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# Caractérisation du contrôle descendant inhibiteur ocytocinergique et de sa modulation par un stress de séparation maternelle néonatale

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# E) Liste des abbreviations

AC : accumbens nuclei

ACC : anterior cingular cortex

ACTH : adrenocorticotropic hormone

AMPc : cyclic adenoise 3',5' monophosphate

AN : accesory nuclei

AP : allopregnanolone

AR : artificial rearing

AWR : abdominal withdrawal response

BBB : blood brain barrier

BNST : bed nucleus of the stria terminalus

CCK : cholecystokinin

CeA : central amygdala

CFA : complete freund adjuvant

CNS : central nervous system

CRD : colorectal distension

CRF : corticotropin-releasing hormone

DRG : dorsal root ganglion

E x : embryonar day n° x

EEG : electroencephalography

ELP : early life pain

ELS : Early life stress

EMG : Electromyography

ENS : enteric nervous system

EPSC : excitatory post synaptic current

ER : estrogen receptor

ERE : estrogen response element

EW: early weaning

FSS : forced swim stress

GI : gastro intestinal

HPA : hypothalamic–pituitary–adrenal axis

HT : hypothalamus

i.p : intraperitoneal

i.t : intrathecal

IBS : irritable bowel syndrome

ICV : intra cerebro ventricular

IL : interleukine

IPSC : inhibitory post synaptic current

KMC : kangaroo mother care

KO : knock out

L.reuteri : lactobacillus reuteri

LB : limited bedding

LPS : lipopolysaccharide

LTP : long term potentiation

MagnOT : magnocellular oxytocinergic neurons

MD : maternal deprivation

mPOA : medial preoptic area of the hypothalamus

MS : maternal separation

NA : noradrenaline

NAc: nucleus accumbens

NICU : neonatal intensive care unit

NTS : Nucleus Tractus Solitarius

OB : olfactory bulb

OT : Oxytocin

OTR : oxytocin receptor

PAG : periaqueducal grey

ParvOT : parvocellular oxytocinergic neurons

PKA / C: protein kinase A / C

PKx: protein kinase x

PLC : phospholipase C

PVN : paraventricular nuclei

RCPG : coupled with G protein receptor

RVM : rostral ventromedial medulla

s.c : subcutaneous

SC : spinal cord

SIA : stress induced analgesia

SNL : sciatic nerve ligation

SON : supraoptic nuclei

UBD : urinary bladder distension

VMH : ventromedial nucleus of the hypothalamus

VMR : viscero motor response

VP : vasopressin

WA : water avoidance

WDR : wide dynamic range

# F) Résumé de la thèse en Français

## Introduction

L'ocytocine (OT) est un neuropeptide contenu dans les neurones hypothalamiques, initialement découvert pour son rôle de neurohormone facilitant l'accouchement et l'éjection du lait maternel lors de la lactation. Au niveau du système nerveux central, elle possède un rôle facilitateur de plusieurs fonctions, notamment des processus sociaux (Lee et al., 2009). Elle a effectivement un effet anxiolytique, facilite le comportement social et permet la mise en place et le maintien du comportement maternel. Plus récemment, un rôle anti-douleur a également été associé à ce peptide (Boll et al., 2017). Dans ce cas, l'OT peut induire son effet analgésique via (i) une libération dans la circulation sanguine par une sous population de neurones magnocellulaires situés dans les noyaux supraoptiques et paraventriculaires de l'hypothalamus et (ii) une libération directe au niveau de la moelle épinière, centre important d'intégration de l'information nociceptive, par une sous population de neurones parvocellulaire du noyau paraventriculaire.

Ainsi l'objectif premier de cette thèse a été de caractériser plus en détail le fonctionnement du contrôle ocytocinergique de la douleur, en s'intéressant en particulier à la coordination entre les noyaux supraoptiques et paraventriculaires et à la communication entre les deux types cellulaires, pour mettre en place une analgésie efficace dans des cas de douleur inflammatoire ou neuropathique. Ce projet a été mené en collaboration avec l'équipe du Dr Valery Grinevich de l'institut Max Planck à Heidelberg.

Dans un second temps, il est connu que l'OT joue un rôle fondamental autour de la naissance, en particulier dans l'interaction mère-enfant et la création du sentiment d'attachement. Les données bibliographiques suggèrent qu'une séparation maternelle précoce (SMP) peut avoir des conséquences à long terme sur les réponses à la douleur une fois adulte (Sengupta, 2009). En particulier chez l'homme, l'état de santé des enfants nés prématurés requiert souvent une isolation en couveuse et une séparation précoce avec les parents. Ces enfants présentent un risque élevé de développer des pathologies neurologiques à l'âge adulte. Parmi les déficits observés, il a été mis en évidence une hypersensibilité à la douleur et tout particulièrement sur le plan viscéral. Une augmentation de la prévalence des douleurs chroniques est également noté, accompagnés de réponses émotionnelles excessives (Grunau et al., 2006). Dans ce contexte, le deuxième objectif de cette thèse a été de déterminer si les altérations des réponses à la douleur observées suite à une SMP pouvaient être dues à une perturbation du fonctionnement du contrôle analgésique médié par l'ocytocine. Nous avons également tenté de contrecarrer les effets de la SMP par une approche pharmacologique visant le système ocytocinergique et de possibles mécanismes épigénétiques sous-jacents.

## Résultats principaux et conclusions

- *Projet de caractérisation du contrôle OT de la douleur*

Pour le premier projet, l'équipe du Dr Grinevich a mis en évidence l'existence d'une sous population de neurones OT situés dans le noyau paraventriculaire par une approche neuro-anatomique utilisant des outils viraux. Cette sous-population de neurones est à la base d'une double projection, centrale vers la moelle épinière d'une part, mais également sur les neurones OT du noyau supraoptique. Lors de ma thèse, j'ai étudié cette population à l'aide de la technique d'optogénétique couplée à des

enregistrements électrophysiologiques *in vivo* sur moelle épinière et à une étude de comportement (test de la pince calibrée et du plantar). Nous avons démontré que l'activation de cette sous population de neurones OT est suffisante pour diminuer l'activité des neurones nociceptifs présents au sein de la moelle épinière (neurones Wide Dynamic Range). Ceci résulte en une analgésie efficace dans un modèle de douleur inflammatoire chez le rat, mais pas dans un modèle de douleur neuropathique. Cet effet est partiellement bloqué par l'application spinale d'un antagoniste des récepteurs de l'ocytocine, et totalement en co-appliquant un bloquant des récepteurs glutamatergiques de type AMPA. L'inactivation de cette population, par une technique de DREADD, augmente cette fois ci les symptômes de douleur inflammatoire, confirmant le rôle anti-nociceptif de cette sous population spécifique de neurones. Ces expériences ont été couplées à des enregistrements électrophysiologies des neurones OT au sein du noyau supraoptique, ainsi qu'à des dosages sanguins d'OT.

Finalement, nous avons donc démontré que cette sous-population d'une trentaine de neurones parvocellulaires est capable de diminuer les symptômes de douleur inflammatoire, via une action directe sur les neurones nociceptifs de la moelle épinière, et via une activation des neurones magnocellulaires du noyau supraoptique qui vont libérer l'OT dans la circulation sanguine. Nous avons également confirmé que ces neurones agissent au niveau de la moelle épinière via une co-libération d'OT et de glutamate, qui participe à son effet antinociceptif.

Ces travaux ont abouti à la publication présentée dans cette thèse (ARTICLE 1)

- *Projet séparation maternelle et altération du contrôle descendant OT*

Pour ce deuxième projet, nous avons utilisé un modèle de séparation maternelle chez le rat, de 3H par jour de P2 à P12. A l'aide de tests comportementaux, j'ai pu confirmer que la SMP induit effectivement une hypersensibilité à la fois mécanique et thermique au chaud à l'âge adulte, couplée à une sensibilité accrue lors d'une inflammation douloureuse. Nous avons étudié le fonctionnement du contrôle analgésique OT lorsqu'il est recruté dans un modèle de douleur inflammatoire à la carragénine et suite à un stress de nage forcée. Cette étude a été menée à l'aide de tests comportementaux associés à l'utilisation d'antagonistes sélectifs du récepteur à l'ocytocine (OTR) pour révéler la présence du contrôle endogène ocytocinergique. Une approche similaire a été menée avec des enregistrements électrophysiologiques in vivo des neurones nociceptifs de la moelle épinière. Dans les deux cas, notre étude démontre un déficit d'analgésie ocytocinergique chez des animaux adultes ayant subis une SMP, dans les douleurs inflammatoires et lors de stress de nage forcée.

D'un point de vue mécanistique, nous avons pu mettre en évidence que le déficit n'est pas lié à un défaut d'expression ou de fonctionnement des récepteurs OTR spinaux, puisque l'application exogène d'OT ou d'un agoniste sélectif de son récepteur permet d'obtenir une analgésie. Nous avons ainsi émis l'hypothèse que le déficit observé réside dans un défaut d'activation ou d'activité des neurones OT, ou dans un défaut de libération spinale d'OT. En parallèle, nous avons démontré que les effets à long terme de la SMP sont associés à des altérations épigénétiques, puisqu'elle induit une modification du profil d'expression de certains facteurs épigénétiques au niveau spinal, notamment des HDAC et des micro ARN.

Dans un second temps, nous avons utilisé une approche pharmacologique pour empêcher le développement des altérations associées à la SMP. Nous avons tenté deux stratégies, la première visant la voie de signalisation induite par les OTR et la deuxième inhibant les mécanismes

épigénétiques impliquant les histones déacétylases (HDAC). Les animaux ont été traités pendant la période de SMP par des injections intrapéritonéales ou sous cutanées d'OT, d'allopregnanolone (AP), un neurostéroïdes dont la production est stimulée par l'activation des OTR, ou de SAHA, un inhibiteur non sélectif des HDAC. Ces traitements ont pu restaurer une sensibilité normale à la douleur à l'âge adulte, ainsi qu'une analgésie OT efficace à la suite d'un protocole de nage forcée. Cependant, l'analgésie OT normalement présente pour lutter contre les symptômes de douleur inflammatoire n'a pu être restaurée chez les animaux SMP malgré nos tentatives de traitements postnataux.

En conclusion, la SMP induit une altération des voies nerveuses qui contrôlent les réponses à la douleur. Ceci s'observe par une hypersensibilité à la douleur et un dysfonctionnement des contrôles descendants de la douleur, comme démontré ici avec le système inhibiteur ocytocinergique. Ces altérations peuvent être partiellement évitées avec un traitement préventif avec de l'OT, de l'AP ou du SAHA. Ceci suggère d'une part qu'une altération de l'activation des OTR chez le nouveau-né pourrait perturber le développement du contrôle ocytocinergique de la douleur, et d'autre part, que des mécanismes épigénétiques sont mis en jeu suite à la SMP. Il reste nécessaire d'identifier plus précisément ces mécanismes épigénétiques ainsi que le mécanisme précis qui sous-tend le dysfonctionnement de l'analgésie OT.

Ces travaux ont été soumis en décembre 2017 à la revue Pain. Le manuscrit soumis est ainsi présenté dans cette thèse (ARTICLE 2)

## Liste des publications

- *Articles de recherches dans des journaux à comité de lecture*

Juif PE, **Melchior** M, Poisbeau P (2015). Characterization of the fast GABAergic inhibitory action of etifoxine during spinal nociceptive processing in male rats. Neuropharmacology. 91:117-22. (IF=5.01)

Juif PE, Salio C, Zell V, **Melchior** M, Lacaud A, Petit-Demouliere N, Ferrini F, Darbon P, Hanesch U, Anton F, Merighi A, Lelièvre V, Poisbeau P (2016) Peripheral and central alterations affecting spinal nociceptive processing and pain at adulthood in rats exposed to neonatal maternal deprivation. European Journal of Neuroscience. 44(3):1952-62. (IF=2.94)

Eliava M*, **Melchior** M*, Knobloch-Bollmann HS*, Wahis J*, da Silva Gouveia M, Tang Y, Ciobanu AC, Triana Del Rio R, Roth LC, Althammer F, Chavant V, Goumon Y, Gruber T, Petit-Demoulière N, Busnelli M, Chini B, Tan LL, Mitre M, Froemke RC, Chao MV, Giese G, Sprengel R, Kuner R, Poisbeau P, Seeburg PH, Stoop R, Charlet A, Grinevich V (2016) A New Population of Parvocellular Oxytocin Neurons Controlling Magnocellular Neuron Activity and Inflammatory Pain Processing. Neuron 89(6):1291-304. *** Co-first author** . (IF=14.02)

- *Articles de revue dans des journaux à comité de lecture*

**Melchior** M, Poisbeau P, Gaumond I, Marchand S (2016) Insights into the mechanisms and the emergence of sex-differences in pain. Neuroscience. 338:63-80. (IF=3.27)

- *Chapitre de livre*

2016 Tang Y*, Wahis J*, **Melchior** M*, Grinevich V, and Charlet A. Optogenetics for neurohormones and neuropeptides: focus on oxytocin. OPTOGENETICS: From Neuronal Function to Mapping & Disease Biology, Cambridge University Press (CUP) book. * Co-first author

- *Articles en français dans des journaux à comité de lecture*

2015 **Melchior** M, Poisbeau P. (2015) Conséquences des perturbations périnatales sur les réponses douloureuses. Douleurs: Evaluation - Diagnostic - Traitement, 16(2).

- *Posters et communications brèves*

**Melchior** M, Juif PE, Petit-Demoulière N, Chavant V, Goumon Y, Lelièvre V, Charlet A, Poisbeau P (2016, November 16) Consequences of neonatal maternal separation on the efficacy of the oxytocinergic control of pain. Society of Neuroscience San Diego USA.

**Melchior** M (2016, March 17-18) Consequences of neonatal maternal separation on the oxytocinergic control of pain. 12th Symposium National Réseau de recherche sur la douleur 17-18 march, Nice, France.

**Melchior** M, Juif PE, Petit-Demoulière N, Charlet A, Poisbeau P (2015, Mai 22) Long term consequences of neonatal maternal separation on the oxytocinergic control of pain mediated by oxytocin. Actes du 12ème colloque de la Société des Neurosciences, Montpellier, France

## Communications orales

- *Conférences invitées*

**Melchior** M (2017, November 16) 17ème congrès de la SFETD, symposium jeune chercheur du réseau INSERM Français de la recherche en Douleur. Nice, France.

**Melchior** M (2016, October 14) Journée douleurs néonatales. Erasme Hospital Bruxelles, Belgium.

**Melchior** M (2016, October 8) Effects of neonatal maternal separation on the oxytocinergic control of pain. 41ème congrès de la société de neuroendocrinologie.

**Melchior** M (2015, November 17) Journée régionale Douleur de l'enfant, Paul Pierre Riquet Hospital, Toulouse, France.

- *Conférence grand public*

**Melchior** M (2017, November 9) « Douillet » ou « Dur à cuire » ? Et si notre sensibilité à la douleur s'écrivait juste après la naissance. Conférences du Jardin des sciences, Schirmeck.

# G) Introduction

## I) Oxytocin, general information about the peptide and its expression throughout the body

### 1) History of the peptide and evolutionary aspect

The neuropeptide oxytocin (OT) is widely studied for its implication in numerous brain and peripheral functions. Its impressive profile has even lead to describe OT as "the great facilitator of life" (Lee et al., 2009). Its discovery dates back to 1906 when Henry Dale first described, on the cat, the uterine contracting properties of pituitary extracts (Dale, 1906). However, the molecule responsible for the effects highlighted by Dale was isolated only a few years after (Kamm et al., 1928) and named oxytocin, from the Greek meaning "swift birth". At that time a compound acting on blood pressure and renal activity was also identified, which has been characterized to be vasopressin (VP). Vincent du Vigneaud went a step further in OT study and was awarded a Nobel Prize in chemistry in 1955 for the artificial synthesis of OT. (DU VIGNEAUD et al., 1953).

