compounds acting on the CNS and that could provide good candidates as treatments for T2D and obesity. The review covers compounds related to leptin, melanocortins and brain-derived neurotrophic factor signaling pathways, as well as compound related to nesfatin-1 and melanin-concentrating hormone.

Future perspective

- This section provides a brief conclusion highlighted the point of view of the authors concerning the future perspectives in T2D management.

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Annexe 4

Revue : Advances in Deoxynivalenol toxicity mechanisms: the brain as a target.

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Review

Advances in Deoxynivalenol Toxicity Mechanisms: The Brain as a Target

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Abstract: Deoxynivalenol (DON), mainly produced by *Fusarium* fungi, and also commonly called vomitoxin, is a trichothecene mycotoxin. It is one of the most abundant trichothecenes which contaminate cereals consumed by farm animals and humans. The extent of cereal contamination is strongly associated with rainfall and moisture at the time of flowering and with grain storage conditions. DON consumption may result in intoxication, the severity of which is dose-dependent and may lead to different symptoms including anorexia, vomiting, reduced weight gain, neuroendocrine changes, immunological effects, diarrhea, leukocytosis, hemorrhage or circulatory shock. During the last two decades, many studies have described DON toxicity using diverse animal species as a model. While the action of the toxin on peripheral organs and tissues is well documented, data illustrating its effect on the brain are significantly less abundant. Yet, DON is known to affect the central nervous system. Recent studies have provided new evidence and detail regarding the action of the toxin on the brain. The purpose of the present review is to summarize critical studies illustrating this central action of the toxin and to suggest research perspectives in this field.

Keywords: mycotoxins; deoxynivalenol; hypothalamus; brainstem; anorexia; anapyrexia; cytokines; POMC; nesfatin-1

1. Introduction

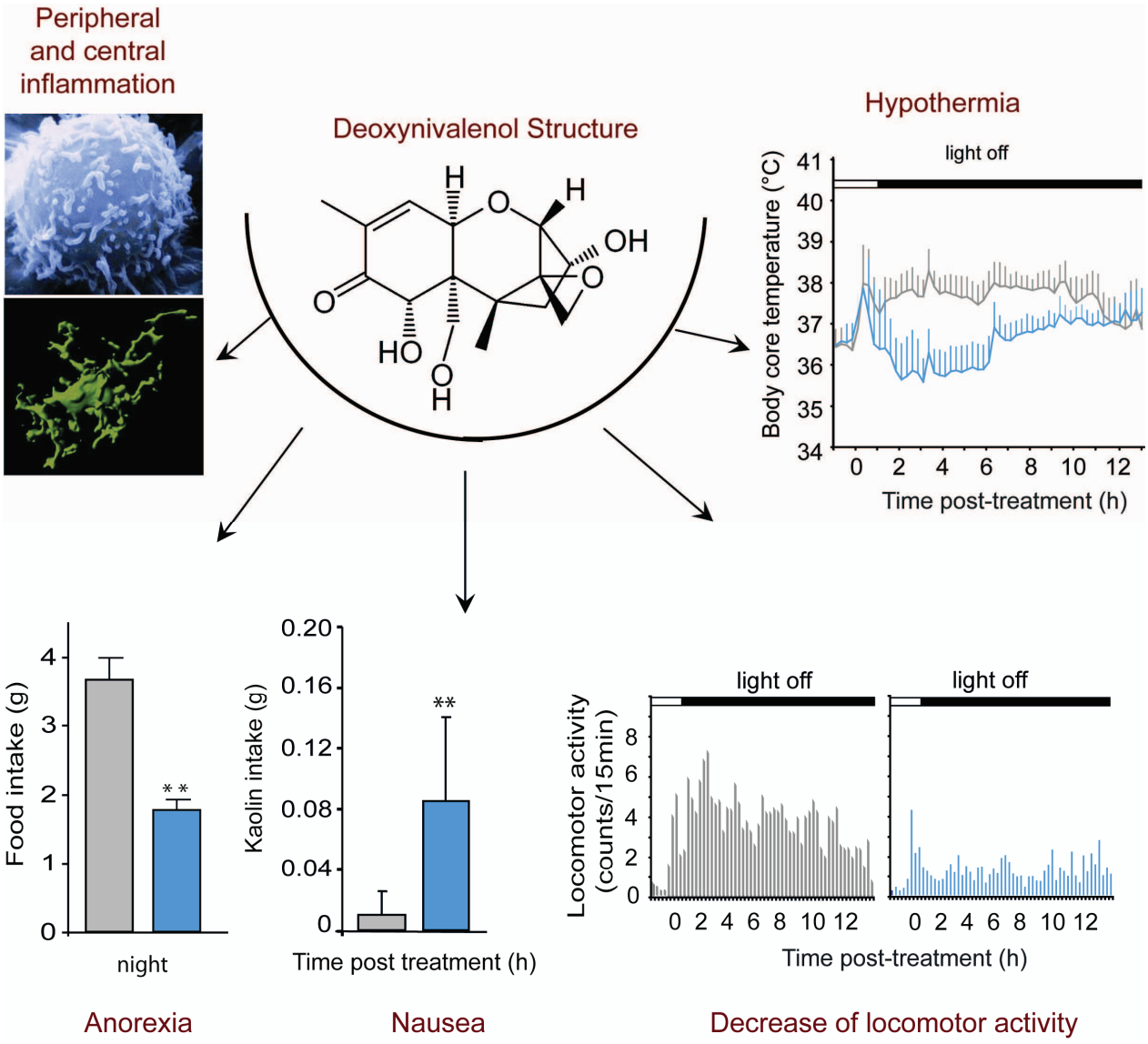
Deoxynivalenol (DON) is a secondary metabolite mycotoxin produced more especially by *Fusarium graminearum* and *culmorum.* These fungi can grow on various cereals such as wheat, barley, oat, rye, maize and rice. The extent of cereal contamination is strongly associated with rainfall and moisture at the time of flowering and with grain storage conditions. Deoxynivalenol represents the major fungus metabolite which can be found on feed grains. The chemical name of DON is DON 12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-en-8-on, ($C_{15}H_{20}O_6$); it has a polar organic structure and a molecular weight of 296.36 g/mol. The keton position in C_8 is a feature of the class B trichotecenes [1]. Due to its high melting point (151 °C–153 °C), DON withstands cereal processing and cooking and thus constitutes an important food contaminant [2–5]. The consumption of DON-contaminated food induces mycotoxicoses in farm animals as well as in humans. DON toxicity is characterized by a set of symptoms including diarrhea, vomiting, anorexia, reduced weight gain, neuroendocrine and immunological changes, leukocytosis, hemorrhage, circulatory shock, and can ultimately lead to death (Figure 1). Its capacity to induce vomiting episodes in various species including humans explains its commonly used nickname "vomitoxin" [6]. During the last two decades, many studies have described DON toxicity using diverse species as a model, where DON consumption was shown to affect numerous physiological functions such as food intake, reproduction or immunity. While the toxin action on peripheral tissues and organs is well documented, studies aiming to identify the impact of DON on the brain are relatively less abundant. Based on evidence showing that DON ingestion could disrupt brain neurochemistry, recent studies have brought new information regarding its central effects especially in relation to anorexia/nausea. The purpose of the present review is to summarize recent critical studies illustrating the central action of the toxin and to propose future research directions in this field.

2. DON, Food Refusal, Emesis and Anorexia

In humans, mycotoxicosis observed after consumption of feed contaminated with DON, leads to vomiting, reduced food intake, abdominal pain and diarrhea (for review [7]). It is well established that DON consumption also results in decreased weight gain and food intake and altered nutritional efficiency in different models including poultry, rodents and pigs. The key studies evaluating emesis, growth and food intake in various species and in response to different DON doses were gathered by Pestka and Somlinski [8]. The intensity of the effects depends on the dose, the species, the duration of consumption, DON purity and the route of administration. For instance, 50 to 100 µg/kg orally administered DON induced vomiting in swine [9–11]. This emetic effect can also be produced by ingestion of feed contaminated with DON at a dose of 20 ppm (equivalent to 0.15 mg/kg BW/day; [12]. The ingestion of naturally contaminated food with 1 to 2 ppm DON caused partial feed refusal, whereas 12 ppm caused complete refusal [13,14]. In mice, consumption of 2 ppm purified DON during

8 weeks (equivalent to 0.3 mg/kg BW/day) provoked decreased growth and 0.5 ppm (equivalent to 0.075 mg/kg) reduced food intake [15]). A drastic decrease in food intake and a related loss of weight was observed in mice consuming a diet containing 2.5 ppm purified DON (equivalent to 0.36 mg/kg) during one week [16]. Similarly, in the rat, the consumption of 0.5 mg/kg purified DON during 9 weeks resulted in growth reduction and decreased food intake [17]. Similar observations have been made in dogs and cats fed for 2 weeks with diet naturally contaminated with 6 to 8 ppm DON (equivalent to 0.4 mg/kg) [18].

Figure 1. Symptoms associated with acute Deoxynivalenol (DON) intoxication. In addition to a peripheral and central inflammation, when administered *per os* at the end of the light phase at a dose of 12.5 mg/kg, DON induces a decrease in food intake observed overnight following the treatment, and an increase in Kaolin intake measured 24 h after administration suggesting a gastric discomfort. Moreover, telemetric analyses show a 2 °C decrease of body core temperature and a locomotor activity decrease during the night in DON-treated animals compared with control animals.



3. DON-Induced Anorexia in Mice Models

Thus, although it is clear that DON ingestion induces a reduction of food intake in different animals models, the feature of this DON-induced anorexia remains poorly characterized. Recently, a noticeable effort was made to describe more precisely the DON-induced anorexia using mice as a model [19–23]. While swine was reported to be the most sensitive species to DON, numerous *in vivo* and *in vitro* DON toxicity studies were conducted on rodents and especially on mice which can be considered as a good model for the study of DON toxicity. In a model of high fat diet-induced obesity (HFD; 60% kcal from fat), mice consuming 5 or 10 ppm DON for 10 weeks exhibited a clear reduction of weight gain [19]. In another set of data originating from the same group using HFD-induced obesity (54% and 60% kcal from fat), DON consumption (equivalent to 10 mg/kg) decreased body weight, fat mass and food intake. These physiological modifications were associated with reduced plasma insulin, leptin, insulin-like growth factor 1, and an increase in hypothalamic mRNA level of orexigenic agouti-related protein [23]. Flannery and collaborators [20] measured noctural food intake on mice fasted during light cycle and treated with i.p. 1 and 2.5 mg/kg or oral gavage 0.5 to 5 mg/kg DON. Food intake was measured 2 h and 16 h after toxin exposure. These authors report that DON caused a rapid feed refusal that was evident 2 h after toxin administration for the highest DON doses. Interestingly, this effect was dose-dependent and transient, since 16 h after DON-administration, an increase in food intake occurred in DON-treated mice when compared with vehicle-treated animals. Using a different experimental paradigm, we recently obtained additional information regarding DON-induced anorexia [21,22]. In these studies, we performed acute *per os* DON administration (6.25, 12.5 and 25 mg/kg) at the end of the light phase and monitored noctural food intake in mice fed *ad libitum*. In this context, *per os* intoxication induced a dose-dependent reduction in daily food intake by especially decreasing night-time food consumption in animals fed *ad libitum* (Figure 1). Anorexia was observable as soon as 3 h after toxin exposure for 12.5 and 25 mg/kg and 6 h for the lowest dose. This anorexigenic effect lasted up to 24 h with 6.25 and 12.5 mg/kg DON and on over 72 h with the highest dose. When performing a meal pattern analysis, which provides an accurate description of the eating behavior through a continuous and automated recording of food intake, we revealed a DON-induced reduction in meal frequency (satiety) and meal size (satiation) by 44.2% and 68% respectively. In accordance, the inter-meal interval increased by 68% in the presence of the toxin. The effect on meal frequency was evocative of nausea-induced anorexia as revealed by studies showing that meal frequency is especially affected by toxicological compounds such as lithium and lipopolysaccarids (LPS; [24,25]). In rodents, emesis cannot occur but DON can induce nausea. Indeed, we measured DON-induced nausea in mice by measuring kaolin consumption, a non-nutritive substance, the intake of which serves to evaluated nausea in rodents [26]. The results showed that DON at 12.5 mg/kg induces a significant increase in kaolin intake for the 24h following toxin administration (Figure 1). This result suggests a nauseous effect of DON in mice. However, kaolin intake was poorly correlated with standard chow intake, suggesting that nausea does not totally explain feed refusal. On the other hand, the consequence of DON treatment on meal size supports the view that DON acts on meal termination mechanisms. A reduction in meal size but not in meal frequency is typically observed with physiological inhibitors of food intake such as leptin [27], cholecystokinin [28], peptide YY 3-36 [29], insulin [30]. Hence, taken together these results reveal a complex action of DON on food intake where DON-induced anorexia

depends not only on modification of satiety but also of satiation. In parallel to food intake a modulation of body temperature and locomotor activity was also recorded in DON-treated mice (Figure 1). This set of symptoms *i.e.*, anorexia/nausea, reduction of locomotor activity and modulation of body core temperature is evocative of sickness behavior occurring in response to inflammatory challenges (for review, [31]).

4. Sickness Behavior and Central Cytokines Expression

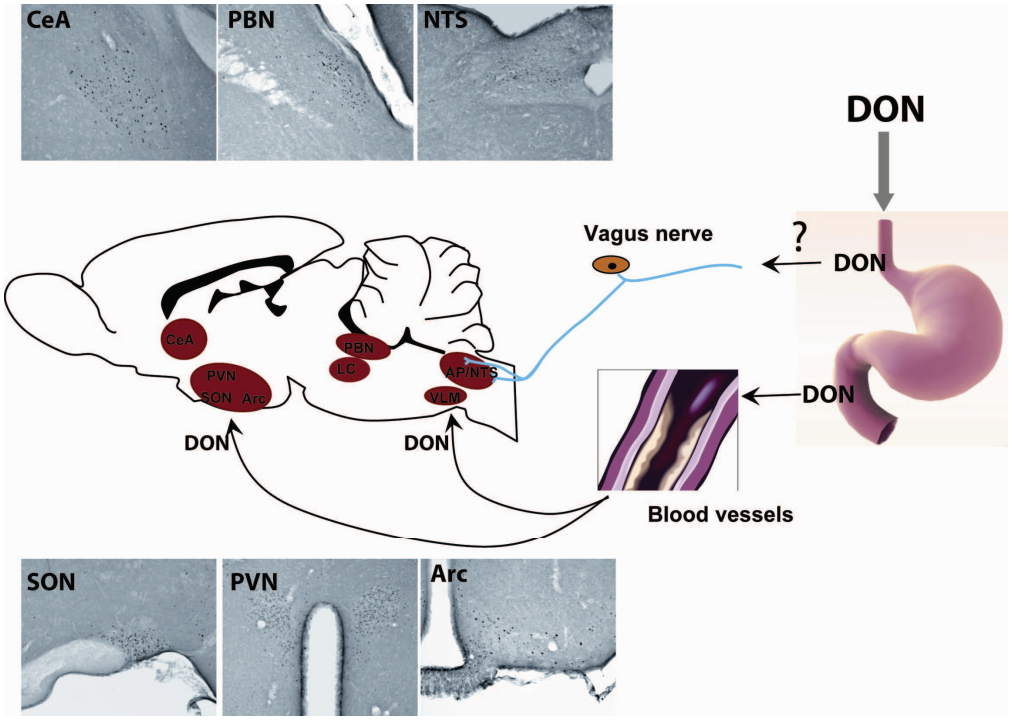
The survival of a pluricellular organism depends on its capacity to fight against infections and to store energy. The immune and metabolic systems, among the most essential ones of the animal kingdom, are closely linked and interdependent. Numerous hormones, cytokines, or bioactive lipids can exert metabolic and immune functions. Inflammation is a response coordinated by an organism that allows it to struggle against an attack. This reaction leads to deep physiological and behavioral changes due to the activation of the innate immune system and the recognition of molecular motives linked to the pathogen. The classical inflammatory response called "acute phase reaction" implicates metabolic modifications, redistribution of energy and the use of lipid supplies. These modifications require redefining physiological preferences for an efficient adaptation of organism with the intervention of the central nervous system (CNS) which coordinates the installation of the central element of the acute phase reaction. This central component of the acute phase reaction consists of deep behavioral changes called "sickness behavior" which includes fever, activation of the stress axis and reduction of food intake. In this context during an infection and inflammation, pro-inflammatory cytokines produced by a variety of cells reach and interact with brain regions that control ingestion *i.e.*, the hypothalamus and the dorsal vagal complex (DVC) to induce anorexia. Peripheral immune signals reach the brain via a humoral communication through circumventricular organs such as the anteroventral region of the third ventricle and the area postrema (AP) and via a neuro-immune gut-brain pathway that mainly involves the sensory vagal afferent innervation (see for review [32]). Accordingly, anorexic doses of peripheral cytokines such as IL-1β activate the primary projection area of the vagus nerve which is located in the DVC. In turn, multiple secondary projection sites of the vagus nerve involved in the processing of gut visceral information and in the control of food intake *i.e.*, the hypothalamus, the central nucleus of the amygdala and the bed nucleus of the stria terminalis, are activated [33]. Alternatively, in response to peripheral inflammation, the brain can also be a source of cytokines, as demonstrated by several studies [34]. Using genetic models exhibiting impairment of LPS and IL-1β signaling, these studies have shown that this *de novo* central production of cytokines is required to obtain a sustained anorexia in response to LPS or IL-1β administration [35,36]. Finally, a modulation in the expression of neurotransmitters and anorexigenic or orexigenic neuropeptides in the hypothalamus was proposed to contribute to the anorexic behavior induced by inflammatory signals (for review [32]).

5. DON, Cytokines Expression and Anorexia

In the mid-nineties, Azcona-Olivera and colleagues [37] have reported that an oral exposure to 5 and 25 mg/kg BW of DON results in mice in an increased cytokine production in spleen, Peyer's patches, liver, kidney and small intestine. Using RT-PCR and Southern-blot, the authors demonstrated

the increased abundance of IL-1 β, IL-6, TNF-α and to a lesser extend TGF-β and INF-γ mRNA shortly after DON exposition (2 and 4 h). This pioneer study was confirmed and strengthened by numerous other works performed on different models including mice, pigs or human cell lines [38–42]. For instance, acute oral exposure of B6C3F1 mice to DON (5 and 25 mg/kg BW) was shown to increase, in spleen and Peyer's patches, the expression of INF-γ, IL-2, IL-4 and IL-10 in addition to the pro-inflammatory cytokines mentioned above [42]. In the same way, piglets intravenously injected with 1 mg/kg BW DON exhibited a modulation of IL-1 β, IL-6 and TNF-α expression in lymphoid organs [39]. In human macrophages, 100 to 1000 ng/mL of DON and other 8-ketotrichothecenes significantly upregulated the expression of TNF-α, IL-6 and IL-8 [43]. In Jurkat T-cell, DON (32.5–500 ng/mL) upregulates IL-2 and IL-8 production [44]. Finally, in human monocyte cell line, 1 μg/mL DON stimulated IL-8 mRNA and protein production [45]. Interestingly, several studies reported the amplified proinflammatory cytokine induction during cotreatment with both DON and bacterial LPS [46–48]. Given the well-known anorexigenic effect of proinflammatory cytokines, their increased expression observed in response to DON intoxication was proposed to drive associated symptoms including anorexia and reduced weight gain [49]. Supporting this hypothesis, we have recently shown that *per os* DON administration induced an up-regulation of IL-1β, IL-6 and TNF-α within two central structures *i.e.*, hypothalamus and DVC [22]. These results constituted the first demonstration that *per os* DON administration results in central neuroinflammation. As mentioned above, these structures are involved in the regulation of food intake. Altogether, these data strongly suggest that peripheral and also central-borne cytokines may participate in the onset of reduced food intake observed in response to DON intoxication. Although attractive and plausible, this hypothesis remains to be experimentally demonstrated. Even though inflammation-related effectors are strongly expressed during DON intoxication, the arguments supporting their triggering role in DON-induced anorexia are still limited. First, TNF-α receptors KO and IL-6 KO mice do not exhibit any reduced susceptibility to DON-induced anorexia [50,51]. Prostaglandins (PGs) are key inflammatory mediators acting downstream of proinflammatory cytokines which induce some of the symptoms of sickness behavior such as anorexia ([52]). PGH2, the end product of cyclooxygenase (COX) enzymes, can be converted into various eicosanoids such as PGD2, PGF2α, PGE2, prostacyclin and thromboxane A2 [53], each compound having their own specific biological activities. PGE2 are the most potent PG in inducing anorexia when centrally administered [54]. The microsomal PGE synthase-1 (m-PGES-1) belongs to the MAPEG (Membrane Associated Proteins in Eicosanoid and Glutathione Metabolism) superfamily and catalyses the final step of PGE2 synthesis [55]. mPGES-1 has been described as an inducible enzyme which expression is stimulated by pro-inflammatory agents [56–58] and silencing mPGES-1 gene prevents IL-1 induced anorexia [59]. We recently reported the increased expression of COX-2 and mPGES-1 transcripts within the hypothalamus and brainstem of DON-treated mice [22]. Different transgenic mouse models were used to investigate the involvement of PGs in DON-induced anorexia and reduced weight gain. COX-2 knock-out (KO) mice were reported to exhibit a decrease in body weight comparable to that of wild type littermate when exposed to chronic consumption of DON during 16 weeks [60]. Moreover, silencing PGE2 signaling pathways using mPGES-1 KO mice did not modify the anorexigenic response to DON [22].

Figure 2. Central structures activated by acute DON intoxication. *Per os* administration of DON induces an increase in the number of c-Fos positive neurons in several brain nuclei such as the hypothalamus (paraventricular nucleus, arcuate nucleus, supraoptic nucleus), the dorsal vagal complexe (DVC) at the brainstem level (nucleus tractus solitarius, dorsal motor nucleus of the vagus), the pons (parabrachial nucleus, locus coeruleus) and the central amygdala. During an oral intoxication, DON may be conveyed to the brain where it may activate these structures, leading to the establishment of symptoms.



6. Brain Structures Activated during Acute DON Intoxication

For many years, the expression of the c-fos gene or protein has been considered as a high resolution marker of neuronal activity since c-Fos protein is expressed in neurons whose activity is strongly stimulated by synaptic input [61,62]. Even if one must bear in mind that not all activated neurons show c-Fos induction, and that the threshold of c-Fos protein induction may differ between subpopulations of CNS neurons, the use of c-Fos expression remains a useful approach to identify and investigate neuronal groups activated in response to different challenges throughout the brain. We recently used this approach to identify central structures activated during DON intoxication [21,22]. We revealed the activation of key autonomic areas, hypothalamic nuclei and parts of the amygdala. This c-Fos analysis was performed three hours after peripheral DON administration. At this time point, activated structures could be considered instrumental in DON-induced responses as anorexia and associated symptoms were ongoing. c-Fos immunoreactivity was observed in key autonomic regulatory nuclear groups, including the nucleus tractus solitarius (NTS), area postrema (AP), ventrolateral medulla (VLM), lateral parabrachial nucleus (LPB), locus coeruleus (LC). At the hypothalamic level, a significant increase in c-Fos immunoreactivity was mostly observed in the paraventricular hypothalamic nucleus (PVN), arcuate nucleus (ARC), median eminence (ME), and supraoptic nucleus (SON). The central nucleus of the amygdala (CeA) and the dorsolateral division of the bed nucleus of the stria terminalis

(BST) which are involved in the integration of emotional stimuli, also displayed a strong c-Fos signal in DON-challenged mice (Figure 2). Noticeably, the serotoninergic raphe formation did not exhibit any DON-induced c-Fos expression whatever the rostro-caudal level considered [22]. Interestingly, this pattern of c-Fos distribution closely resembles c-Fos immunoreactivity described in previous studies performed to identify neurocircuitry involved in the coordinated autonomic, endocrine, and behavioral response to immune challenge [63–66]. The strong c-Fos induction in structures strongly involved in food intake regulation *i.e.*, the NTS, Arc, and PVN, can be linked to the DON-induced reduction in food intake. Moreover, the c-Fos induction within the brainstem and particularly the AP was evocative of nausea. Using systemic DON administration in rats, Ossenkopp and colleagues [67] have indeed reported the induction of conditioned taste aversion, which was mediated by the AP. The strong increase in c-Fos immunoreactivity in specific regions of the hypothalamus including the preoptic area/anterior hypothalamus [68], the PVN and SON, together with the activation of LC and NTS [69] could also explain the decreased body temperature observed in response to DON [22]. These novel results, while interesting and informative, remain incomplete. Actually, only one DON dose *i.e.*, 12.5 mg/kg was tested and the c-Fos activation pattern was assessed at a single time-point *i.e.*, 3 h after treatment. Future studies should evaluate the impact of lower doses of toxin and a time-course of brain activation to more precisely understand the impact of DON on various brain regions.

7. Brain Neurochemistry and DON

The effect of DON on brain neurochemistry has been investigated in different species by several authors for many years [11,70–75]. It was hypothesized that alterations of brain neurochemistry might be one of the possible mechanisms underlying *Fusarium* mycotoxin-induced feed refusal, emesis and anorexia. In 1988, Fitzpatrick and colleagues [76] analyzed brain biogenic monoanines (serotonin, 5-HT; 5-hydroxyindole-3-acetic acid, 5HIAA; norepinephrine, NE ; dopamine, DA) in both rats and chickens at different time (2 to 48 h) after an oral dose of 2.5 mg/kg DON. In the rat, while no difference was observed when the whole brain was considered, 5-HT and 5-HIAA were elevated in all brain regions studied including pons and medulla oblongata, cerebellum, hypothalamus, hippocampus and cortex. These alterations of the serotoninergic system were not observed in poultry, where NE and DA decreased in the hypothalamus, hippocampus and pons. Following acute DON administration (0.25 mg/kg intravenous) in swine, modifications of brain amine levels were also detected during the 24 h post-intoxication [73]. More precisely, DON administration was reported to increase NE and decrease DA concentrations in the hypothalamus, frontal cortex and cerebellum while 5-HT content, which initially increased in the hypothalamus (1 h post-treatment), diminished in the hypothalamus and the cortex at 8 h. Likewise, intravenous (10 µg/kg) and *per os* (30 µg/kg) DON administrations were shown to increase 5-HIAA in the cerebral spinal fluid, suggesting an increased CNS serotoninergic activity [72]. More recently, the effects of feeding (21 days) with a blend of grains naturally contaminated with *Fusarium* mycotoxins (containing 5 ppm DON, 0.5 ppm 15-acetylDON and 0.4 ppm zearalenone) on brain regional neurochemistry of starter pigs and chickens were studied [74,75]. In pigs, contaminated food intake also results in modifications of brain bioamine contents. Mainly, this study reports an increased concentration of 5-HT within the cortex and a decreased hypothalamic tryptophan concentration. Moreover, hypothalamic NE and pons DA

concentrations decreased after inclusion of contaminated grains in the diet. In broiler chickens, feeding with contaminated grains induced an increase of 5-HT, 5-HIAA concentrations within the pons and cortex, and an increase in NE and DA concentrations in the pons [74,75]. Finally, one study was recently performed with turkeys fed with grains contaminated with *Fusarium* mycotoxins (equivalent to 2.2 and 3.3 mg DON per kg of feed) during 6 weeks [70]. In this model, 5-HIAA concentration and 5-HIAA: 5-HT ratio decreased in the pons, but no significant effects on 5-HT, 5-HIAA, NE and DA concentrations were observed in the hypothalamus and cortex. Despite the use of different intoxication models (DON doses, routes of administration) and apparent interspecies dissimilarities, these alterations in bioamine turnover, and particularly in the serotoninergic system, were proposed to play a role in the triggering of feeding refusal, emesis and anorexia observed during DON intoxication [7]. 5-HT has well-known emesis and food intake reducing effects [71]. In accordance, 5-HT receptor antagonists, *i.e.*, 5-HT3 and to a lesser extend 5-HT2, significantly prevented DON-induced vomiting (intravenous: 80 µg DON per kg BW or oral: 300 µg DON per kg BW; [11]). To discriminate between peripheral and central serotoninergic systems in DON-induced vomiting and anorexia, Prelusky [77] studied the effect of intravenous or intragastric DON administration on plasma concentrations of 5-HT and related metabolites in pigs. Interestingly, at doses able to evoke emesis, the toxin did not induce any change in blood monoamine levels, suggesting that the increased activity of the central serotoninergic system may account for the altered feeding behavior and emesis observed during DON intoxication. It should be noted that using an *in vitro* membrane receptor binding assay, a functional interaction of the toxin with serotoninergic receptors was ruled out [78]. Nevertheless, whether these alterations of brain neurochemistry are responsible for the DON-induced anorexia remains unknown. In a recent study using c-Fos expression as a marker of neuronal activation and performed on mice acutely intoxicated with 12.5 mg/kg DON, we observed an activation of the A1/C1 and A2/C2 catecholaminergic groups [21], which was evocative of the modulation of NE concentrations observed after DON intoxication in different models [11,70,73–76]. The activation of these catecholaminergic groups by DON also demonstrates the activation of brainstem/hypothalamus connecting networks. On the other hand, we did not observe any activation of the serotoninergic raphe nucleus in this model. This result is quite surprising since the modulation of brain 5-HT concentrations in intoxicated animals has been reported (see above). The specie used *i.e.*, mice and the time point studied in this work *i.e.*, 3 h after intoxication, may explain the discrepancies between our results and previous data. To clarify this point, more complete analyses need to be done in mice but also in other species where monoamine dosages during DON-intoxication are available, e.g., pigs.

8. Feeding Circuits and DON

In order to shed light on the mechanisms involved in DON-induced anorexia and identify cell populations implicated, we have recently searched for the possible activation of central pathways strongly dedicated to the control of food intake after administration of the toxin. In this context, we have reported that a significant proportion of POMC- and nesfatin-1-expressing neurons located both in the hypothalamus and NTS were activated during DON intoxication [21]. Both POMC and nesfatin-1 neurons are strongly involved in the reduction of food intake under physiological condition. POMC-expressing neurons also called “first-order neurons” are well recognized as the major

component in the control of energy homeostasis both in rodents and humans [79]. POMC neurons which relay the anorexigenic action of leptin and insulin, release the α-melanocyte stimulating hormones (αMSH). This hormone acts on melanocortin 3 and melanocortin 4 receptors (MC3R and MC4R) of neurons mainly located in the PVN to reduce appetite. Both insulin and leptin increase the activity of anorexigenic POMC neurons in the ARC. Thus, the increase activity of POMC neuron induces a decrease in food intake and an increase in energy expenditure. Similarly, nesfatin-1, an 82 amino-acid peptide, was identified in 2006 by Oh-I and colleagues [80]. This peptide exerts potent anorexigenic action after either peripheral [81] or central administration [80]. In addition to its expression by peripheral tissues *i.e.*, the stomach, pancreas and adipose tissues [82], nesfatin-1 was also found to be expressed in neurons of various brain areas including hypothalamic (PVN, ARC) and brainstem nuclei (NTS, DMNX) [83]. Nesfatinergic neurons located in these nuclei are activated in response to refeeding or intraperitoneal injection of the anorexigenic hormone cholecystokinin indicating that central nesfatin-1 could participate in the meal termination mechanisms [84,85]. Moreover, nesfatin-1 has a food intake-reducing effect that is linked to the recruitment of the melanocortinergic pathway [80]. The activation of DON-induced POMC and nesfatin-1 expressing neurons in mice suggests that the release of these anorexigenic and related compounds could partake in the anorexigenic action of the toxin. Strengthening these results, we observed that DON modulated mRNA levels of the hypothalamic melanocortinergic system including POMC, CART and MC4R, whereas mRNA of orexigenic effectors *i.e.*, NPY and AgRP remained unaffected. Altogether, these studies provide strong additional support to the view that DON directly or indirectly interferes with neuronal networks devoted to central energy balance and that this action could partly explain the DON-induced hypophagia observed in response to the toxin.

9. DON May Target the Brain

Although DON effects on brain structures and neurochemistry were clearly established by a body of evidence, little is known about the routes used by the toxin to signal from the periphery (gastrointestinal tract, blood) to the brain. The vagal nerve which conveys information from the visceral tract to the brainstem is a good candidate to explain the impact of the toxin on the brain. Unfortunately, very few data are available regarding this hypothesis. In a recent study, we have shown that unilateral cervical vagotomy did not modify the c-Fos activation in the brainstem induced by *per os* 12.5 mg/kg DON [21,22]. This result seems to rule out the involvement of the vagus nerve, at least with the dose employed. Nevertheless, additional experiments with other DON doses are clearly needed to definitively settle this point. Alternatively, it is conceivable that the toxin enters the brain to directly modify neuronal function. In accordance, following i.v. toxin administration, DON is rapidly detected (less than 2.5 min) in the cerebral spinal fluid of swine and sheep demonstrating that this toxin reaches the brain [86]). DON concentrations within the brain reach a maximum after 5–10 min in sheep and 30–60 min in swine. In the same way, DON was detected in hen or mouse brains after acute or chronic (6 days) DON administration [49,87]. Using radiolabelled-DON or ELISA DON detection, these authors detected the toxin within the brain of these species following *per os* administration. Interestingly, after oral administration of 25 mg/kg DON in mice, the toxin concentrations detected in the brain were lower (~1 μg/g) than in other tissues *i.e.*, spleen (~7.3), liver (~19.5), heart (~6.8), and

kidney (~7.6), probably due to the low permeability of the BBB to the toxin. Of course the toxin could also infiltrate the brain through the circumventricular organs. In support of this assumption, DON was shown to induce c-Fos staining in circumventricular organs (AP and ME) and surrounding structures (NTS and ARC; [21,22]). Moreover, using systemic DON administration in rats, Ossenkopp and colleagues [67] reported that area postrema ablation prevents DON-induced conditioned taste aversion. Finally, to assess the direct action of the toxin on the brain, we recently performed the first i.c.v. (lateral ventricle) DON administration in the mouse (2–20 μg/g; [21]). The doses used in these i.c.v. experiments were compatible with DON brain levels found after *per os* administration (25 mg/kg; [38]), but totally inefficient to modify feeding behavior when peripherally administered. In these conditions, DON dose-dependently decreased meal frequency and size and augmented intermeal intervals in the same way as after *per os* administration (12.5 mg/kg DON). Like *per os* administration, DON central injections also result in a modification of body core temperature and locomotor activity. DON-induced c-Fos expression pattern following i.c.v. and *per os* DON administrations were also similar. Lastly, POMC and nesfatin-1 positive neurons of both the NTS and hypothalamus were found activated after i.c.v. DON injections to a same extent as after *per os* DON administration. Taken together, these results suggest that DON might directly target the brain to modify feeding behavior acting on anorexigenic neurocircuitry.

10. Perspectives

Despite significant progress in the identification of neuronal circuitry and neuropeptides recruited during DON intoxication, the precise physiological mechanisms and cellular pathways underlying the central action of the toxin remain mostly unidentified. The hypothesis of the instrumental role of cytokines in the triggering of DON-induced anorexia, while attractive, remains to be established. To date, genetic models displaying an altered inflammatory response, *i.e.*, IL6 KO [51], TNFR KO [50], COX-2 KO [60] and mPGES-1 KO [22], failed to confirm the involvement of inflammatory signals in the reduction of feeding behavior induced by the toxin. Surprisingly, the outcome of lessening IL-1β signaling on DON-induced anorexia has not been tested to date. Yet among the inflammatory cytokines, IL-1β seems to be the most potent in inducing sickness behavior, since its peripheral or central injection reproduces the set of non-specific symptoms of inflammation including decreased motor activity, social withdrawal, anorexia and fever (for review [88]). Accordingly, strategies aiming to limit its action have been shown to attenuate most of the symptoms observed during infection and inflammation [89–91]. So in the future, it should be of interest to test the impact of interleukin-1 receptor antagonist (IL-1Ra) on DON-induced anorexia, or to evaluate the hypophagic response in interleukin-1β-deficient mice. Alternatively, important information could result from the use of mice exhibiting an invalidation of the intracellular protein MyD88, the central adapter protein mediating activation signals emanating from the IL-1 receptor [92]. As mentioned above, we recently demonstrated the activation of POMC-expressing neurons in both the hypothalamus and brainstem [21] and the modulation of POMC expression in the hypothalamus during acute DON intoxication. To test the hypothesis of melanocortinergic pathway involvement in DON-induced hypophagia, the central injection of synthetic MC3R/MC4R antagonists ought to be tested in different models of DON-intoxication. This point is crucial since nesfatin-1 has a food intake-reducing effect

that is also linked to the recruitment of the melanocortinergic pathway [80]. Another intriguing question regarding the central action of the toxin is the pattern of activated structures observed after *per os* DON administration. Surprisingly, DON does not induce a neuronal activation throughout the brain, but on the contrary the number of central structures found activated in response to gastric administration of the toxin are quite limited [21,22]. What is the mechanism underlying this specificity? We believe that the proximity to circumventricular organs *i.e.*, AP and median eminence, which facilitates the toxin penetration into the brain could explain the strong activation in adjacent structures and connected nuclei such as the NTS, Arc or PVN. This hypothesis should be confirmed in the future. Nevertheless, this does not make clear why only anorexigenic pathways are modulated in these structures [21].

The cellular mechanisms through which DON could modify neuronal activity and brain neurochemistry are also still unknown. DON binds to ribosomes and in turn inhibits protein synthesis (for review [7]). In mice, *per os* DON administration (5 to 25 mg/kg) inhibits protein synthesis in the spleen, peyer's patches, kidney, liver, intestine and plasma from 3 h to 9 h post-treatment [37]. It was recently proposed that the ribotoxic effect of the toxin induces a modulation of mitogen activated protein kinases (MAPKs) activity. In turn, MAPKs activation may result in the activation of transcription factors that induce for instance cytokine or COX-2 expression [93–95]. It was proposed that other signaling factors such as double stranded RNA-activated protein kinase or hematopoietic cell kinase relay DON toxicity from ribosome inhibition to MAPKs activation [96–98]. Whether such mechanisms intervene in the brain and may account for the neuronal activation observed in response to orally administered DON remain to be evaluated. Alternatively, other mechanisms were proposed to explain trichothecene toxicity especially T-2 toxin [99,100]. On L-6 myoblasts, T-2 toxin has multiple effects on cell membrane functions independently of protein synthesis inhibition. T-2 Toxin modifies the activity of amino acid and glucose transporters as well as calcium and potassium channel activities [99]. This toxin was also shown to increase cellular calcium concentration in the human promyelotic line HL-60 [100]. The assumption that DON induces such modulations of membrane functions or intracellular pathways remains to be evaluated. Since DON was shown to induce rapid behavioral responses (1 h), *i.e.*, anorexia, anapyrexia, reduced locomotor activity, the possibility of an action independent of protein synthesis inhibition can be reasonably envisaged. In neurons, such alterations can result in modifications of neuronal electrical activity. Electrophysiological experiments performed on brain slices originating from DON-administered animals or on hypothalamic or brainstem slices acutely treated with the toxin could bring essential information regarding the central action of the toxin.

11. Conclusions

From pioneer studies and more recent works, it clearly appears that DON acts at the brain level to modify neurochemistry and neuronal activity. In turn, behaviors regulated by the central nervous system are modified. Notwithstanding these interesting progresses, the study of DON impact on brain functioning is still in its infancy. Among the different aspects that must be addressed in this field in the close future, the study of the possible consequences of chronic DON consumption on long-term brain deregulation will surely constitute a major issue and a great challenge.

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Conflict of Interest

"The authors declare no conflict of interest".

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Annexe 5

Article : The food-contaminant Deoxynivalenol modifies eating by targeting anorexigenic neurocircuitry.

Girardet C*, Bonnet MS*, Jdir R, Sadoud M, Thirion S, Tardivel C, Roux J, Lebrun B, Mounien L, Trouslard J, Jean A, Dallaporta M, Troadec JD.

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The Food-Contaminant Deoxynivalenol Modifies Eating by Targeting Anorexigenic Neurocircuitry

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Abstract

Physiological regulations of energy balance and body weight imply highly adaptive mechanisms which match caloric intake to caloric expenditure. In the central nervous system, the regulation of appetite relies on complex neurocircuitry which disturbance may alter energy balance and result in anorexia or obesity. Deoxynivalenol (DON), a trichothecene, is one of the most abundant mycotoxins found on contaminated cereals and its stability during processing and cooking explains its widespread presence in human food. DON has been implicated in acute and chronic illnesses in both humans and farm animals including weight loss. Here, we provide the first demonstration that DON reduced feeding behavior and modified satiation and satiety by interfering with central neuronal networks dedicated to food intake regulation. Moreover, our results strongly suggest that during intoxication, DON reaches the brain where it modifies anorexigenic balance. In view of the widespread human exposure to DON, the present results may lead to reconsider the potential consequences of chronic DON consumption on human eating disorders.

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Introduction

The capacity to adjust food intake in response to changing energy requirements is essential for survival. Recent progress has provided an insight into the central regulation of energy balance that links changes of body fat stores to adaptive adjustments of feeding behavior [1]. In the central nervous system (CNS), the regulation of appetite relies on complex neurocircuitry. Discrete neuronal pathways within specific brain areas, mainly the hypothalamus and the brainstem, are clearly involved in this control of feeding behavior. Peripheral information linked to fat deposit or nutriment availability are implicated as endogenous signaling molecules in the control of energy expenditure, and initiation and termination of a meal. The primary targets of these peripheral molecules are first-order anorexigenic and orexigenic neurons that express pro-opiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY)/Agouti-related peptide (AgRP) respectively. The physiological importance of this homeostatic control system is highlighted by the severe eating disorders (obesity, anorexia, cachexia) that result from the dysfunction or any of several of its key components.

Deoxynivalenol (DON), also commonly called vomitoxin, is a trichothecene mycotoxin mainly produced by *Fusarium* fungi. DON is one of the most abundant trichothecenes found on cereals such as wheat, barley, oats, rye, and maize, and less often in rice grown in Europe, America and Asia [2]. The extent of cereal contamination is strongly associated with rainfall and moisture at the time of flowering and with grain storage conditions. DON has been implicated in mycotoxicoses in both humans and farm animals. High doses toxicity of DON is characterized by a set of symptoms including diarrhea, vomiting, leukocytosis, hemorrhage, circulatory shock and death whereas low doses toxicity is characterized by anorexia, reduced weight gain, diminished nutritional efficiency, neuroendocrine changes and immunologic effects [2]. In farm animals including poultry and ruminants, intoxication following consumption of cereals and cereal-derived products contaminated with DON results in feed refusal and reduced weight gain. These symptoms lead to growth retardation and can have great economic consequences. In humans, epidemiological studies have reported acute illnesses including vomiting, abdominal pain, diarrhea, headache, dizziness in populations who have consumed *Fusarium*-contaminated grains and in addition, DON is suspected to increased occurrence of more severe diseases such as esophageal cancer [3]. The levels and patterns of human food commodities contamination justify that DON consumption constitutes a major issue for global public health. A survey by Lombeart and colleagues reported the detection of DON in 63% of 363 cereal-based infant foods [4].

Similarly, a European food study carried out in 12 countries revealed that, out of a total of 11.022 samples analyzed, more than a half contained DON [5]. DON stability during processing and cooking explains its widespread presence in human food. Trichothecenes, including DON, are actually stable at 120°C, moderately stable at 180°C, decompose within 30–40 min at 210°C [6], and autoclave processing reduces by only 12% maize contamination [7]. In view of this widespread human exposure to DON, studies improving our knowledge of DON toxicity remain essential and should be conducted. While the peripheral action of DON was extensively studied [1], data illustrating its effects on CNS are significantly less abundant. Despite the described modulation of feeding behavior induced by DON consumption, the data aiming to characterize this effect are limited and the mechanisms by which this toxin exerts its action remain largely unknown. Accordingly, additional information are strongly needed to more accurately understand how such a commonly encountered toxin can so severely modify animal and human food intake.

Using a multidisciplinary approach, we accessed to mechanisms underlying anorexia induced by acute DON intoxication. In conclusion, we showed that, in addition to a peripheral action, the toxin having reached the brain after *per os* administration can act centrally and results in the impairment of anorexigenic/orexigenic balance. These data may lead to reconsider the consequence of the chronic consumption of low DON doses on the development of pathophysiological alteration of food intake behavior.

Results

1- Acute *per os* administration of DON alters night-time food intake and meal microstructure

A single oral administration of DON resulted in a dose-dependent decrease in daily food intake with a notably long-lasting effect for the highest doses (Figure 1A). Note that 6.25, 12.5 and 25 mg/kg of DON diminished respectively by 24, 39

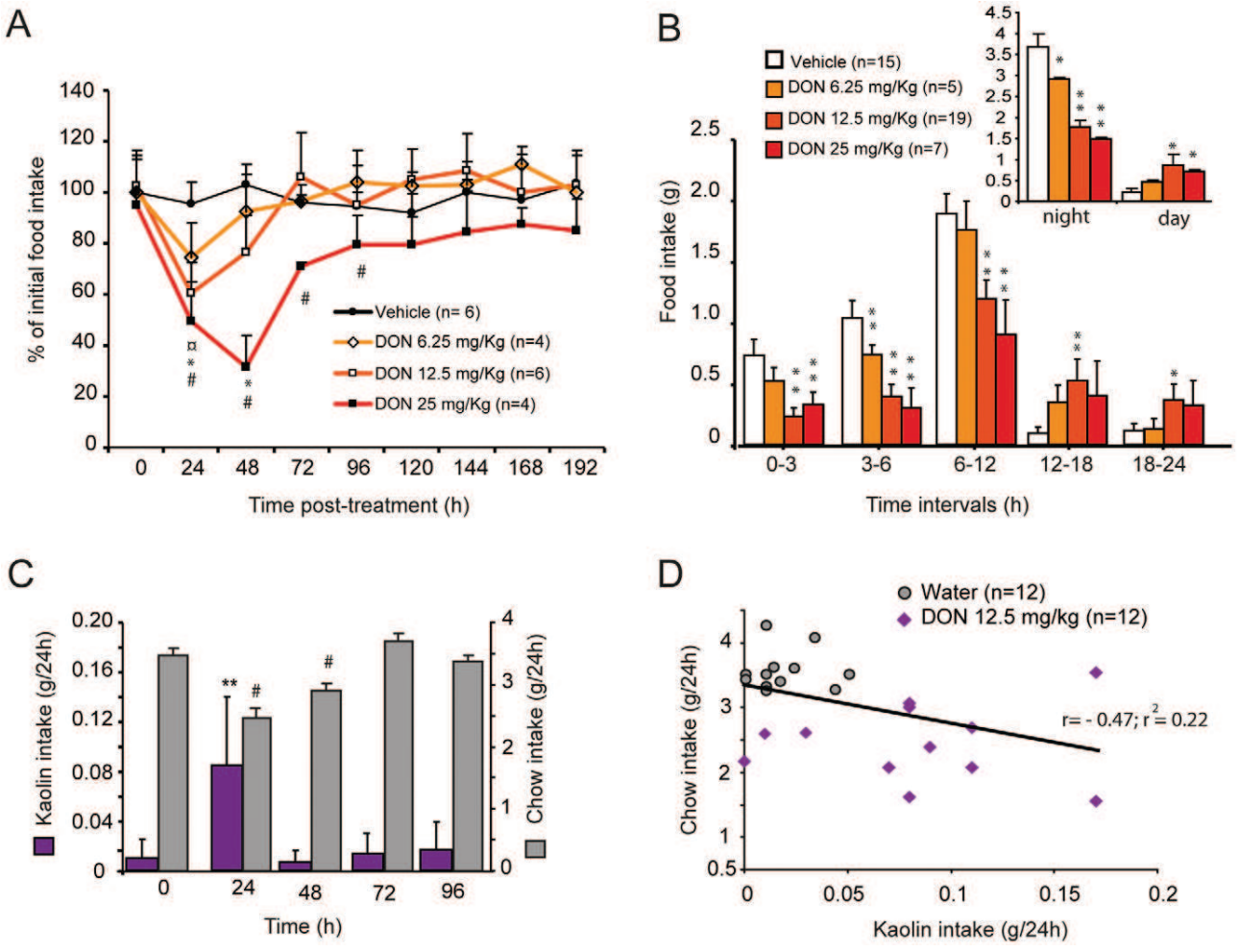


Figure 1. Acute *per os* DON administration modifies night-time food intake. A: Daily food intake (% of initial food intake) measured from 24 to 192 h after oral gavage of either water (vehicle) or DON (6.25, 12.5 and 25 mg/kg) in adult mice. **B:** Food intake (g), measured over the first 24 h period, of mice having received an oral gavage of either water or DON (6.25, 12.5 and 25 mg/kg). **C:** Kaolin intake and regular chow intake measured 0, 24, 48, 72 and 96 h after DON (12.5 mg/kg) administration. **D:** Correlation of kaolin intake and chow intake by mice that received an oral gavage of either water or DON (12.5 mg/kg). In A: ¤; *; # represent $P<0.05$, significantly different from vehicle-treated mice, respectively for 6.25, 12.5 and 25 mg/kg DON-treated mice. In B: *$P<0.05$ and **$P<0.01$, significantly different from vehicle-treated mice. In C: **$P<0.01$, significantly different from control Kaolin intake. # $P<0.05$, significantly different from control chow intake.
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and 47% food intake measured during the first 24 h following administration. Food consumption measured 3, 6, 12 and 18 h after treatment revealed that DON profoundly affected the night-time food intake (Figure 1B). To decipher feeding behavior analysis during DON intoxication, we quantified the consumption of a non-nutritive substance i.e. kaolin. This behavior, known as pica, serves as a model for the study of nausea/emesis in rodents [8]. While mice treated with vehicle consumed 18.3+/−4.8 mg/24 h of kaolin (time 0 on Figure 1C), 12.5 mg/kg of DON caused a significant increase in kaolin intake (83.3+/−16.2 mg/24 h; $P<0.01$). This behavior was not observable any more 48 h post-injection, while anorexia was still ongoing. In the DON treated-mice, daily standard chow and kaolin intakes were not significantly correlated (*r*-value:0.1). Plotting the standard chow and kaolin intakes for the vehicle- and DON-treated animals on the same graph revealed that these two parameters were poorly correlated (*r*-value:−0.47, r^2:0.22; Figure 1D). Using continuous recording of food consumption (Figure 2A), we next analyzed the effect of DON (12.5 mg/kg) administration on meal parameters computed for each animal over all meals during the trial period. This monitoring confirmed that DON essentially modified food intake during the dark phase (Figure 2A). Given the modification of nightly food intake observed in response to DON, meal microstructure analysis was then computed for the dark phase (0–12 h). Compared with vehicle, DON reduced meal frequency by 27.9% (15.0+/−1.3 *versus* 20.8+/−1.7 meals/12 h, $P<0.05$) and meal size by 44.2%(99.4+/−8.4 mg *versus* 178.1+/−26.8 mg, $P<0.01$) and increased intermeal intervals by 68%(47.5+/−8.9 min *versus* 28.2+/−3.5 min, $P<0.01$). During this trial period, the satiety ratio was also increased by 40% in response to the toxin ($P<0.01$; Figure 2B).

2- Brain pattern of central pathways activated in response to DON *per os* administration

Central structures activated in response to *per os* administration of DON were next identified using the immune detection of the c-Fos protein. A very low basal level of c-Fos positive nuclei was observed in the brainstem, pons and forebrain of water-treated mice (Table 1). DON-treated mice exhibited a strong rise in the number of c-Fos positive nuclei within the nucleus tractus solitarius (NTS) and a moderate increase within the area postrema (AP, Figure 3 and Table 1) and ventrolateral medulla

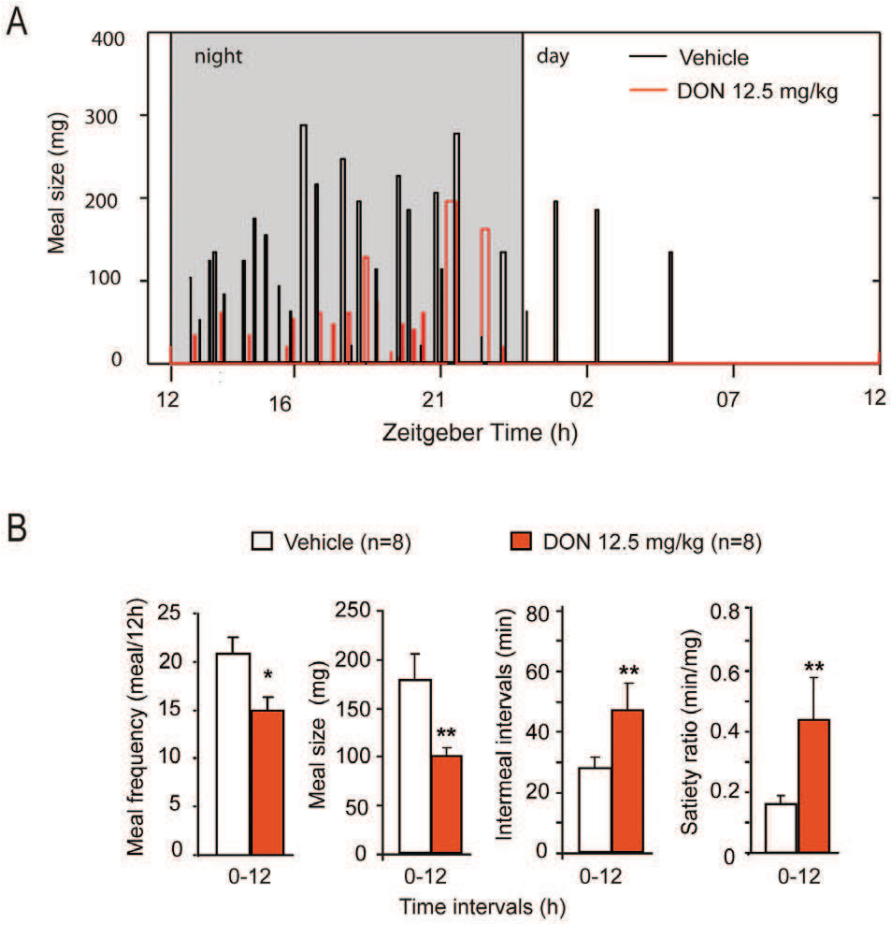


Figure 2. Effect of acute *per os* DON administration on meal microstructure. A: Graph showing meal size over 24 h after an oral gavage of either water (vehicle, black histograms) or DON (12.5 mg/kg, red histograms) in an adult mouse. Note that the width of each bar represents the meal duration. Time is indicated in zeitgeber time, 12 being the transition between lights on and lights off and 0 or 24 the transition between lights off and lights on. The dark period is represented by the shaded box. **B:** Meal microstructure analysis performed during the dark period for mice that received either water (white bars) or DON (12.5 mg/kg, orange bars). *$P<0.05$ and **$P<0.01$, significantly different from vehicle-treated mice.
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Table 1. c-Fos signal in brain structures of vehicle or DON-challenged mice.

	per os vehicle (water)	*per os* DON (12,5 mg/kg)	icv vehicle (NaCl)	icv DON (12,5 mg/kg)
Diencephalon				
Subfornical organ	0/+	**++**	+	**++**
Paraventricular thalamic nuclei	+++	+++	++	++
Central nucleus of the amygdala	0/+	**+++**	0/+	**+**
Laterodorsal part of the bed of the stria terminalis	0/+	**+++**	0/+	**+**
Hypothalamus				
Preoptic area	0/+	**+/++**	+	**+++**
Anterior area	+	**++**	+	**++**
Paraventricular nucleus	0/+	**+++**	+	**+++**
Supraoptic nucleus	+	**+++**	0/+	**++**
Median eminence	0/+	**++**	+	+
Dorsomedian nucleus	0/+	0/+	0/+	**+**
Ventromedian nucleus	0/+	0/+	0/+	0/+
Lateral area	+	**++**	+	**++**
Posterior area	+	**++**	+	**++**
Parasubthalamic nucleus	0/+	**+++**	0/+	**++**
Arcuate nucleus	0/+	**++**	0/+	**+++**
Brainstem				
Dorsal raphe nucleus	0/+	0/+	0/+	0/+
Laterodorsal tegmental nucleus	+	**++**	+	**++**
Parabrachial nucleus	0/+	**+++**	0/+	**+++**
Raphe magnus nucleus	0/+	0/+	0/+	0/+
Locus coeruleus	+	**+++**	0/+	**+++**
Motor trigeminal nucleus	0/+	**++**	0/+	0/+
Nucleus of trapezoid body	+++	+++	0/+	0/+
Area postrema	0/+	**+/++**	0/+	**+**
Caudal NTS	0/+	**++**	0/+	**++**
Postremal NTS	0/+	**+++**	0/+	**+++**
Rostral NTS	0/+	**+++**	0/+	**+++**
Dorsal motor nucleus of the vagus	0/+	**+**	0/+	**++**
Raphe obscursus nucleus	0/+	0/+	0/+	0/+
Raphe pallidus nucleus	0/+	0/+	0/+	0/+
Caudal Ventolateral medulla	+	**++**	0/+	**++**
Rostral ventolateral medulla	+	**++**	0/+	**++**
Spinal nucleus trigeminal	0/+	0/+	0/+	0/+
Other				
Ependymal cells	0/+	0/+	0/+	**++**
Choroïd plexus	0/+	**++**	+	**++**

0 = no stained cell; + = 1−30 cells per section; ++ = 31−60 cells; +++ = 61 and more cells. Bold indicates brain structures where c-Fos signal is significantly different between vehicle and DON-treated mice.

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(VLM). Noticeably, the serotoninergic raphe formation did not exhibit any DON-induced c-Fos expression whatever the rostro-caudal level considered (Figure 3 and Table 1). Animals challenged with DON also displayed a strong rise in c-Fos immunoreactivity in the lateral part of the parabrachial nucleus (LPB) and in the locus coeruleus (LC), and a moderate-to-strong c-Fos staining within forebrain structures such as the paraventricular hypothalamus nucleus (PVN), arcuate nucleus (ARC), median eminence (ME), bed nucleus of the stria terminalis (BST) and central nucleus of the amygdala (CeA) (Figure 3; Table 1).