OT is composed of nine amino acids (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly$NH_2$); the two cysteines being linked with a sulfur bond. It is important to note here that OT is quite similar, in terms of structure, gene and neuroanatomy, to the neuropeptide VP. They differ only by two amino acids on the 3$^{rd}$ and 8$^{th}$ position and their gene is located on the same chromosome. Distinct OT and VP receptors exists, but they both can bind each receptor, as will be described later in this introduction. From an evolutionary point of view, they both emerged early in evolution since homologs of OT and VP can be found in invertebrates, fishes, amphibians, reptiles or birds (Figure 1) (Grinevich et al., 2015). It is suggested that the OT and VP neuronal populations shared a common ancestor who later specialized in the synthesis of either mesotocin (an OT analog) or vasotocin. Similarly, OT/VP gene emerged from a common vasotocin precursor gene, and OTR and various vasopressin receptors emerged from a vasotocin receptor ancestor. In all the species where these peptides are found, they are supposed to have conserved functions linked to social or mating behaviour.

| *Peptide* | *Sequence* | *Vertebrates among which peptide is found* |
|---|---|---|
| | **Oxytocin-Like Peptides** | |
| | 1 2 3 4 5 6 7 8 9 | |
| Oxytocin | Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly ($NH_2$) | Placentals<br>Some marsupials<br>Lungfishes |
| Mesotocin | Cys-Tyr-Ile-Gln-Asn-Cys-Pro-**Ile**-Gly ($NH_2$) | Nonmammalian tetrapods<br>Marsupials<br>Lungfishes |
| Seritocin | Cys-Tyr-Ile-Gln-**Ser**-Cys-Pro-**Ile**-Gly ($NH_2$) | *Bufo regularis* |
| Isotocin | Cys-Tyr-Ile-**Ser**-Asn-Cys-Pro-**Ile**-Gly ($NH_2$) | Bony fishes |
| Glumitocin | Cys-Tyr-Ile-**Ser**-Asn-Cys-Pro-**Gln**-Gly ($NH_2$) | Rays |
| Valitocin | Cys-Tyr-Ile-Gln-Asn-Cys-Pro-**Val**-Gly ($NH_2$) | Spiny dogfish |
| Aspargtocin | Cys-Tyr-Ile-**Asn**-Asn-Cys-Pro-Leu-Gly ($NH_2$) | Spiny dogfish |
| Asvatocin | Cys-Tyr-Ile-**Asn**-Asn-Cys-Pro-**Val**-Gly ($NH_2$) | Spotted dogfish |
| Phasvatocin | Cys-**Phe**-Ile-**Asn**-Asn-Cys-Pro-**Val**-Gly ($NH_2$) | Spotted dogfish |
| | **Vasopressin-Like Peptides** | |
| | 1 2 3 4 5 6 7 8 9 | |
| Vasopressin | Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly ($NH_2$) | Mammals |
| Lysipressin | Cys-Tyr-Phe-Gln-Asn-Cys-Pro-**Lys**-Gly ($NH_2$) | Pig<br>Macropodids<br>Didelphids<br>Peramelids |
| Phenypressin | Cys-**Phe**-Phe-Gln-Asn-Cys-Pro-Arg-Gly ($NH_2$) | Macropodids |
| Vasotocin | Cys-Tyr-**Ile**-Gln-Asn-Cys-Pro-Arg-Gly ($NH_2$) | Nonmammalian vertebrates |

*Figure 1: Aminoacid structure of OT and VP peptides and their analogues in different animal species.*

*Adapted from* (McEwen, 2004)

### *(i) Synthesis*

OT is coded by a gene of 850 base pairs long, located on chromosome 2 in mice and chromosome 20 in human. OT and VP genes are located on the same chromosomal site but are in opposite transcriptional direction (Figure 2). It contains three exons and two introns. The first exon encodes for a translocator signal and for the peptide as well as a part of the associated vector protein neurophysin. The second exon encodes for the main part of neurophysin and the last exon for the carboxy-terminal region of neurophysin (Gimpl and Fahrenholz, 2001). This lead to the synthesis of a pre-pro-peptide which is cleaved during its axonal transport into a pro-peptide and finally to the peptide itself associated with its chaperone protein neurophysin. OT is stored in large dense core vesicles, as for any other classical neuropeptide, distributed in axonal terminals, soma and dendrites. The number of OT molecules in a vesicle has been estimated to range from 60 000 to 85000 (van den Pol, 2012). The dissociation of OT from neurophysin only occurs after its release from vesicles to the extraneuronal area, due to the low binding stability of the OT- neurophysin complex in more basic environments (Blumenstein et al., 1979).

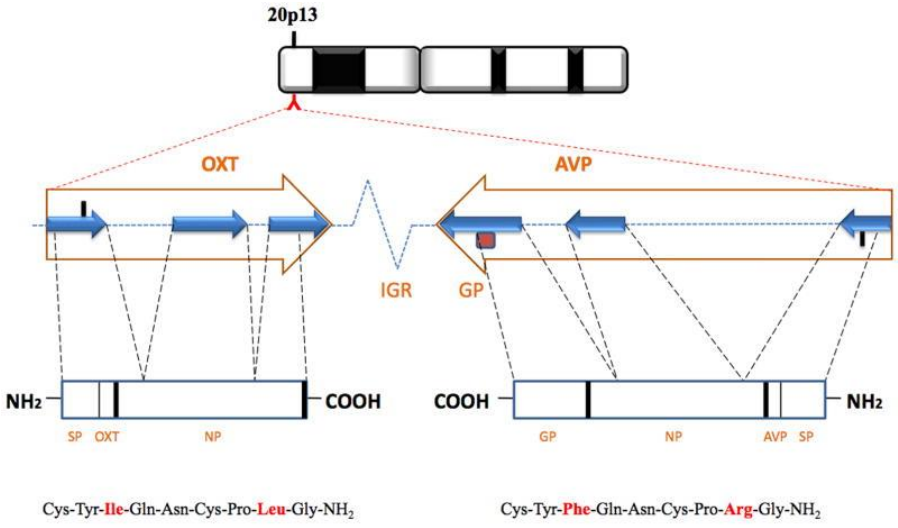


*Figure 2 : OT and VP genes and related neuropeptides in human.*

SP = signal peptide, NP = neurophysine, GP = glycopeptide. Adapted from (Lee et al., 2009)

### *(ii) Degradation and half life*

OT is degraded by an aminopeptidase of the M1 group called ocytocinase (also referred to as insulin-regulated aminopeptidase, Leucyl-cystinyl aminopeptidase or placental leucine aminopeptidase), a membrane-bound zinc-dependent metallo-exopeptidase (Tobin et al., 2014). This enzyme can be found both in the CNS and in the periphery, especially in the placenta where it is synthesised during pregnancy to avoid premature uterine contractions and preterm birth (Klimek, 2001; Ishii et al., 2009). In the CNS, it can be found in various brain regions, with a high expression in olfactory regions, hypothalamus, hippocampus, cerebral cortex and motor nuclei (Fernando et al., 2005). In that case, it is expressed only by neuronal cells. This results in a half-life of OT of around 4 minutes in the blood (Rydén and Sjöholm, 1969) and 20 minutes in brain tissues and cerebrospinal fluid (Mens et al., 1983).

*(iii) Regulation of gene expression*

**Upstream gene regulation**. The expression of OT gene is highly regulated, as seen in figure 3 (Burbach, 2002). One of the early observation about OT has been that its synthesis is stimulated by oestrogens (McCarthy, 1995). At the end of pregnancy, OT mRNA expression is highly increased by oestrogens. This is likely due to the fact that the OT gene promotor possesses an oestrogen response element (ERE) as well as multiple motifs resembling to ERE motifs in the proximal 5' flanking region, among which hormone response elements (HRE) (Stedronsky et al., 2002). OT neurons also express the nuclear oestrogen receptor beta (Alves et al., 1998). The hormone response element also allows a modulatory action of thyroid hormone and retinoic acid, as well as other orphan receptors (Richard and Zingg, 1991; Adan et al., 1992, 1993). Moreover, a POU-Homeodomain is present upstream of the OT gene at -1535 to -1270, allowing POU class III proteins to bind and regulate OT expression (Burbach, 2002). This region seems to be implicated in cell-specific regulation of OT expression.

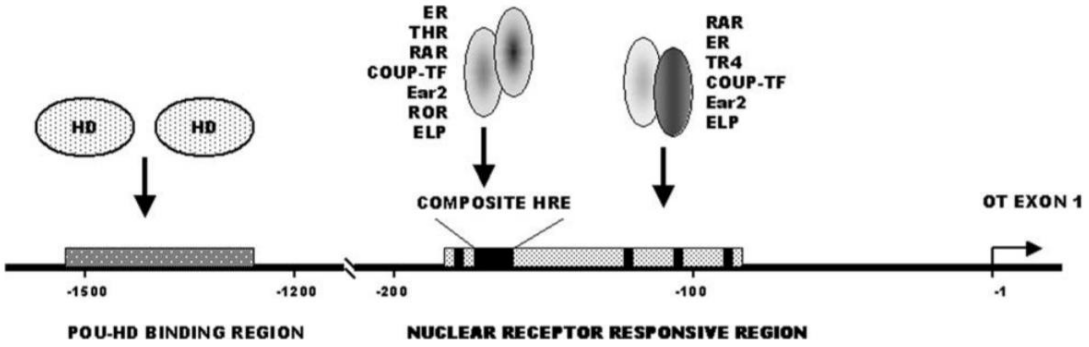


*Figure 3: Regulatory elements in the 5' flanking region of the OT gene.*

The schematic representation of the main known regulatory regions includes a complex domain which confers activities of nuclear hormone receptors, and a distal region binding POU-homeodomain proteins (POU-HD). The nuclear responsive region contains multiple half site motifs of the AGGTCA-type in different orientations and with different spacing. The strongest element is composed of three such motifs, the composite hormone response element (HRE) and allows action of hormone receptors, the oestrogen receptor ER, thyroid hormone receptors (THRs), and retinoic acid receptors (RAR), as well as a number of orphan receptors, as indicated. Adapted from (Burbach, 2002)

**Cell type specificity**. Many efforts have been made to identify the region responsible for the cell specific expression of OT in magnocellular neurons, as reviewed a few years ago by Gainer (Gainer, 2012). It has been proposed that the intergenic region is a critical site for enhancer elements. The analysis of heterologous cell lines and the use of transgenes in transgenic mice has indeed led to the identification of a region located < 0.6 kbp upstream of OXT exon I and about 3 kb upstream of AVP exon I, which is responsible for the cell-specific expression of OT and VP ( see also reviews (Murphy and Wells, 2003; Young and Gainer, 2003)).

## 2) Pattern of expression of OT throughout the body

### *(i) In the periphery*

OT is not only present in hypothalamic neurons but is also synthesized by several peripheral cell types. Local OT synthesis have been detected in the female reproductive system, especially in the ovary, placenta, uterus, corpus luteum and foetal membranes (Ivell et al., 1990, 1993). This OT expression is detected mostly at term just before delivery. But it is also present in the male genital tract (Guldenaar and Pickering, 1985). OT has also been detected in the skin, in particular in the epidermis, where it can released by keratinocytes (Denda et al., 2012). This can play a role in social interactions such as early attachment between mother and child, as described with skin to skin contact (Vittner et al., 2017) (also called kangaroo mother care), or in analgesia (Grinevich and Charlet, 2017). Other peripheral sites such as adrenal medulla, thymus, pancreas, bone, gastrointestinal tract or cardiovascular tissues have been identified (Gimpl and Fahrenholz, 2001). It is also secreted by macrophages and monocytes, possibly linked to the anti-inflammatory actions of OT.

### *(ii) OT neurons in supraspinal regions*

The main source of OT synthesis is localized in hypothalamic nuclei, and more precisely in the paraventricular nuclei (PVN), supraoptic nuclei (SON) and accessory nuclei (AN) (Swanson and Sawchenko, 1983). They are composed of small (soma diameter: 10-12 µm) parvocellular neurons and larger (soma diameter: 20-35 µm) magnocellular neurons. The number of magnocellular neurons in the hypothalamus is estimated at 100 000 in the human brain (Manaye et al., 2005) and around 10 000 in the rat brain (Rhodes et al., 1981). The magnocellular and parvocellular neurons can be easily distinguished based on morphological, anatomical, and functional markers. First, the PVN contains both parvocellular and magnocellular neurons whereas the SON is composed only of magnocellular neurons. The early studies suggested that magnocellular OT (magnOT) located both in the SON and the PVN project to the pituitary and release OT through the blood stream (Brownstein et al., 1980). However, parvocellular OT neurons (parvOT) located in the PVN were described to be responsible for long-distance projections in the SNC. More recent studies showed that MagnOT are also responsible for central release of OT, at least in the prefrontal area, accumbens nuclei and amygdala (Knobloch et al., 2012). For example, Ross and colleagues demonstrated using retrograde fluorogold labelling that OT fibers in accumbens nuclei (AC) of the prairie vole are collaterals from MagnOT neurons in PVN and SON, and play a role in attachment behaviour and monogamy (Ross et al., 2009a). But MagnOT and parvOT also differ from their electrophysiological properties, essentially residing on different expression of voltage gated K+ and Ca2+ currents (Luther and Tasker, 2000). Using whole cell patch clamp recordings in PVN slices, it has been demonstrated that magnocellular neurons display a transient outward rectification and a large A type K+ (Ia) current, but lack low-threshold spike and have very little T type Calcium current. On the contrary, parvocellular neurons do not display this transient outward rectification and have small Ia current, but they present low threshold spike and often have t type Calcium current. Using the appropriate stimulation protocol, it is then possible to distinguish between the two types of neurons in a patch clamp experiment.

Overall, OT released by neurons can act via synaptic sites or, at distance, by paracrine and endocrine-type communications, as illustrated in figure 4 which shows the example of social behaviour.

OT expressing neurons and VP expressing neurons were originally supposed to be two distinct population in hypothalamic nuclei (van Leeuwen and Swaab, 1977; Mohr et al., 1988), and to display specific electrophysiological properties and distinct responses to some pharmacological agents (Renaud and Bourque, 1991; Armstrong, 1995). However later studies suggest that they both express OT and VP mRNA but in different ratios, and that there is a small population of neurons that co-

express both mRNA (Xi et al., 1999). Moreover, OT neurons can also co-express many other neurotransmitter or neuropeptides, for example glutamate, CCK, dynorphin, Leu-enkephalin or Met-enkephalin, as it is described in figure 5 for magnocellular OT and VP neurons. The release of OT can hence be associated with the release of other classical transmitter or peptides. However, peptides are stores in LDCV, whereas classical neurotransmitter are stored in small vesicles. It has been proposed that the release of these distinct vesicles might require different neuronal activities. In that context, burst of action potentials are more likely to induce the release of LDCV, whereas single isolated action potentials would be sufficient to release small synaptic vesicles containing classical neurotransmitters. Co-release might also occur, when the two substances are stored in the same vesicular compartment (Vaaga et al., 2014). For example, a recent study showed that the co-release of OT and glutamate in the central amygdala is important for the modulation of fear response (Knobloch et al., 2012)

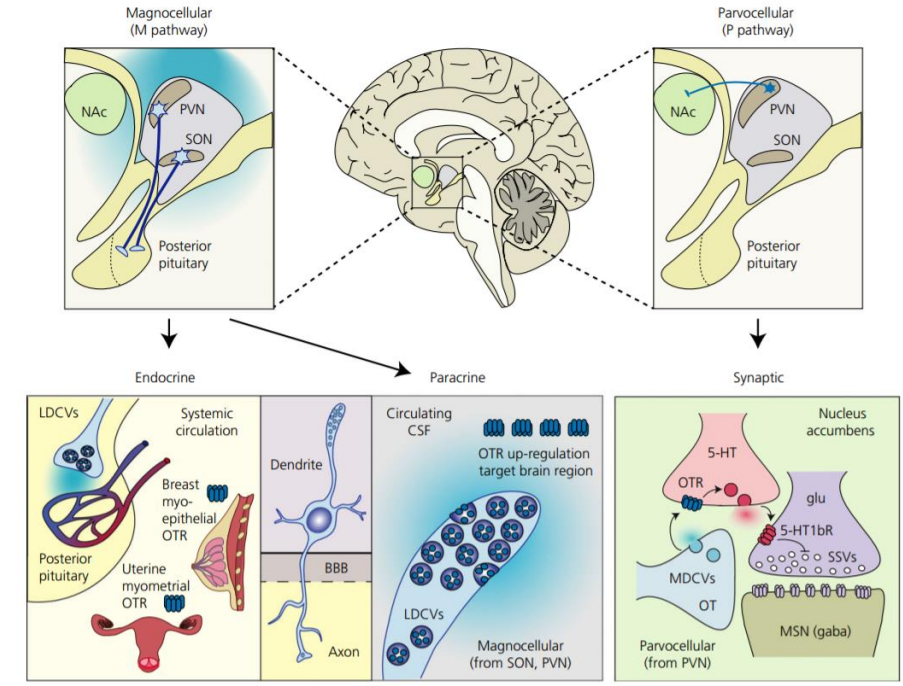


*Figure 4: Different pathways for OT release by magnocellular and parvocellular cells to regulate behaviours.*

*The example of the regulation of social behaviour is depicted here. NAc, nucleus accumbens; LDCV, large dense core vesicle; OTR, oxytocin receptor; BBB, blood–brain barrier; CSF, cerebrospinal fluid; 5-HT, serotonin; MDCV, medium dense core vesicle; OT, oxytocin; OTR, oxytocin receptor; MSN, medium spiny neurone; SSV, small synaptic vesicle; 5-HT1bR, serotonin receptor subtype 1b; glu, glutamate. Adapted from* (Dölen, 2015)

| Gene | MC Type | Reference Nos. |
|---|---|---|
| CRH | OT | 85, 121, 846, 859 |
| CCK | OT | 792, 794 |
| Galanin | VP | 207, 261, 412, 413, 641, 712, 590 |
| Neuropeptide FF | VP | 75 |
| Prodynorphin | VP | 260, 857, 882 |
| TRH | OT | 776 |
| Neuropeptide Y | VP | 69, 331, 371, 418 |
| Pro-enkephalin | VP + OT | 491, 492, 793 |
| VIP/PHI | VP | 571, 642 |
| PACAP | ND | 398 |
| Neurotensin | ND | 846 |
| Endothelin-1 | OT | 332, 407, 894 |
| PTH-related peptide | | 768 |
| Angiotensin | VP | 345, 380, 400, 440 |
| Chromogranin | VP + OT | 482, 697 |
| Secretogranin II | VP | 29, 478, 479 |
| Neuroendocrine polypeptide 7B2 | VP | 246, 489 |
| 14K prolactin | | 512 |
| VGF | OT | 479 |
| RESP18 (18K regulated endocrine specific protein) | VP + OT | 168 |
| CART | VP + OT | 836 |

*Figure 5: Diversity of co-expression of different peptides in OT and VP magnocellular neurons.*

*ND : non determined. Adapted from* (Burbach et al., 2001) *(see in the papers for further references)*

*(iii) OT levels*

The analysis of OT levels in blood, saliva, urine or CSF has been performed mostly using EIA or RIA in different animal species. However, different values have been obtained depending on the method used to extract samples, as illustrated in figure 6 obtained from a recent review highlighting the issues in OT peripheral levels measurement (McCullough et al., 2013). Altogether, methods using unextracted samples lead to higher values than methods using extracted samples, probably detecting other products than OT. Other methods, such as a two dimensional liquid chromatography separation with tandem mass spectrometry detection have also been used to measure human and rat OT plasmatic levels (Zhang et al., 2011). OT plasmatic levels were evaluated to range from 398 to 2440pg/mL in rats and from 1.05 to 3.67 pg/mL in humans. Using EIA, Carter and colleagues measured plasmatic levels of around 500 pg/mL in female prairie voles and 265 pg/mL in males (SUE CARTER et al., 2007). In the rat, they detected lower OT levels, estimated at 190 pg/mL in females and 80 in males. Using EIA, Martinez-Lorenzana and colleagues detected about 33 pg/mL OT in rat CSF and about 600 pg/mL in plasma (Martínez-Lorenzana et al., 2008).

| Plasma oxytocin estimates for... | Method | |
|---|---|---|
| | RIA on extracted samples | EIA on unextracted samples |
| Healthy, non-pregnant, non-lactating women (basal levels) | 1–5 pg/ml (Amico et al., 1981; Cyranowski et al., 2008; Domes et al., 2010; Grewen et al., 2005; Jokinen et al., 2012; Salonia et al., 2005; Tabak et al., 2011) | 200–359 pg/ml (Feldman et al., 2012; Gordon et al., 2008; Schneiderman et al., 2012; Taylor et al., 2010; Weisman et al., 2013) |
| Healthy men (basal levels) | 0–5 pg/ml (Amico et al., 1981; Chicharro et al., 2001; Grewen et al., 2005; Jokinen et al., 2012; Lee et al., 2003) | 240–405 pg/ml (Bello et al., 2008; Feldman et al., 2010, 2012; Gordon et al., 2008; Schneiderman et al., 2012; Taylor et al., 2010; Weisman et al., 2013) |
| Healthy, pregnant women | 1–10 pg/ml (Amico et al., 1986; Dawood et al., 1978; Sellers et al., 1981) | 264–329 pg/ml (Feldman et al., 2007; Levine et al., 2007) |
| Healthy women in normal early-stage labor | 7–45 pg/ml (Dawood et al., 1978; Fuchs et al., 1982; Lindow et al., 1998; Rahm et al., 2002; Sellers et al., 1981) | – |
| Healthy women in normal later-stage labor | 9–114 pg/ml (Dawood et al., 1978; Fuchs et al., 1982; Sellers et al., 1981; Thornton et al., 1988) | – |
| Lactating mothers (prior to breastfeeding session) | 2–13 pg/ml (Lucas et al., 1980; Nissen et al., 1996; Yokoyama et al., 1994) | – |
| Lactating mothers (peak during breastfeeding session) | 11–24 pg/ml (Lucas et al., 1980; Nissen et al., 1996; Yokoyama et al., 1994) | 166 pg/ml (Jonas et al., 2009) |

*Figure 6 : Plasmatic oxytocin levels. (RIA on extracted samples vs. EIA on unextracted samples).*

*Adapted from* (McCullough et al., 2013)

### 3) Anatomy of the hypothalamus and properties of OT neurons

In human, the hypothalamus (HT) account for 0.3 % of the brain, measuring only 4 $cm^3$. However, this small area is crucial for many physiological, behavioural, and endocrine processes during the whole lifespan. The HT is one of the regions originating from the diencephalon. It is located right above the pituitary gland, and under the thalamus, while the 3$^{rd}$ ventricle lies in the middle of the structure. Globally, the HT is subdivided in 3 sub regions, the lateral, medial and periventricular HT, and they are further divided along the rostro-caudal axis into the anterior or chiasmatic regions, median or tuberal region and posterior or mammillary region. Since OT neurons are located in PVN, SON and AN, only these particular areas of the HT will be described in this introduction. For more information about the hypothalamus, please refer to the recent chapter by Lechan and Toni (Lechan and Toni, 2000)

#### *(i) The paraventricular nucleus*

The PVN has a volume of 6 $mm^3$ in human and contains about 56 000 neurons. About half of them synthesize OT whereas the other half synthesize VP (Swaab et al., 1993). In the rat, PVN is about 0.5 $mm^3$ on each side of the 3$^{rd}$ ventricle and contains about 10000 neurons (Simmons and Swanson, 2008). 8 subdivisions of the PVN have been described by Swanson and Kruypers, 3 magnocellular and 5 parvocellular, which contain both OT and VP neurons (Swanson and Kuypers, 1980). Later it has been subdivided in eleven subdivisions (Simmons and Swanson, 2008). The lateral part of the PVN is composed of clusters of magnocellular neurons, whereas the medial part of composed of clusters of parvocellular neurons. A few years ago, Simmons and Swanson created a 3D model of the anatomy of the PVN that is presented in figure 7. Within the nuclei, Rhodes and colleagues described that the medial and posterior part of the PVN is mostly composed of OT neurons, whereas the lateral part is composed of both neurons with “a core of vasopressin-producing cells with a rim of oxytocin cells” (Rhodes et al., 1981)

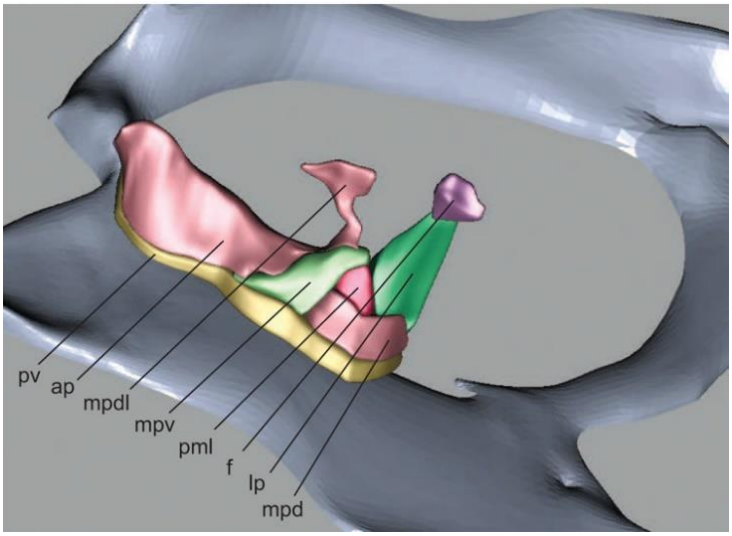


*Figure 7: 3D model of the PVN surface.*

ap, anterior parvicellular; f, forniceal; lp, lateral parvicellular; mpd, dorsal zone of medial parvicellular; mpdl, lateral wing of mpd; mpv, ventral zone of medial parvicellular; pml, lateral zone of posterior magnocellular; pv periventricular part. Adapted from (Simmons and Swanson, 2008)

*(ii) The supraoptic nucleus*

The SON has been subdivided in 3 major parts. The dorsolateral is the biggest part and is composed of 53000 neurons, among which 90% are VPergic and 10% are OTergic. The dorsomedial and ventromedial regions are smaller and contain about 23000 neurons together (Swaab et al., 1993). The major difference with the PVN is that the SON only contains magnocellular neurons, and no parvocellular neurons. Within the nuclei, OT neurons are mostly located rostrally and dorsally, whereas VP neurons are located more caudally and ventrally (Rhodes et al., 1981).

*(iii) The accessory nuclei*

These nuclei are located between the SON and PVN, and are composed by 6 nuclei : the antero-commissural, circular, forniceal, dorsolateral, ventrolateral (or nucleus of the medial forebrain bundle) and extra-hypothalamic (Knobloch and Grinevich, 2014). It is considered that 1/3 of magnocellular neurons are actually located in the AN (Rhodes et al., 1981). These AN probably emerged throughout evolution during the process of separation of the ancestral preoptic nucleus into PVN and SON, as depicted in figure 8. More recently, it has been showed that AN neurons projects to the central amygdala and play a role in the modulation of fear response in rodents (Knobloch et al., 2012).

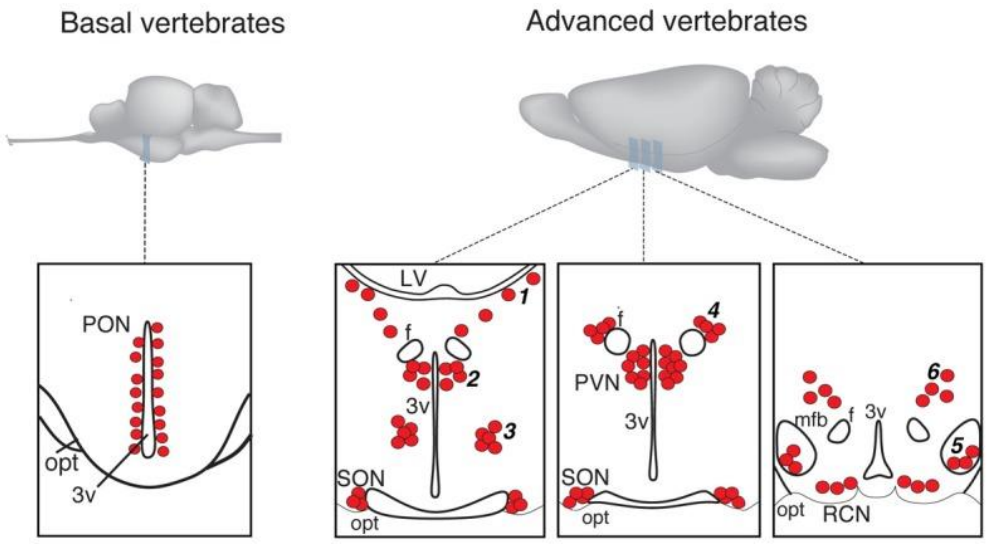


*Figure 8: Organisation of magnocellular hypothalamic nuclei in basal and advanced vertebrates.*

*3v, third ventricle; F, columns of fornix; LV, lateral ventricle; MFB, medial forebrain bundle; OC, optic chiasm; OT, optic tract; PON, preoptic nucleus; PVN, paraventricular nucleus; SON, supraoptic nucleus. Accessory nuclei: 1—extrahypothalamic; 2—anterior commissural; 3—circular; 4—fornical; 5—nucleus of the medial forebrain bundle; 6—dorsolateral. Adapted from* (Knobloch and Grinevich, 2014).

*(iv) OT neuron efference*

MagnOT neurons project mostly to the pituitary gland to release the peptide into the systemic circulation. It is considered that about 50 % of these projections come from PVN and SON, and that the other half originates from the AN. Although magnOT neurons do release OT in the blood stream, their axon collaterals project to various brain regions. Mapping studies using retrograde tracing with fluorogold and viral based techniques identified MagnOT projections in forebrain regions, especially in the prefrontal area, accumbens nuclei and amygdala. This is not restricted to one rodent specie, since it has been demonstrated in mice, rats and voles (Ross et al., 2009a; Knobloch et al., 2012). Parvocellular neurons main projections are targeting brainstem nuclei and the spinal cord. In the brainstem, they mostly project to autonomic nuclei, the NTS, dorsal motor of the vagus and the RVM (Swanson and Kuypers, 1980; Sawchenko and Swanson, 1982). In the spinal cord, each segment of the spinal cord displays OT fibers, but the cervical segment displays lower OT content and the autonomic centers at the thoracolumbar and sacral segments are highly innervated by OT projections. OT neurons projects in particular to superficial laminae, intermediate autonomic areas (IMM, IML) and to regions around the central canal (Swanson and McKellar, 1979; Jójárt et al., 2009). They also send intra-hypothalamic projections, in the external zone of the medial eminence, and projections onto blood vessels where they possibly influence vascular dynamics. As described in ARTICLE 1 of the thesis, we recently identified a subpopulation of about 30 parvOT neurons that projects to the SC and to magnOT neurons in the SON. A summary of OT projections fibers from the HT is presented in figure 9.

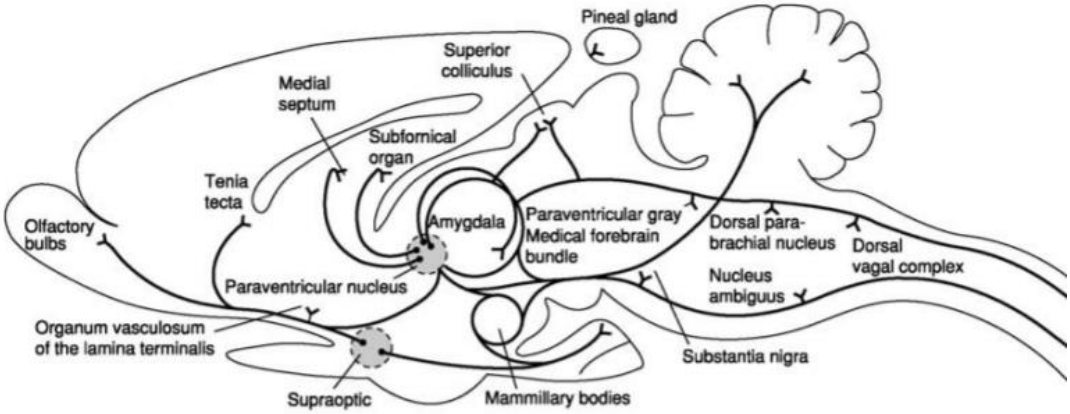


*Figure 9: Mapping of OT projections within the rat CNS.*

Adapted from (McEwen, 2004)

Interestingly, OT projections do not perfectly overlap with the expression of OTRs. As seen in figure 10, a few regions do express OTR but are not targeted with OT fibers, the olfactory bulb, the ventral pallidum, the medial preoptic area and the ventromedial nucleus of the hypothalamus. In these regions, OT release by either a transventricular pathway or a dendritic release is suggested (Grinevich et al., 2015).