3- Phenotypic characterization of DON-activated neurons

The use of POMC-GFP transgenic mice revealed that oral DON administration (12.5 mg/kg) significantly increased the number of POMC neurons positive for c-Fos, both in the NTS and in the ARC (Figure 4). Interestingly, more than 40% of POMC neurons were found immunoreactive for c-Fos within the NTS (Figure 4B). Similarly, the number of nesfatin-1 neurons labeled for c-Fos in the NTS and hypothalamic nuclei (ARC, PVN, SON) was greatly increased by the treatment (Figure 5). For these structures, the percentage of double labeled neurons was

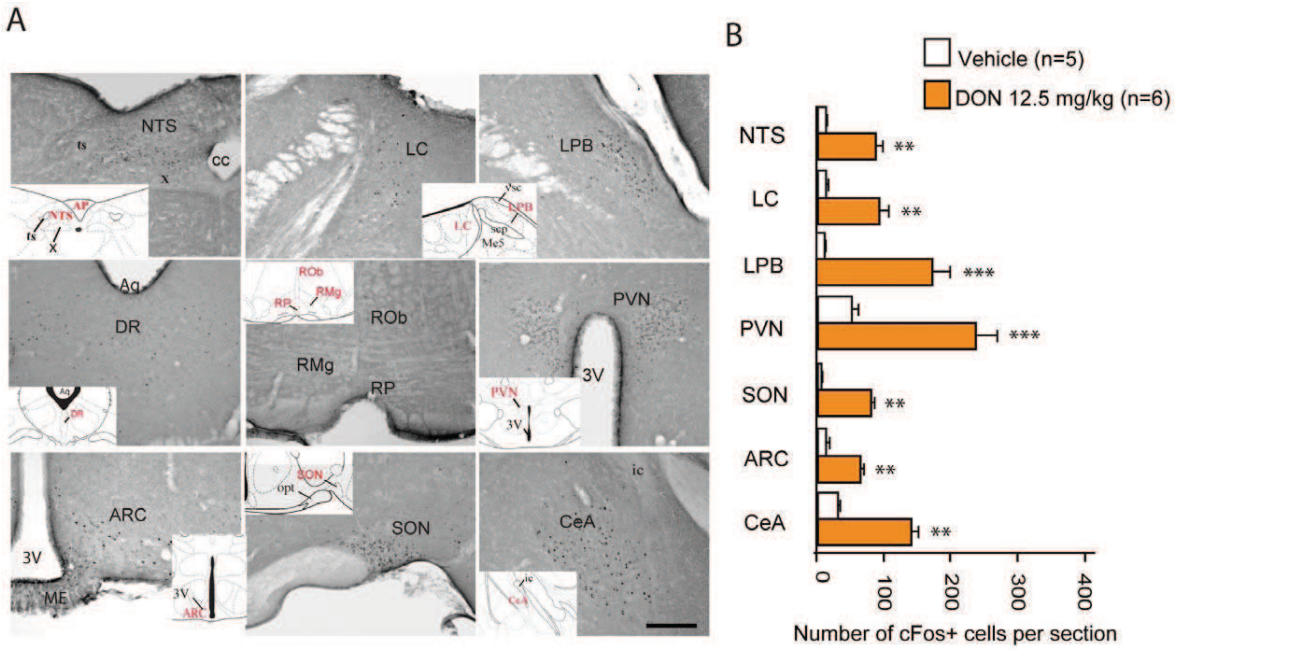


Figure 3. Effects of *per os* DON administration on c-Fos immunoreactivity. A: Representative coronal sections illustrating the c-Fos labeling observed within brainstem, pons and forebrain regions of mice treated with DON (12.5 mg/kg) and sacrificed 3 h post-treatment. Inserts show the level and the location where the photomicrographs originate from **B:** Quantification of the number of c-Fos immunoreactive nuclei within brainstem, pons and forebrain nuclei observed in mice treated either with vehicle (water, white bars) or DON (12.5 mg/kg, orange bars). *$P<0.05$, **$P<0.01$, ***$P<0.001$ significantly different from vehicle-treated mice. 3V, third ventricle, AP, area postrema; ARC, arcuate nucleus; Aq, Aqueduct; cc, central canal; CeA, central amygdala; DR, dorsal raphe; LPB, lateral parabrachial; LC, locus coeruleus; ic, insular cortex; Me5, mesencephalic trigeminal nucleus; NTS, nucleus tractus solitarius; opt, optic tract; PVN, paraventricular nucleus; RMg, raphe magnus; ROb, raphe obscursis; RP, raphe pallidus; scp, superior cerebellar peduncle; SON, supraoptic nucleus; X, dorsal motor nucleus of the vagus; ts, tractus solitarius; vsc, ventral spinocerebellar tract. Scale bar: 100 µm.
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comprised between 24 and 58% of the entire nesfatin-1 neuron population (Figure 5B). Finally, double immunostaining of c-Fos/Tyrosine Hydroxylase (TH) cells in the brainstem sections revealed that about 15% of catecholaminergic (TH-containing) neurons in the NTS were c-Fos immunoreactive after DON treatment (Figure 6). Note that control animals (water-treated) were virtually devoid of double labeled cells. Using real-time RT-PCR analysis on hypothalamic samples, we next quantified POMC, CART, melanocortin-4 receptor (MC4R), NPY and AgRP mRNA expression. We observed that POMC, CART and MC4R mRNA expression strongly increased following the treatment while NPY and AgRP mRNA remained unaffected (Figure 7).

4- Unilateral cervical vagotomy does not alter DON-induced c-Fos expression within the brainstem

The contribution of the vagus nerve in the conveyance of DON signaling from the gastrointestinal tract toward the brainstem was evaluated by studying the impact of unilateral cervical vagotomy (UCV) on DON-induced c-Fos expression within the brainstem. Seven days before DON administration and sacrifice, animals were given an intraperitoneal injection of Fluorogold for verification of the lesion. The absence of the retrograde neuronal tracer fluorogold in the cell columns of the dorsal motor nucleus (DMNX) corresponding to the sectioned branch of the vagus attested of the correct lesion of the nerve (Figure 7A). c-Fos immunoreactivity within the NTS, consecutive to DON treatment, was not significantly altered by UCV. Furthermore, NTS ispsi- and contra-lateral sides did not differ significantly, whatever the rostro-caudal NTS level analyzed ($P>0.05$; Figure 8).

5- Intracerebroventricular (icv) DON injection on induced anorexia and neuronal activation

To address the possible direct central action of DON, we next performed icv injections (lateral ventricles) of the toxin on chronically cannulated mice. At doses ineffective at the periphery, central DON injections dose-dependently (2–20 µg/mouse) reduced food intake measured during the dark phase (Figure 9A). It should be noted that these DON doses are about 20–100 fold lower than previously used *per os* administered DON doses (equivalent to 125–500 µg/mouse). Interestingly, at the dose of 20 µg/mouse, DON reduced night-time food intake as soon as 1 h after injection and the effect was long-lasting during the following 12 h post-injection. Compared with vehicle, this dose decreased meal frequency by 40.1% (15.7+/−4.3 *versus* 9.4+/−1.8 meals/12 h, $P<0.05$) and meal size by 53.3% (233.0+/−19.7 mg *versus* 108.7+/−31.4 mg, $P<0.01$; Figure 9B). Finally, c-Fos analysis performed on animals centrally injected with the toxin revealed a pattern of labeled structures quite similar to that observed in animals having received a *per os* administration of the toxin (Figure 9C and Table 1). c-Fos labeling was indeed observed within the NTS, AP, LPB, LC and hypothalamic nuclei including ME, ARC and PVN. Central DON injection resulted also in a significant increase in c-Fos positive POMC and nesfatin-1 expressing neurons located both in the brainstem and hypothalamus (Figure 9C).

Discussion

The mechanisms underlying the modulation of food intake behavior by DON and its central targets are until now unclear. To

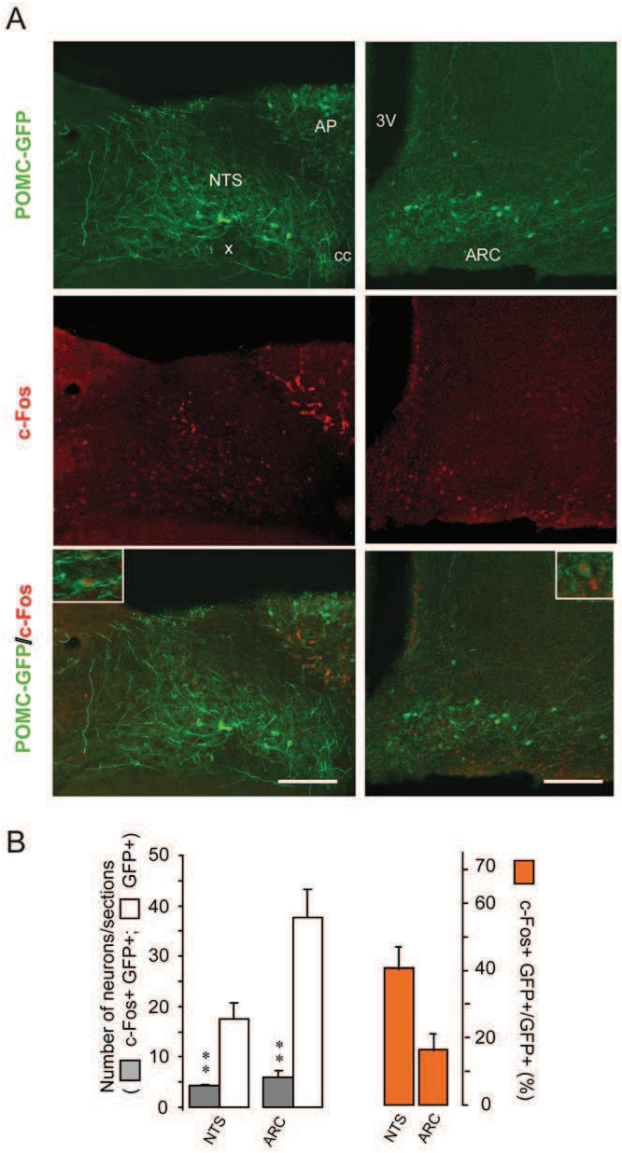


Figure 4. Impact of *per os* DON administration on POMC neurons activation. A: Representative photomicrographs of POMC-GFP (green) and c-Fos (red) double labeling performed on hypothalamic and brainstem coronal sections of mice treated with DON. Inserts show high magnification images of double-labeled neurons. **B:** Quantification of the number of POMC-GFP positive neurons (white bars) and c-Fos double-labeled cells (gray bars) in the NTS and ARC after DON treatment. The orange bars represent the percentage of double-labeled neurons in each area. All immunostaining were obtained from animals sacrificed 3 h after a 12.5 mg/kg DON administration. 3V, third ventricle; AP, area postrema; ARC, arcuate nucleus; cc, central canal; NTS, nucleus of the solitary tract; X, dorsal motor nucleus of the vagus. Scale bars: 150 µm. $^{**}P<0.01$, significantly different from vehicle-treated mice. ns, non significantly different from vehicle-treated mice.
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investigate these mechanisms, we performed acute DON administrations. We characterized DON intoxication by showing that the toxin affects feeding behavior by modulating both satiety and satiation. Using c-Fos expression mapping, we first identified that part of neuronal pathways activated by the toxin belong to prototypic anorexigenic pathways. Finally, by performing central injection, we demonstrated that DON could directly act on the brain to modify eating. This new results may lead to reconsider the impact of chronic consumption of this toxin on human health.

Acute DON intoxication results in the alteration of satiety and satiation

While pig was reported to be the most sensitive species to DON, numerous *in vivo* and *in vitro* DON toxicity studies were conducted on rodents and especially on mice which can be considered as a good model for the study of DON toxicity [9–10] and DON-induced anorexia [11]. The doses of DON used here are classically employed to study acute intoxication in mice [12–13]. *Per os* intoxication induced a dose-dependent reduction in daily food intake by decreasing night-time food consumption. By performing meal pattern analysis, which allows the continuous study of *ad libitum* eating and provides a detailed description of the elements of eating behavior, we revealed that *per os* DON decreased both meal frequency (satiety) and size (satiation). The observed effect on meal frequency was evocative of nausea-induced anorexia [14–15]. On the other hand, the consequence of DON treatment on meal size suggested the action of DON on meal termination mechanisms. A reduction in meal size but not in meal frequency is classically observed with physiological regulators that potently inhibit food intake such as leptin [16], cholecystokinin [17], peptide YY 3–36 [18], insulin [19] or melanocortin agonists [20]. Moreover, the clay (kaolin) intake, used here as an indicator of nausea/emesis [8], was poorly correlated with standard chow intake. This suggesting that malaise and nausea did not totally explain anorexia. Hence, altogether our results revealed a complex action of DON on food intake where DON-induced anorexia depends not only on modification of satiety but also on satiation modulation.

DON activates central anorexigenic pathways

To date, central structures activated in response to DON were not clearly identified. Using c-Fos staining which remains a useful approach to identify activated neuronal groups, we revealed the activation of key autonomic areas and hypothalamic nuclei. A significant elevation of c-Fos immunoreactivity was also observed in the central nucleus of the CeA and the dorsolateral division of the BST which are involved in the integration of emotional stimuli. This pattern is consistent with a coordinated autonomic, endocrine, and behavioral (anorexia) response to DON.

Regardless of sometimes conflicting results and evident interspecies differences, it was initially proposed that the toxin modifies serotoninergic and catecholaminergic activities in the CNS, thereby altering neurotransmission in brain structures such as the hypothalamus, frontal cortex, and cerebellum. Accordingly, this observed elevated brain serotonin turnover was supposed to be responsible for the vomiting effects of DON [1]. The capacity of serotonin receptor antagonists to prevent DON-induced emesis in swine supported this assumption [21]. Surprisingly, it should be noted that the serotoninergic raphe nuclei did not display any increased c-Fos labeling in response to DON, suggesting that on the contrary to previous assumption (see [2] for review), central serotonin release does not significantly contribute to DON-induced hypophagia.

Moreover, we report here that a significant proportion of the DON-activated neurons belong to central pathways strongly dedicated to the control of food intake. These cell groups include POMC- and nesfatin-1-expressing neurons located both in key hypothalamic nuclei and NTS. The demonstration of POMC-expressing neurons activation is crucial since these neurons are well recognized to intervene in normal energy homeostasis both in rodents and humans [22]. POMC neurons which co-express

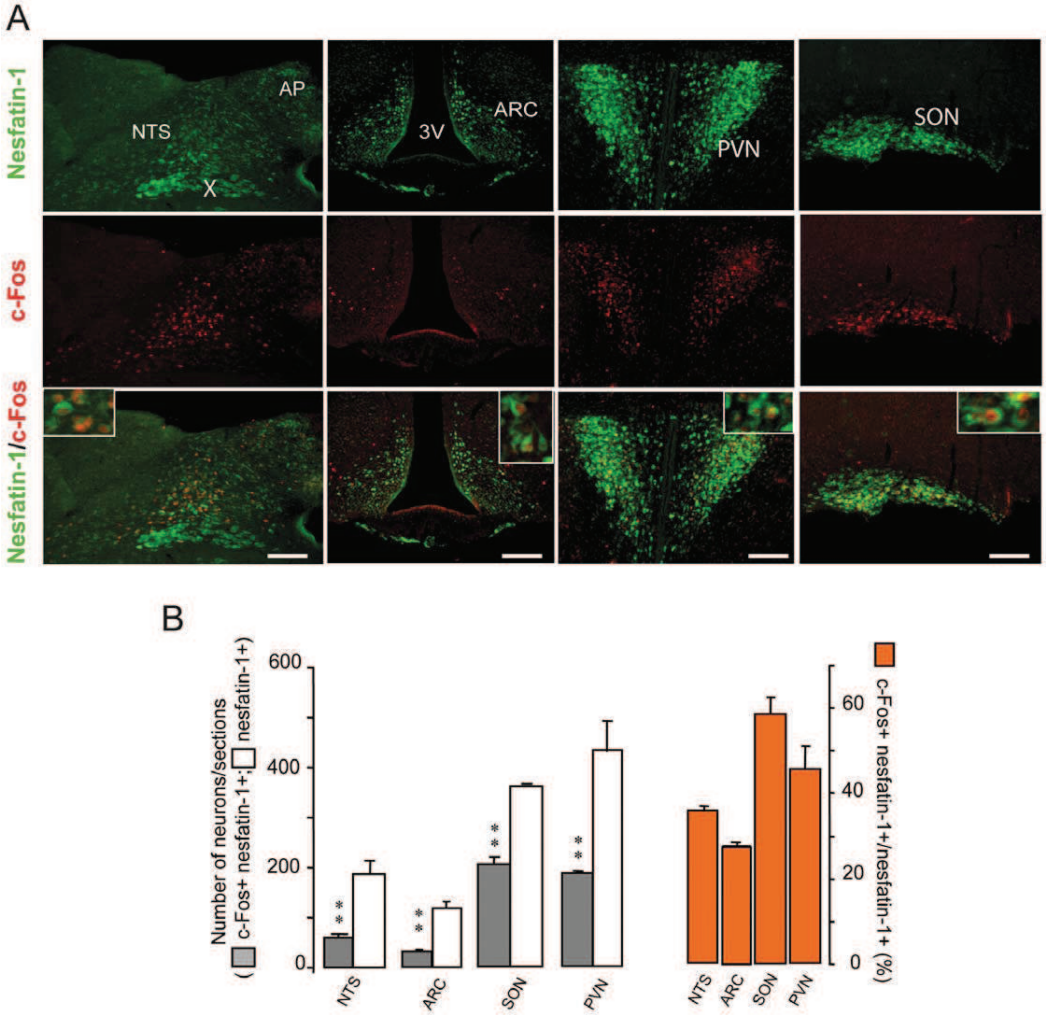


Figure 5. Nesfatin-1 and c-Fos double immunochemistry performed on DON-treated mice. A: Co-detection of nesfatin-1 expressing neurons (green) and c-Fos (red) positive cells on brainstem and hypothalamic coronal sections of mice treated with DON. Note the presence of double-labeled neurons (inset). **B:** Quantification of the number of nesfatin-1-positive neurons (white bars) and c-Fos double-labeled cells (gray bars) in the NTS, ARC, PNV and SON after DON treatment. The orange bars represent the percentage of double labeled neurons per phenotype in each area. All immunostaining were obtained from animals sacrificed 3 h after a 12.5 mg/kg DON administration. Inserts show high magnification images of double labeled neurons. 3V, third ventricle; AP, area postrema; ARC, arcuate nucleus; cc, central canal; PVN, paraventricular nucleus; NTS, nucleus of the solitary tract; SON, supraoptic nucleus; TH, tyrosine hydroxylase; X, dorsal motor nucleus of the vagus. Scale bars: 200 μm. $^{**}P<0.01$, significantly different from vehicle-treated mice. ns, non significantly different from vehicle-treated mice.
doi:10.1371/journal.pone.0026134.g005

CART neurons relay the anorexigenic action of emblematic adiposity signals leptin or insulin. In these neurons, POMC is cleaved to α-melanocyte stimulating hormones (α-MSH) which acts on melanocortin 3 and melanocortin 4 receptors (MC3R and MC4R) of neurons mainly located in the PNV to reduce appetite. In accordance, endogenous and synthetic agonists for MC3R/MC4R have been shown to cause hypophagia and weight loss in experimental animals [23]. The catabolic action of both leptin and insulin relies upon α-MSH signaling since the central administration of MC3R/MC4R antagonists was shown to block their actions in the brain. Both insulin and leptin increase the activity of anorexigenic POMC/CART neurons in the ARC. POMC/CART neuronal population, also called "first-order neurons", widely project to "second-order neurons" located in the PVN, the LHA, the DMH and to areas of the hindbrain involved in satiation and satiety control [24–25]. The POMC/CART-originating tract has an overall catabolic effect whose increased activity induces a decrease in food intake and increased energy expenditure. Similarly, Nesfatin–1, an 82 amino-acid peptide derived from the cleavage of the precursor NUCB2, was identified in 2006 by Oh-I and colleagues [26]. This peptide exerts potent anorexigenic action after either peripheral or central administration [26]. The interest generated by this peptide is based on its anorexigenic action performed independently of leptin signalization after injection in both the cerebral ventricles [26] and the peritoneal cavity [27]. Nesfatin-1 was found expressed in neurons of various brain areas including hypothalamic nuclei (PVN, ARC) and at the brainstem level (NTS, DMNX) [28–29]. Nesfatinergic neurons located in these structures are activated in response to refeeding or intraperitoneal injection of CCK suggesting that central nesfatin-1 could participate in the meal termination mechanisms [30]. Moreover, Nesfatin-1 has a food intake-reducing effect that is

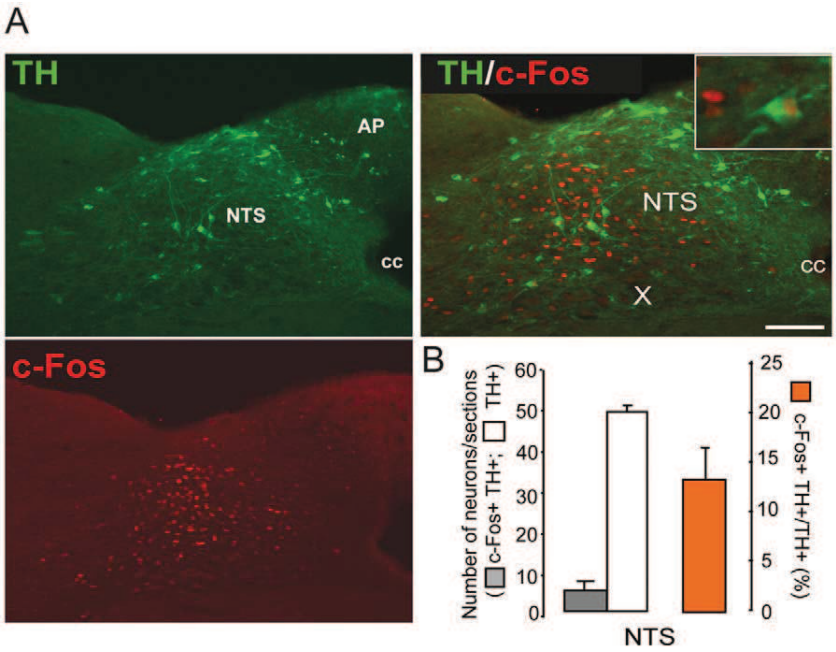


Figure 6. Effect of *per os* DON administration on brainstem A2/C2 neurons activation. A: Visualization of TH-expressing neurons (green) and c-Fos (red) positive cells on brainstem coronal sections of mice treated with DON. Inserts show high magnification images of double-labeled neurons. **B:** Quantification of the number of TH positive neurons (white bars) and c-Fos double-labeled cells (gray bars) in the NTS after DON treatment. The orange bars represent the percentage of double labeled neurons per phenotype in each area. All immunostaining were obtained from animals sacrificed 3 h after a 12.5 mg/kg DON administration. AP, area postrema; cc, central canal; NTS, nucleus of the solitary tract; TH, tyrosine hydroxylase; X, dorsal motor nucleus of the vagus. Scale bars: 100 μm. $^{**}P<0.01$, significantly different from vehicle-treated mice. ns, non significantly different from vehicle-treated mice.
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linked to the recruitment of the melanocortinergic pathway [31]. The observation of DON-induced POMC and nesfatin–1 expressing neurons activation suggest that the release of these anorexigenic signals and related compounds could partake in the feeding reducing action of the toxin. Strengthening these results, we observed that DON modulated mRNA levels of the hypothalamic melanocortinergic pathway including POMC, CART and MC4R, whereas mRNA of orexigenic effectors i.e. NPY and AgRP remained unaffected. Interestingly, POMC and CART mRNA expression is increased in response to leptin while they are reduced in fasted or obese (ob/ob or db/db) animals [32]. Hence, these results confirmed that in response to DON intoxication anorexigenic pathways are recruited. Finally, DON activated brainstem/hypothalamus connecting networks as attested by the activation of the A2/C2 catecholaminergic group. Altogether, these data strongly support the assumption that DON interferes directly or indirectly with neuronal networks devoted to central energy balance and that this action could partly explain the DON-induced hypophagia and the modification of meal frequency and size we observed in response to the toxin.

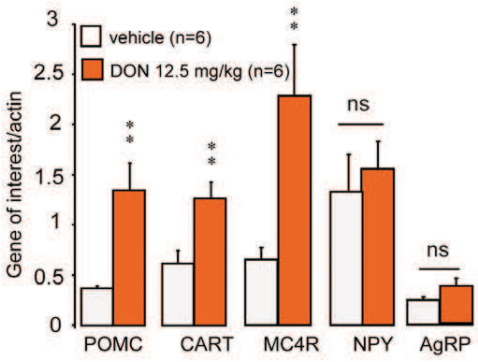


Figure 7. Effects of *per os* DON administration on hypothalamic mRNA expression. Quantification of POMC, CART, MC4R, NPY and AgRP transcript expression within the hypothalamus of water- (white bars) or DON-treated mice (12.5 mg/kg, orange bars). $^{**}P<0.01$, significantly different from vehicle-treated mice. ns, non significantly different from vehicle-treated mice.
doi:10.1371/journal.pone.0026134.g007

DON can directly target the brain

While we reported the activation of central neurocircuitry in response to *per os* DON administration, the question is how does DON signal to the brain? The stimulation of the vagus nerve by DON itself or peripheral compounds released in response to the toxin could be assumed. To investigate whether *per os* DON administration and probable consecutive mucosal gastric irritation signal to the brain through vagal terminals innervating the gastrointestinal tract, we submitted mice to UCV. UCV, previously used to consider the contribution of the vagus nerve to c-Fos expression within the NTS in response to various conditions [33–34], failed to reduce DON-evoked c-Fos expression in the ipsilateral NTS of all animals which excludes the involvement of the vagal pathway in the DON-related information conveyed from the gut to the brain, at least in our experimental model and at the DON doses used. This puzzling result pointed out the existence of an alternative, non characterized mechanism supporting the anorexigenic action of the toxin. We hypothesized a possible action on specific central structures of the orally

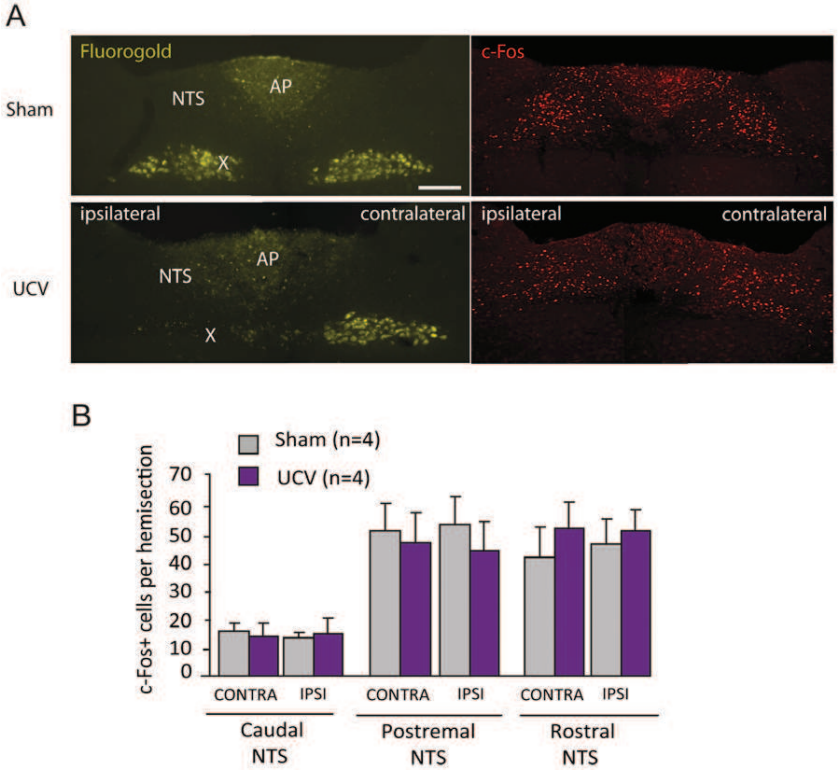


Figure 8. Effect on vagotomy on brainstem DON-induced c-Fos expression. A: Detection of fluorogold (yellow) and c-Fos (red) on brainstem coronal sections of sham or unilaterally vagotomized (UCV) mice after an oral administration of DON (12.5 mg/kg). Note that after vagotomy no fluorogold labeling can be visualized on the ipsilateral DMNX. **B:** Quantification of DON-induced (12.5 mg/kg) c-Fos immunoreactivity in subareas of the NTS in sham (grey bars) or vagotomized (purple bars) mice.
doi:10.1371/journal.pone.0026134.g008.

administered toxin having reached the brain through circumventricular organs. The DON-induced c-Fos staining observed in circumventricular organs and surrounding structures (AP, NTS, ME, ARC) supported this hypothesis. Moreover, DON was shown to be rapidly distributed in various organs including the brain within a short time after exposure [35]. We bring here the first evidence showing that centrally injected DON, at doses ineffective at the periphery, was able to dose-dependently reproduce anorexia observed after *per os* intoxication. DON affected meal pattern in a way similar to that previously observed after peripheral administration i.e. significant decrease of meal frequency and size and parallel increase in intermeal intervals and satiety ratio. Interestingly, the pattern of activated structures was similar after *per os* and icv administration and central DON injection also resulted in the activation of anorexigenic networks including POMC and nesfatin–1 expressing neurons both in the NTS and hypothalamus. The mechanism by which DON activates neuronal networks remains however to be clarified but its action seems faster than would be expected from its inhibitory effect on protein synthesis. DON has been reported to modulate the immune system in murine models and to increase cytokine production in both *in vitro* and *in vivo* murine models (see [2] for review). It is likely that pro-inflammatory mediators could partake in the anorexigenic response to the toxin. In accordance, we recently reported that *per os* DON administration induced an up-regulation of IL-1β, IL-6 and TNF-α mRNA within central structures involved in food intake control [36]. The induction of such pro-inflammatory cytokines within the brain may explain the DON-induced anorexia observed here. A similar increased cytokine expression was indeed observed during anorexigenic immune challenge [37].

In summary, the present work provides the first demonstration that DON modifies feeding behavior by interfering with central neuronal networks dedicated to food intake regulation. Moreover, our results, with particular attention to icv DON injection, strongly suggest that DON reaches the brain where it modifies anorexigenic circuitry activity. These results were obtained with an animal model using acute intoxication with relatively high DON doses. Accordingly, extrapolation on human health should be made with caution. However, we believe these new results are sufficient to reconsider the impact of DON on human population chronically exposed to low toxin doses. The consequences of a possible central energy balance modulation should be evaluated, especially on vulnerable and predisposed individuals suffering from unwanted body weight loss (such as cachexia) or eating disorders (such as anorexia nervosa). Using animal models, future investigations will in particular determine whether chronic consumption of low DON doses worsens such eating disorders. Finally, these points should be addressed in the future for DON alone and consumed in combination with other toxins that may be present in human food.