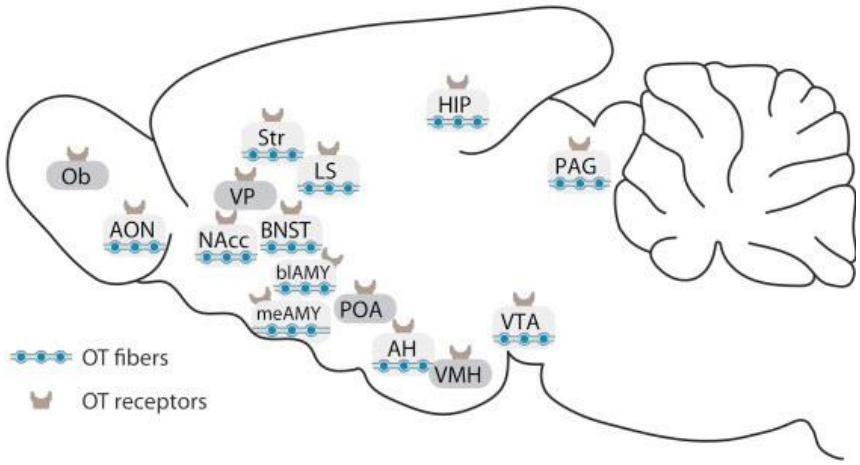


*Figure 10: distribution of OT fibers and OTR in the rat brain.*

*AH, anterior hypothalamus; AON, anterior olfactory nucleus; blAMY, basolateral amygdala; BNST, bed nucleus of the stria terminalis; HIP, hippocampus; LS, lateral septum; NAcc, nucleus accumbens; meAMY, medial amygdala; Ob, olfactory bulv; PAG, periaqueductal gray; POA, preoptic area; Str, striatum; VMH, ventromedial nucleus of the hypothalamus; VP, ventral pallidum; VTA, ventral tegmental area. Adapted from* (Grinevich et al., 2015)

### 4) OTR and its signalling pathway

The OTR belongs to the class I G-protein coupled receptors family (GPCRs), composed of 7 transmembrane domains. The gene encoding OTR is located in human on chromosome 3 in the gene locus 3p25-3p26.2 (Kimura et al., 1992), chromosome 4 in rats (Rozen et al., 1995) and chromosome

6 in mice (Kubota et al., 1996). The OTR gene is 17kb long and is composed of 3 introns and 4 exons, as presented in figure 11. The amino acid sequence of the receptor is encoded by exon 3 and 4, while exon 1 and 2 corresponds to the 5' non-coding region.

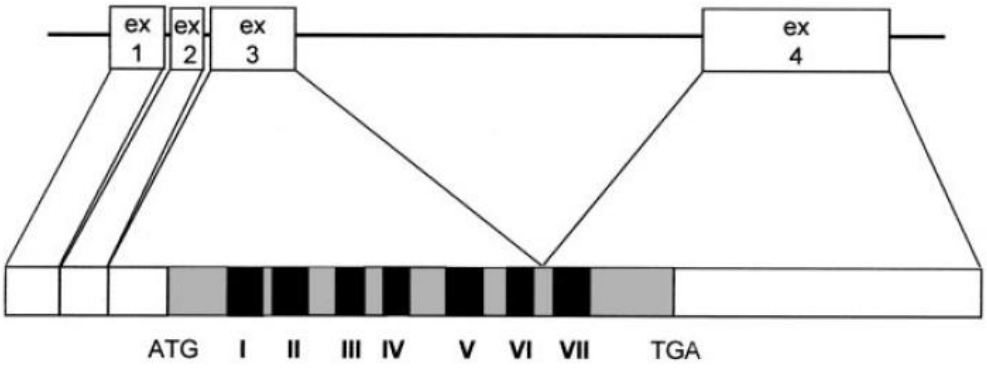


*Figure 11: Organization of the human OTR gene, composed of its 4 exons.*

The start (ATG) and stop (TGA) sequences are indicated. The black areas correspond to the DNA sequence encoding the transmembrane regions of the receptor. Adapted from (Gimpl and Fahrenholz, 2001)

### *(i) OTR pharmacological properties*

The binding site of OT to OTR is composed of residues located in the extracellular domains and in transmembrane domains, as seen in figure 12. The first and second extracellular loops are in particular very important for the specificity of binding to OTR. The NH2 terminal part of OTR interacts with the COOH-terminal tripeptidic part of the OT peptide, and the second extracellular loop rather interacts with the cyclic part of the peptide. If OT and VP are very similar peptides, their receptors also share some common features. Indeed, about 100 amino acid are invariants in the human receptors for OT, V1a/b and V2 (Gimpl and Fahrenholz, 2001). The extracellular loops and transmembrane helices are very similar between receptors. VP can hence also bind to OTR and act as a partial agonist, since 100-fold concentrations of the peptide are need to produce the same effects as OT(Freund-Mercier et al., 1988b; Chini et al., 1996). OT itself can also bind V1a receptor, but with a much lower affinity than VP (Akerlund et al., 1999). Synthetic agonists and antagonists with high selectivity to OTR have hence been developed, such a TGOT which is widely used as a specific agonist for OTR (Elands et al., 1988a).

Different variants of OTR have been identified and may play a role in neuropathological disorders. In the autistic population, single nucleotide polymorphisms rs7632287, rs237887, rs2268491 and rs2254298 have been identified (LoParo and Waldman, 2015). In an evolutionary point of view, OTR is very similar to mesotocin or isotocin receptor. Moreover, OTR is highly conserved between mammalian species, but some residues vary between species, as seen in figure 12.

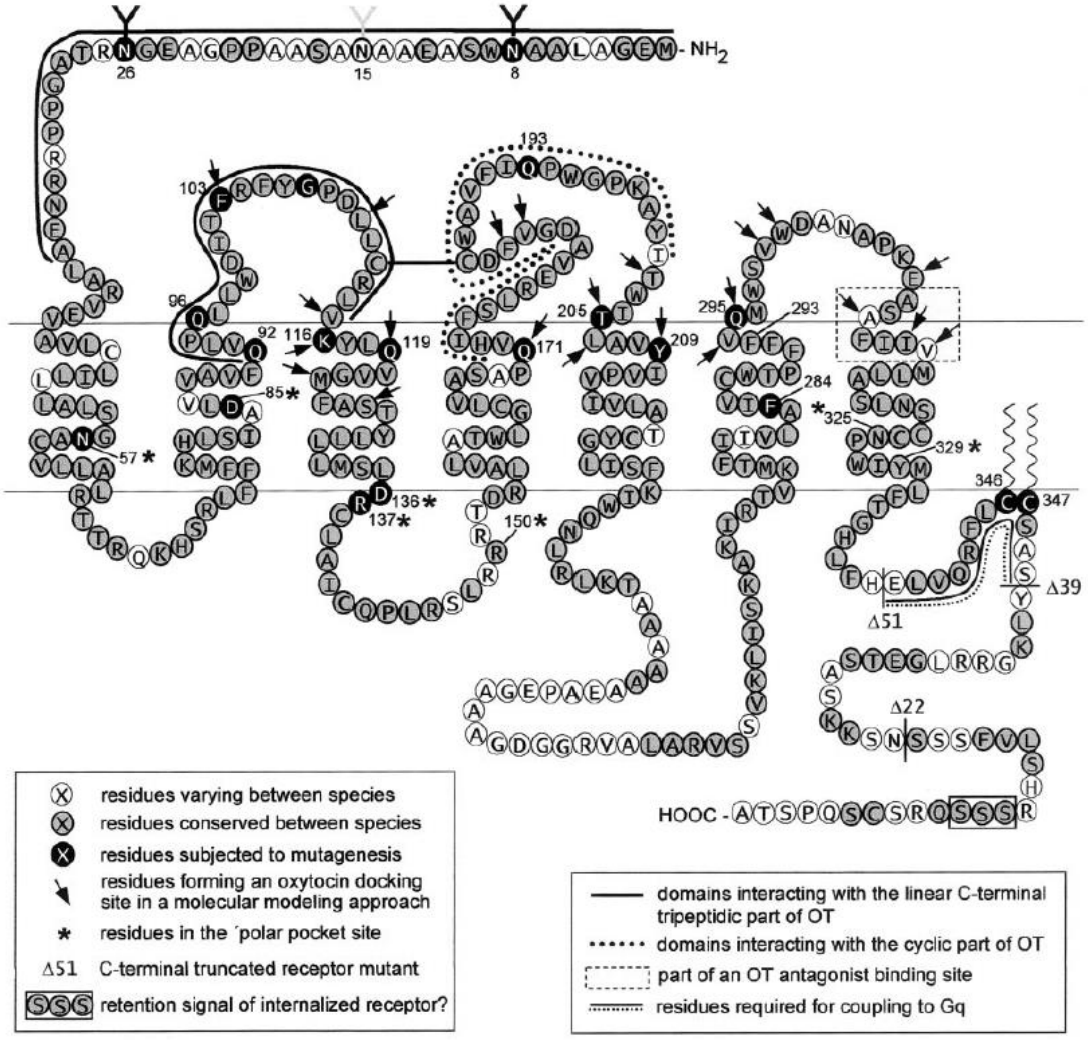


*Figure 12: Human OTR structure and its binding domains.*

*Adapted* from (Gimpl and Fahrenholz, 2001)

### *(ii) Intracellular signalling*

The first descriptions of the OTR told us that it is coupled to $G\alpha_{q/11}$, and induces the activation of phospholipase C (PLC) leading to the generation of diacylglycerol and IP3, which in turn induce the recruitment of intracellular calcium and the activation of protein kinase C (PKC) (Gimpl and Fahrenholz, 2001). The increase in intracellular calcium then leads to numerous intracellular events, including the binding to calmoduline, critical for the contraction of myometrial and mammary myoepithelial cells. The stimulation of phospholipase A2 production is also induced by the activation of $G_q$ protein, resulting in an increased prostaglandin production. In neuronal and endocrine cells, the increase in cellular calcium can also lead to the release of transmitters and hormones. In DRG neurons, the calcium increase following OTR activation activates NO production by nNOS, in fine leading to the activation of ATP sensitive $K^+$ channels, resulting in membrane hyperpolarization of the nociceptive neurons (Gong et al., 2015).

However it is now known that OTR is actually coupled to various Gα types including $G\alpha_i$, $G\alpha_h$ and possibly $G\alpha_s$ (Gimpl and Fahrenholz, 2001). Gravati and colleagues demonstrated that different G-

protein coupling can be found it the same cell type, with the example of the GN11 cell line, who presents the properties of immature olfactory neurons. In these cells, OTR activation can have differential effects depending on the activation of either $G_q$ or $G_{i/o}$ protein (Gravati et al., 2010). The $G_i$ pathway mostly has anti-proliferation effects whereas the $G_q$ pathway stimulates cell proliferation. Moreover, the localization of OTR in specific microdomains of the plasma membrane might be involved in the differential G-protein coupling. In caveolin-1-enriched microdomain, OTR activation has a mitogenic effect whereas it has an inhibitory effect on cell proliferation when located outside these microdomains (Guzzi et al., 2002; Rimoldi et al., 2003). A very detailed representation of intracellular pathways induced by OTR activation can be found in a recent review by Chatterjee and colleagues (Chatterjee et al., 2016). A simpler representation is presented in figure 13.

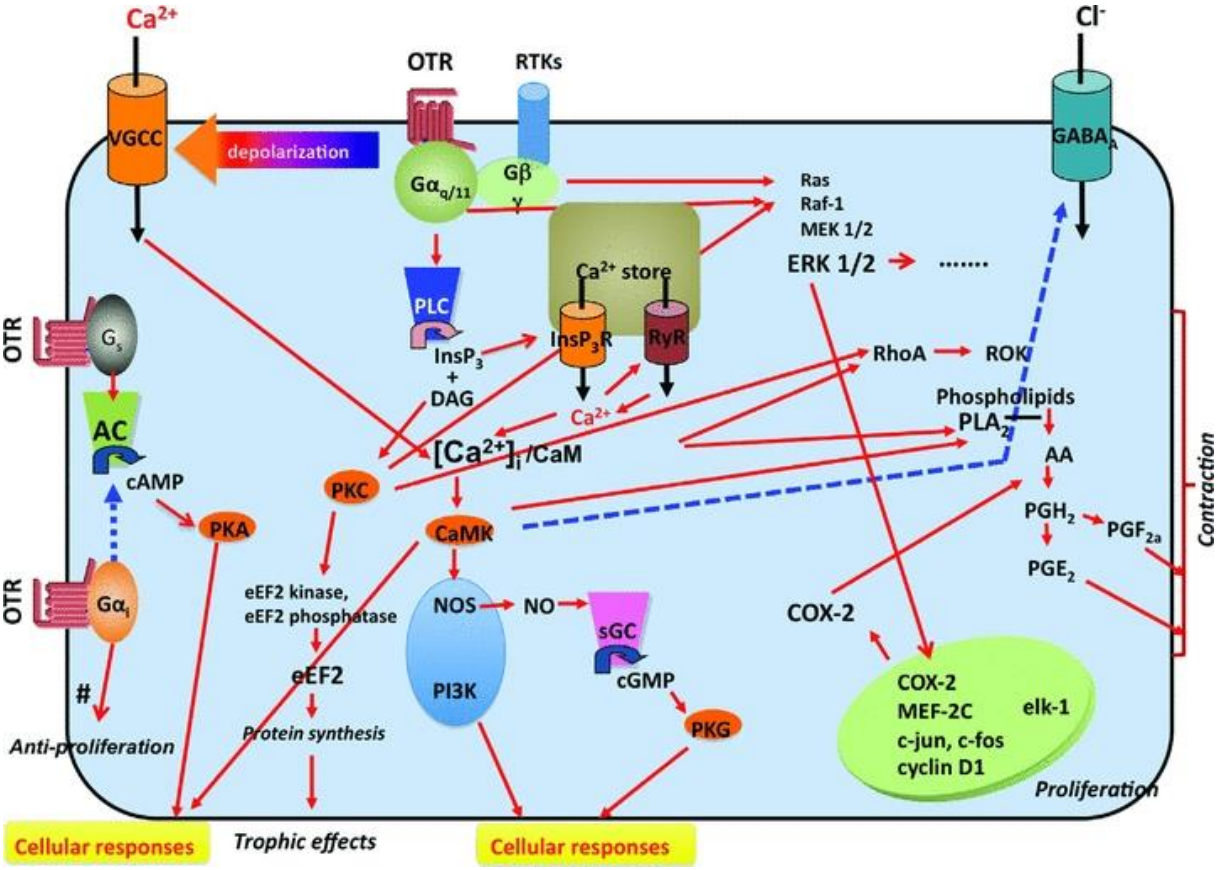


*Figure 13: Overview of OTR signalling pathways.*

Adapted from (Viero et al., 2010)

The therapeutic targeting of OTR can hence be a challenge, and the development of agonists and antagonists specific for a define intracellular reaction would be of great interest (Busnelli et al., 2012). For example, to prevent pre-term labour atosiban, an antagonist of OTR is often used. Interestingly, it acts as an antagonist on OTR linked to a $G_q$ protein but display agonistic effects on OTR linked to $G_i$. It has hence been considered as a biased agonist for the OTR (Reversi et al., 2005). Carbetocin on the other hand, an analog of OT used to prevent postpartum haemorrhage, selectively activates the OTR-mediated $G_q$ pathway (Passoni et al., 2016).

*(iii) Inactivation of OTR*

Desensitization of the OTR can occur after strong and persistent stimulation, similarly to what is occurring for other GPCRs. Indeed, when the myometrial OTR is exposed to OT for 24H the binding capacity of OT is reduced by 10 times and OTR mRNA is downregulated (Phaneuf et al., 1994). Even with shorter OT exposure (from 1 to 6H), the pre-treatment of cultured human myocytes by OT induces a reduction in the percentage of cells responding to OT exposure, as measured with calcium imaging (Robinson et al., 2003). Actually, OTR desensitizes rapidly, even only within 5 minutes (Willets et al., 2009). Receptors are inactivated by phosphorylation followed by arresting binding and the receptor is then internalized. In the case of OTR, the G-protein-coupled receptor kinase 2 is implicated in the process and promotes beta-arrestin 1 or 2 recruitment and endocytocis via clathrin-coated pits (Hasbi et al., 2004; Smith et al., 2006). Another kinase, the G protein-coupled receptor kinase 6 seems to be also involved in the desensitization process of OTR (Willets et al., 2009).
After internalization, the future of OTR has raised a few interrogations. Indeed, the association between OTR and beta arrestin is very stable, which is specific to "Class B "receptors described to be mostly transported to lysosomes for degradation or to be only slowly recycled back to the cell membrane (Oakley et al., 2001). But Conti and colleagues suggested that the OTR is recycled to the plasma membrane through a short cycle of about 4 hours, in vesicles containing Rab5 and Rab4 small GTPases which are specific to short cycles (Conti et al., 2009). A possibility could be that the desensitization process of OTR is dependent on the ligand. A study indeed showed that carbetocin does promote OTR internalisation but via a beta-arrestin independent pathway, and does not induce OTR recycling to the membrane (Passoni et al., 2016). Atosiban on the other hand does not induce any beta arrestin recruitment nor receptor internalization (Busnelli et al., 2012).

*(iv) Regulation of gene expression*

In the promotor sequence of the OTR gene several regulatory sequences are present. The flanking 5' region of the gene possesses a cAMP element of response, a palindromic oestrogen-response element (ERE) as well as ERE half sites and interleukine elements of response, as showed in figure 14 (Rozen et al., 1995; Bale and Dorsa, 1998; Gimpl and Fahrenholz, 2001).

The regulation of the expression of OTR gene by gonadal hormones has been investigated in many studies. In the periphery, oestrogens elevation during parturition or exogenous oestrogen application also induce an elevation in OTR mRNA levels (Zingg et al., 1995; Quiñones-Jenab et al., 1997). During the oestrous cycle, OTR expression also varies in the rat uterus, increasing by 2 times between met oestrus and pro oestrus in the rat (Larcher et al., 1995). On the contrary, progesterone treatment leads to decreased uterine OTR expression. Oestrogen action is hence mediated by an increase in OTR mRNA transcription or mRNA stabilization, whereas progesterone acts directly on the receptor, possibly inhibiting its binding to the ligand (Zingg et al., 1998). In the mammary gland, a 5.4 kb mRNA specific to this tissue has been identified, and its expression does not change upon steroid treatment (Breton et al., 2001). In the brain, OTR expression also seems to be dependent on the oestrous cycle, as observed in the ventromedial hypothalamus (VMH). It also increases at parturition, suggesting a regulation by oestrogen (Bale et al., 1995; Young et al., 1997). However, many studies highlight the fact that gonadal steroid effect on OTR might be region and specie-specific (Insel et al., 1993).

Besides, OTR expression seems also to be regulated by cAMP levels. The OTR gene indeed possesses a cAMP response element and Forskolin, a substance inducing an elevation in intracellular cAMP levels induces OTR upregulation (Bale and Dorsa, 1998). Inflammatory mediators were also proposed to modulate OTR expression. The OTR gene contains a nucleofactor-interleukin 6 binding consensus sequence and an acute phase reactant-responsive element (Inoue et al., 1994). In cultured human myometrium, OTR expression is indeed upregulated by IL-6 treatment (Rauk et al., 2001). However, IL-1β induces a down regulation of OTR expression in cultured uterine myocytes (Rauk and Friebe-

Hoffmann, 2000). This has been proposed to be the result of an increased secretion of OT that down-regulates OTR expression (Friebe-Hoffmann et al., 2007)

Epigenetic mechanisms are also involved in the regulation of OTR expression. The OTR gene is highly subjected to regulation by methylation. In particular methylation around intron 1 and in intron 3 is implicated in the tissue specific suppression of the gene. A rich CpG regions is indeed present and less methylated in OTR expressing tissues (Kusui et al., 2001). In the brain, the methylation profile of the OTR gene similarly modulates OTR expression levels (Mamrut et al., 2013; Harony-Nicolas et al., 2014). Human studies showed that OTR methylation levels can have an impact on social behaviour and the recognition of facial emotions, as well as play a role in social anxiety disorder (Puglia et al., 2015; Ziegler et al., 2015). The link between OTR epigenetic regulation and mental disorders such as autism is also part of the actual hypothesis of the research community (Gregory et al., 2009; Kumsta et al., 2013). Some of the behavioural outcomes associated with early life events or maternal adversities have also been linked to differential methylation profiles of the OTR gene (Beery et al., 2016; Unternaehrer et al., 2016; Gouin et al., 2017).

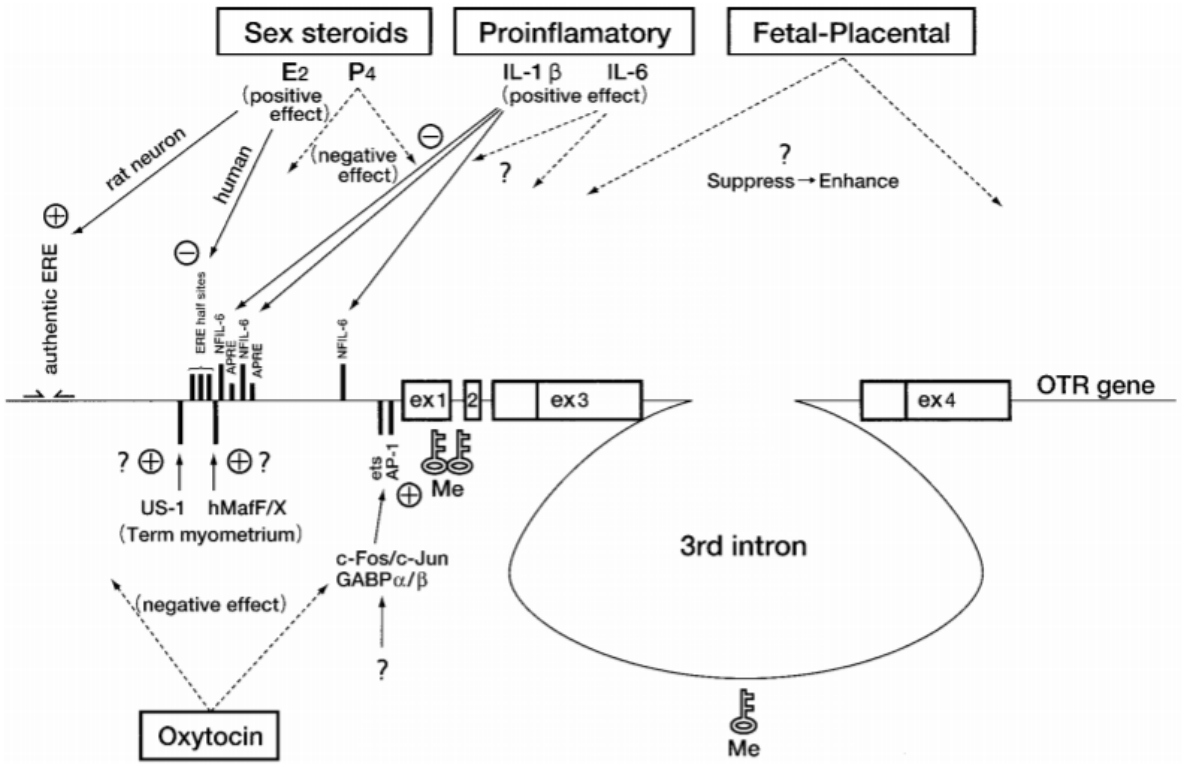


*Figure 14: Regulation processes controlling the expression of the OTR gene.*

Ex : exon, E2: oestradiol, P4:progesterone, Me: methylation. Adapted from (Kimura et al., 2003).

### *(v) Pattern of expression of OTR*

The distribution of OTR within the brain has been investigated using various techniques, including immunohistochemistry, histoautoradiography or knock in of fluorescent proteins in the OTR gene. Radiolabelled ligands have been extensively used to identify OT binding sites in the brain. Tritiated OT was used for the early studies, but raised the problem of a potential cross-reactivity with VP

receptors (Freund-Mercier et al., 1988b; Veinante and Freund-Mercier, 1997). Selective radioligands such as $^{125}$I-ornithine vasotocin analogue ($^{125}$I-OVTA) allowed a more precise evaluation of OTR distribution in the brain (Elands et al., 1988b). More recently, the development of a new specific antibody also helped to a more precise description of OTR expression in the mouse brain (Mitre et al., 2016)

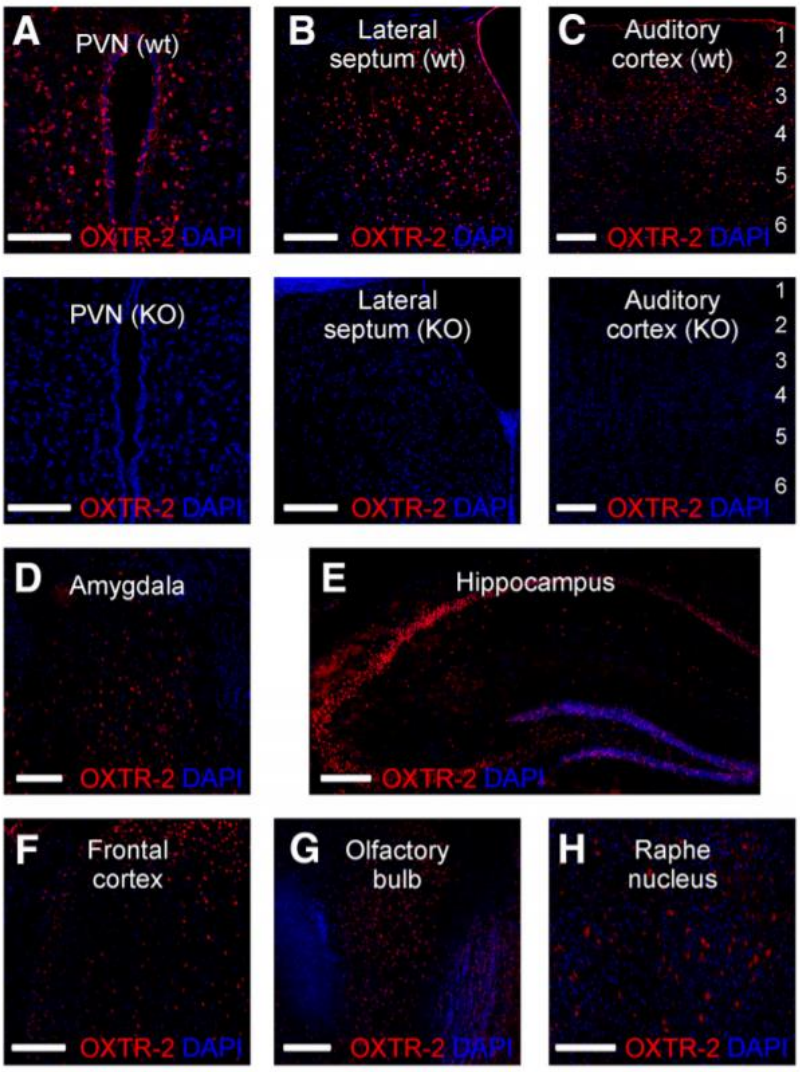


*Figure 15: : OTR immunostaining in the virgin female mouse brain.*

Red : OTR, Blue : DAPI. A. Imaged X20, scale bar 100µm. B images 10X, scale bar 15.µm. C images 10X, scale bar 100µM. D. imaged 20X, scale bar 100µm. E imaged 10X, scale bar 500µm. F imaged 20X, scale bar 150µm. G imaged 10X, scale bar 150µm. H imaged 20X, scale bar 150µm. Adapted from (Mitre et al., 2016)

Altogether, these studies demonstrate that OTRs are widely distributed in the CNS (Elands et al., 1988b; Tribollet et al., 1988; Adan et al., 1995; Veinante and Freund-Mercier, 1997; Gould and Zingg, 2003). In rodents OTRs have been detected in the olfactory bulb, neocortex, endopiriform cortex, hippocampus, amygdala, BNST, nucleus accumbens, ventral tegmental area hypothalamus (ventromedial nucleus in particular) and dorsal motor nucleus of the vagus nerve. In their review, Gimpl and Farenholz combined literature data in a table reproduced in figure 17. Finally, OTRs are expressed in regions classically associated with reproductive and social behaviours, but also in areas

of the brain involved in pain modulation. It is also detected in the rodent and human spinal cord in the most superficial layers of the dorsal horn, known to have a key role in pain processing, and in autonomic areas, as illustrated in figure 16 (Loup et al., 1989; Uhl-Bronner et al., 2005; Wrobel et al., 2011a). OTRs are also expressed in DRG neurons expressing IB4 or CGRP, a subpopulation of small unmyelinated C-type sensory neurons (Moreno-López et al., 2013).

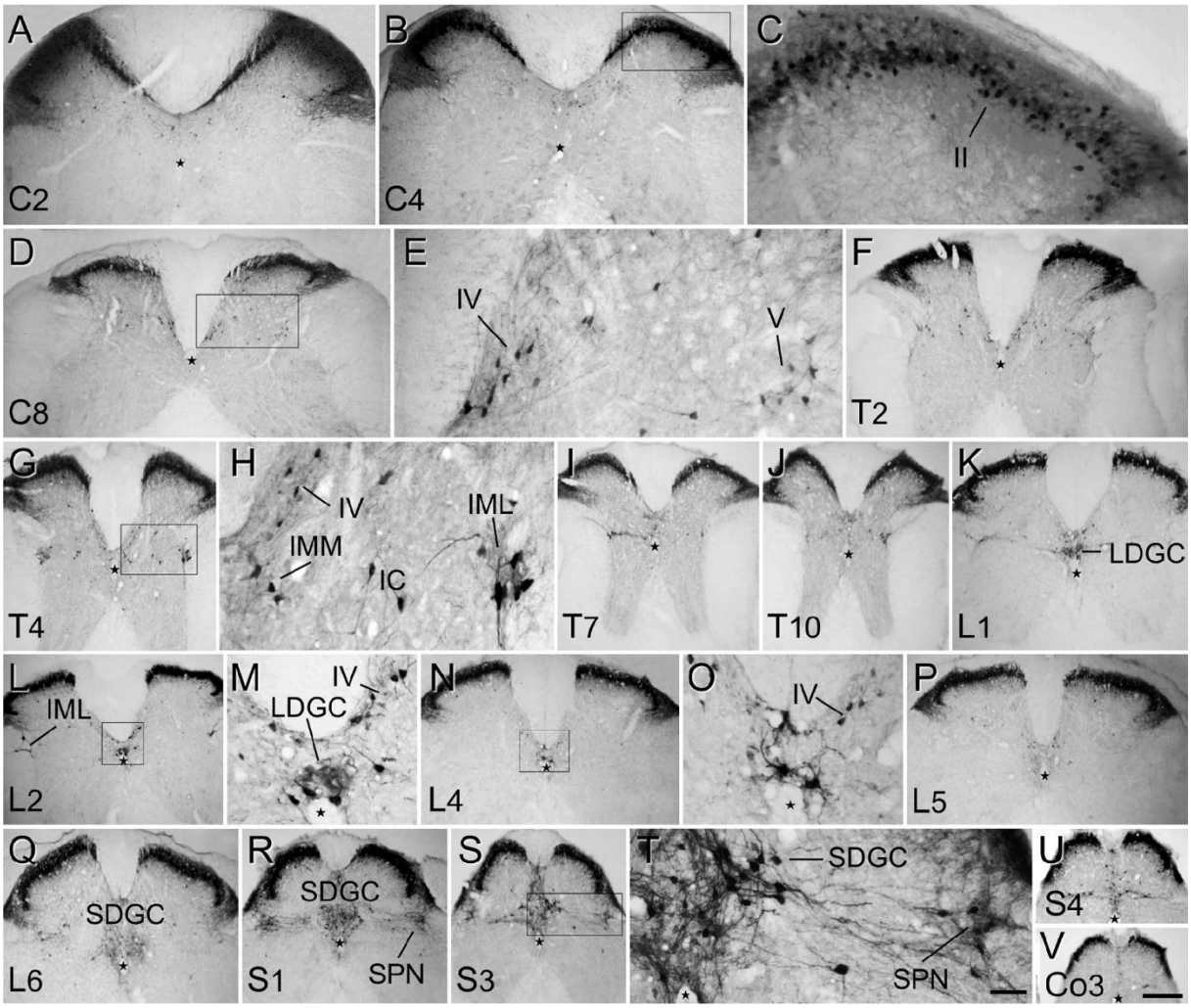


*Figure 16: OTR expression in the spinal cord of male mouse.*

OTR are stained using knock in of the Venus sequence in OTR gene and DAB procedure to reveal Venus immunoreactiviwroty. C, cervical; T, thoracic; S, sacral and Co, coccygeal). II, IV and V, laminae II, IV and V of the spinal gray; IMM, intermediomedial nucleus; IML, intermediolateral nucleus; LDGC, lumbar dorsal gray commissure and SDGC, sacral dorsal gray commissure. Star: central canal. Bars: low magnification photographs, 500 µm and high magnification photographs, 100 µm. Adapted from (Wrobel et al., 2011a)

The distribution of OTRs in the brain can highly vary depending on the specie and of the sex of the animal. These differences in OTR expression are proposed to explain differences in behaviour. For example, prairie voles and montane voles have different social and parental behaviour. Prairie voles form stable pair bond and display shared parental behaviour towards the pups, with is not true of their cousin montane voles. This differences have been attributed to differential OT and VP receptor expression in specific brain regions, nucleus accumbens in particular, but not to other system linked to social behaviour (Insel et al., 1997). Sex-differences are also observed in the expression of OTRs.