Materials and Methods

Animal housing

Experiments were performed on adult male DBA/1lac J mice of 20–25 g body weight (Janvier). All animals were individually

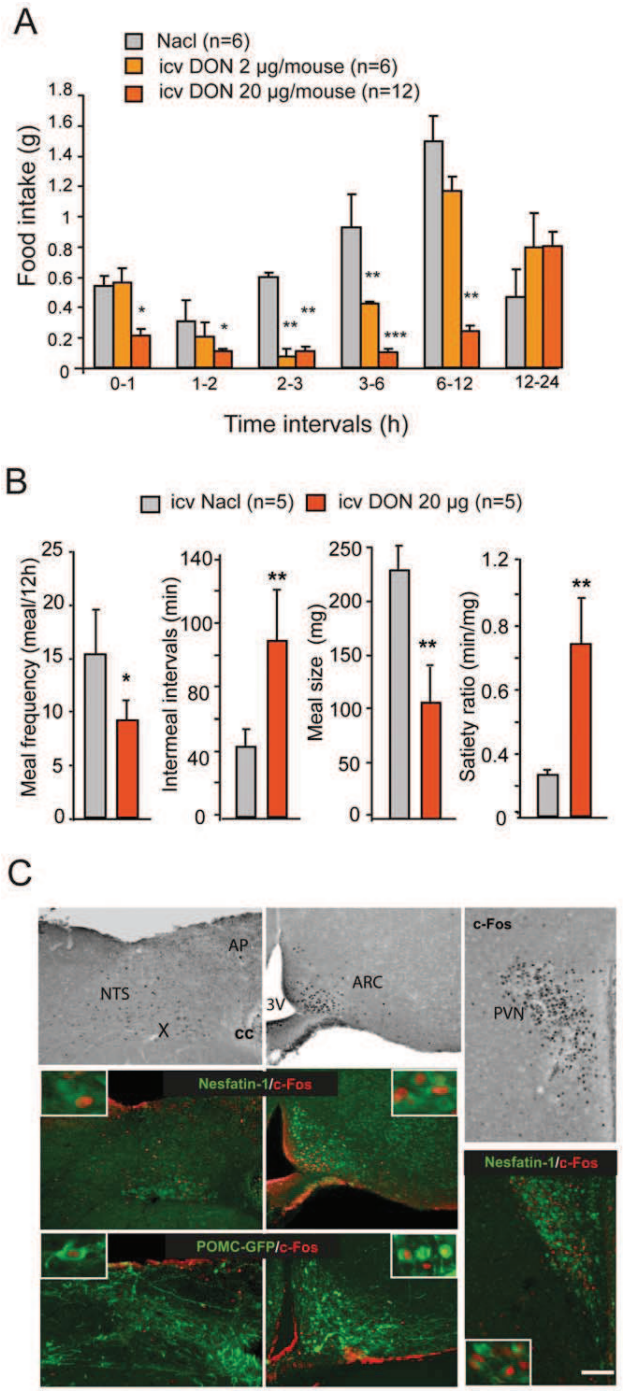


Figure 9. Effect of intracerebroventricular DON injection. **A:** Food intake of mice having received either an icv injection of saline (vehicle) or DON at different doses (2 or 20 µg/mouse). **B:** Effect of icv DON injection (20 µg/mouse) on meal parameters. **C:** c-Fos, nesfatin−1/c-Fos and POMC-GFP/c-Fos labeling on brainstem and hypothalamic coronal sections 1 h after an icv injection of DON (20 µg/mouse). Inserts in E show higher magnification images of double labeled neurons. 3V, third ventricle; AP, area postrema; ARC, arcuate nucleus; cc, central canal; NTS, nucleus of the solitary tract; X, dorsal motor nucleus of the vagus. Scale bars 100 µm *$P<0.05$, **$P<0.01$ and significantly different from vehicle-treated mice.
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housed in a pathogen-free facility at controlled temperature on a 12/12-h light/dark cycle (lights on at 7 am) with standard powder diet (AO4 P2.5, SAFE UAR) and water available *ad libitum*. Individual cages were designed in order to limit spillage [38]. Mice had free access to standard powder diet *via* two holes made at the bottom of the cage. For habituation, animals were housed in these cages at least 10 days before experiments. All experiments were performed at 21°C. Additional experiments were performed using male proopiomelanocortin (POMC)-Tau-Topaz GFP transgenic mice developed by Pinto and collaborators [39] using the BAC transgenic technology.

Ethics Statement

Experiments carried out in this study were performed in strict accordance with European Economic Community guidelines (86/609/EEC) and the local committees' recommendations (C-13-055-6, Aix-Marseille University) for the care and use of laboratory animals.

Per os administration of DON

One hour prior to the beginning of the dark phase, mice were administered orally 6.25 to 25 mg/kg body weight (bw) DON (D-0156, Sigma Chemical Co.), dissolved in 100 µl per 10 g body weight of distilled water via gavage, using a 22 gauge intubation needle (Popper and Sons). Prior to DON treatment, mice received the same volume of distilled water using the similar oral administration procedure for a habituation period of seven consecutive days.

Surgery and intracerebroventricular injection of DON

Cannula implantation. Animals were anaesthetized by an intraperitoneal injection of ketamine (100 mg/kg; Imalgen 1000, Merial) and xylazine (6 mg/kg; Rompun, Bayer), and placed in a digital stereotaxic apparatus (Model 502600, WPI) coupled to the neurostar software (Neurostar GmbH). A 26-gauge stainless steel cannula was implanted into the lateral ventricle at the following coordinates: 0.3 mm posterior to bregma, 1.1 mm lateral to the midline, and 2.6 mm ventral to the skull surface. The cannula was secured to the skull with dental cement and sealed with removable obturators. The animals were sutured, placed in individual cages and allowed to recover for seven days. During this resting period, animals were injected with physiological saline every other day for habituation. One week post-surgery, mice were administered either 10 µl (2 µl/min) of physiological saline or DON solution at the beginning of the dark phase. The correct cannula positioning was checked for each animal at the end of the experiment by cresyl violet staining of brain sections. Subgroups of mice were anaesthetized as previously described and fixed with paraformadehyde 4% one hour after injections for immunohistochemistry procedures.

Food intake Measurements

Powdered food consumption. One hour before lights off, mice received either intra-esophageal or icv administration of DON or vehicle. Immediately after treatment, a fresh supply of pre-weighed food was given. The measurement of powdered food intake was the same as in previous studies [33]. Food intake was calculated as the difference between the pre-weighed and the remaining powder measured with a precision balance (0.01 g; Denver Instrument from Bioblock).

Continuous food intake measurements and meal pattern analysis. To measure food intake in mice, we customized commercially available polypropylene cages (Cage S; Charles River Laboratories) with a feeding station allowing access to a food jar, placed below the cage, and filled with powdered food (powder

AO4; SAFE UAR). The feeding station was adapted from Kurokawa and colleagues [40] and designed in such a manner that neither feces nor urine could drop onto the food jar and that food spillage was reduced to a negligible amount. To analyze meal pattern, the food jar was placed on a precision balance (Ohaus Scout Pro; 200 g range; 0.01 g precision) connected to a computer with a USB/RS232 interface. Custom software, developed with Delphi, allowed the multiplexed continuous data acquisition from 1 to 8 cages. Time series of food jar weight data consisted in stepwise reductions with high amplitude and high frequency oscillations, separated by stabilized values, with low amplitude infrequent oscillations. Analysis first proceeded through a Matlab developed software, to filter out high frequency oscillations in food jar weights and collect the starting time, end time and weight amplitude of the stepwise reductions in food jar weight. Analyzed data were exported to Excel for further analysis. Meals were defined as stepwise reductions in food jar weight of at least 0.02 g in amplitude (i.e. twice the balance precision) and 30 seconds in duration.

Measurement of pica behavior (kaolin intake). Kaolin pellets were prepared from pharmacological grade kaolin and gum Arabic (Sigma Chemical Co) mixed at a 99:1 ratio in distilled water. The kaolin paste was rolled and cut into pieces similar in shape to mice chow pellets. The pellets were dried in an oven at 37°C for 72 h and then placed into individual cages. Mice were allowed access to regular food and kaolin pellets during a 5 days adaptation period before the beginning of the study. On the day of the experiment the last 24 h kaolin consumption was recorded. Mice were then administered DON (12.5 mg/kg bw) *per os* as described above and kaolin intake was measured daily for the four following days by subtracting preweighed and remaining kaolin pellets with a precision balance (Denver Instrument from Bioblock).

Vagotomy Surgeries

Animals were anaesthetized with an ip injection of ketamine (100 mg/kg; Imalgen 1000, Merial,) and xylazine (6 mg/kg; Rompun, Bayer), and unilateral cervical vagotomies (UCV) were performed according to previously established procedures [41]. The left vagus nerve, which transfers the majority of gastrointestinal signals to the CNS, was severed. In sham-operated animals, the carotid trunk was exposed but the vagus nerve was not touched. Verification of vagotomy was performed by injecting into the peritoneal cavity a retrograde neuronal tracer, which is transported to cell columns of the DMNX. Seven days before DON administration and sacrifice, animals were given 0.5 ml intraperitoneal injections of 0.4% Fluorogold (# FP46766A, Interchim). Fluorogold staining was observed with a Nikon Eclipse E600 light microscope and images were acquired using a DXM 1200 camera (Nikon) coupled to ACT-1 software. The absence of Fluorogold staining in the left DMNX attested of the correct sectioning of the nerve.

Quantitative RT-PCR analysis

Animals used for RT-PCR analysis were not refed after DON administration and were sacrificed 3 h after treatment. mRNA expression within the brainstem and the hypothalamus was quantified as described previously [33]. Briefly, total RNA was extracted from frozen hypothalamus using RNeasy Mini kit and RNeasy Micro Kit (Qiagen) respectively. After RNA reverse transcription, gene expression analysis by real time PCR was performed using the ABI Thermocycler 7500 fast (Applied Biosystems). The equivalent of 6.25 ng initial RNA was subjected to PCR amplification in a 10 µl final volume using specific 2.4 µM primers and SYBR Green PCR master mix (Applied Biosystems). Product formation (primers in Table 2) was detected at 60°C in the fluorescein isothiocyanate channel. The generation of specific PCR products was confirmed by melting-curve analysis. For each PCR, cDNA were run in duplicate in parallel with serial dilutions of a cDNA mixture tested for each primer pair to generate a standard linear curve, which was used to estimate relative quantities of gene of interest and of β-actin (internal reference gene) mRNA.

Immunohistochemistry procedures

As mentioned for PCR analysis, *per os* treated-animals used for immunostaining procedure were sacrificed 3 h after treatment without free access to food. Animal perfusion was achieved with 10 ml of 0.1 M PBS followed by 50 ml of 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were post-fixed for 1 h in 4% PFA at room temperature, rinsed in PBS and then cryoprotected for 24–48 h in 30% sucrose at 4°C. After freezing of the brains in isopentane (−40°C), coronal sections (40 µm thick) were cut on a cryostat (Leica CM3050, France) and collected serially in PBS (0.1 M; pH 7.4). Brains were cut from caudal brainstem (Bregma−8.24 mm) to forebrain (Bregma+0.75 mm).

c-Fos immunohistochemistry. c-Fos immunohistochemistry was performed on free-floating sections using an anti-c-Fos rabbit antiserum synthesized against amino acids 4–17 of human protein (1:10000, Ab-5, Calbiochem) as previously described [41]. Briefly, the free-floating sections were incubated 10 min in a solution containing 0.3% H_2O_2 in PBS 0.1 M for quenching of endogenous peroxidase activity. After 1 h in PBS containing 3% normal goat serum (NGS) and 0.3% Triton X-100, sections were incubated for 48 h at 4°C in PBS containing 3% NGS, 0.3% Triton X-100 and anti-c-Fos antibody. A biotinylated goat anti-rabbit IgG (1:400, Vector Labs) was used as secondary antibody. After incubation with the avidin-biotin complex (1:200, Vector Labs), horseradish

Table 2. Primers for SYBR Green assays.

Gene product	RefSeq	Primers	
		Fw	Rv
AgRP	NM_007427.2	AGCTTTGGCGGAGGTGCT	GCGACGCGGAGAACGA
CART	NM_013732.6	GCCAAGGCGGCAACTTC	TCTTGCAACGCTTCGATCTG
MC4-R	NM_016977.3	AAGCTGCCCAGATACAACTTATGA	ACGCGCTCCAGTACCATAACA
NPY	NM_023456.2	TCCGCTCTGCGACACTACAT	TGCTTTCCTTCATTAAGAGGTCTG
POMC	NM_008895.3	TGAACATCTTTGTCCCCAGAGA	TGCAGAGGCAAACAAGATTGG

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peroxydase activity was visualized using a nickel-enhanced diaminobenzidine (DAB) as the chromogen. The reaction was closely monitored and terminated when optimum intensity was achieved (3–5 min) by washing the sections in distilled water. Non-specific labeling was assessed on alternate slices that were treated identically to the above but in which the primary antibody was omitted. Alternatively, when associated with phenotyping labeling, c-Fos staining was performed using an anti-c-Fos goat IgG (1:5000; sc-52-G, Santa Cruz) and revealed by means of a donkey-Alexa 594 conjugated anti-goat IgG (1:400, A11058, Invitrogen). In these cases, the sections were incubated 2 h at room temperature with the secondary antibody.

Phenotypic identification. To determine potential activation of POMC, nesfatin-1 and TH expressing neurons within the hypothalamus and brainstem in response to DON (12.5 mg/kg) administration, we performed double immunohistochemistry labeling on c-Fos stained sections. For POMC detection, we used mice expressing the fluorescent GFP under the promoter of POMC gene. GFP was labeled using an anti-GFP rabbit primary antibody (1:1000, A11122, Molecular Probes). Similarly, identification of nesfatin-1 neurons was performed by incubating sections with a rabbit primary antibody raised against nesfatin-1 (1:10000, H-003-22-B, Phoenix Pharmaceuticals Inc.). For TH detection purpose, brainstem sections were incubated with an anti-TH rabbit antibody (1:1000, MAB318, Chemicon). Each labeling was revealed using an Alexa-488-conjugated secondary antibody (1:400, A11034, Molecular Probes). Finally, all sections were mounted on gelatin-coated slides, air dried, and coverslipped with mounting medium for fluorescence microscope preparation (DAKO).

Microscopy, image analysis and cell count

c-Fos immunostaining was further analyzed by counting the positive nuclei on four sections. c-Fos positive nuclei counting was performed on photomicrographs acquired using a 10 fold lens with a DXM 1200 Camera (Nikon) coupled to ACT-1 software. The microscope was set at a specific illumination level, as was the camera exposure time. c-Fos positive nuclei were then counted on these pictures by computer-assisted morphometry using the NIH image J software. Images were normalized by subtracting the background determined for each structures studied. The c-Fos stained elements were identified by setting a threshold value (140 grey levels above the background on a 0–255 intensity scale). Counts were manually corrected for overlapping cell nuclei that were counted by the software as single. The software-generated counts of c-Fos stained profiles were also manually corrected by excluding positive objects whose surface area did not exceed 10 pixels (image resolution 150 pixels/inch) corresponding to objects with a surface equal or inferior to 3 square μm. For double labeling, fluorescent images were first acquired on a confocal microscope (Leica TCS SP2) using the 488-nm band of an Ar-laser and the 543-nm band of a He/Ne-laser. Images were sequentially acquired to check marker co-localization. Then, images were routinely acquired with a DXM 1200 Camera (Nikon) coupled to ACT-1 software. Fluorescent images acquired at 520 and 594 nm were merged with phase contrast images to allow visualization of activated neurons. For each brain structure studied and each staining, the counts performed on 4 distinct sections from both hemispheres were averaged.

Statistical analysis

Data are represented as mean ± S.E.M. Comparisons between data from vehicle- and DON-treated mice were performed using unpaired 2-tailed Student's *t*-test. One-way ANOVA were performed for comparison between data from mice treated with different doses of DON. Tukey's HSD (Honestly Significant Difference) test was used for post hoc analysis. *P* values less than 0.05 were considered significant. The correlations between the kaolin and chow intakes were calculated by chi-square correlation test.

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Author Contributions

Conceived and designed the experiments: JT ST AJ MD JDT. Performed the experiments: CG MSB RJ MS CT ST LM MD. Analyzed the data: CG MSB CT JT ML ST AJ MD JDT. Contributed reagents/materials/analysis tools: NW JR BL. Wrote the paper: MD JDT.

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Annexe 6

Article : Central inflammation and sickness-like behavior induced by the food contaminant Deoxynivalenol: A PGE2-independent mechanism.

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Central Inflammation and Sickness-Like Behavior Induced by the Food Contaminant Deoxynivalenol: A PGE2-Independent Mechanism

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Deoxynivalenol (DON), one of the most abundant trichothecenes found on cereals, has been implicated in mycotoxicoses in both humans and farm animals. Low-dose toxicity is characterized by reduced weight gain, diminished nutritional efficiency, and immunologic effects. The levels and patterns of human food commodity contamination justify that DON consumption constitutes a public health issue. DON stability during processing and cooking explains its large presence in human food. We characterized here DON intoxication by showing that the toxin concomitantly affects feeding behavior, body temperature, and locomotor activity after both *per os* and central administration. Using c-Fos expression mapping, we identified the neuronal structures activated in response to DON and observed that the pattern of neuronal populations activated by the toxin resembled those induced by inflammatory signals. By real-time PCR, we report the first evidences for a DON-induced central inflammation, attested by the strong upregulation of interleukin-1β, interleukin-6, tumor necrosis factor-α, cyclooxygenase-2, and microsomal prostaglandin synthase-1 (mPGES-1) messenger RNA. However, silencing prostaglandins E2 signaling pathways using mPGES-1 knockout mice, which are resistant to cytokine-induced sickness behavior, did not modify the responses to the toxin. These results reveal that, despite strong similarities, behavioral changes observed after DON intoxication differ from classical sickness behavior evoked by inflammatory cytokines.

Key Words: **mycotoxin; cytokines; brain; mPGES-1; c-Fos.**

Fusarium fungi are common pathogen growing on cereals cultivated in Europe, America, and Asia. The extent of cereal contamination is strongly associated with rainfall and moisture and with grain storage conditions. These fungi produce various mycotoxins, which contaminate animals and human food commodities. The trichothecene deoxynivalenol (DON), also commonly called vomitoxin, is one of the most abundant mycotoxin found on contaminated cereals, and its stability during processing and cooking explains its widespread presence in human food (Lombaert *et al.*, 2003; Schothorst and van Egmond, 2004; Turner *et al.*, 2008, 2010a). Moreover, farm workers who handle grain or silage may be at additional risk exposure to DON (Turner *et al.*, 2010b). DON has been shown to be implicated in acute and chronic illnesses in both humans and farm animals. Whereas high-dose toxicity of DON is characterized by a set of symptoms including diarrhea, vomiting, leukocytosis, hemorrhage, circulatory shock, and death, low-dose toxicity is characterized by anorexia, reduced weight gain, diminished nutritional efficiency, neuroendocrine changes, and immunologic effects (for review, see Pestka, 2010). In view of this widespread human exposure to DON, studies improving our knowledge of DON toxicity are essential and should be conducted. DON was reported to have modulatory effects on the immune system. Low doses of DON upregulate the expression of cytokines and chemokines, whereas high doses exert immune suppression action via the induction of leukocyte apoptosis. Oral exposure to DON at 5 mg/kg was shown to increase, shortly after treatment (2 h), the expression of proinflammatory cytokines such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) in the spleen, liver, lung, and kidney (Amuzie *et al.*, 2008; Pestka and Amuzie, 2008). This stimulatory effect of low DON doses could be explained by both an increased cytokine gene transcription and an enhancement of their messenger RNA (mRNA) stability. It is now well admitted that the proinflammatory cytokines produced during infection or inflammation allow communication between the immune and central nervous systems (CNS). In response to cytokine actions, the CNS coordinates a set of nonspecific symptoms referred to as sickness behavior, which notably includes anorexia, modulation of body core temperature, and reduced activity. During infection, cells of

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the innate immune system located at the periphery or in the CNS recognize specific components of microorganisms, which in turn induce the synthesis and release of inflammatory cytokines such as IL-1β, TNF-α, and IL-6. These soluble mediators coordinate the local and systemic inflammatory responses and act on the brain to induce the central symptoms specific to sickness. Among the inflammatory cytokines, IL-1β seems to be the most potent in inducing sickness behavior because its peripheral or central injection reproduces the set of nonspecific symptoms of inflammation including decreased motor activity, social withdrawal, anorexia, and fever (Kelley *et al*., 2003). The injection of TNF-α or IL-6 also induces anorexia, fever, decreased activity, and social withdrawal. Although the direct action of blood-borne cytokines on brain structures has been proposed to mediate sickness behavior, the *de novo* cytokine synthesis in discrete areas of the brain in response to peripheral inflammation has also been largely illustrated. Moreover, it is well recognized that a complex neuronal circuitry activated in response to inflammation coordinates the behavioral responses observed during infection and inflammation.

The concomitant induction of anorexia and upregulation of proinflammatory cytokines by DON emphasize the strong similarities between sickness behavior resulting from inflammatory challenge and DON intoxication. Some authors have indeed proposed that, given the well-characterized anorexigenic action of these inflammatory molecules, they could partake in the toxin-induced anorexia (Pestka *et al*., 2004). However, although the peripheral action of DON was extensively studied (Pestka, 2010), data illustrating its effects on CNS are significantly less abundant. Despite the well-described modulation of feeding behavior induced by DON consumption, the data aiming to characterize the symptoms associated with this loss of appetite and the central mechanisms by which this toxin exerts its actions remain largely unknown.

In the present study, combining a multidisciplinary approach and a mice model of DON intoxication, we characterized the symptoms associated with anorexia during acute oral and central administrations of DON and identified the inflammation biomarkers whose central expression is upregulated by the toxin. Finally, using a mice genetic model characterized by an impaired Prostaglandins E2 (PGE2) synthesis, we demonstrated that DON-induced sickness-like behavior is independent of PGE2 action.

MATERIALS AND METHODS

Animal Housing

Experiments were performed either on adult male wild-type (WT) (20–25 g) or microsomal prostaglandin synthase-1 (mPGES-1) −/− (18–25 g) DBA/1Lac J mice. mPGES-1 −/− mice exhibit a deletion of the *Ptges* gene, which encodes mPGES-1 (Trebino *et al*., 2003). mPGES-1 −/− colony was provided by Prof. F. Berenbaum (Pierre and Marie Curie University, Paris) and has been characterized previously (Pecchi *et al*., 2006). Historically, DBA/1Lac J mice strain was used to develop arthritis models induced by immunization with type II collagen. This genetic background was next chosen for mPGES-1 invalidation and subsequent characterization of mPGES-1 −/− phenotype in response to various inflammatory conditions. In the present study, both WT and knockout (KO) mice were obtained from heterozygous mPGES-1 mice mating. All animals were individually housed in a pathogen-free facility at controlled temperature on a 12/12-h light/dark cycle (lights on at 7 A.M.) with standard powder diet (AO4 P2.5, SAFE UAR) and water available *ad libitum*. Individual cages were designed in order to limit spillage (Pecchi *et al*., 2008). Mice had free access to standard powder diet via two holes made at the bottom of the cage. For habituation, animals were housed in these cages at for least 10 days before experiments. All experiments were performed at 21°C except when specified. Experiments carried out in this study were performed in strict accordance with European Economic Community guidelines (86/609/EEC) and the local committee's recommendations (C-13-055-6, Aix-Marseille University) for the care and use of laboratory animals.

Per os *Administration of DON*

One hour prior to the beginning of the dark phase, mice were orally administered 6.25, 12.5, and 25 mg/kg body weight (bw) DON (D-0156, Sigma Chemical Co., 98% purity) and its purity verified by high-performance liquid chromatography with diode-array detection set at 220 nm. Mice received an injection volume of 100 μl/10 g bw of DON dissolved in distilled water via gavage, using a 22-gauge intubation needle (Popper and Sons). Prior to DON treatment, mice received the same volume of distilled water using the similar oral administration procedure for a seven consecutive days of habituation period.

Surgery and Intracerebroventricular Injection of DON

Cannula implantation. Animals were anesthetized by an ip injection of ketamine (100 mg/kg; Imalgen 1000, Merial) and xylazine (6 mg/kg; Rompun, Bayer) and placed in a digital stereotaxic apparatus (Model 502600, WPI) coupled to the StereoDrive software (Neurostar GmbH). A 26-gauge stainless steel cannula was implanted into the lateral ventricle at the following coordinates: 0.3 mm posterior to bregma, 1.1 mm lateral to the midline, and 2.6 mm ventral to the skull surface. The cannula was secured to the skull with dental cement and sealed with removable obturators. The animals were sutured, placed in individual cages, and allowed to recover for 7 days. During this resting period, animals were injected with physiological saline every other day for habituation. One week after surgery, mice were administered either 10 μl (2 μl/min) physiological saline or DON solution (20 μg per mouse) at the beginning of the dark phase. This dose of DON was chosen because it is known to not cause anorexia when administered at the periphery. The correct cannula positioning was checked for each animal at the end of experiment by cresyl violet staining of brain sections. In some cases, a TA10TA-F20 telemetry probe (Data Sciences International) was implanted ip as soon as the cannula implantation procedure was finished.

Food Intake Measurements

Powdered food consumption. Experiments were performed on animals allowed to eat throughout the day. One hour before lights off, mice received either intraesophageal or intracerebroventricular (ICV) administration of DON or vehicle. Immediately after treatment, a fresh supply of preweighed food was given. The measurement of powdered food intake was the same as in previous studies (Pecchi *et al*., 2008). Food intake was calculated as the difference between the preweighed and the remaining powder measured with a precision balance (0.01 g; Denver Instrument from Bioblock).

Telemetry Measurements

Body temperature and locomotor activity were recorded using TA10TA-F20 telemetry probes (Data Sciences International). Mice were anesthetized as previously described. A telemetry probe was implanted ip in each animal. After surgery, mice were housed individually, maintained at a constant temperature of 21°C or 31°C and placed on a receiver RPC-1 (Data Sciences International).

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Telemetry radio signals emitted by the implanted transmitter were relayed to the data acquisition system via a consolidation matrix, converted into temperature and locomotor activity data using Dataquest ART 4.2 data acquisition software, and recorded every 5 min. Mice were then administered water by oral gavage daily at 6 pm for habituation. One week after surgery, mice received water as a control and 3 days later, they received DON, both by oral gavage.

Quantitative RT-PCR Analysis

Animals used for real-time polymerase chain reaction (RT-PCR) analysis were not refed after DON administration and were sacrificed 3 h after treatment. mRNA expression within the brainstem and the hypothalamus was quantified as previously described (Pecchi *et al*., 2008). Briefly, total RNA was extracted from frozen hypothalamus and brain stem using RNeasy Mini Kit and RNeasy Micro Kit (Qiagen), respectively. Reverse transcription of the RNA was performed using random primers and M-MLV reverse transcriptase (Promega). Gene expression analysis by real-time PCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). The equivalent of 6.25 ng initial RNA was subjected to PCR amplification in a 10 µl final volume using 2.4µM specific primers and SYBR Green PCR Master Mix (Applied Biosystems). Product formation (primers in Table 1) was detected at 60°C in the fluorescein isothiocyanate channel. The generation of specific PCR products was confirmed by melting-curve analysis. For each PCR, complementary DNAs (cDNAs) were run in duplicate in parallel with serial dilutions of a cDNA mixture tested for each primer pair to generate a standard linear curve, which was used to estimate relative quantities of each gene of interest and of β-actin (internal reference gene) mRNA.

Immunohistochemistry Procedures

As mentioned for PCR analysis, *per os*–treated animals used for the immunostaining procedure were sacrificed 3 h after treatment without any access to food. Animal perfusions were achieved with 10 ml of 0.1M PBS followed by 50 ml of 4% paraformaldehyde (PFA) in 0.1M PBS. Brains were postfixed for 1 h in 4% PFA at room temperature, rinsed in PBS, and then cryoprotected for 24–48 h in 30% sucrose at 4°C. After freezing of the brains in isopentane (−40°C), coronal sections (40 µm thick) were cut on a cryostat (Leica CM3050, France) and collected serially in PBS (0.1M, pH 7.4) from caudal brain stem (Bregma −8.24 mm) to forebrain (Bregma + 0.75 mm). c-Fos immunohistochemistry was performed on free-floating sections using an anti-c-Fos rabbit antiserum synthesized against amino acids 4–17 of human protein (1:10,000, Ab-5, Calbiochem) as previously described (Pecchi *et al*., 2007). Briefly, the free-floating sections were incubated for 10 min in a solution containing 0.3% H_2O_2 in 0.1M PBS for quenching of endogenous peroxidase activity. After blocking with PBS containing 3% normal goat serum and 0.3% Triton X-100, sections were incubated for 48 h at 4°C with primary anti-c-Fos antibody diluted in the same solution. A biotinylated goat anti-rabbit IgG (1:400, Vector Labs) was used as secondary antibody. After incubation with the avidin-biotin complex (1:200, Vector Labs), horseradish peroxidase activity was visualized using a nickel-enhanced diaminobenzidine as the chromogen. The reaction was terminated when optimum intensity was achieved (3–5 min) by washing the sections in distilled water.

TABLE 1
References of Primers Used (Qiagen) for SYBR Green Assays

Gene product	RefSeq	Commercial references	
IL-1β	NM_008361	Mm_Il1b_2_SG	QT01048355
IL-6	NM_031168	Mm_Il6_1_SG	QT0098875
TNF-α	NM_013693	Mm_TNF-a_1_SG	QT00104006
COX-2	NM_011198	Mm_Ptgs2_1_SG	QT00165347
mPGES-1	NM_022415	Mm_Ptges_1_SG	QT00118223
β-Actin	NM_031144	Rn_Actb_1_SG	QT00193473

Microscopy, Image Analysis, and Cell Count

c-Fos immunostaining was analyzed by counting the positive nuclei on four adjacent sections. c-Fos–positive nuclei counting was performed on photomicrographs acquired using a 10-fold lens with a DXM 1200 Camera (Nikon) coupled to ACT-1 software. The microscope was set at a specific illumination level, as was the camera exposure time. c-Fos–positive nuclei were then counted on these pictures by computer-assisted morphometry using the NIH image J software as previously described (Dallaporta *et al*., 2007). Briefly, images were normalized by subtracting the background determined for each structure studied, and c-Fos–stained elements were identified by setting a threshold value. Counts were manually corrected to avoid overlapping cell nuclei and count of positive objects whose surface area did not exceed 3 μm^2.

Statistical Analysis

Data are represented as mean ± SEM. Comparisons between data from vehicle- and DON-treated mice were performed using unpaired two-tailed Student's *t*-test. One-way ANOVA were performed for comparison between data from mice treated with different doses of DON. For experiments on KO mice and at 31°C, data were compared with respective controls (WT and 21°C) with two-way ANOVA, with treatment (DON vs. vehicle) and, respectively, either genotype (WT vs. KO) or ambient temperature (21°C vs. 31°C) as factors. Tukey's Honestly Significant Difference test was used for *post hoc* analysis. p Values less than 0.05 were considered significant.