Dumais and colleagues demonstrated lower OTR binding densities in females compared to males in many forebrain regions. This seems to be associated with higher social interest towards unfamiliar conspecific in males (Dumais et al., 2013). Similar observations were obtained by Uhl-Bronner and colleagues, who specify that the sexually-dimorphic regions are steroid sensitive (Uhl-Bronner et al., 2005). A variation in OTR binding in NACC, BNST, MPOA and VMH during the oestrus cycle has also been detected in females (Dumais et al., 2013). Lower expression in the dorsal horn of the spinal cord has also been observed in females (Tribollet et al., 1997; Uhl-Bronner et al., 2005).

| | Rat | | | Human |
|---|---|---|---|---|
| Brain Regions | mRNA | OT binding (infant)* | OT binding (adult)* | OT binding |
| Olfactory system | | | | |
| Olfactory bulb | + | ? | ? | ND |
| Anterior olfactory nucleus | +++ | ++ | ++ | ? |
| Olfactory tubercle | +++ | ? | ++ | ? |
| Islands of Calleja | ND | ND | +++ | + |
| Piriform cortex | ++ | ? | ? | ? |
| Entorhinal/perirhinal area | + | + | + | ND |
| Cortical areas | | | | |
| Peduncular cortex | ? | ++ | +++ | (+) |
| Insular cortex | ? | + | + | ? |
| Cingulate cortex | + | +++ | ND | ? |
| Retrosplenial cortex | ? | +++ | ND | ? |
| Frontal cortex | ++ | ? | (+) | ND |
| Temporal cortex | (+) | ? | + | ND |
| Taenia tecta | +++ | ? | (+) | ? |
| Diagonal band of Broca | + | ? | ? | + |
| Basal nucleus of Meynert | ND | ND | ND | +++ |
| Basal ganglia | | | | |
| Caudoputamen | +++ | +++ | ++ | ND |
| Ventral pallidum cell groups | ++ | ND | +++ | ++ |
| Globus pallidus | ND | +++ | ND | ++ |
| Nucleus accumbens | + | ? | + | ND |
| Limbic system | | | | |
| Lateral septal nucleus | + | + | + | +++ |
| Bed nucleus of stria terminalis (BNST) | +++ | ++ | +++ | ND |
| Amygdaloid-hippocampal area | +++ | + | + | ND |
| Central amygdaloid nucleus | +++ | ++ | +++ | ND |
| Medial amygdaloid nucleus | ++ | + | + | ND |
| Basolateral amygdaloid nucleus | +++ | + | + | ND |
| Parasubiculum and presubiculum | ND | ++ | ++ | ND |
| Dorsal subiculum | +++ | +++ | (+) | ND |
| Ventral subiculum | +++ | + | +++ | ND |
| Thalamus and hypothalamus | | | | |
| Anteroventral thalamic nucleus | ND | + | ND | ND |
| Paraventricular thalamic nucleus | ++ | ++ | + | + |
| Ventromedial hypothalamic nucleus[b] | +++ | ND | ++ | ND |
| Anterior medial preoptic area | +++ | ND | ND | ++ |
| Supraoptic nucleus (SON) | +++ | ND | (+) | ND |
| Paraventricular nucleus (PVN) | ++ | ND | (+) | ND |
| Medial tuberal nucleus | ND | ++ | ++ | + |
| Posterior hypothalamic area | + | ND | ND | ++ |
| Supramammillary nucleus | ++ | + | + | ND |
| Lateral mammillary nucleus | ND | +++ | + | ++ |
| Medial mammillary nucleus | ND | +++ | ND | + |
| Brain stem | | | | |
| Substantia nigra pars compacta | ++ | ND | ND | +++ |
| Ventral and dorsal tegmental area | ++ | ND | ND | ND |
| Central gray | + | ND | ND | + |
| Dorsal raphe nucleus | + | ND | ND | + |
| Reticular nuclei | + | ND | ND | ND |
| Medial vestibular nucleus | + | ND | ND | ND |
| Hypoglossus nucleus | ++ | ND | ND | ++ |
| Nucleus of the solitary tract | ND | ND | (+) | +++ |
| Dorsal motor nucleus of the vagus nerve | +++ | + | + | + |
| Inferior olive nucleus | ND | + | + | (+) |
| Substantia gelatinosa of trigeminal nucleus | + | +++ | + | +++ |
| Pituitary gland | ND | + | + | ND |

*Figure 17: Distribution of OTR in the central nervous system in rats and human.*

Adapted from (Gimpl and Fahrenholz, 2001)

## II) OT functions

OT, through its neuroendocrine and neuromodulatory mode of release, can regulate both central and peripheral functions, which will be described in this part of the introduction. A more detailed focus will be done on pain modulation, since it is the main subject of my PhD project.

### 1) OT action on peripheral targets

#### *(i) On female reproductive organs*

OT has been first discovered and described for its function on the female reproductive system. OT is of great importance around birth when it participates in labour. In obstetrics, OT agonists associated with prostaglandins are hence used to provoke labour. OT antagonists, mostly atosiban (Tsatsaris et al., 2004), are also used to prevent preterm labour while carbetocin is used to prevent uterine atony and haemorrhage after caesarean section (Su et al., 2012).

Before birth, OT and OTR levels in the uterus increase in response to higher oestrogen levels, which have been demonstrated both in rodents and in humans. OTR expression increases up to 200 times the level in non-pregnant women, and more than 25 times in the rat (Fuchs and Fuchs, 1984; Larcher et al., 1995). Activation of myometrium OTR activates a $G\alpha_{q/11}$ protein, leading to an increase in intracellular calcium concentration and resulting in muscle contractions through calcium dependant calmodulin (Arrowsmith and Wray, 2014). After birth, OTR levels rapidly decrease, a mechanism which might be protective to avoid residual contraction of the uterus during lactation (Larcher et al., 1995). Moreover, in the uterine epithelium and decidua, OTR activation leads to a different pathway, beeing an increase in prostaglandin level, also important for labour initiation. Arrowsmith reviewed the different signalling pathways possibly recruited after OTR activation, as illustrated in figure 18.

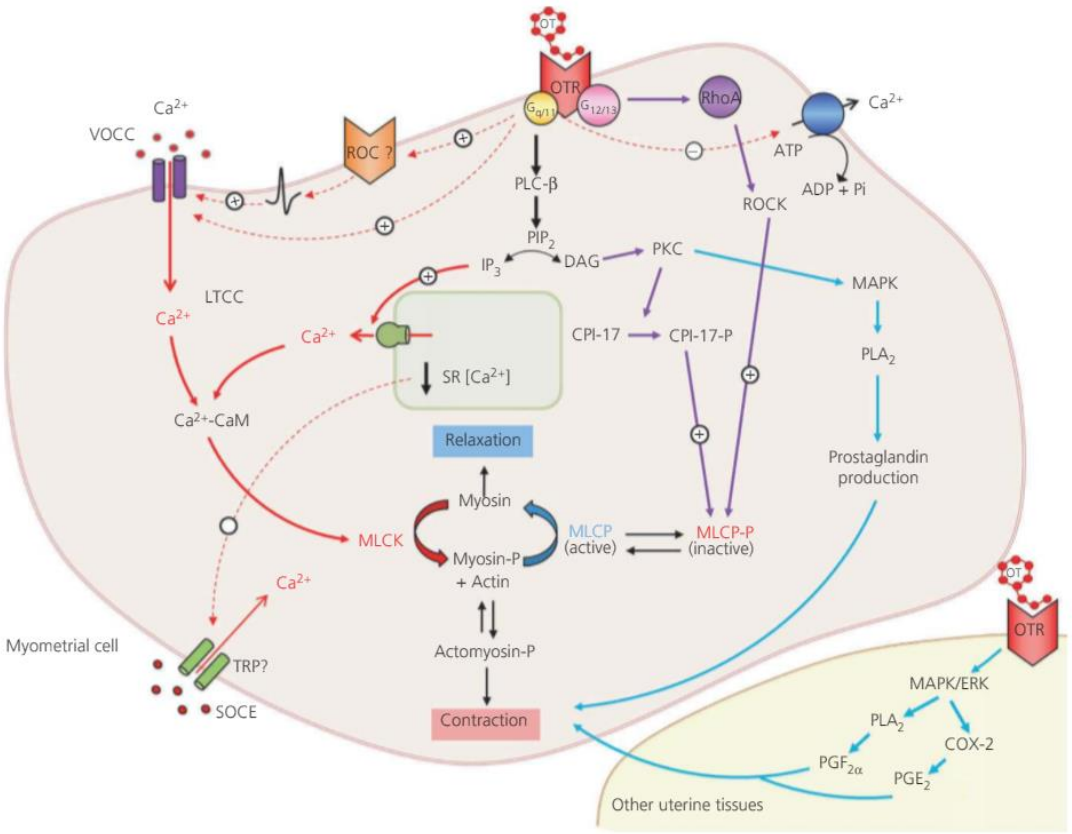


*Figure 18: Intracellular pathways after the activation of OTR by OT on myometrial cells, leading to contractile activity during labor.*

*Red pathways indicate signalling pathways with direct influences on $[Ca]_i$, whereas purple and turquoise lines indicate $Ca^{2+}$-independent pathways to contraction, including $Ca^{2+}$ sensitisation (purple lines) and the production of prostaglandins (blue pathways). Dotted lines indicate where mechanisms are not yet fully determined. COX-2, cyclooxygenase-2; ROC, Receptor operated channel; VOCC, voltage operated $Ca^{2+}$ channel; LTCC, L-type $Ca^{2+}$ channel; TRP, transient receptor potential channel; $Ca^{2+}$-CaM, $Ca^{2+}$-calmodulin complex; ERK, extracellular signal regulated protein kinase; SOCE, store-operated $Ca^{2+}$ entry. Adapted from* (Arrowsmith and Wray, 2014)

However, animal studies showed that mice deleted for OT gene are still capable of giving birth to viable pups (Nishimori et al., 1996). In humans, OT is sometimes ineffective to induce labour, whereas low doses are sufficient in other mothers (Budden et al., 2014). Of course, other mechanisms seem as important as OT to initiate delivery, such as prostaglandin pathways for example.

In addition to its first described functional role on labour induction and milk ejection, OT can modulate other aspects of female sexual behaviour. Indeed, the PVN is contacted by sensory inputs from genital areas both in males and females (Marson and Murphy, 2006; Normandin and Murphy, 2011) . Hypothalamic neurons also project to brain areas known to control genital reflexes, such as the nucleus paragigantocellularis in the brain stem (Normandin and Murphy, 2008). Activation of OT neurons in the PVN has been detected after sexual activity in female rats (Flanagan et al., 1993). (Flanagan et al., 1993). In rodents, female sexual behaviour, assessed by measuring lordosis behaviour in response to male mounting, is regulated mostly by oestrogens (Kow and Pfaff, 1998). Central injection of OT, in particular in the medial preoptic area (mPOA) and ventromedial hypothalamus (VMH), facilitates lordosis behaviour in oestrogen-primed females rats (Schulze and Gorzalka, 1991). Similarly, blocking OTR activation in the mPOA reduces the frequency and duration of lordosis behaviours (Caldwell et al., 1994). However, it is important to notice that the facilitating effect of OT is dependent on endogenous oestrogen and progesterone levels. It is observed only in intact females during oestrus, where progesterone levels are high, or in females primed with oestrogen or progesterone (as reviewed in (Lee et al., 2009))

*(ii) <u>On Mammillary glands</u>*

Like in the uterus, OTR expression in the mammary gland is increased up to 150 times through the whole pregnancy but stays high after birth during lactation. The neuronal pathways underlying milk secretion by the mammary gland have been widely studied, since it relies on a reflex response to nipple suckling. The tactile stimulation of the nipples activates sensory neurons contacting spinal cord neurons which further activate oxytocinergic neurons in the HT. After activation, OT is then released in the blood flow leading to a contraction of myoepithelial cells surrounding lactiferous ducts. This is made possible because of a synchronisation of hypothalamic OT neurons emitting high frequency action potential bursts every 15 minutes. Other sensory stimulations are known to induce OT release, such as the sound of the baby crying and the observation of specific behaviour of the baby (McNeilly et al., 1983). In OT KO mice, milk ejection is not seen (Nishimori et al., 1996).

*(iii) <u>Male reproductive tissues</u>*

OT has been detected in the testis and prostate gland of different species, including goats, monkeys, and a mesotocin-like peptide in birds. It is produced locally, in Leydig and Sertoli cells and is proposed to have an autocrine/paracrine role of modulation of steroidogenesis and on the contraction of seminiferous tubules (Ivell et al., 1997). However, differences between species have been observed

(Pickering et al., 1989). In mice for example, OT does not seem to be expressed in the testes (ANG et al., 1991), and OT deletion does not impact reproductive function (Nishimori et al., 1996).

As in females, the OT system is implicated in male sexual behaviour, such as penile erection and ejaculation. Subsequent c-fos activation is detected in OTergic PVN neurons after sexual activity in the rat (Witt and Insel, 1994). Lesions of OTergic hypothalamic nuclei increase erection latency in rats (Liu et al., 1997). Icv injection of OT decreases ejaculation latency and also decreases post-ejaculatory interval (Arletti et al., 1985). However, lesioning only the parvocellular neurons does not alter ejaculation but decreases the amount of semen emitted (Ackerman et al., 1997). OT action can rely on the release of OT at different central areas, such as the autonomic region of the SC. The blockade of OTR at the L6 level of the SC indeed reduces bulbospongious muscle activity and further ejaculation (Clément et al., 2008). Besides, an increase in systemic OT is detected after ejaculation and might be linked to post-ejaculatory refractoriness (Ogawa et al., 1980; Stoneham et al., 1985).

*(iv) Renal function*

Changes in hydromineral homeostasis are known to promote the release of hypothalamic neurohormones. In response to an increase in plasmatic sodium, OT and VP are released in the blood stream (Verbalis and Dohanics, 1991). OTRs are expressed in rodent kidneys, in Henle's loop, although VP binding sites are present in much higher quantity (Arpin-Bott et al., 2002). OT acts as a nonhypertensive natriuretic agent, since intraperitoneal injection of OT in the rat induces an increase in urinary osmolarity, natriuresis and also kaliuresis (Haanwinckel et al., 1995). It acts mostly by a decrease in tubular $Na^+$ reabsorption, mediated by the stimulation of NO synthetase and release of ANP , both leading to release of cGMP (Soares et al., 1999).

*(v) Cardiovascular function*

OT can regulate cardiac function both by a central and by a local effect(Gutkowska et al., 2000) (Gutkowska et al., 2000). OTRs are indeed expressed by cardiomyocytes in all chambers of the heart, and their activation lead to an increased release of atrial natriuretic hormone in the heart (Favaretto et al., 1997; Gutkowska et al., 1997). OT peripheral injection can hence decrease basal blood pressure in rats, but does not affect heart rate (Petersson et al., 1996, 1997). The heart and vasculature are also responsible for a local OT production, acting in a paracrine/autocrine manner (Jankowski et al., 1998; Gutkowska et al., 2000). Icv OT has similar effects as peripheral OT (Petersson et al., 1996, 1997). In response to stress, icv infusion of OTR antagonist exacerbates the cardiovascular response to the stressor (Wsol et al., 2008).

OT hypothalamic neurons are closely connected to autonomic nuclei in the brain stem and the spinal cord and respond to increases in blood volume. In particular, hypothalamic OT neurons can contact cardiac vagal neurons in the dorsal motor nucleus of the vagus, hence modulating cardiovascular response and decreasing heart rate (Rogers and Hermann, 1985). Chronic activation of these neurons is hence able to prevent hypertension linked to chronic intermittent hypoxia-hypercapnia exposure (Jameson et al., 2016). OT can also modulate cardiovascular response by an indirect action through other brain areas. For example OT infusion in the amygdala increases arterial pressure, heart rate and ventilation (Granjeiro et al., 2014).

Interestingly, when used in early life, OT can have long term effects on cardiac function. OT subcutaneous injections during the first two post-natal weeks reduce systolic and diastolic blood pressure at adulthood (Holst et al., 2002). It seem also to be protective against the consequences of prenatal stress, which results in an increased blood pressure at adulthood, restored by neonatal OT treatment (Holst et al., 2002).