RESULTS

Effect of Acute per os *DON Administration on Food Intake, Locomotor Activity, and Body Core Temperature*

A single oral administration of DON resulted in a dose-dependent decrease in daily food intake with a notably long-lasting effect for the highest doses (Fig. 1A). Although food consumption returned to basal level after 24 h with the 6.25 mg/kg dose, eating was affected up to 48 h after treatment with 12.5 and 25 mg/kg. Cumulative food intake measured over a period of 24 h, i.e., 3, 6, 12, 18, and 24 h after treatment, revealed that DON profoundly affected the nighttime food intake (Fig. 1B). In parallel to the alteration of feeding behavior, DON also caused a dose-dependent reduction of nightly locomotor activity (Fig. 2). The rhythm of locomotor activity was durably affected by the DON treatment especially with the 12.5 and 25 mg/kg DON doses with normal activity amplitude reappearing during the night of the third day (Fig. 2A). Because changes in body temperature are features associated with anorexia and reduced locomotor activity during sickness behavior consecutive to infection, we next examined the impact of DON on body core temperature. At an ambient temperature of 21°C, *per os* DON administration at the end of the light phase resulted in a decrease in body temperature during the first 2 h after DON challenge (Fig. 3B). This effect was more pronounced with the highest doses, i.e., 12.5 and 25 mg/kg (Figs. 3A and 3B). With these doses, the DON-induced decrease in temperature reached up to 2.5°C ± 0.4°C in the first 2 h followed by a long-lasting hypothermia, which affected daily temperature rhythm amplitudes during the following 72 h (Fig. 3A). To evaluate the dependence of DON-induced hypothermia on ambient temperature, additional experiments

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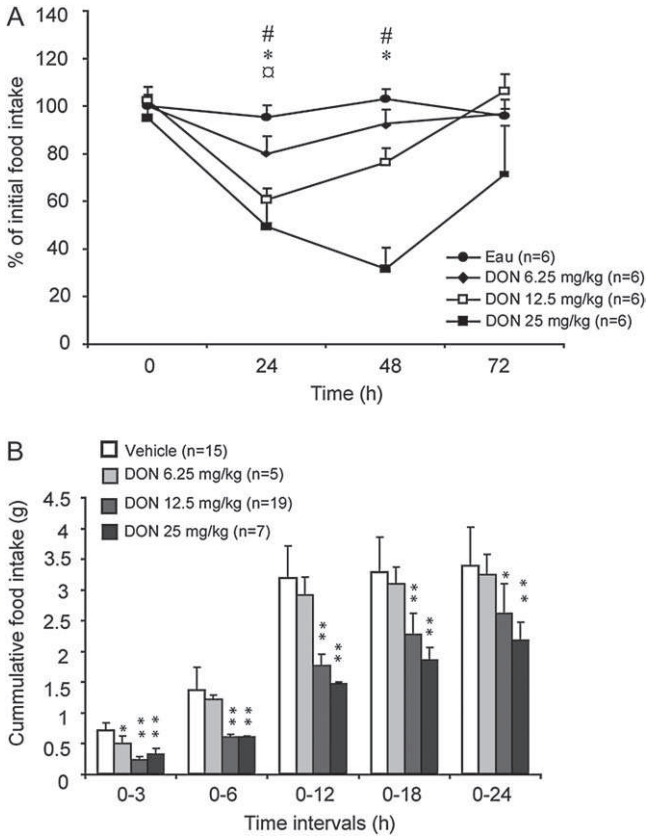


FIG. 1. Effect of acute *per os* DON administration on food intake. (A) Daily food intake (percentage of initial food intake) measured from 0 to 72 after oral gavage of either water (vehicle) or DON (6.25, 12.5, and 25 mg/kg) in adult mice. (B) Cumulative food intake (g) measured over the first 24-h period of mice having received an oral gavage of either water or DON (6.25, 12.5, and 25 mg/kg). In (A), ''¤,'' ''*,'' and ''#'' represent $p < 0.05$, significantly different from vehicle-treated mice, respectively, for 6.25, 12.5, and 25 mg/kg DON-treated mice. In (B), $*p < 0.05$ and $**p < 0.01$, significantly different from vehicle-treated mice.

were performed with an ambient temperature of 31°C. Under these conditions, DON still induced a decrease in body temperature (Fig. 3C).

Effect of ICV DON Injection on Sickness-Like Behavior

To determine whether a central action of DON could result in the same symptoms as those observed after *per os* administration, we next performed ICV injections (lateral ventricles) of the toxin on chronically cannulated mice. As observed after *per os* administration, central DON injections (20 μg per mouse) reduced food intake measured during the dark phase (Fig. 4A). Cumulative food intake measured over a 12-h period revealed that DON profoundly affected food intake as soon as the first hour and the effect was long lasting during the following 12 h after injection. Food intake was indeed reduced by more than 70% six and 9 h after injection. The anorexigenic effect of centrally injected DON was still observable 12 h after injection. When administered peripherally, the same DON dose, which is about 10- to 30-fold lower than the *per os* administered DON doses (equivalent to 150–600 μg per mouse), failed to reduce food intake. It should be noticed that ICV DON injection resulted in a decrease in body core temperature with a maximum of −3. 5 ± 0.9°C reached 7 h after injection (Fig. 4B). Regarding locomotor activity, ICV DON injection resulted in a biphasic modulation of this parameter. Whereas locomotor activity appeared to be increased during the 3 h following DON injection, a strong decrease of this behavior was observed in the second part of the dark phase (Fig. 4C).

Central Expression of Inflammation Biomarkers during per os *DON Intoxication*

Because DON was previously shown to increase proinflammatory cytokine gene expression in peripheral tissues, we next investigated whether DON administration (12.5 mg/kg) could result in an upregulation of these genes in the brain. Real-time PCR was performed on transcripts isolated from two central structures: the dorsal vagal complex (DVC), which constitutes a gateway for immune information from the periphery to the brain, and the hypothalamus, which coordinates feeding behavior. Three hours after DON administration, IL-1β, TNF-α, and IL-6 mRNAs were strongly upregulated both in the hypothalamus and the DVC (Fig. 5). PGE2 are key effectors of sickness behavior observed during inflammatory states. Analysis of transcripts revealed that DON induced a strong upregulation of the PGE2-synthesizing enzymes cyclooxygenase-2 (COX-2) and mPGES-1 mRNA expression, 3 h after the injection, in both structures studied (Fig. 5).

DON-Induced Intoxication in mPGES-1 KO Mice

Based on these observations, we next evaluated the impact of DON treatment on mPGES-1–lacking mice (mPGES-1 −/−). A single oral administration of 12.5 mg/kg DON resulted in a similar reduction of food intake up to 48 h after treatment in both WT and mPGES-1 −/− (Fig. 6A). The quantification of food consumption 3, 6, 12, 18, and 24 h after DON administration revealed that DON affected the dark phase food consumption similarly in WT and mPGES-1 −/− mice (Fig. 6B). Locomotor activity and body core temperature analysis did not reveal any difference in response to *per os* DON administration between WT and mPGES-1 −/− treated mice (Fig. 7). Central structures activated in response to *per os* DON administration were next identified using the immune detection of the early gene product c-Fos. A very low basal level of c-Fos–positive nuclei was observed in the brain stem, pons, and forebrain of water-treated mice whatever the genotype considered (Table 2). DON-treated WT mice exhibited a strong rise in the number of c-Fos–positive nuclei within the nucleus tractus solitarius (NTS) and a moderate increase within the area postrema (AP, Fig. 8, Table 2) and ventrolateral medulla. Noticeably, the serotoninergic raphe formation did not exhibit any DON-induced c-Fos expression whatever the rostrocaudal level considered (Fig. 8,

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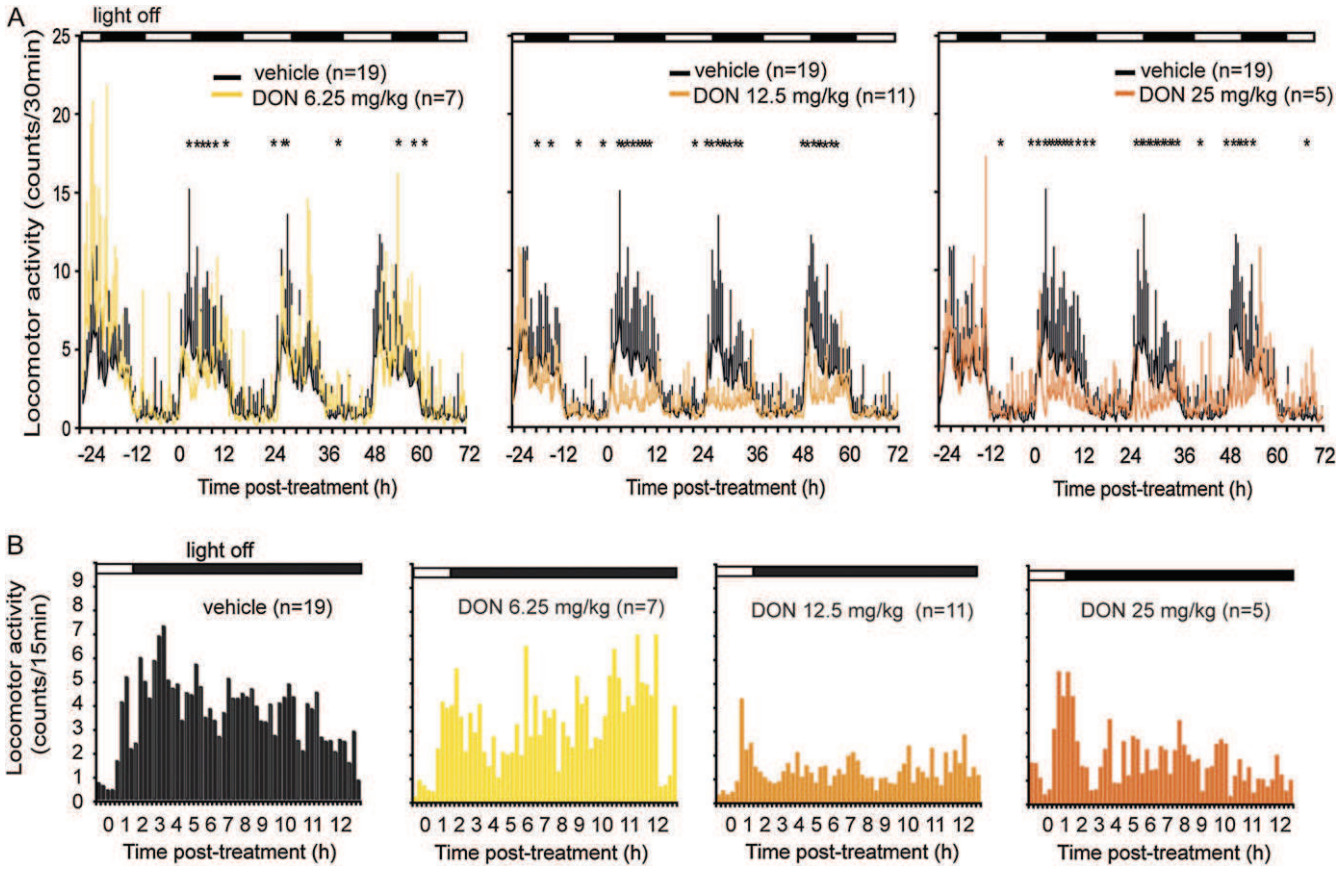


FIG. 2. DON-induced intoxication reduced locomotor activity. (A) Recording of locomotor activity over 96 h of mice treated with either vehicle (water) or DON (12.5 mg/kg). Black and white bars represent the dark and light periods. (B) Effect of increasing DON doses (6.25, 12.5, and 25 mg/kg) on nightly locomotor activity. In (A), $*p < 0.05$, significantly different from vehicle-treated mice.

Table 2). WT animals challenged with DON also displayed a strong rise in c-Fos immunoreactivity in the lateral part of the parabrachial nucleus and in the locus coeruleus (LC) and a moderate-to-strong c-Fos staining within forebrain structures such as the paraventricular hypothalamus nucleus (PVN), arcuate nucleus, median eminence (ME), bed nucleus of the stria terminalis, and central nucleus of the amygdala (CeA) (Fig. 8, Table 2). c-Fos analysis failed to reveal any large differences in the pattern and intensity of cellular activation between DON-treated WT and mPGES-1 −/− mice (Fig. 8, Table 2). Only the CeA of mPGES-1 −/− mice exhibited a higher number of c-Fos–positive nucleus in response to DON when compared with WT counterparts.

DISCUSSION

DON-Induced ''Sickness Behavior'' Symptoms in Mice

The present study was performed using mice as a model. Although less sensitive to DON than pig, mice can nevertheless be considered as a good model for *in vivo* DON toxicity studies (Arnold *et al.*, 1986; Flannery *et al.*, 2011; Rotter *et al.*, 1992). We report here that *per os* DON intoxication modified food intake and affected body core temperature and spontaneous locomotor activity. The anorexic action of DON measured here is in accordance with previous works (for review, Pestka, 2010). Recently, Flannery *et al.* (2011) reported the characterization of DON-induced anorexia using mouse as a model. These authors reported that the reduction of food intake induced by DON exposure is followed by an orexigenic effect observed 14 h after the treatment. Such a rebound in food intake was not observed in the present study. The dissimilar protocols used in these works may explain this difference. Whereas Flannery *et al.* (2011) proceed to a fasting during the light cycle before DON exposure, the present study was performed on animals with free access to food throughout the day. We showed that concomitantly to anorexia, DON induced a fall in locomotor activity and body temperature shortly after administration. The hypothermia observed here in rodents was evocative of the decreased skin temperature observed in young pigs fed low dietary concentrations of DON (Rotter *et al.*, 1994) and of the hypothermia monitored after an exposure to the mycotoxin T-2 (Taylor *et al.*, 1991) or to cisplatin (Vera *et al.*, 2006). We bring here evidence showing that centrally

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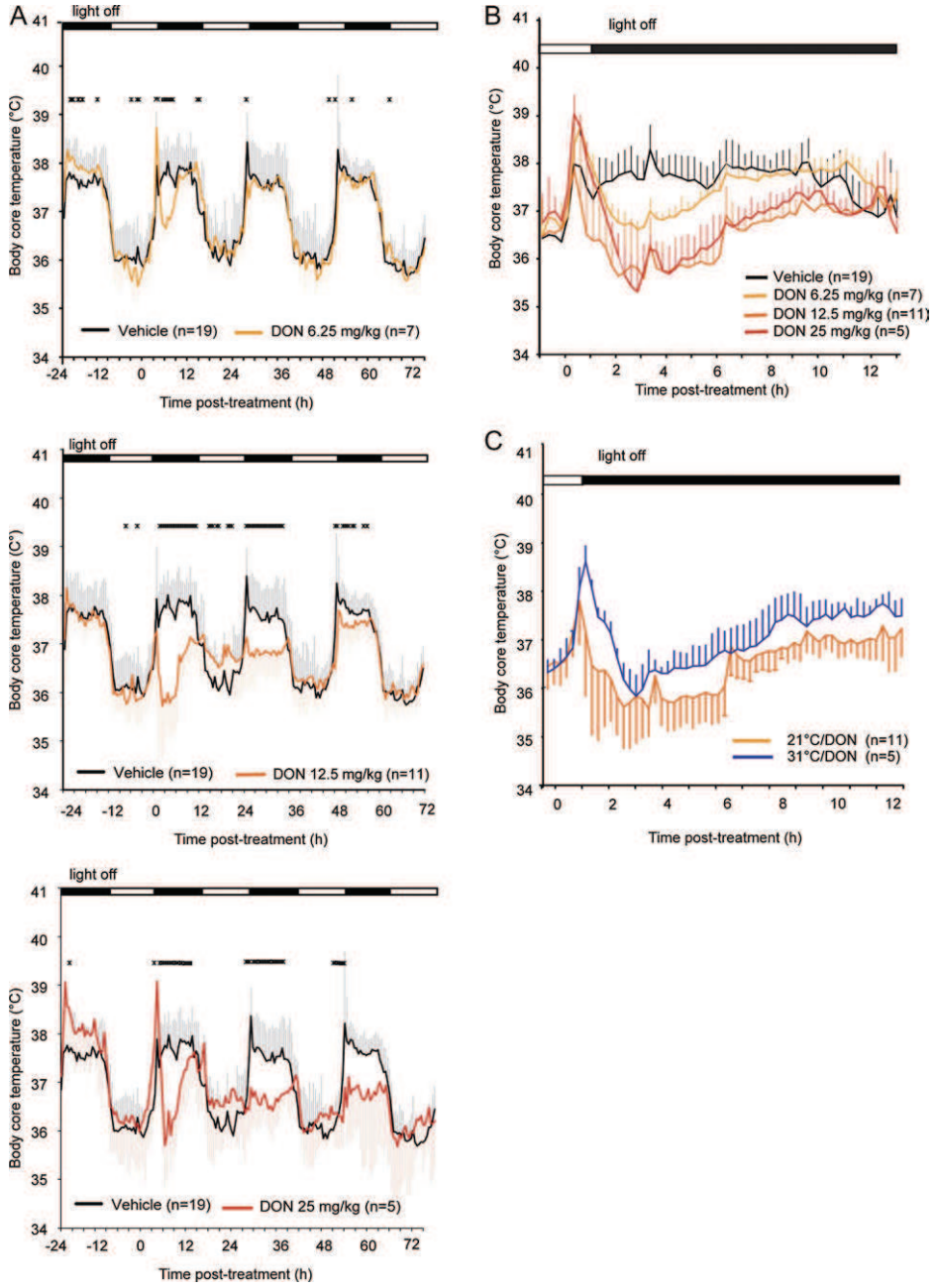


FIG. 3. Monitoring of body core temperature during *per os* DON intoxication. (A) Recording of body core temperature over 96 h of mice treated with either vehicle (water) or DON (12.5 mg/kg). (B) Effect of increasing DON doses (6.25, 12.5, and 25 mg/kg) on nightly body core temperature. (C) Effects of DON (12.5 mg/kg) administration performed at different ambient temperature (21°C vs. 31°C) on nighttime body core temperature. In (A), $*p < 0.05$, significantly different from vehicle-treated mice.

injected DON, at doses ineffective at the periphery, was able to reproduce behavioral features observed after *per os* intoxication. Centrally administered DON induced hypothermia and modulation of locomotor activity in addition to a rapid (< 1 h) reduction in food intake. These results suggested that a part of the orally administered toxin could reach the brain through circumventricular organs. The DON-induced c-Fos staining observed in circumventricular organs and surrounding structures (AP, NTS, ME, and Arc) supported this hypothesis. Moreover, DON was shown to be rapidly distributed in various organs including the brain within a short time after peripheral exposure (Pestka *et al.*, 2008). The symptoms observed in response to DON were evocative of nonspecific symptoms induced by inflammatory challenges. Acute infections and other immune challenges trigger in the host a generalized defense response termed "acute phase reaction," which

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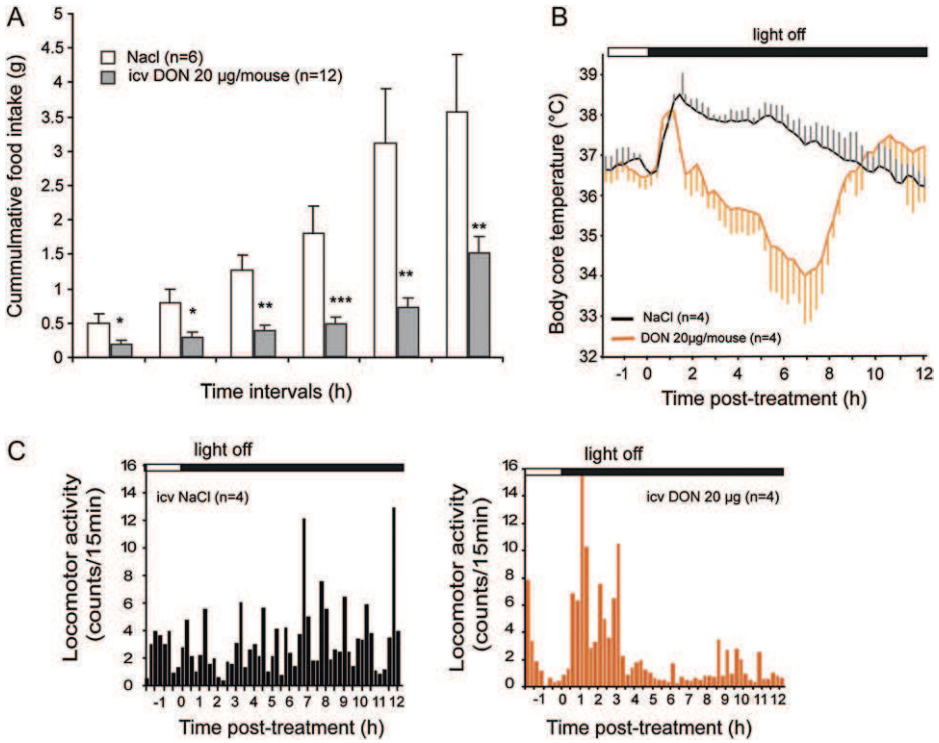


FIG. 4. Effect of ICV DON injection. (A) Food intake of mice having received an ICV injection of either saline (vehicle) or DON (20 µg per mouse). Effect of ICV DON injection (20 µg per mouse) on body core temperature (B) and locomotor activity (C). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, significantly different from vehicle-treated mice.

comprises immune, physiological, and behavioral changes. Sick individuals develop a set of nonspecific symptoms, which includes emesis, anorexia, fever, lethargy, and reduction of social interactions, referred to as sickness behavior (for review, see Hart, 1988). It is well established that a complex neuronal circuitry activated in response to inflammation coordinates sickness behavior. Using c-Fos staining, which remains a useful approach to identify activated neuronal groups (Dragunow and Faull, 1989), we revealed the activation of key autonomic areas, hypothalamic nuclei, and parts of the amygdala. This analysis was performed 3 h after peripheral DON administration. At this time point, structures found activated could be considered instrumental in DON-induced responses as anorexia and associated symptoms were ongoing. The c-Fos pattern observed is consistent with a coordinated autonomic, endocrine, and behavioral response to DON. c-Fos

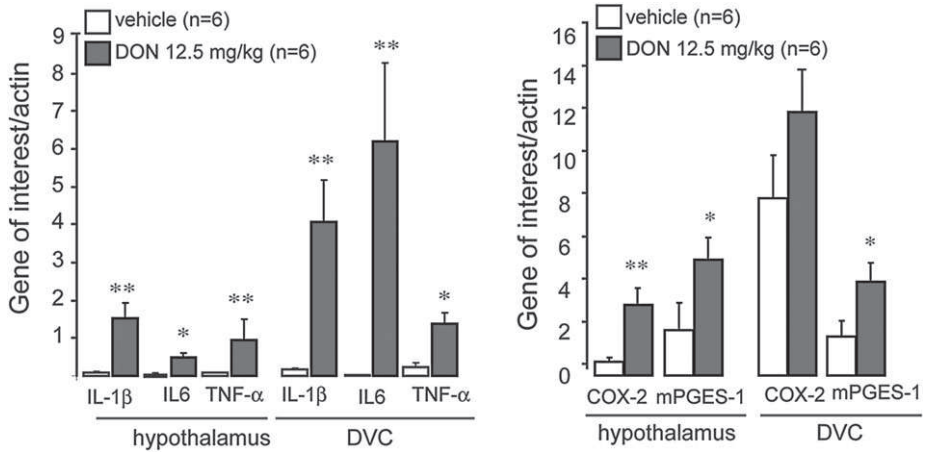


FIG. 5. Central expression of inflammatory biomarkers during DON-induced intoxication. IL-1ß, IL-6, TNFα, COX-2, and mPGES-1 transcript expressions in the hypothalamus and the DVC quantified 3 h after the oral administration of vehicle (water, white bars) or DON (12.5 mg/kg, purple bars). *$p < 0.05$ and **$p < 0.01$, significantly different from vehicle-treated mice.

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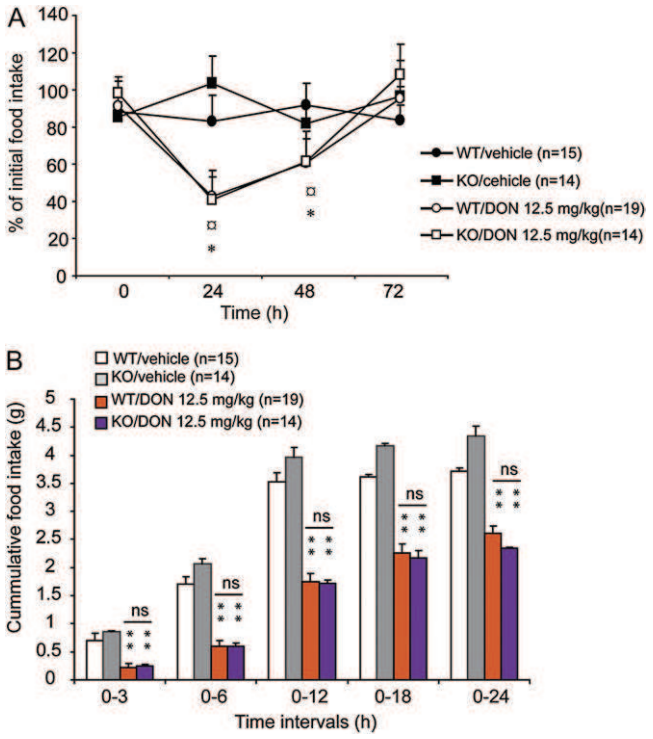


FIG. 6. DON-induced anorexia in mPGES-1 KO mice. (A) Daily food intake (percentage of initial food intake) measured from 0 to 72 h after oral gavage of either water (vehicle) or DON (12.5 mg/kg) in WT and mPGES-1 −/− mice. (B) Cumulative food intake measured over the first 24 h for WT or mPGES-1 −/− mice treated with either vehicle or DON (12.5 mg/kg). In (A), ''¤'' and ''*'' represent $p < 0.05$, significantly different from vehicle-treated mice, for WT and mPGES-1 −/−, respectively. In (B) $^{**}p < 0.01$, significantly different from vehicle-treated mice; ns, nonsignificantly different from DON-treated WT mice.

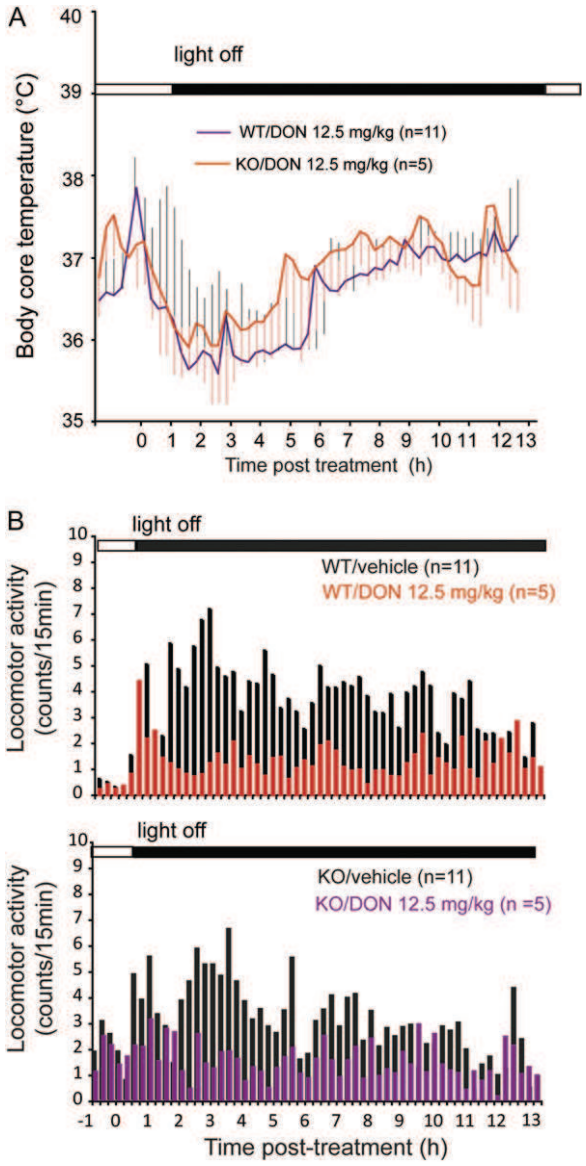


FIG. 7. Effect of DON on locomotor activity and body core temperature in mPGES-1 −/− mice. (A) Recording of body core temperature during the dark phase following *per os* DON (12.5 mg/kg) administration in WT and mPGES-1 −/− mice. (B) Recording of locomotor activity during the dark phase following *per os* DON (12.5 mg/kg) administration in WT and mPGES-1 −/− mice. Black and white bars represent the dark and light periods.

immunoreactivity observed in key brain stem and hypothalamic nuclei such as NTS, Arc, and PVN is consistent with the DON-induced reduction in food intake. Moreover, the observed c-Fos induction within the brain stem and particularly the AP was evocative of nausea-induced anorexia. Using systemic DON administration in rats, Ossenkopp *et al.* (1994) have indeed reported the induction of conditioned taste aversion, which was mediated by the AP. The strong increase in c-Fos immunoreactivity in specific regions of the hypothalamus including the preoptic area/anterior hypothalamus (Boulant, 2000), the PVN and supraoptic nucleus, together with the activation of LC and NTS (Takahashi *et al.*, 2001), could explain the decreased body temperature observed in response to DON. As mentioned earlier, the observed responses to DON, i.e., nausea/emesis, anorexia, modulation of body temperature, and reduced locomotor activity, are also features of sickness behavior that follows an infection. In accordance, the pattern of c-Fos distribution throughout the brain observed after *per os* DON administration was also consistent with the findings of studies performed to identify immunosensitive neurocircuitry involved in the coordinated responses to inflammatory challenges (Lacroix and Rivest, 1997). However, unlike in the case of a response to inflammation, we did not observe any significant elevation of c-Fos immunoreactivity in the ventromedial preoptic area, a cell group adjacent to the organum vasculosum of the lamina terminalis. Finally, c-Fos expression observed in the brain after DON exposure is evocative of previous results showing that in swine, DON intoxication induced changes in extracellular levels of neurotransmitters such as norepinephrine, dopamine, and serotonin in discrete areas of the brain including the hypothalamus and the medulla (Prelusky *et al.*, 1992).

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TABLE 2
c-Fos Signal in Brain Structures of Vehicle- or DON-Challenged Mice

	WT		mPGES-1 −/−	
	Water	DON (12.5 mg/kg)	Water	DON (12.5 mg/kg)
Diencephalon				
Subfornical organ	0/+	++	0/+	++
Paraventricular thalamic nuclei	+++	+++	+++	+++
CeA	0/+	+++	0/+	+++
Laterodorsal part of the bed of the stria terminalis	0/+	+++	0/+	+++
Hypothalamus				
Preoptic area	0/+	+/++	0/+	+/++
Anterior area	+	++	+	++
Paraventricular nucleus	0/+	+++	0/+	+++
Supraoptic nucleus	+	+++	+	+++
ME	0/+	++	0/+	++
Dorsomedian nucleus	0/+	0/+	0/+	0/+
Ventromedian nucleus	0/+	0/+	0/+	0/+
Lateral area	+	++	+	++
Posterior area	+	++	+	++
Parasubthalamic nucleus	0/+	+++	0/+	+++
Arcuate nucleus	0/+	++	0/+	++
Brain stem				
Dorsal raphe nucleus	0/+	0/+	0/+	0/+
Laterodorsal tegmental nucleus	+	++	+	++
Parabrachial nucleus	0/+	+++	0/+	+++
Raphe magnus nucleus	0/+	0/+	0/+	0/+
LC	+	+++	+	+++
Motor trigeminal nucleus	0/+	++	0/+	++
Nucleus of trapezoid body	+++	+++	+++	+++
AP	0/+	+/++	0/+	+/++
Caudal NTS	0/+	++	0/+	++
Postremal NTS	0/+	+++	0/+	+++
Rostral NTS	0/+	+++	0/+	+++
Dorsal motor nucleus of the vagus	0/+	+	0/+	+
Raphe obscurus nucleus	0/+	0/+	0/+	0/+
Raphe pallidus nucleus	0/+	0/+	0/+	0/+
Caudal ventrolateral medulla	+	++	+	++
Rostral ventrolateral medulla	+	++	+	++
Spinal nucleus trigeminal	0/+	0/+	0/+	0/+
Other				
Ependymal cells	0/+	0/+	0/+	0/+
Choroid plexus	0/+	++	0/+	++

Note. 0, no stained cell; +, 130 cells per section; ++, 31-60 cells; +++, 61 and more cells. Red color indicates brain structures where c-Fos signal is significantly different between vehicle- and DON-treated mice.

Neuroinflammation Induced by DON Exposure

DON has been reported to modulate the immune system in murine models and to increase cytokine production in both *in vitro* and *in vivo* murine models (for review, see Pestka, 2010). However, the data illustrating the possible stimulating effect of DON on cytokine production within the CNS are still lacking. Yet, the development of sickness behavior in response to infection is strongly associated not only with the peripheral production of cytokines but also with their central induction. The role of *de novo* cytokine synthesis in discrete areas of the brain has also been illustrated by recent works (Chakravarty and Herkenham, 2005; Wisse *et al.*, 2007). Using genetic models exhibiting impairment of lipopolysaccharide (LPS) and IL-1β signaling, these studies have shown that the central production of cytokines is required to obtain a sustained anorexia in response to LPS or IL-1β. Moreover, we have shown that DON administered at the CNS level provoked anorexia, hypothermia, and activity modulation, suggesting that the peripheral action of DON is not required to observe behavioral changes. In accordance, we showed here that *per os* DON administration induced an upregulation of IL-1β, IL-6, and TNF-α within two central structures, i.e., hypothalamus and DVC. As mentioned earlier for c-Fos study, mRNA analysis was performed relatively shortly after DON

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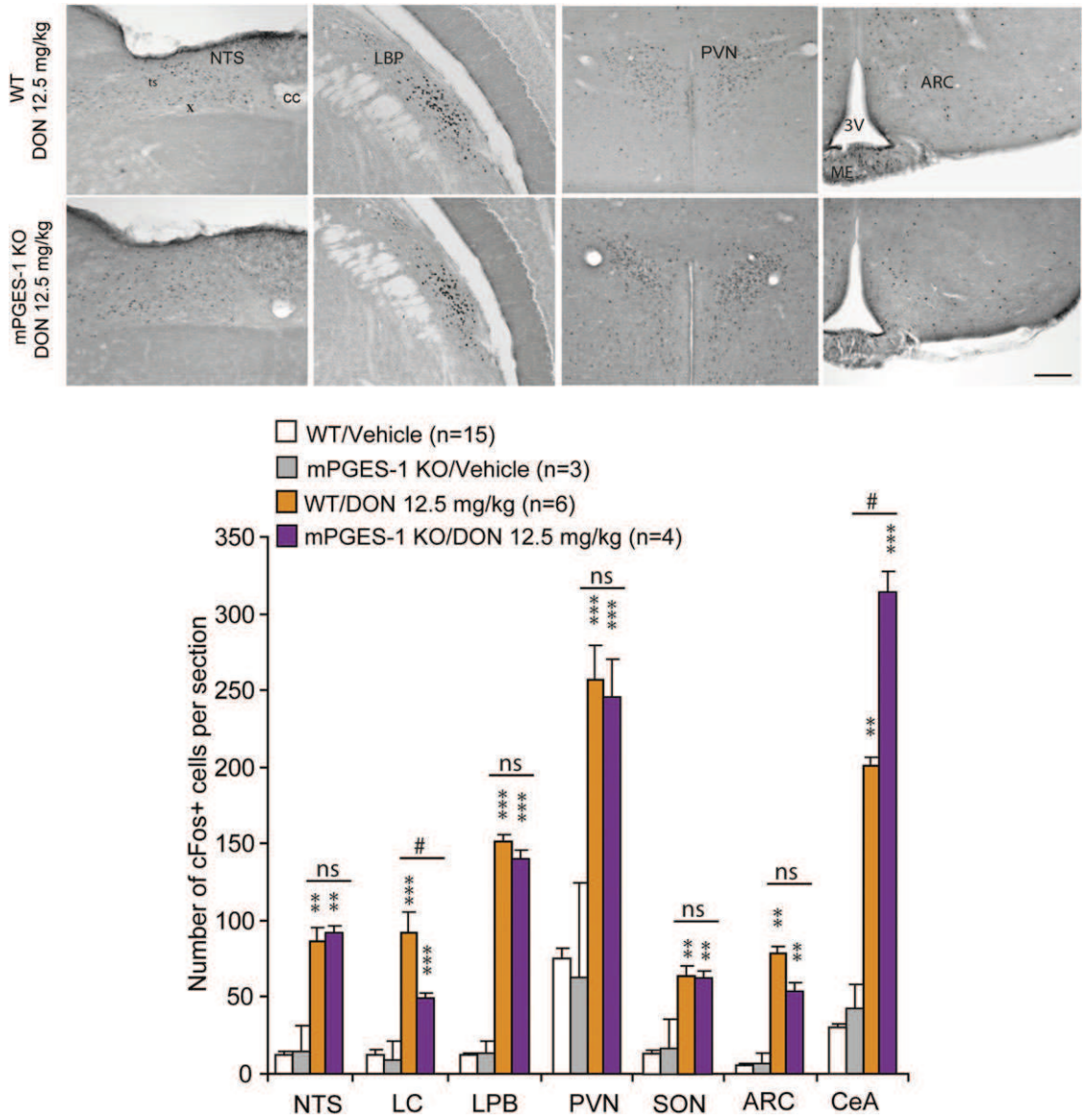


FIG. 8. Effects of *per os* DON administration on c-Fos immunoreactivity in WT and mPGES-1 −/− mice. (A) Representative coronal sections illustrating the c-Fos labeling observed within brain stem, pons, and forebrain regions of WT (upper panel) and mPGES-1 −/− (lower panel) mice treated with DON (12.5 mg/kg) and sacrificed 3 h after treatment. (B) Quantification of the number of c-Fos immunoreactive nuclei within brain stem, pons, and forebrain nuclei observed in WT and mPGES-1 −/− mice treated either with vehicle (water, white bars) or with DON (12.5 mg/kg, orange bars). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from vehicle-treated mice. 3V, third ventricle; ARC, arcuate nucleus; cc, central canal; CeA, central amygdala; LPB, lateral parabrachial; LC, locus coerelus; NTS, nucleus tractus solitarius; PVN, paraventricular nucleus; SON, supraoptic nucleus; X, dorsal motor nucleus of the vagus; ts, tractus solitarius. Scale bar: 100 μm.

administration at a time point where symptoms are clearly visible. These results constitute the first demonstration that *per os* DON administration results in central neuroinflammation. Interestingly, both studied structures, i.e., DVC and hypothalamus, are involved in the regulation of food intake and constitute gateways for immune information from the periphery to the brain. Moreover, c-Fos immunostaining performed in the present work has confirmed their strong activation in response to *per os* DON treatment. The induction of proinflammatory cytokines within these key structures may explain the DON-induced symptoms. A similar increased cytokine expression within these structures was also observed during anorexigenic immune challenge (Pecchi *et al.*, 2006). TNF-α or IL-1β may act as endogenous cryogen and have been proposed to participate in hypothermia during inflammation (Leon, 2004). The increased expression of TNF-α or IL-1β mRNA within the hypothalamus might be linked to the reduced body core temperature observed here after DON administration. Altogether, these results strongly suggest that not only peripheral but also central-borne cytokines may partake in the onset

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of sickness-like behavior observed in response to DON intoxication. The cellular targets of DON within the brain remain to be identified. Given the well-known action of this toxin on innate immune cells such as monocytes and macrophages (Bae and Pestka, 2008; Bae *et al*., 2010; Islam *et al*., 2006), microglial cells that are the resident macrophages in the CNS constitute a likely DON target.

DON-Induced Behaviors Are Independent to PGE2 Production

In addition to cytokine induction, we reported the central upregulation of PGs synthesizing pathways, i.e., COX-2 and mPGES-1 mRNA. The induction of COX-2 gene expression observed here is consistent with previous studies showing that DON upregulates COX-2 expression *in vitro* (macrophage) and *in vivo* (Peyer's patches and spleen; Moon and Pestka, 2002, 2003). On the other hand, our results constitute the first evidences of a stimulatory effect of DON on mPGES-1 mRNA expression. mPGES-1 has been described as a regulated enzyme whose expression is stimulated by inflammatory agents at the periphery and within the CNS. Because COX-2 and mPGES-1 cooperate to synthesize PGE2, an emblematic mediator of inflammation (Pecchi *et al*., 2009), our results suggest a high level of central PGE_2 synthesis during DON challenge. Given the well-known action of PGE2 on the CNS during immune challenge, it can be hypothesized that this centrally produced PGE2 could, at least partially, contribute to the emergence of the DON-induced behavioral symptoms. Indeed, during inflammation and the subsequent production of cytokines, PGE_2 modifies the central neuronal activity to elicit the symptoms of the sickness behavior (Pecchi *et al*., 2009). Moreover, the reduction of PGE_2 production by indomethacin has been shown to attenuate some of the symptoms of sickness behavior such as anorexia (Lugarini *et al*., 2002). Accordingly, we next investigated the potential instrumental role of mPGES-1 and related PGE2 in DON-induced neuronal activation and associated symptoms by using mPGES-1 −/− mice. Mice lacking the mPGES-1 gene have more than 95% reduction in PGE_2 production during inflammation (Trebino *et al*., 2005) and exhibit impaired febrile (Engblom *et al*., 2003) and anorexic responses (Pecchi *et al*., 2006) during IL-1β challenge. Moreover, invalidation of mPGES-1 enzyme strongly reduces inflammation-induced c-Fos expression in immunosensitive brain regions (Dallaporta *et al*., 2007). In the present study, neither cell activation nor behavioral changes observed in response to DON were prevented or modified by mPGES-1 deletion. These results highlight the fact that mPGES-1 and related PGE2 were not crucial for DON-induced symptoms. This result reinforces previous observations showing that interfering with inflammatory signaling does not reduce the susceptibility to DON-induced symptoms and especially anorexia. TNF-α receptors KO and IL-6 KO mice do not exhibit any reduced susceptibility to DON-induced anorexia (Pestka and Zhou, 2000, 2002). Yet, it should be mentioned that TNF-α KO and IL-6 KO studies were performed using DON-contaminated food, which might have reduced food appetence and masked a potential protective effect of TNF-α receptors KO and IL-6 invalidation. COX-2 KO mice were reported to exhibit a decrease in body weight comparable to that of WT after 16 weeks consumption of DON, although food intake was not monitored in this study (Jia and Pestka, 2005). Even though inflammation-related effectors are strongly expressed in key central structures during DON intoxication, the arguments supporting their triggering role in DON-induced anorexia remain limited. Emphasizing this discrepancy, we showed that unlike cytokine-induced hypothermia, which was shown to be dependent of ambient temperature in rodents (Krall *et al*., 2010), DON intoxication resulted in hypothermia both at 21° and 31°C. Overall, our results shed light on the fact that, despite high similarities, infection- and DON-induced sickness behavior involve partially different signaling pathways. This suggests that anti-inflammatory therapeutics targeting the arachidonic acid pathways should not reap huge benefits against behavioral symptoms (anorexia, anapyrexia, and lethargy) associated with acute DON intoxication.

In summary, the present work provides the first demonstration that DON induces a set of nonspecific symptoms and a concomitant central inflammation. Although these results suggest that the central expression of inflammatory effectors could partake in the onset of symptoms as proposed for sickness behavior induced by infection, anti-inflammatory strategies aiming to reduce PGE2 production failed to prevent DON-induced symptoms. Finally, in view of the widespread human exposure to DON, future studies should evaluate the impact of DON-induced central inflammation on brain signaling, especially in individuals suffering from neurological pathologies that involve a clear inflammatory component.

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Annexe 7

Article : c-Fos immunoreactivity in the Pig brain following deoxynivalenol intoxication : focus on NUCB2/nesfatin-1 expressing neurons.

Gaigé S, Bonnet MS, Tardivel C, Pinton P, Trouslard J, Jean A, Guzylack L, Troadec JD, Dallaporta M.

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c-Fos immunoreactivity in the pig brain following deoxynivalenol intoxication: Focus on NUCB2/nesfatin-1 expressing neurons

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ABSTRACT

Deoxynivalenol (DON), produced by the cereal-contaminating *Fusarium* fungi, is a major trichothecene responsible for mycotoxicoses in farm animals, including swine. The main effect of DON-intoxication is food intake reduction and the consequent body weight loss. The present study aimed to identify brain structures activated during DON intoxication in pigs. To this goal, we used c-Fos staining which constitutes a useful approach to identify activated neurons. We showed that *per os* administration of *Fusarium graminearum* extracts (containing the equivalent of 1 mg DON per kg of body weight) induced an increase in c-Fos immunoreactivity in several central structures, including the ventrolateral medulla (VLM), dorsal vagal complex (DVC), paraventricular nucleus of the hypothalamus (PVN), arcuate nucleus (Arc), supraoptic nucleus (SON) and amygdala (CeA). Moreover, we coupled c-Fos staining with phenotypic markers detection in order to specify the neuronal populations activated during DON intoxication. This phenotypic characterization revealed the activation of catecholaminergic but not of serotoninergic neurons in response to the toxin. In this context, we also paid a particular attention to NUCB2/nesfatin-1 positive cells, since nesfatin-1 is known to exert a satiety effect. We report here, for the first time in the pig brain, the presence of NUCB2/nesfatin-1 neurons in the VLM, DVC, PVN, Arc and SON, and their activation during DON intoxication. Taken together, these data show that DON stimulates the main structures involved in food intake in pigs and suggest that catecholaminergic and NUCB2/nesfatin-1 neurons could contribute in the anorexigenic effects of the mycotoxin.

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1. Introduction

Fusarium fungi are common parasites growing on cereals cultivated in Europe, America and Asia. The extent of cereal contamination is strongly associated with rainfall and moisture and with grain storage conditions. These fungi produce various mycotoxins which contaminate animals and human food commodities. Among these mycotoxins, deoxynivalenol (DON) is one of the most abundant trichothecenes found in cereals such as wheat, barley, oats, rye, and maize, and less often in rice (Pestka, 2010a). DON has been shown to be implicated in mycotoxicoses in both humans and farm animals. DON intoxication is characterized by a set of symptoms including diarrhea, anorexia, diminished nutritional efficiency, vomiting and reduced weight gain. The nausea episodes observed in pigs having consumed corn contaminated with *Fusarium graminearum* lead to the isolation of DON which was called vomitoxin (Vesonder et al., 1973). During severe intoxications, the toxin can induce hemorrhages and circulatory shocks leading to death (Pestka, 2010a). The immune system activation was proposed by several authors to contribute in vomitoxin toxicity. DON induces a vigorous expression of proinflammatory genes such as interleukin (IL)-6, tumor necrosis factor (TNF)-alpha or IL-1 beta in different animal models (Harvey et al., 1991; Zhou et al., 1998; Mikami et al., 2010; Pestka, 2010b). Among the farm animals exposed to DON, the pig is the most sensitive species, and consumption of cereals and cereal-derived products contaminated with DON results in feed refusal and reduced weight gain (Trenholm et al., 1984). These symptoms lead to growth retardation and can have serious economic consequences. Numerous cases of swine intoxication after consumption of *Fusarium*-infected food were described especially in the USA (Osweiler et al., 1990), Canada (Abramson et al., 1997) or Australia (Moore et al., 1985). In the pig, food intake reduction is observed from 1 mg DON per kg of body weight (bw), and beyond this value a 4.3% decrease in food consumption is observed for each

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additional mg/kg of DON (Forsyth et al., 1977). While the peripheral action of DON was extensively studied (Pestka, 2010a), data illustrating its effects on the central nervous system (CNS) are significantly less abundant. Despite the described modulation of feeding behavior induced by DON consumption, the data illustrating the mechanisms by which this toxin exerts its anorexigenic action remain partial. It is now clearly established that appetite regulation relies on a complex neurocircuitry that links body fat stores changes to adaptive adjustments of feeding behavior (Morton et al., 2006). In the CNS, specific neuronal pathways within specific brain areas, mainly the hypothalamus and the brainstem, are clearly involved in this control of feeding behavior. Recently, in previous studies based on c-Fos immunostaining, we clearly observed the activation of key central areas and anorexigenic networks during DON intoxication in mice (Girardet et al., 2011a,b). This pattern was consistent with a coordinated behavioral (anorexia) response to DON. These studies conducted on mice needed to be strengthened on the pig which remains the most relevant model to study DON toxicity. In the present study, we performed the first functional neuroanatomic study using c-Fos expression in DON-intoxicated-pig. In addition, we paid a special attention to the activation of NUCB2/nesfatin-1 neuronal population in response to the toxin. Nesfatin-1, which derives from the cleavage of the precursor NUCB2, consists in an 82 amino acid peptide with a potent anorexigenic action on rodents after both central (Oh-I et al., 2006) and peripheral (Shimizu et al., 2009) administration. At the central level, nesfatin-1 is expressed by neuronal populations located in different brain areas including hypothalamic and brainstem nuclei (Goebel et al., 2009a). These nesfatinergic neurons were reported to be activated in response to refeeding or intraperitoneal injection of cholecystokinin (CCK), thereby contributing to satiety signaling (Kohno et al., 2008; Noetzel et al., 2009). Moreover, these neurons were shown to be activated in response to other stimuli including restraint stress (Goebel et al., 2009b; Könczöl et al., 2010; Okere et al., 2010; Yoshida et al., 2010), abdominal surgery (Stengel et al., 2010), and lipopolysaccharides (LPS)-induced inflammation (Bonnet et al., 2009). Thus, we performed here the first characterization of NUCB2/nesfatin-1 expression in the piglet brain and looked for evidence of an activation of this neuronal population during DON intoxication in this model.

2. Materials and methods

2.1. DON production

DON was produced as previously described (Duverger et al., 2011). Briefly, production assays were carried on Petri dishes (15 cm diameter) containing 50 g of wheat in 50 ml distilled water. Cultures were incubated at 22 °C for 4 weeks. DON was extracted from homogenized culture material by mechanical agitation overnight with 150 ml acetonitrile/water (90:10, v/v). Extracts were then filtered through Whatmann no 1 filter and tested for mycotoxin content. DON was quantified by liquid chromatography–mass spectrometry method (Monbaliu et al., 2010). The final DON concentration in the *F. graminearum* extracts was 2.1 g per kg of powder. In addition to DON, traces of other trichothecenes, expressed in mg per kg of powder, were found: de-epoxy-DON-1: 0.010, 15 acetyl-DON: 0.006, 3 acetyl-DON: 0.001, fusarenon X: <0.001, nivalenol: 0.009 and zearalenone: 0.003.

2.2. Experimental design

4-Week-old weaned female piglets (12 kg; Pietrain/Duroc/Large-white) were acclimatized for 1 week (animal facility, INRA ToxAlim Unit-Toulouse, France), prior to being used in experimental protocols. Young animals were used in the present study since young animals from different species i.e. mouse (Pestka and Amuzie, 2008), poultry (Andretta et al., 2012b) and pig (Andretta et al., 2012a) were reported to be more sensitive than older. Moreover, the use of young animals limits the toxin quantity necessary for the experiments. All animal experimentation procedures were carried out in accordance with the European Guidelines for the Care and Use of Animals for Research Purposes (Directive 86/609/EEC). Each animal (n = 3 per condition) received by gavage one of the different solutions (control water or DON at the concentration of 1 mg/kg bw). Animals were sacrificed 3 h after treatment (without free access to food) by intramuscular injection with ketamine (10 mg/kg; Imalgen, Merial) and xylazine (0.1 mg/kg; Rompun, Bayer). Animal perfusion was achieved with 100 ml of 0.1 M phosphate buffered saline (PBS) followed by 1 l of 4% paraformaldehyde (PFA, Sigma) in 0.1 M PBS, 10 U/ml of heparine (Choay, Sanofi-Aventis). Brains were post-fixed for 1 h in 4% PFA at room temperature, rinsed in PBS and then cryoprotected for 24–48 h in 30% sucrose at 4 °C. For each piglet, plasma, urine and intestinal fluid were collected for DON and cytokine quantifications.

2.3. DON quantification in body fluids

Prior to DON measurement, plasma was prepared from blood by centrifugation and stored at −20 °C. Supernatant fractions were first heated at 100 °C for 5 min to inactivate endogenous enzymes and precipitate proteins and then centrifuged at 15,000 × g for 10 min. The resulting supernatant was used with appropriate dilution for DON analysis. DON was quantified using a Veratox High Sensitivity (HS) ELISA (Neogen) as previously described (Pestka and Amuzie, 2008). Briefly, 100 μl aliquots of DON standards (1–200 ng/ml) or diluted samples were mixed with 100 μl of diluted enzyme conjugates and then incubated in antibody-coated microtiter wells for 45 min. After incubation and wash with distilled water, DON-horseradish peroxidase substrate (100 μl) was added and incubated for 20 min. The reaction was ended by adding 100 μl of stop reagent and plates read at 690 nm on an ELISA plate reader (Spectra thermo).

2.4. Cytokine quantification in plasma

Prior to DON measurement, plasma was prepared from blood by centrifugation and stored at −20 °C. Concentrations of IL-6, IL-1beta and TNF-alpha were measured by enzyme linked immunoabsorbent assays (ELISA) using porcine specific commercial kits according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

2.5. Immunohistochemistry procedures

After freezing the brains in isopentane (−40 °C), coronal sections (40 μm thick) were prepared with a cryostat (Leica CM3050) and collected serially in PBS (0.1 M; pH 7.4). Brains were cut from caudal brainstem to forebrain. Each structure was identified using previous architectonic descriptions of the pig brain (Félix et al., 1999; Ettrup et al., 2010; Równiak et al., 2010).

c-Fos immunohistochemistry: c-Fos immunohistochemistry was performed on free-floating sections using an anti-c-Fos rabbit antiserum synthesized against amino acids 4–17 of the human protein (1:5000, Ab-5, Calbiochem) as previously described (Dallaporta et al., 2007). Briefly, the free-floating sections were incubated 10 min in a solution containing 0.3% H_2O_2 in 0.1 M PBS for quenching of endogenous peroxidase activity. After 1 h in PBS containing 3% normal horse serum (NHS) and 0.3% Triton X-100, sections were incubated for 48 h at 4 °C in PBS containing 3% NHS,

0.3% Triton X-100 and anti-c-Fos antibody. A biotinylated goat anti-rabbit IgG (1:600, Vector Labs) was used as secondary antibody. After incubation with the avidin–biotin complex (1:200, Vector Labs), horseradish peroxydase activity was visualized using a nickel-enhanced diaminobenzidine (DAB). The reaction was closely monitored and terminated when optimum intensity was achieved (3–5 min) by washing the sections in distilled water. Non-specific labeling was assessed on alternate slices treated identically to the above but in which the primary antibody was omitted. Finally, sections were mounted on gelatin-coated slides, air dried, dehydrated with increasing ethanol

Table 1
Dosage of plasmatic cytokines (IL-6 and IL-1 beta) and DON concentrations in body fluids of control and intoxicated-pigs. Significantly different from control animals, $^{*}p < 0.05$; $^{**}p < 0.01$.

	per os vehicle (water)	*per os* DON (1 mg/kg)
DON (plasma)	0.02 μg/ml (±0.01)	0.52 μg/ml (±0.29)*
DON (urine)	0.64 μg/ml (±0.04)	8.61 μg/ml (±0.85)**
DON (intestinal fluid)	0.27 μg/ml (±0.34)	1.51 μg/ml (±030)**
IL-1 beta (plasma)	6.00 pg/ml (±0.81)	12.47 pg/ml (±1.60)*
IL-6 (plasma)	107.20 pg/ml (±2.59)	370.93 pg/ml (±46.43)*

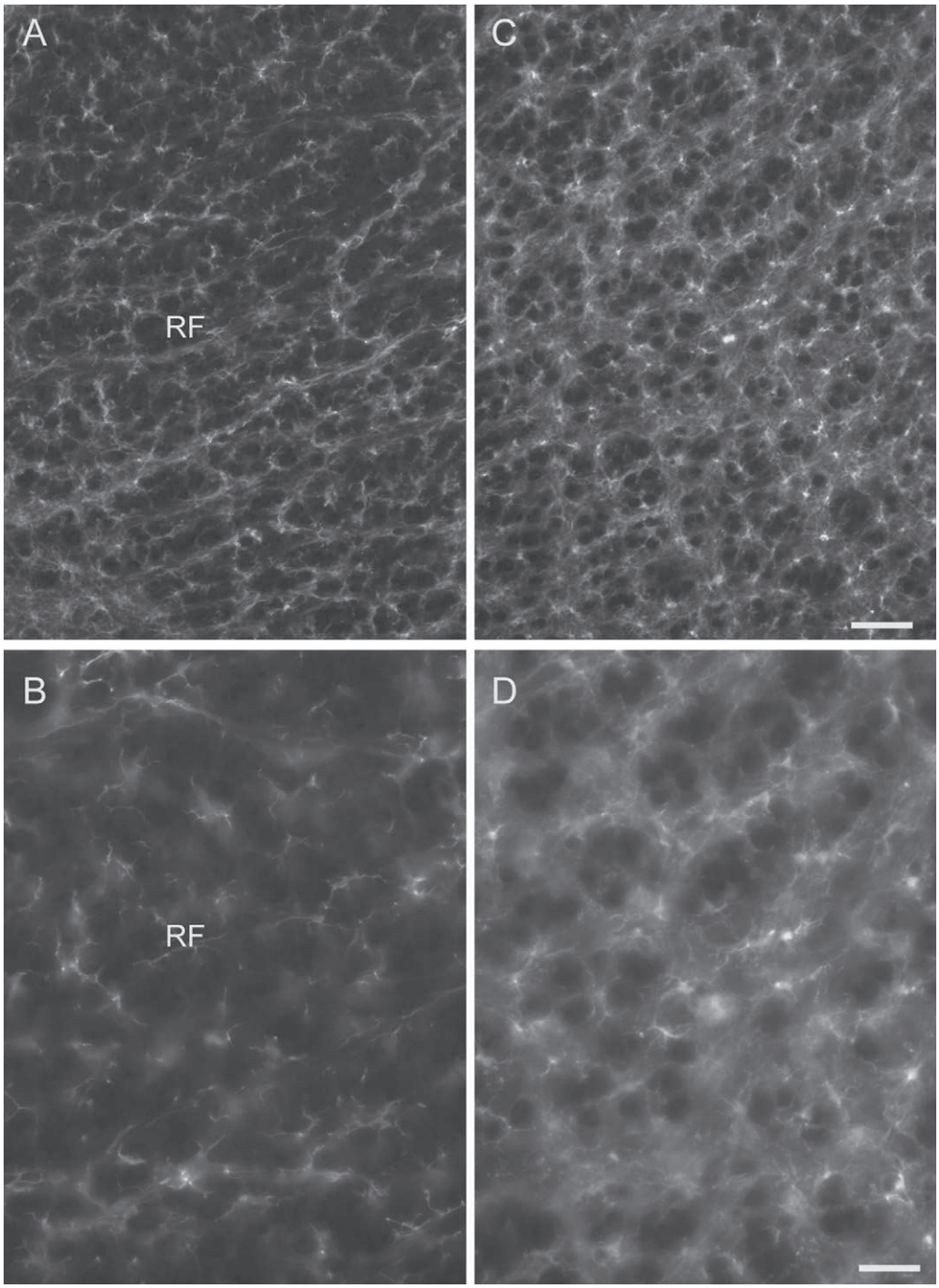


Fig. 1. Representative photographs show Iba1 immunostained microglia in brainstem coronal sections. DON-treated animal ((A) and (B)) shows a clear microglia activation compared to the control animal ((C) and (D)). RF: reticular formation. Scale bars: 100 μm in (A) and (C); 20 μm in (B) and (D).

concentrations (70%, 95%, twice in 100% [v/v] – for 3 min each), incubated for a further 6 min in xylene, and coverslipped with Eukitt embedding medium. When associated with phenotyping labeling, c-Fos staining was performed using the same anti-c-Fos rabbit antiserum at the same dose and revealed by means of a donkey-Alexa 488 conjugated anti-rabbit IgG (1:400, A21206, Invitrogen). In this case, the sections were incubated for 2 h at room temperature with the secondary antibody.