*(vi) Pancreatic function*

OT as well as VP have been detected in human and rat pancreatic extracts (Amico et al., 1988), and OTRs have been detected in A and B cells in the islets of Langerhans (Suzuki et al., 2013). OTRs expressed in rat pancreatic cells have been further cloned and characterized, and identified as the same receptor as the one found in the reproductive tract (Jeng et al., 1996). Early study showed that OT administration into the splenic part of the pancreas can induce the release of insulin and glucagon (Dunning et al., 1984; Stock et al., 1990). Subcutaneous injection of OT is efficient to induce insulin and glucagon release in the blood. However, if icv injection does not change glucagon levels, it increases insulin levels (Björkstrand et al., 1996). The same study showed that nipple suckling in lactating rodents is also efficient to increase glucagon levels.

*(vii)Bone*

Recent studies helped the scientific community to gain knowledge about the interaction between pituitary hormones and bones and the term pituitary-bone axis has recently emerged in the literature. Among "pituitary hormones" (i.e. OT is present in the hypothalamic axons of the posterior pituitary), OT is important for bone mass regulation (Colaianni et al., 2014). Genetic studies reveal that the deletion of OT or its receptor in mice has drastic effects on bone function, since it leads to osteoporosis, due to reduced bone formation in both males and females. OT can indeed act on osteoblasts to promote mineralization and on osteoclast formation itself. It also prevents bone resorption through an action on mature osteoclasts (Tamma et al., 2009). This seems to rely on a peripheral action of OT, which receptors are expressed by osteoblasts and osteoclasts (Copland et al., 1999; Colucci et al., 2002). On top of that, OT can also be released directly by osteoblasts, and regulate bone function by a paracrine/autocrine effect (Colaianni et al., 2014).

*(viii) Immune system*

OT actions on the immune system have been review recently (Li et al., 2017). First of all, OT seems to be involved in the development of immune organs, in particular the thymus. OT is expressed in high concentration in the thymic epithelium, as well as other neuropeptides. A study measured OT levels ranging from 1.5 to 35.5 ng/g wet weight of thymus depending on the patient. It is not classically released by thymic epithelium cells, but is present as a membrane antigen where it interacts with CD8+ maturing pre-T cells (Geenen et al., 1998; Martens et al., 1998). Moreover, a blockade of OTR receptors inhibits the differentiation of thymic T-cells in mice (Hansenne, 2005)

In response to an immune challenge, cultured macrophages increase their OTR expression, making them more sensitive to OT (Szeto et al., 2017). In humans, OT decreases the elevation of pro-inflammatory cytokines and the stress response (i.e. ACTH elevation) induced by LPS injection (Clodi et al., 2008). Immune challenge in rodents has been showed to activate OT hypothalamic neurons and to induce OT release. For example, in response to sepsis, SON magnOT neurons are activated and reorganize OT reserve pools to the axonal compartment, suggesting an adaptation to intense OT secretion (Sendemir et al., 2013). Exogenous OT treatment in a rat model of sepsis seems protective against oxidative damages, as it reduces neutrophil infiltration, lipid peroxidation and inhibits the decrease of antioxidant levels in several organs (İşeri et al., 2005). In response to peripheral carrageenan inflammation, OT subcutaneous application is able to reduce hindpaw oedema, as well as neutrophil recruitment (Petersson et al., 2001). OT level variations and OT anti-inflammatory effects have also been demonstrated in various other pathological models (Li et al., 2017).

An interesting study in Siberian hamster showed that OT may promote wound healing. A model of cutaneous wound has been used in isolated or socially housed hamsters. It shows that wound healing

is impaired in isolated animals and that exogenous OT treatment facilitates recovery time (Detillion et al., 2004).

*(ix) OT and intestinal functions*

The gastrointestinal tract (GI) contains specific endocrine cells called entero-endocrine cells, who release a huge number of peptides, including classical neuropeptides found in the brain, such as cholecystokinin (CCK), CRF, serotonin or ghrelin (Latorre et al., 2016). These peptides can act locally on surrounding cells or on receptors located on nerve terminals, as showed in figure 19. The gut mucosa is close to enteric plexus of neurons, the myenteric and the submucosal plexus, and contacted by axonal terminals of the vagus nerve and by peripheral axonal endings of sensory neurons. In that context, OT may act as a developmental modulator of the enteric nervous system (ENS) and gut function (Welch et al., 2009). OT action could be mediated by receptors expressed at the membrane of smooth muscle cells, enteric neurons or vagal afferences.

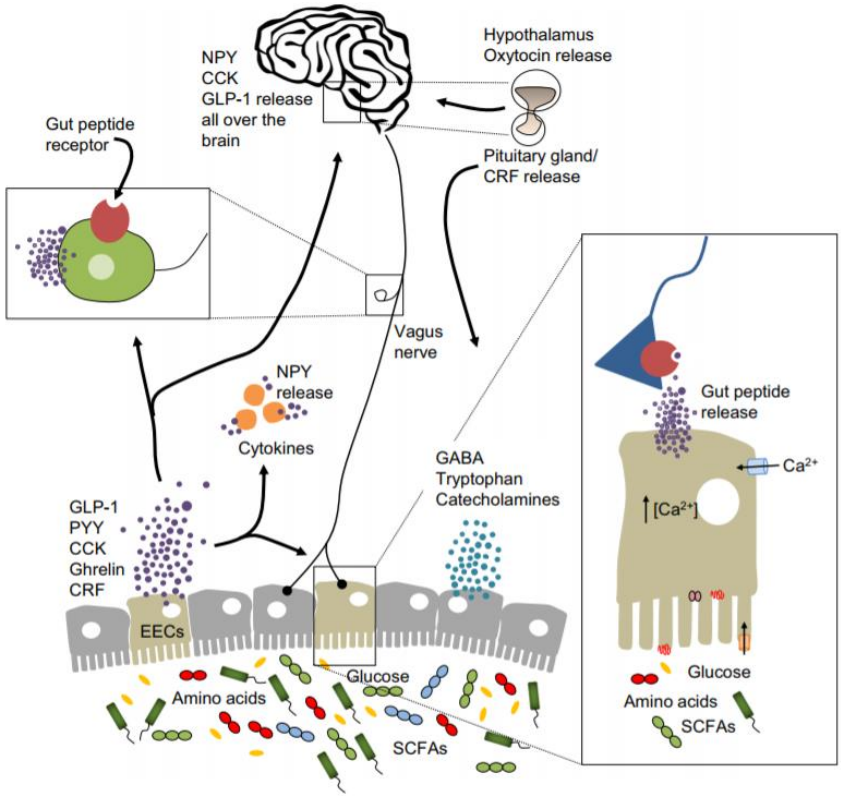


*Figure 19: Communication pathways of the brain-gut axis.*

*CCK = cholecystokinin; EEC = enteroendocrine cells; GLP-1 = glucagon-like peptide; CRF = corticotropin-releasing factor; PYY = peptide YY; GABA = γ-aminobutyric acid; NPY = neuropeptide Y; SCFA = short-chain fatty acid. Adapted from* (Lach et al., 2017)

The first observation supporting this hypothesis is that OT and OTR mRNA are detected all along the human GI tract (Monstein et al., 2004) and in the dorsal motor nucleus of the vagus nerve in the rat (Dreifuss et al., 1988). OT transcripts have been detected in nerve cell bodies and fibers in the myenteric and submucous ganglia, suggesting that OT could act directly on ENS in human and animals (Ohlsson et al., 2006b; Yu et al., 2011). OTRs are expressed by neurons in the myenteric plexus and submucosal plexus in adult rats and mice (Welch et al., 2009, 2014). Vagal sensory

neurons innervating the intra-abdominal viscera also express OTR in the rat. It has hence been proposed that OT could be directly synthesised in a subpopulation of myenteric neurons. It is important to note here that OT and OTR gut expression seems to be developmentally regulated and undergoes post-natal maturation (Welch et al., 2009). This might be important considering the high rate of visceral dysfunction often observed in human and animals subjected to early life stress (ELS), as introduced in paragraphs V.2 and V.4.iii

Another observation supporting the idea that OT could be a key regulator of the ENS resides in the analysis of gut function in OTR KO mice (Welch et al., 2014). First, the number of neurons in the enteric plexus of the small intestine is lower in OTR KO mice, indicating a possible role of OT in the development of the ENS. GI function is also altered in OTR KO mice, which display a faster GI motility, an abnormal intestinal mucosa associated with a limited proliferation of transit amplifying cells and with an increase in the permeability of the intestinal barrier.

Altogether, animal and human studies suggest that OT can modulate several GI functions. Gastric emptying is for example increased by OT in human. However, this might be specie-specific since contradictory results have been observed in animal studies (Petring, 1989; Ohlsson et al., 2006a). In guinea pigs, for example, OT induces a relaxation of the smooth muscles in the gastric antrum (Duridanova et al., 1997). Similar results have been obtained in the rat (Flanagan et al., 1992). Moreover, nasal OT may be protective against constipation, even if it still has to be confirmed since its effect does not differ from the one of the placebo. But it seems to be protective against abdominal pain and discomfort in women suffering from refractory constipation (ohlsson et al., 2005). Besides, pathologies linked to the GI tracts can be affected by the absence of OT signalling pathways, or by exogenous OT treatment. For example, the severity of experimental colitis is higher in OTR KO mice (Welch et al., 2014). Clinical and histological damage scores are indeed higher, associated with increased inflammatory factors. In this experiment, OT was also efficient to alleviate colitis symptoms in normal mice.

On top of these effects of OT on the GI tract, recent studies identified that the gut microbiota can modulate the function of the OT system, leading to a bidirectional gut-OT pathway. Treating adolescent mice with antibiotics reduces OT and VP expression in the brain at adulthood (Desbonnet et al., 2010) and alters OT-related adult behaviour (anxiety, social and cognitive behaviour) as showed also in germ-free mice (Crumeyrolle-Arias et al., 2014; Desbonnet et al., 2014; Fröhlich et al., 2016). A component of the microbiota, Lactobacillus reuteri (L. reuteri) has been identified to interact with the OT system. Feeding animals with the bacteria induces modification in some OT-dependent behaviours, such as grooming activity, maternal behaviour or wound healing (Ibrahim et al., n.d.; Levkovich et al., 2013; Poutahidis et al., 2013). This is associated with an increase in systemic OT levels and an increase in OT-positive cells in the PVN (Poutahidis et al., 2013; Varian et al., 2017). It is hypothesized that this modulation of the OT system is controlled by the vagal pathway, as it has been showed also for other gut-brain interaction (Cryan and Dinan, 2012; Lach et al., 2017). In accordance with the idea that maternal microbiota can influence later brain function in its offspring (Tochitani et al., 2016), maternal high fat diet decreases L.reuteri levels in the offspring, as well as the number of OT neurons in the hypothalamus, which is a possible explanation for the social deficit detected in these animals (Buffington et al., 2016). In agreement, these alterations can be restored by L. reuteri treatment in the offspring.

### 2) OT central functions

In this part, we will review OT functions on the CNS with a focus on nociception and functions linked to social behaviour and anxiety since they are the most relevant in the context of early life stress and

maternal separation. Figure 20, from (Gimpl and Fahrenholz, 2001), gives a summary of central OT function identified in different animal species.

| Behaviors | Species | | | | | Reference Nos. |
|---|---|---|---|---|---|---|
| | Rat | Mouse | Prairie vole | Sheep | Human | |
| Maternal behavior | ↑ | (↑) | ? | ↑ | ? | 163, 256, 289, 367, 440, 441, 599, 616 |
| Female sexual behavior | ↑ | ↔ | ↑ or ↓ | ↓ | ↑ | 13, 87, 122, 256, 288, 416, 599, 601 |
| Male sexual behavior | ↑ or ↓ | ↔ | ↓ | ? | ↑ | 21, 24, 90–92, 286, 356, 416, 537, 599 |
| Female affiliative behavior | ? | ↔ | ↑ | ↑ | ? | 256, 286, 416, 593 |
| Male affiliative behavior | ↑ | ↔ | ↑ or ↔ | ? | ? | 111, 256, 416, 602, 613 |
| (Auto)grooming | ↑ | ↑ | (↑) | ? | (↑) | 83, 127, 140, 324, 356, 375, 568, 600, 602 |
| Social memory | ↑ | ↑ | ↑ | ↑ | ? | 94, 125, 137, 154, 256, 286, 459, 598 |
| Male aggression | ? | ↑ | ↑ or ↔ | ? | ? | 130, 286, 356, 597, 613 |
| Female aggression | ↑ or ↓ | ? | ↓ | ? | ? | 172, 213, 286, 600 |
| Nociception | ↓ | ↓ | ? | ? | ↓ | 27, 350, 606 |
| Anxiety | ↓ | ↓ | ? | ? | ? | 368, 371, 566, 595 |
| Feeding | ↓ | ? | ? | ? | ? | 25, 26, 382, 574, 576 |
| Memory and learning | ↓ | ↑ or ↓ | ? | ? | ↓ | 56, 60, 76, 133, 134, 155, 170, 310, 431 |
| Tolerance to opiates | ↓ | ↓ | ? | ? | ? | 313, 498 |

*Figure 20: Effects of OT on behaviours in different species.*

↗ indicates a faciliatory effect, ↘ indicates an inhibitory effect and ↔ indicates no effect. Symbols in parentheses indicate weak effects or tendencies. Adapted from (Gimpl and Fahrenholz, 2001).

### *(i) Pain modulation*

#### *(a) Short overview of the pain system*

Pain is the result of the activation of nociceptive neurons by peripheral mechanical, thermal, or chemical noxious stimulations. These stimulations are detected by peripheral nociceptors located on nerve endings in skin, viscera and other organs. They transmit the nociceptive information throughout the axons of the DRG neurons to the dorsal horn of the SC. Primary afferent neurons can be either non-myelinated C-fibers of small diameter (0.1-1.2 µm), thinly myelinated Aδ fibers (2-6 µm), or myelinated Aβ fibers (> 10 µm). However, Aβ fibers transmit non-nociceptive sensory information like touch. C fibers mostly innervate superficial layers of the spinal cord (I and II), whereas Aδ fibers also target deeper layers (see figure 21 and (Todd, 2010)). In the SC, nociceptive information is processed by a complex network of interneurons and then transmitted to supraspinal centers by projection neurons, located mostly in lamina I and lamina III, IV. Lamina II is mostly composed of interneurons, of excitatory and inhibitory neurochemical nature. Projections ascend through the contralateral spinothalamic tract or other tract responsible for the autonomous or emotional response to pain. Through these pathways, the nociceptive information is distributed to the thalamus and cortex, as well as RVM, PAG, dorsal reticular nucleus (not exhaustive, see figure 22). Through a projection onto autonomous brainstem nuclei like the nucleus of the solitary tact, projection neurons induce the cardiovascular reflex response to noxious stimulation. The emotional response to pain is supposed to rely mostly on ipsilateral projections reaching the lateral parabrachial areas, which are interconnected with the amygdala and hypothalamus. Locally, the spinal neuronal network activated by noxious stimulation also induces the activation of ventral motoneurons which lead to a withdrawal reflex of the targeted area.

The integration properties of dorsal horn neurons are also modulated by descending information from different supraspinal regions, being either inhibitory or excitatory, as showed in figure 22 (Millan, 2002; Heinricher et al., 2009). Descending inputs from the RVM and PAG have been extensively studied, but other regions like the hypothalamus also send descending direct or indirect projections to the spinal cord in order to modulate nociceptive processing. Many neuropeptides

synthesised in the HT, including OT and VP, have been described to modulate pain processing and behaviour.

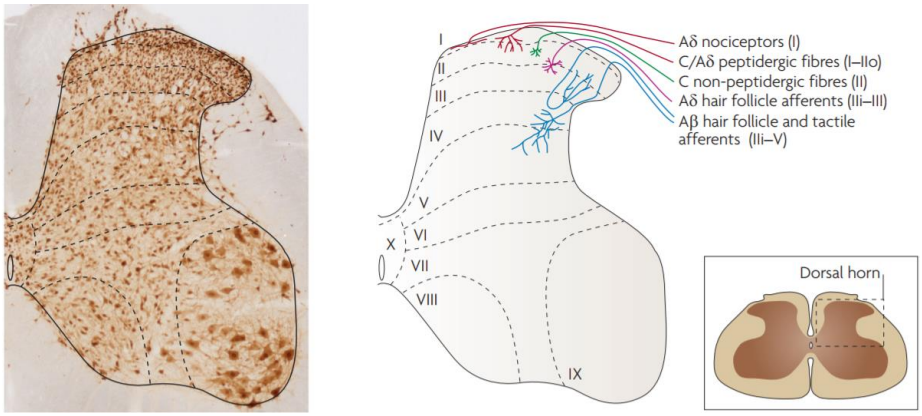


*Figure 21: Laminar organization of the dorsal horn and primary afferent inputs.*

Adapted from (Todd, 2010).