Phenotypic identification: To determine potential activation of NUCB2/nesfatin-1, tyrosine hydroxylase (TH), serotonin (5-HT) and somatostatin (SOM) expressing neurons in response to DON (1 mg/kg) administration, we performed double immunohistochemistry labeling on c-Fos stained sections. The identification of NUCB2/nesfatin-1 neurons was performed by incubating sections with a mouse monoclonal primary antibody raised against nesfatin-1 and its precursor NUCB2 (1:5000, mAb, Alexis

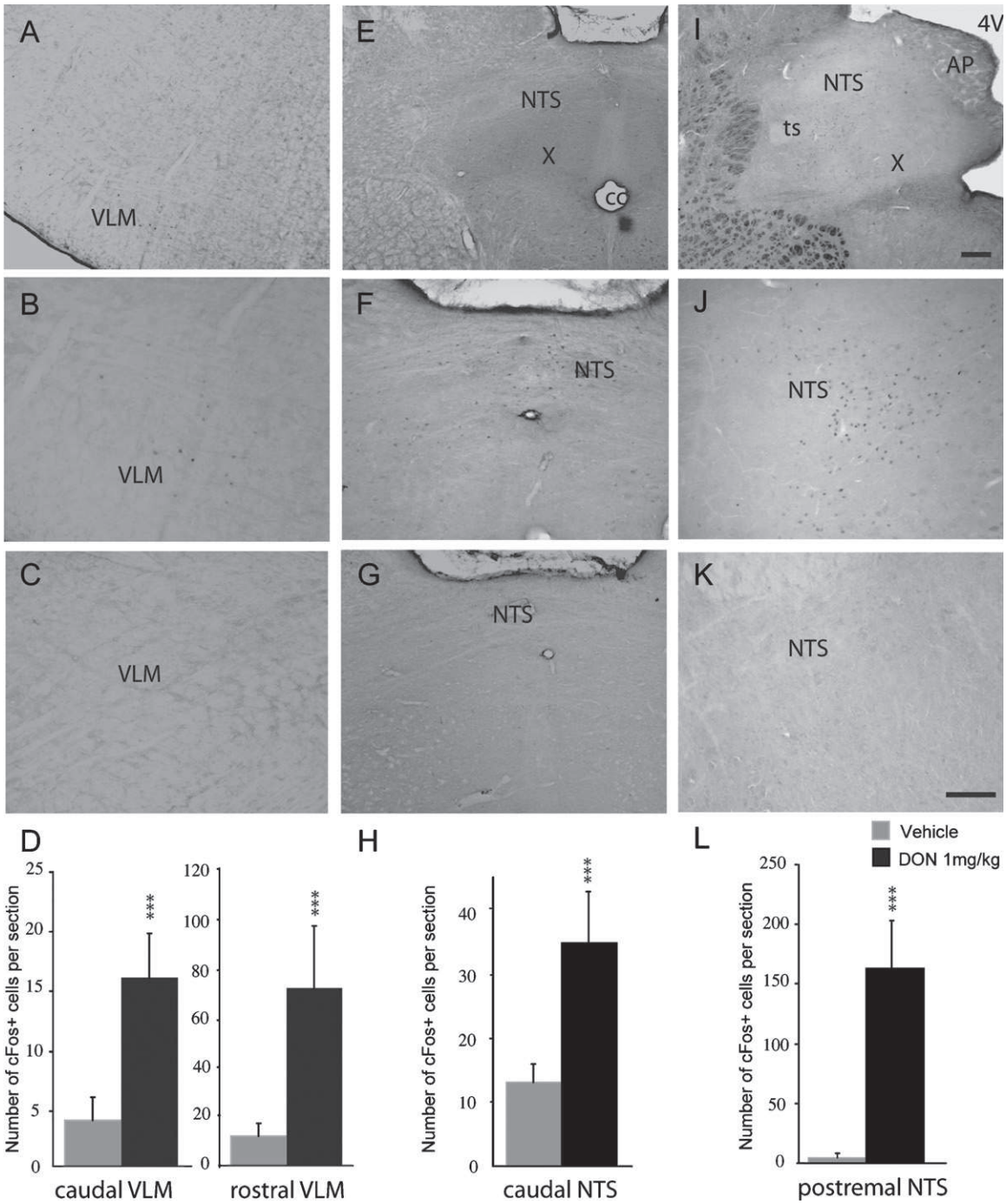


Fig. 2. Effect of DON treatment on c-Fos immunoreactivity within the ventrolateral medulla (VLM) and dorsal vagal complex (DVC). (A)–(C) Representative coronal sections of the VLM of DON-treated pigs at low (A) and high magnification (B). The absence of labeling in control animal is illustrated in (C). Two different anatomical levels of the DVC were illustrated: the postremal DVC ((E)–(G)) and the rostral DVC ((I)–(K)). (E) and (I) Low magnification of representative coronal sections of DON-treated animals. High magnifications of brainstem sections illustrating c-Fos labeling in the nucleus tractus solitarius (NTS) of DON-treated ((F), (J)) and control pigs ((G), (K)) allow clear visualization of c-Fos induction in treated animals. Number of c-Fos immunoreactive nuclei in the VLM (D) and NTS ((H) and (L)) of control (gray bars, $n = 3$) and DON-treated (black bars, $n = 3$) pigs. Significantly different from control animals, $^{***}p < 0.001$. AP, area postrema; X, dorsal motor nucleus of the vagus; ts, tractus solitarius; 4 V, fourth ventricle; cc, central canal. Scale bars: 200 μm in (A), (D) and (G); 100 μm in (B)–(C), (E)–(F) and (H)– (I).

Biochemicals). For TH detection purpose, brainstem and pontine sections were incubated with an anti-TH mouse monoclonal antibody (1:1000, MAB318, Chemicon). 5-HT expressing neurons of the raphe nuclei were identified using an anti-5-HT goat polyclonal antibody (1:600, ImmunoStar Inc.). An anti-SOM goat polyclonal antibody (1:400, D20, Santa Cruz Biotechnology) was used to visualize SOM-expressing neurons located within the central nucleus of amygdala (CeA). NUCB2/nesfatin-1 and TH labelings were revealed using a goat-Alexa 594 conjugated anti-mouse IgG (1:400, A11032, Life Technologies) whereas 5-HT and SOM were revealed with a donkey-Alexa 594 conjugated anti-goat IgG (1:400, A11058, Invitrogen).

Iba1 labeling: For Iba1 detection, brainstem sections were incubated with an anti-Iba1 rabbit monoclonal antibody (1:500, 019-19741, Wako Chemicals). Iba1 labeling was revealed using a donkey-Alexa 488 conjugated anti-rabbit IgG (1:400, A21206, Invitrogen).

Finally, all sections were mounted on gelatin-coated slides, air dried, and coverslipped with homemade Mowiol (calbiochem) mounting medium for fluorescence microscope preparation.

2.6. Microscopy, image analysis and cell count

c-Fos immunostaining was further analyzed by counting the positive nuclei on DAB-revealed sections. For each brain structure studied and each staining, the counts performed on 4–6 distinct hemisections were averaged. c-Fos positive nuclei counting was performed on photomicrographs acquired using a 10 fold lens with a DXM 1200 Camera (Nikon) coupled to ACT-1 software. The microscope was set at a specific illumination level, as was the camera exposure time. c-Fos positive nuclei were then manually counted on these pictures. For double labeling, fluorescent images were acquired on a confocal microscope (ZEISS LSM 710) using the 488 nm band of an Ar-laser and the 543 nm band of an He/Ne laser for excitation of FITC and TRITC, respectively. Images were sequentially acquired and merged to allow visualization of activated neurons. Images were analyzed and prepared using ZEN2009 light Edition (Zeiss software) and Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA, USA). Only contrast and brightness were adjusted and the figures were not otherwise altered.

2.7. Statistical analysis

Data are represented as mean ± S.E.M. Comparisons between data from vehicle- and DON-treated piglets were performed using unpaired 2-tailed Student's *t*-test. $p < 0.05$ was considered significant.

3. Results

3.1. Assessment of DON intoxication

To evaluate the extent of DON contamination following *per os* administration of *F. graminearum* extracts (containing the

Table 2
c-Fos signal and NUCB2/nesfatin-1/c-Fos colocalization in brain structures of control and DON-challenged pigs. +++: strong signal or colocalization, ++: moderate signal or colocalization, +: low signal or colocalization, 0: no detectable signal or colocalization. +++, ++, + and 0 refer to the average number observed in replicate samples. Bold indicates brain structures where c-Fos signal is significantly different between vehicle- and DON-treated pigs. Red indicates brain structures where c-Fos and NUCB2/nesfatin-1 strongly colocalize in DON-treated pigs.

	per os vehicle (water)	*per os* DON (1 mg/kg)	*NUCB2/Nesfatin-1*	*NUCB2/Nesfatin-1/c-fos colocalization*
Diencephalon				
Central nucleus of the amygdala	0/+	+++	++	0/+
Hypothalamus				
Periventricular nucleus	0/+	+++	+++	++
Paraventricular nucleus	0/+	+++	+++	++
Supraoptic nucleus	0/+	+++	+++	+++
Median eminence	0	0/+	0	0
Dorsomedian nucleus	0	0/+	0	0
Ventromedian nucleus	0/+	++	+++	++
Lateral area	0/+	+/++	++	+
Posterior area	0	0	0	0
Arcuate nucleus	0/+	+/++	++	+
Brainstem				
Dorsal raphe nucleus	0	0/+	0	0
Parabrachial nucleus	0	0	0	0
Raphe magnus nucleus	0/+	0/+	0/+	0/+
Locus coeruleus	0	0/+	+	0
Nucleus of trapezoid body	0	+/++	+/++	+/++
Area postrema	0	+/++	0	0
Caudal NTS	0/+	++	++	+
Postremal NTS	0/+	+++	++/+++	++
Rostral NTS	0/+	++	0/+	+
Dorsal motor nucleus of the vagus	0	0	++	0
Caudal Ventolateral medulla	0/+	+/++	++	+/++
Rostral ventolateral medulla	0	++	0	0
Raphe pallidus nucleus	0	0/+	0/+	0/+
Other				
Ependymal cells	0	+/++	0	0
Choroïd plexus	0/+	+/++	0	0

equivalent of 1 mg DON per kg of body weight), a quantification of toxin content was performed in different fluids 3 h after treatment. In the plasma, urine and intestinal fluids, DON concentration was strongly increased in intoxicated piglets when compared with control animals (Table 1). DON concentration was increased by a factor of 22.3, 13.3 and 5.8 in the plasma, urine and intestine respectively in DON-treated animals. Moreover, as expected from previous works (Mikami et al., 2010, 2011), DON treatment induced a systemic inflammation in piglets as attested by the increased expression of pro-inflammatory cytokines. The plasmatic concentration of IL-1 beta increased from 6.0 pg/ml in control animals to 12.4 pg/ml in treated piglets (Table 1). Similarly, the plasmatic concentration of IL-6 was augmented by a factor of 3.4 (Table 1). However, in our conditions i.e. 3 h after intoxication, no modification in plasmatic TNF-alpha content was observed (data not shown). Immunohistochemistry against the microglia marker

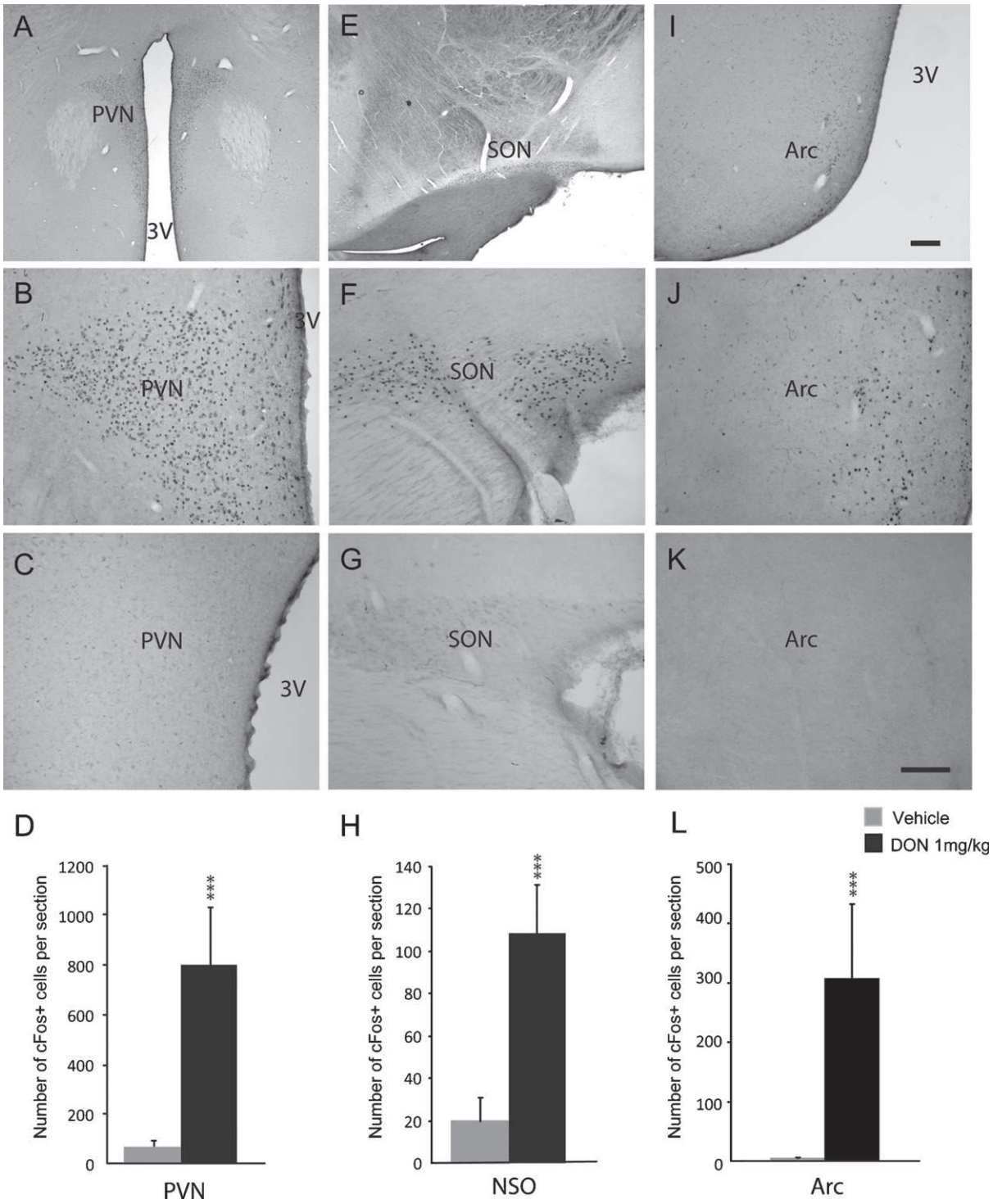


Fig. 3. Representative photomicrographs of c-Fos immunoreactivity in representative coronal sections of the paraventricular nucleus of the hypothalamus (PVN, (A)–(C)), supraoptic nucleus (SON, (E)–(G)) and arcuate nucleus (Arc, (I)–(K)). Images derived from DON-treated ((A)–(B), (E)–(F) and (I)–(J)) and control pigs ((C), (G) and (K)). Number of c-Fos immunoreactive nuclei in the hypothalamus ((D), (H) and (L)) of control (gray bars, $n = 3$) and DON-treated (black bars, $n = 3$) pigs. Significantly different from control animals, $^{***}p < 0.001$. 3 V, third ventricle. Scale bars: 200 μm in (A), (D) and (G); 100 μm in (B)–(C), (E)–(F) and (H)– (I).

Iba1 was performed to detect a microglia activation which could indicate an inflammatory reaction in the brain of DON-treated animals. An intensified Iba1 immunostaining density and an activated-form of microglia which display an increased cell size and irregular shape were observed in DON-treated pig brain compared to the control group (Fig. 1).

3.2. *Effect of* F. graminearum *extracts intoxication on c-Fos expression in the brainstem and pontine areas*

A very low basal level of c-Fos positive nuclei was observed in the caudal brainstem of control (water) animals (Fig. 2 and Table 2). DON-treated piglets exhibited an elevated number of c-Fos positive nuclei in the ventrolateral medulla (VLM) and throughout the dorsal vagal complex (DVC) including the nucleus tractus solitarius (NTS) and the area postrema (AP, Fig. 2). Counts of positive nuclei in the VLM and DVC revealed significant increases in the number of c-Fos labeled nuclei in treated animals when compared with control animals (Fig. 2). In DON-challenged animals, the induction of c-Fos staining was higher in the rostral part of the VLM than in the caudal part of this nucleus (Fig. 2A–C). In sub-regions of the DVC, we observed a weak c-Fos induction in the caudal part of the NTS and a more striking increase in the postremal division of the nucleus (Fig. 2E–K). As an example of

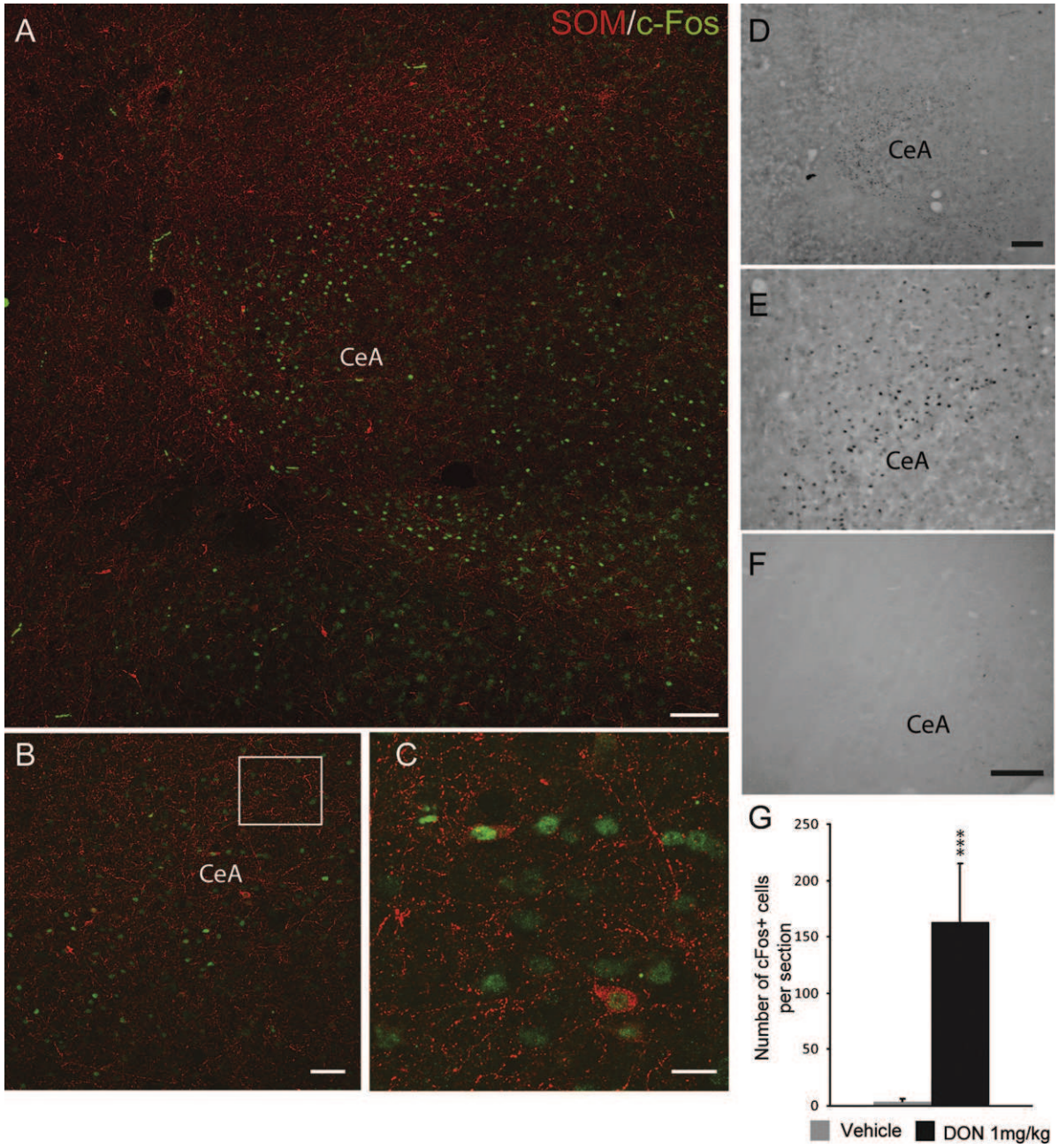


Fig. 4. Effect of DON treatment on c-Fos immunoreactivity within the central nucleus of the amygdala (CeA). (A)–(C) Confocal analysis of somatostatin (SOM) and c-Fos double-labeling in sections of DON-treated pig. (A) Low magnification of immunostaining revealed the dense SOM immunoreactivity in the CeA. (B)–(C) High magnification of SOM/c-Fos labeling within the CeA revealed double labeled neurons. The area where the higher magnified image (C) originates from is indicated by a bounded box in the low power micrograph (B). (D)–(F) Representative coronal sections of the CeA of DON-treated pigs at low (D) and high magnification (E). The absence of labeling in control animals is illustrated in (F). (G) Number of c-Fos immunoreactive nuclei in the CeA of control (gray bars, $n = 3$) and DON-treated (black bars, $n = 3$) pigs. Significantly different from control animals, $^{***}p < 0.001$. Scale bars: 100 μm in (A); 50 μm in (B); 20 μm in (C), 200 μm in (D) and 100 μm in (E)– (F).

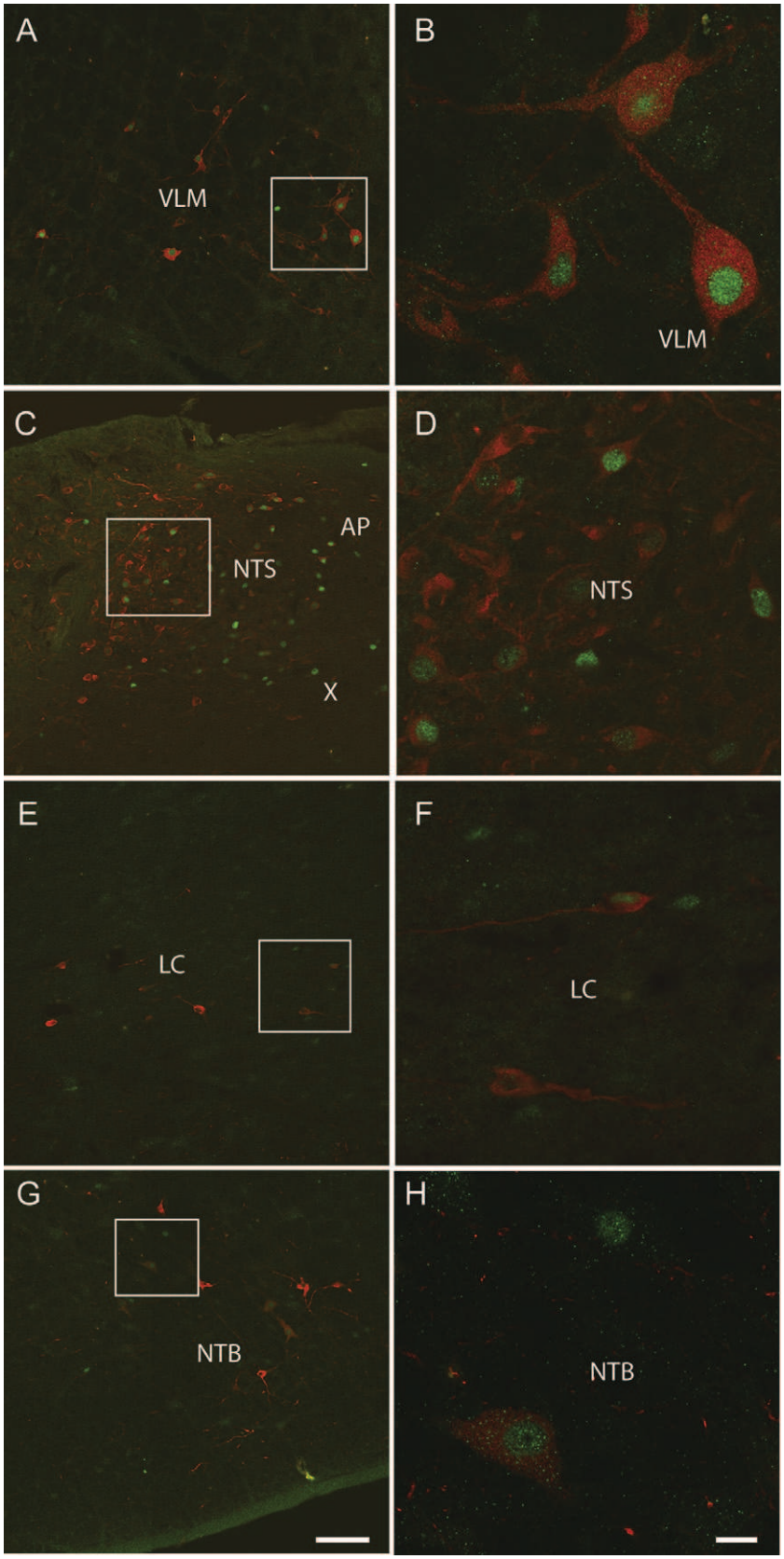


Fig. 5. Confocal analysis of thyrosine hydroxylase (TH) and c-Fos double-labeling in the ventrolateral medulla (VLM, (A) and (B)), nucleus tractus solitarius (NTS, (C)–(D)), locus coeruleus (LC; (E)–(F)) and nucleus of trapezoid body (NTB, (G)–(H)) of DON-treated pig. (A), (C), (E) and (G) Low magnification of TH and c-Fos immunolabeling. (B), (D), (F) and (H) High magnification of TH/c-Fos labeling revealed the presence of double labeled neurons in all structures. The areas where the high magnified images originate from are indicated by a bounded box on the low power photomicrographs. Scale bars: 100 μm in (A), (C), (E) and (G): 20 μm in (B), (D), (F) and (H).

DON-induced c-Fos expression, the number of c-Fos positive nuclei in the postremal NTS reached 163.4 ± 39.35 in DON-treated animals versus 4.7 ± 3.9 in control animals (Fig. 2I–K). Whatever the level considered (postremal or rostral) the c-Fos labeling within the AP remained moderate (Fig. 2 and Table 2). More rostrally, a small number of c-Fos positive nuclei was seen in the pontine structures of control animals (Table 2) and DON-treated animals displayed only a moderate rise in c-Fos immunoreactivity in the locus coeruleus (LC; Table 2). Finally, other structures exhibited a moderate to low increase in the c-Fos signal in response to DON administration including the spinal nucleus of the trigeminal and cochlear nucleus (Table 2).

3.3. *Forebrain patterns of c-Fos immunoreactivity in* F. graminearum *extracts-challenged piglets*

In the forebrain of control animals, only few regions exhibited low to moderate c-Fos immunoreaction (Fig. 3 and Table 2). Interestingly, in DON-treated animals, a strong c-Fos staining was observed within forebrain structures either involved in the regulation of food intake behavior or classically recognized as immuno-sensitive regions such as the paraventricular nucleus of the hypothalamus (PVN), the arcuate nucleus (Arc) and the supraoptic nucleus (SON; Fig. 3). A more moderate increase was observed in the hypothalamic lateral area (HLA) of DON-treated animals. Finally, the central nucleus of amygdala (CeA) identified by means of SOM immunoreactivity (Równiak et al., 2010;Fig. 4A–C) also exhibited an increase in c-Fos expression after DON intoxication (Fig. 4D–G). Finally, non neuronal cells such as ependymal and choroïd plexus cells of either the third or fourth ventricles exhibited, in restricted areas, a moderate c-Fos immunostaining after treatment.

3.4. F. graminearum *extracts induced c-Fos expression in cathecholaminergic but not in serotoninergic neurons*

Phenotypic labeling performed at the brainstem level revealed that a subpopulation of c-Fos immunoreactive neurons within the NTS and VLM was positive for TH, indicating that a strong part of DON-activated neurons belonged to the A1/C1 and A2/C2 catecholaminergic groups (Fig. 5A–D). At the pontine level, only few TH+ neurons that constitute the A6, A7 and A8 areas were also immunoreactive for c-Fos (Fig. 5E–H). On the other hand, whatever the level considered, the raphe nucleus, identified with 5-HT immunostaining, was virtually devoid of c-Fos-activated neurons and double immunolabeling totally failed to reveal any colocalization of the two markers (Fig. 6).

3.5. *Effect of* per os *administration of* F. graminearum *extracts on c-Fos expression in NUCB2/nesfatin-1 neurons*

We detected NUCB2/nesfatin-1 immunoreactivity at different levels of the pig brain. The most intensive NUCB2/nesfatin-1 immunoreactivity was observed in the hypothalamus, in particular within the PVN, Arc, SON and the HLA (Figs. 7 and 8). In addition, outside of the hypothalamus, labeling was observed in the CeA, ventral raphe system, NTS, dorsal motor nucleus of the vagus (DMNX) and VLM (Figs. 9 and 10). Double c-Fos/NUCB2/nesfatin-1 immunohistochemistry revealed that a significant proportion of neurons activated by DON treatment were NUCB2/nesfatin-1 positive whereas the same structures were virtually devoid of c-Fos/NUCB2/nesfatin-1 neurons in control conditions. In DON-treated animals, c-Fos/NUCB2/nesfatin-1 colocalization was found in most NUCB2/nesfatin-1 expressing nuclei including the NTS, VLM, PVN, Arc and SON with the exception of the CeA, the LC and raphe system (Figs. 7–10). The highest colocalization incidence

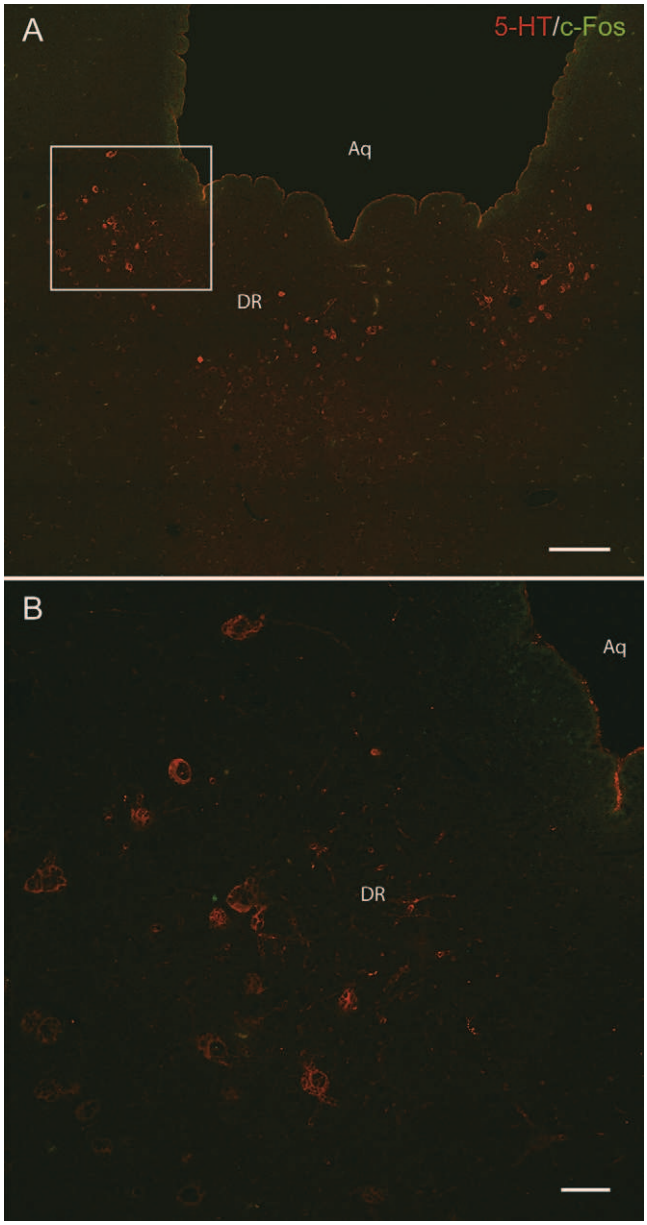


Fig. 6. Confocal analysis of serotonin (5-HT) and c-Fos double-labeling in the dorsal raphe of a DON-treated pig. (A) Low magnification of 5-HT and c-Fos immunolabeling allows visualization of the entire dorsal raphe nucleus. (B) High magnification of 5-HT/c-Fos labeling revealed the absence of double labeled neurons in this structure. The area where the high magnification originates from is indicated by a bounded box in the low power micrograph. Scale bars: 200 μm in (A) and (G): 50 μm in (B).

was found in the SON where virtually all c-Fos positive neurons were immunoreactive for NUCB2/nesfatin-1 (Fig. 8). In the PVN, Arc and NTS the percentage of c-Fos positive nuclei immunoreactive for nesfatin-1 is 40.6 ± 13.29%, 45.08 ± 6.62% and 35.72 ± 4.43% respectively.

4. Discussion

In the present study, we performed *per os* intoxication with *F. graminearum* extracts (containing the equivalent of 1 mg DON per kg of body weight) in piglets. This *F. graminearum* extract administration resulted in a clear systemic contamination with DON attested by high concentrations of the toxin in body fluids. Moreover, given the preponderance of DON compared with other

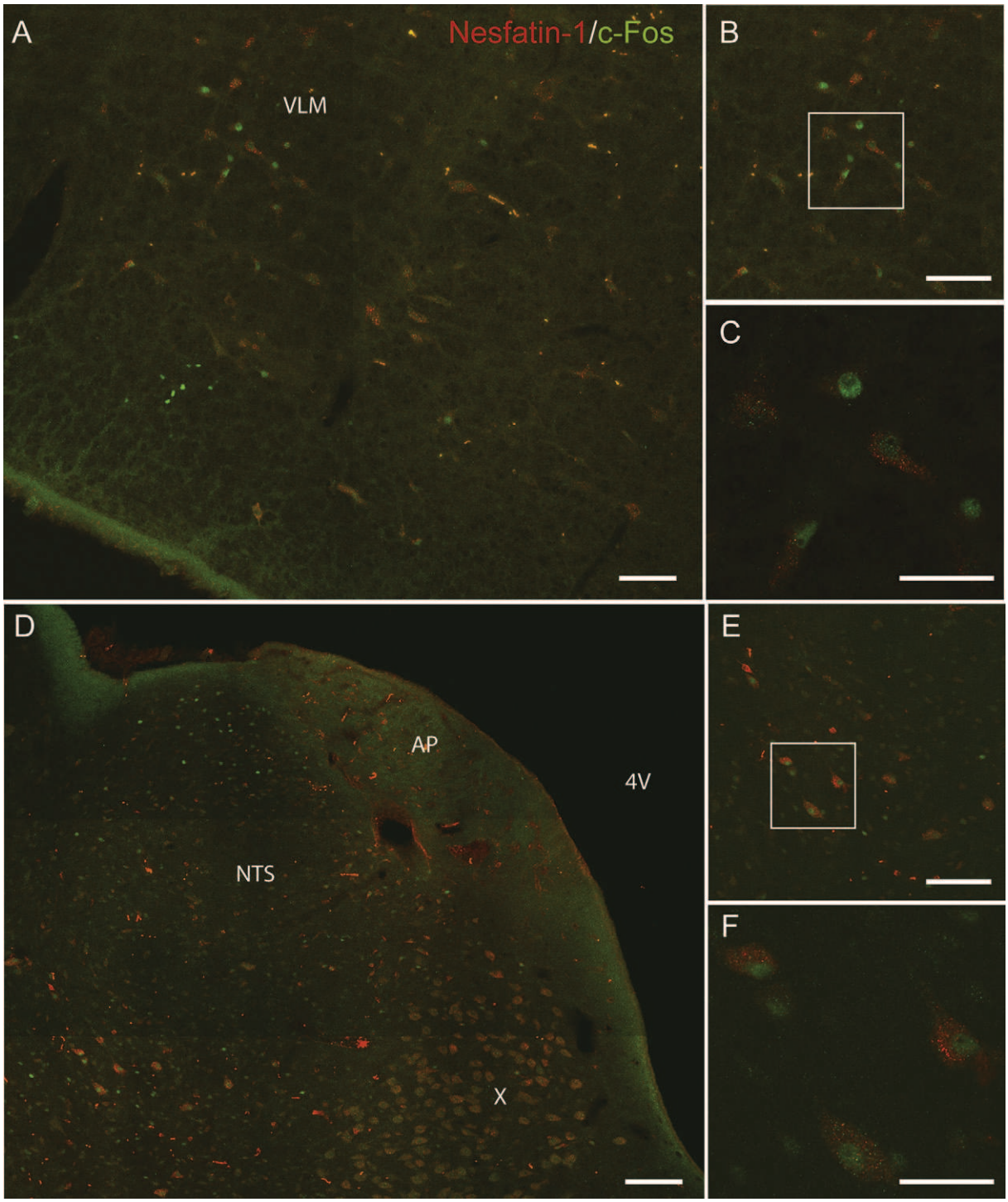


Fig. 7. DON-activated neurons in the ventrolateral medulla (VLM, (A)–(C)) and nucleus tractus solitarius (NTS, (D)–(F)) expressed NUCB2/nesfatin-1. (A) and (D) Representative confocal photomicrographs at low magnification of NUCB2/nesfatin-1 and c-Fos immunolabeling at the VLM and NTS levels. (B)–(C) and (E)–(F) High magnification of NUCB2/nesfatin-1/c-Fos labeling revealed the presence of double labeled neurons in these structures. The areas where the high magnification originates from are indicated by a bounded box on the low power photomicrographs. AP, area postrema; X, dorsal motor nucleus of the vagus; 4 V, fourth ventricle. Scale bars: 200 μm in (A), (D); 100 μm in (B), (E) and 50 μm in (C), (F).

trichothecenes concentrations in the *F. graminearum* extracts, it can be reasonably postulated that DON is responsible for the effects observed during intoxication. In these conditions, we sought to identify the central brain structures activated in response to such an intoxication. The present investigation is indeed the first one that assesses DON-induced changes in c-Fos expression within the brainstem and forebrain of piglets. c-Fos staining remains a useful approach to identify activated neuronal groups (Dragunow and Faull, 1989). In pigs, c-Fos immunohistochemical method was used to evaluate neuronal activation in response to various stimuli including stress (Parrott and Vellucci, 1994), hypercapnia (Ruggiero et al., 1999) or adrenomedullin 5 (Otsubo et al., 2009). These studies confirm the potential value of the c-Fos immunohistochemical method for the detection of activated neurons in pigs. In the present study, in response to DON, c-Fos immunoreactivity was observed in specific neurocircuitry

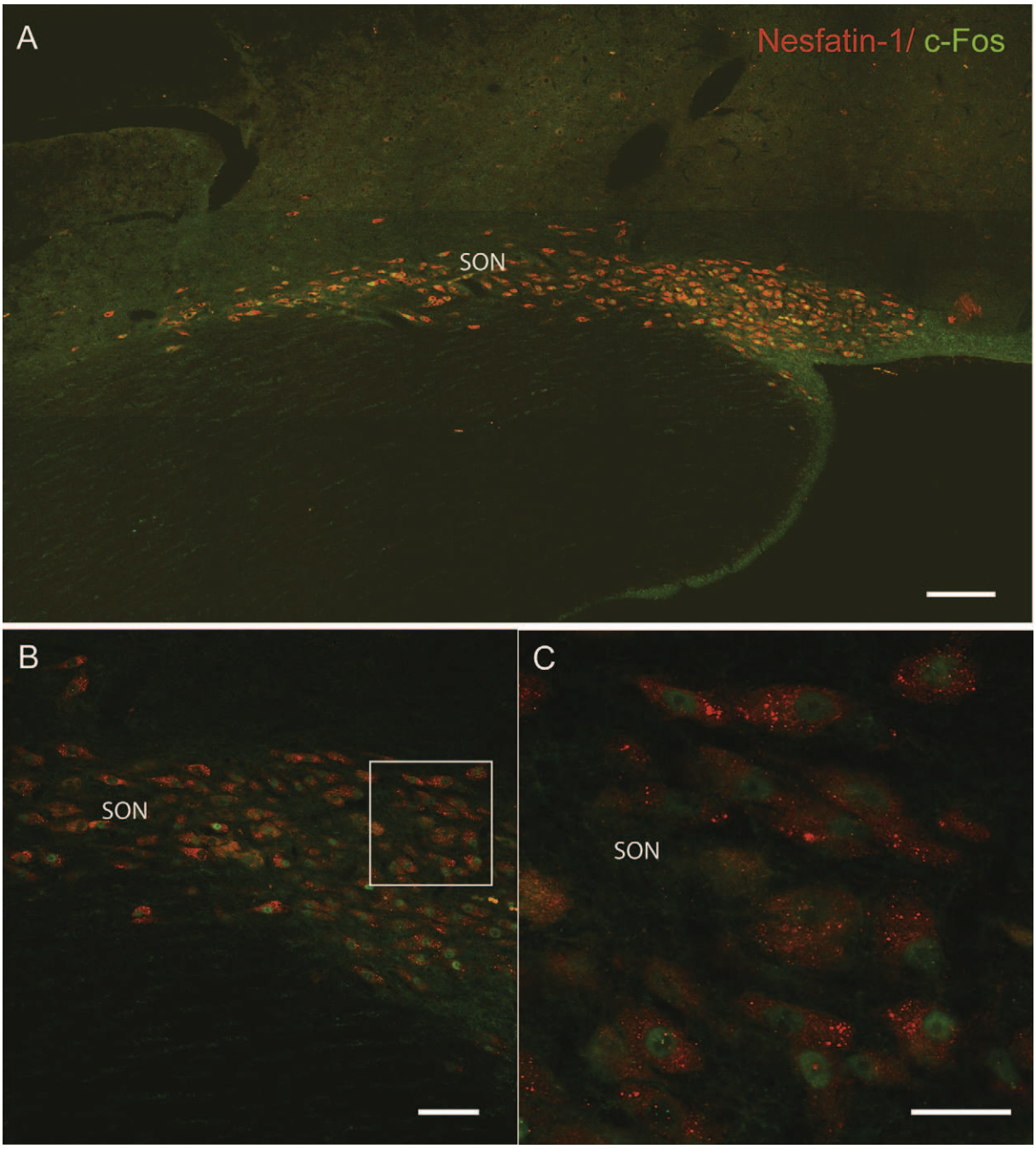


Fig. 8. Confocal analysis of NUCB2/nesfatin-1 and c-Fos double-labeling in the supraoptic nucleus (SON) of DON-treated pig. (A) Representative low magnification confocal photomicrograph of NUCB2/nesfatin-1/c-Fos immunolabeling at the SON level allows visualization of the entire nucleus. (B) and (C) High magnifications of NUCB2/nesfatin-1/c-Fos labeling revealed the presence of double labeled neurons in this structure. The area where the high magnification (C) originates from is indicated by a bounded box in (B). Scale bars: 200 μm in (A); 100 μm in (B) and 50 μm in (C).

including key regulatory nuclear groups such as the NTS, AP, VLM, LC, SON, Arc and PVN, i.e. structures involved in the coordinated autonomic, endocrine, and behavioral response. The CeA, which is involved in the integration of emotional stimuli and identified here by means of SOM immunoreactivity (Równiak et al., 2010), also displayed a strong c-Fos signal in DON-challenged piglets. Under our experimental conditions, the pattern of c-Fos distribution observed throughout the brain of DON-injected piglets was consistent with our previous study performed in mice (Girardet et al., 2011b). The present analysis was performed 3 h after peripheral DON administration. At this time point, activated structures can be considered as instrumental in DON-induced responses such as anorexia and associated symptoms were ongoing. It should also be noted here that the c-Fos expression pattern observed throughout the brain after *per os* DON administration in piglets was also consistent with the activation of immuno-sensitive neurocircuitry involved in the coordinated responses to inflammatory challenges in rodents (Lacroix and Rivest, 1997). In accordance, exposure to DON was shown to increase proinflammatory cytokine expression and induce immunomodulatory effects in pigs (Mikami et al., 2010, 2011). We report here the increase in circulating pro-inflammatory cytokines which may contribute to the activation of central structures. The up-regulation of Iba1 expression observed in DON-treated animals in

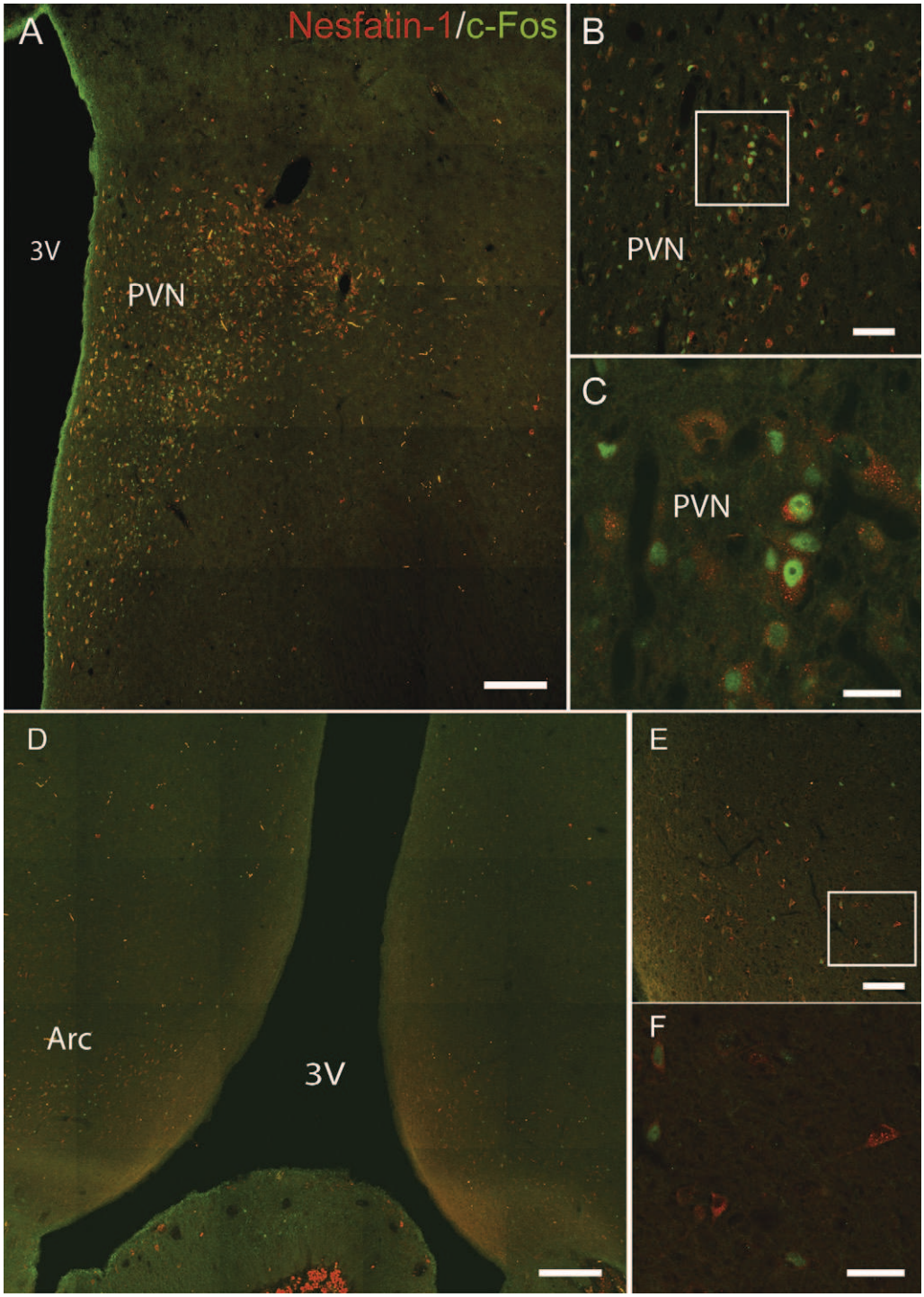


Fig. 9. DON-activated neurons in the paraventricular nucleus of the hypothalamus (PVN, (A)–(C)) and arcuate nucleus (Arc, (D)–(F)) expressed NUCB2/nesfatin-1. (A) and (D) Representative low magnification confocal photomicrographs of NUCB2/nesfatin-1 and c-Fos immunolabeling at the PVN and Arc levels. (B)–(C) and (E)–(F) High magnifications of NUCB2/nesfatin-1/c-Fos labeling revealed the presence of double labeled neurons in these structures. The areas where the high magnification images originate from are indicated by a bounded box on the low power photomicrographs. 3 V, third ventricle. Scale bars: 200 μm in (A), (D); 100 μm in (D), (E) and 50 μm in (C), (F).

addition the modification of cell shape strongly suggest the existence of inflammatory reaction in the brain of treated animals. This result is also in agreement with data previously obtained on mice (Girardet et al., 2011b).

Modifications of central neurotransmitter and metabolite concentrations in pigs were reported in several works. Starter pigs chronically fed (21 days) *F. graminearum*-contaminated grains exhibit increased cortex 5-HT concentrations associated with a decrease in hypothalamic norepinephrine (NE) and pons dopamine (DA) concentrations (Swamy et al., 2004). Following acute DON administration to pigs (0.25 mg/kg, intravenous), modifications in NE, DA and 5-HT levels were observed in the hypothalamus, frontal cortex and cerebellum. While DON treatment increases NE and decreases DA concentrations in all these regions, the levels of 5-HT which increase in the hypothalamus 1 h after injection, fall below the baseline 8 h after treatment in both the hypothalamus and frontal cortex (Prelusky et al., 1992). Finally, the effect of repeated intravenous or intragastric administrations of low-level DON doses

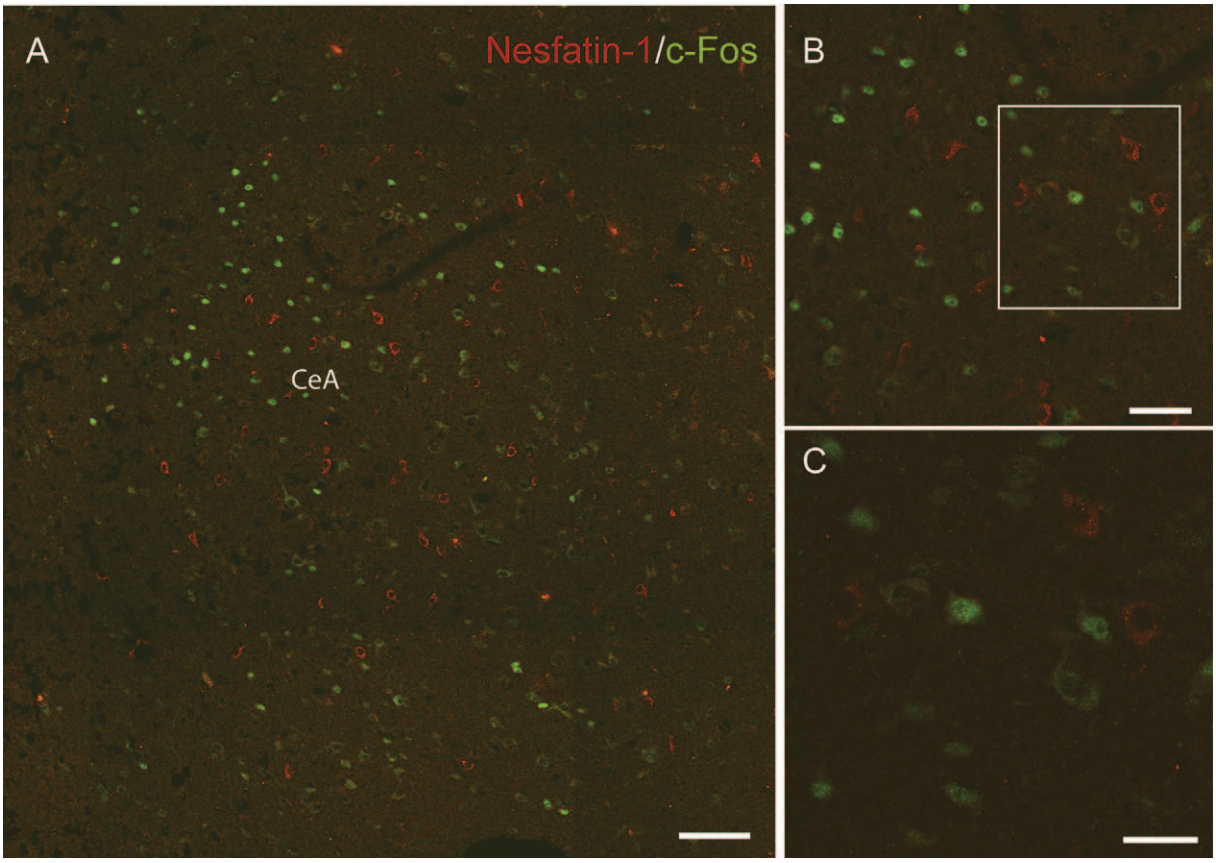


Fig. 10. Confocal analysis of NUCB2/nesfatin-1 and c-Fos double-labeling in the central nucleus of amygdala (CeA) of DON-treated pig. (A) Representative low magnification confocal photomicrograph of NUCB2/nesfatin-1/c-Fos immunolabeling at the CeA level allows visualization of the entire nucleus. (B)–(C) High magnification of NUCB2/nesfatin-1/c-Fos labeling revealed the absence of double labeled neurons in this structure. The area where the high magnification originates from is indicated by a bounded box in (B). Scale bars: 200 μm in (A); 100 μm in (B) and 50 μM in (C).

on neurotransmitter concentrations within the cerebral spinal fluid was investigated in the pig (Prelusky, 1993). This author reported a change in serotoninergic activity, suggesting an increased CNS serotoninergic turnover. These alterations in the serotoninergic system within the brain were proposed to play a role in the triggering of the feeding behavior reduction and emesis observed during DON intoxication. Nevertheless, whether these alterations of brain neurochemistry are responsible for the DON-induced anorexia remains unknown. In the present study, we observed an activation of the A1/C1, A2/C2 and A7/A8 catecholaminergic groups which may account for the modulation of NE concentrations observed after DON intoxication (Prelusky et al., 1992). The activation of these catecholaminergic groups by DON also demonstrates the activation of brainstem/hypothalamus connecting networks. These data strongly support the assumption that DON directly or indirectly interferes with interconnected neuronal networks which may participate in the coordinated responses observed following toxin administration. On the other hand, we did not observe any activation of the serotoninergic raphe nuclei. This result is quite surprising since the modulation of 5-HT concentrations within the brains of intoxicated animals has been reported (Prelusky et al., 1992) and central 5-HT release has a well known food intake reducing effect (Lam et al., 2010). Moreover, in addition to peripheral release, the central 5-HT discharge subsequent to raphe nuclei activation was reported to contribute to drug-induced nausea and vomiting that are symptoms observed in DON-intoxicated pigs (Endo et al., 2000). The time point used here, i.e. 3 h after intoxication, may explain discrepancies between our results and previous data.

In the present study, we have also studied the piglet brain distribution, from brainstem to diencephalon and basal telencephalon, of NUCB2/nesfatin-1 which has been ascribed anorexigenic properties. We observed a wide distribution in the hypothalamus and in a limited set of other brain regions. This brain distribution is summarized in Table 2. NUCB2/nesfatin-1 immunoreactivity was mostly concentrated in the hypothalamus, especially within the PVN, Arc, SON and the HLA. Additionally, besides the hypothalamus, NUCB2/nesfatin-1 immunoreactivity was located within the CeA, LC, ventral raphe system, NTS, DMNX and VLM. Our results are the first to report the expression of NUCB2/nesfatin-1 in the pig brain. This distribution is consistent with previous data obtained in rodents (Oh-I et al., 2006; Brailoiu et al., 2007; Kohno et al., 2008; Foo et al., 2008). Moreover, we have shown the activation, mainly in the hypothalamus and the brainstem, of a strong proportion of NUCB2/nesfatin-1 expressing neurons in response to DON intoxication. On the other hand, nesfatinergic neurons located in the LC, raphe system or CeA did not exhibit any activation in response to the toxin. These results confirm our recent report obtained in mice, where we showed an activation of NUCB2/nesfatin-1 neurons in the PNV and NTS (Girardet et al., 2011a), but significantly extend these data by showing that (i) NUCB2/nesfatin-1 neurons located in the Arc, SON and VLM are also sensitive to the toxin and (ii) in the LC, raphe system and CeA, DON did not activate NUCB2/nesfatin-1 neurons.

Nesfatin-1 exerts an anorexigenic effect on rodents (Oh-I et al., 2006; Shimizu et al., 2009). Nesfatinergic neurons were reported to be activated in response to refeeding or intraperitoneal injection of CCK, thereby contributing to satiety signaling (Kohno et al., 2008;

Noetzel et al., 2009). The activation of NUCB2/nesfatin-1 in nuclei neurons known to be involved in the control of feeding behavior, i.e. PVN, Arc or NTS, in response to DON treatment, provides arguments suggesting that nesfatinergic neurocircuitry could be involved in the anorexic response observed during DON intoxication. Nesfatin-1 was also shown to control water intake and body temperature (Yosten et al., 2012; Könczöl et al., 2012) and to reduce gastric emptying (Stengel et al., 2009). Moreover, this peptide exerts cardiovascular actions (Yosten and Samson, 2009, 2010) and modulates peripheral arterial contractility (Yamawaki et al., 2012). One cannot exclude that nesfatin-1, released in response to the toxin, may exert a more complex action in the pig than the simple reduction in food intake. The activation of NUCB2/nesfatin-1 neurons observed in a large number of nuclei, i.e. VLM, NTS, SON, Arc, and PNV, may account for a multifaceted action. DON used at 1 mg/kg is known to induce emetic response in pigs (Trenholm et al., 1984). Emetic episodes were indeed observed in the present study with a latency period of 1 h 30 min. Therefore, we cannot totally rule out the possibility that some of the c-Fos positive neurons activated during emetic episodes played a part in emesis even through at the time of sacrifice, i.e. 3 h post-treatment, vomiting was not observable any more. Whether released nesfatin-1 is implicated in DON-induced vomiting remains nevertheless an open question. To date, no report mentions such an action of nesfatin-1 after peripheral or central administration. Moreover, we previously reported evidence showing that NUCB2/nesfatin-1-expressing neurons are activated in the brainstem and hypothalamus during an inflammation induced by LPS administration (Bonnet et al., 2009). This neuronal activation observed in key nuclei emphasizes the potential role played by nesfatinergic neurons in the transfer of immune information and in the coordinated physiological and behavioral changes characteristic of LPS-induced sickness behavior. Here in response to the toxin, we observed a systemic and central inflammation attested by an increased cytokines expression and microglia activation and the recruitment of NUCB2/nesfatin-1 neurons. Hence, it can be reasonably predicted that immune messages produced in response to the toxin could partake in the recruitment of NUCB2/nesfatin-1 network in immunosensitive nuclei.

In conclusion, the data presented in this study identify central structures activated during DON intoxication in the piglet. This work also demonstrates that catecholaminergic and nesfatinergic systems belong to this neurocircuitry. Given the brain areas identified as DON-sensitive and the nature of activated neurons, one may suggest that hypothalamic and brainstem nuclei strongly contribute to the food intake modulation induced by the toxin. In particular, the anorexigenic properties described for nesfatin-1 could account for the DON-induced reduction in food intake.

Conflict of interest

None.

Acknowledgments

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