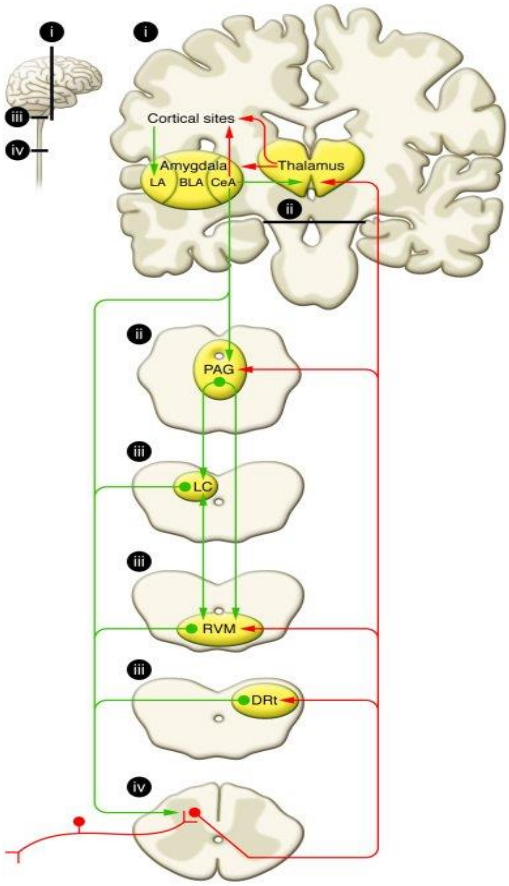


*Figure 22:Schematic representation of pain modulatory circuits.*

Ascending pathways are in red and descending pathways in green. BLA = basolateral amygdala, CeA = central amygdala, Drt = dorsal reticular nucleus, LA = lateral amygdala, LC = locus coeruleus, PAG = periaqueducal grey, RVM = rostral ventromedial medulla. Adapted from (Ossipov et al., 2010).

*(b) Development of the nociceptive system*

In human and rodents, the nociceptive system develops before birth but undergoes an important maturation after birth. The development of nociceptive system in human is shown in figure 23. Cutaneous innervation by sensory neurons starts during the 2nd trimester of gestation in human. Spinal connections mature until the end of gestation, with C fibers being the ultimate sensory fibers to establish functional connections with SC neurons. During the last trimester, functional connections with higher brain centers are created. The myelination of ascending fibers starts during pregnancy and is complete up to the thalamus at the 30th week of gestation. Evoked cortical potentials can hence be recorded in response to a stimulation of peripheral receptive field as soon as the 30th week of gestation (Slater et al., 2010b). The capacity of the cortex to integrate sensory information, including nociceptive, has also been confirmed with near infrared spectroscopy (Bartocci et al., 2006). Finally, the integration of nociceptive vs non nociceptive stimulation seems to rely on distinct brain areas between the 35th and 37th week of gestation (Fabrizi et al., 2011). Imaging studies clearly reveal that the infant brain, a few days after birth, is able to process sensory and pain information, and that brain activation increases with the stimulus intensity (Williams et al., 2015).

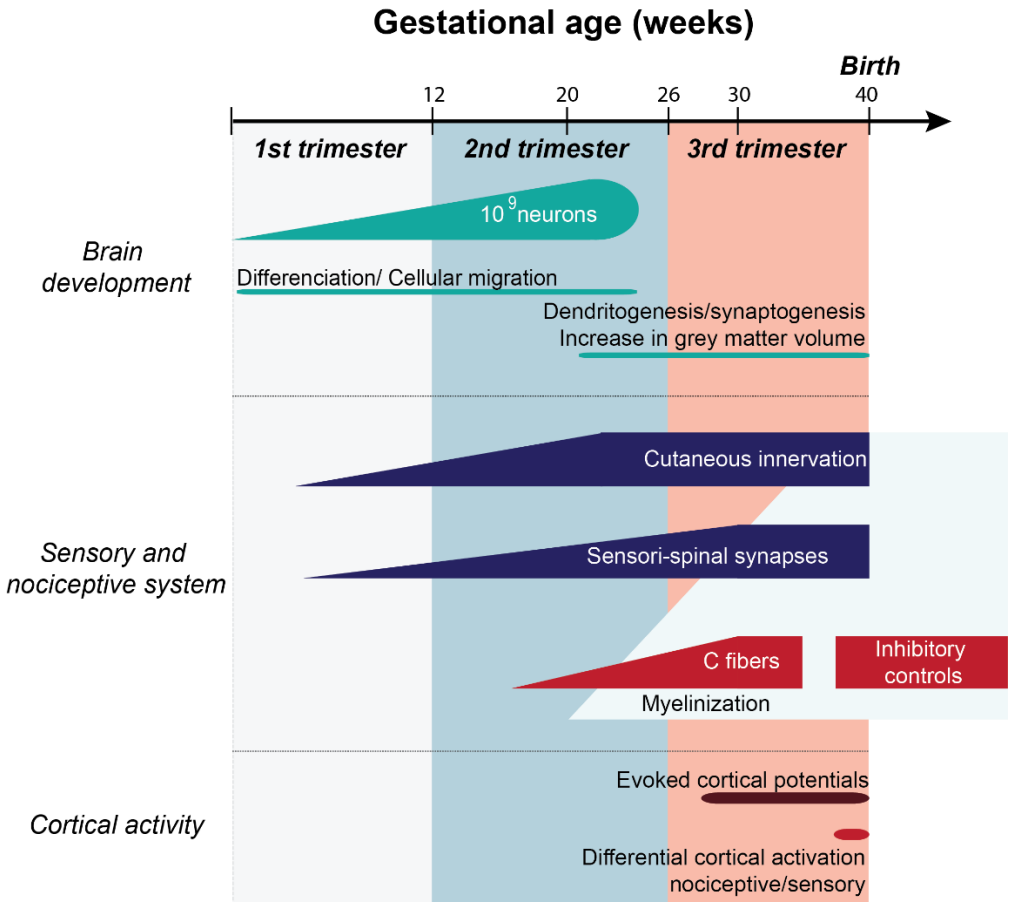


*Figure 23: Development of the nociceptive brain in the human foetus.*

*Adapted from* (Melchior and Poisbeau, 2015)

In the rat, gestation lasts 3 weeks. At birth, the rat nociceptive system is at the same developmental stage as the human foetus at the beginning of the 3rd trimester. DRG neurons are born at E12, A-type neurons first and then C-type neurons. Peripheral and central innervation from these neurons starts soon after E12, and they enter the SC at E15. The expression of voltage gated channels by DRG

neurons begins during gestation but also goes on after birth. For example, Nav1.8 expression starts at E15 but reaches adult levels only at P21. As in the human foetus, the projections to higher brain centers are functional at birth (Man et al., 2012).

After birth, the SC undergoes a major re-organization in rodents. A fiber inputs are predominant in the neonatal SC and are more diffuse compared to what is seen in the adult SC. Primary afferent inputs re-organize during the first three post-natal weeks, with A fibers withdrawing from lamina I and II to relocalize in deeper lamina, by an activity-dependent process (Beggs et al., 2002). In the SC, lamina I projection neurons mature before local interneurons (Fitzgerald, 2005). But for a proper and sharp regulation of SC nociceptive information by interneurons, chloride homeostasis is also a key factor. This parameter is also developmentally regulated (Cordero-Erausquin et al., 2005; Ben-Ari, 2014). Contrary to the adult SC, NKCC1 transporter is highly expressed after birth, resulting in high intracellular chloride levels. The other chloride transporter KCC2 is expressed in low levels in the post-natal period. This lead to a depolarizing effect of GABA in the neonatal SC. Endogenous controls of pain are also extensively immature at birth, as it has been described for example with the opioidergic system. Kwok and colleagues showed that in the Sprague Dawley rat, microinjection of Mu opioid receptor agonist in the PAG increases the sensibility to mechanical stimulation in P21 rats, but inhibits nociceptive responses at adulthood (Kwok et al., 2014). Similar observations have been obtained with injection of MOR agonist in the RVM. It is noticeable also that the regulation of RVM descending activity by nociceptive input is ineffective in the neonatal rat before P8 (Schwaller et al., 2016). The OT system also develops partially after birth, as will be described in paragraph IV.

Functionally, these maturation processes have direct consequences on pain behaviour in the newborn. Especially newborn babies, as well as newborn rodents, are actually hypersensitive to pain (Fitzgerald and Beggs, 2001). This is even more true with preterm babies, born during the 3$^{rd}$ trimester of gestation. Due to their maturation state at birth, these systems are particularly sensitive to any change in the environment of the newborn. Early life events can hence modify strongly the development of the nervous system and the function of the brain, possibly leading to a range of sensory, behavioral or cognitive deficits at adulthood, as seen in ARTICLE 2 with the example of maternal separation.

### *(c) OT action on central processes*

- Spinal modulation

OT neurons send projections and can modulate the activity of many brain areas involved in pain processing (Boll et al., 2017). In particular, a direct spinal projection is ensured by parvOT neurons which establish synaptic contact with superficial dorsal horn neuron in lamina I and II, as well as with neurons located in the autonomous nuclei and in lamina X around the central canal (Swanson and McKellar, 1979; Rousselot et al., 1990; Puder and Papka, 2001; Jójárt et al., 2009). The localisation of these projections corresponds to areas where OTRs have been detected (Reiter et al., 1994; Breton et al., 2008)

An electrical stimulation of the anterior part of the PVN increases OT levels in the SC, confirming that OT is directly released there (Martínez-Lorenzana et al., 2008). Recording the activity of WDR neurons in the SC showed that the electrical stimulation of the PVN decreases the activity of theses neurons, which was reversed by spinal application of a selective OTR antagonist (Condés-Lara et al., 2006). The direct application of OT onto the SC had the same effect. Another study investigated OT effect on the long-term potentiation of WDR nociceptive evoked responses, which facilitates nociceptive evoked responses. (DeLaTorre et al., 2009). PVN stimulation and OT application inhibited

the LTP induced facilitation of nociceptive evoked responses of WDR neurons. Using patch clamp experiments, Robinson and colleagues showed that bath application of OT inhibits glutamatergic EPSC induced by stimulation of dorsal root nerves. On the behavioural level, PVN stimulation as well as OT application can decrease hyperalgesia and allodynia symptoms in a neuropathic model of loose ligature of the sciatic nerve (Condés-Lara et al., 2006; Miranda-Cardenas et al., 2006). The LTP-mediated hyperalgesic symptoms are also suppressed by PVN stimulation and spinal OT application (DeLaTorre et al., 2009).

This analgesic OT system is endogenously activated in response to different stimulations, either in response to nociceptive challenge, during lactation, or in response to environmental stress. In response to cold forced swim stress (10 degrees 3 minutes) or restraint stress, OT neurons are activated in the HT (Ceccatelli et al., 1989; Robinson et al., 2002). After swim stress, mice display a transient analgesia which is blocked by i.t injection of OTR antagonist and reduced in OT KO mice (Robinson et al., 2002) . In a rat model of inflammatory pain induced by carrageenan, OT neurons activation lead to increased OT levels in the spinal cord 24H after inflammation (Juif et al., 2013). In that case, OT has a potent analgesic effect on mechanical and thermal responses. In response to hindpaw formalin injection, parvOT neurons in the anterior part of the PVN are activated, as well as the magnocellular portion of PVN and SON, as demonstrated with c-fos expression (Matsuura et al., 2016a). In that study the blockade of OT signalling in the SC via an i.t injection of OT-saporin induced a behavioural nociceptive hypersensitivity.

Gutierrez and colleagues investigated the nociceptive sensitivity of pregnant female rats before and after delivery (Gutierrez et al., 2013). They performed spinal nerve ligation at mid-pregnancy which induced similar behavioural symptoms as in non-pregnant females. But interestingly, after delivery, when OT levels in spinal CSF are increased, these symptoms partially resolved. This is reversed by an i.t injection of the OTR antagonist atosiban, suggesting that spinal OT is responsible for protection against chronic pain after delivery. When the SNL is performed at the time of delivery, symptoms start to develop similarly to non-pregnant animals, but decrease soon after. A supplementary information is given in this article and will be further discussed later in regard to our results with the maternal separation model. Indeed, separation of the mother from the pups after delivery seems to prevent the OT-related improvement of pain symptoms, and the increase in OT levels in spinal CSF.

*Spinal mechanism of OT action*

Spinal application of bicuculline prevents the effect of PVN stimulation on lamina II neuron responses, suggesting that GABAergic interneurons mediates OT anti-nociceptive effect (Condés-Lara et al., 2009). On top of that, during PVN stimulation, an activation of previously silent neurons has been detected in the SC, and is increased with bicuculline (Condés-Lara et al., 2009). Breton and colleagues further demonstrated that OT activates a subpopulation of glutamatergic interneurons, presumably expressing OTRs, located in superficial lamina II of the SC. These neurons, while activated by OT, recruit inhibitory GABAergic interneurons in lamina II and decrease spinal nociceptive processing triggered by the activation of C and Aδ fibers, preferentially via a presynaptic effect on primary afferent neurons, as seen in figure 24.

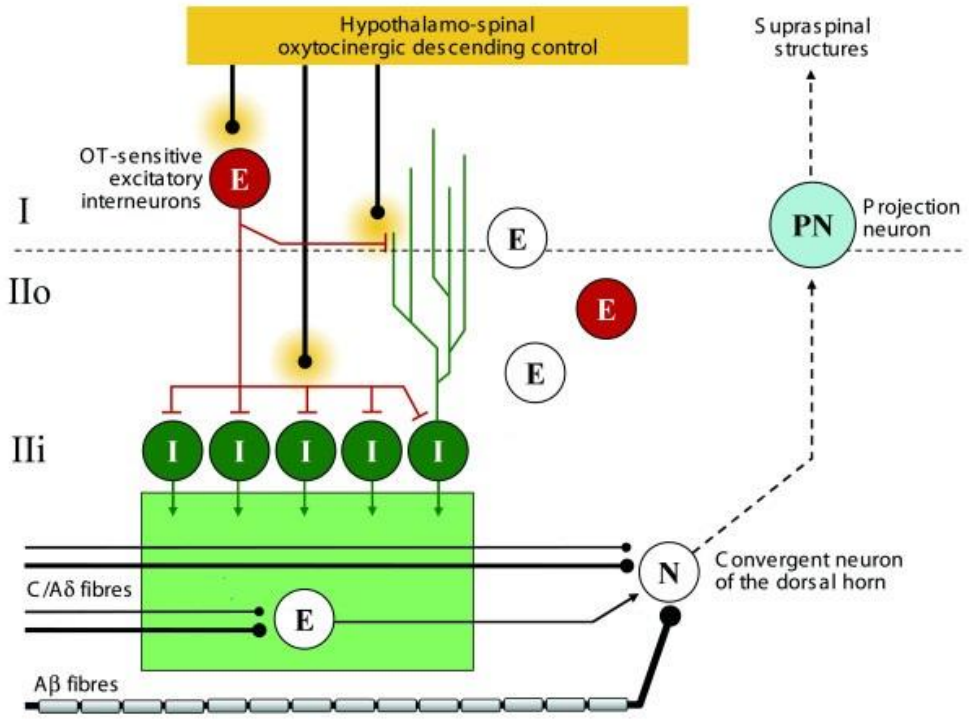


*Figure 24 : OT effects in lamina I and II sensory neurons.*

*E = excitatory interneurons, I = inhibitory interneurons. Adapted from* (Breton et al., 2008)

OTR activation can also modify the firing response of a subset of lamina II interneurons displaying repetitive firing action potential discharges. In these neurons, OTR activation leads to a change of firing pattern, from repetitive to delayed profiles, and from delayed to phasic. This seems to be the result of a decrease in the amplitude of transient Ia current (Breton et al., 2009). Apart from these immediate effects, OT seems to also exert long lasting effects involving the stimulation of neurosteroidogenesis. This leads to the production of AP, a positive allosteric modulator of chloride-permeable GABAa receptor-channels which increase inhibitory function in the SC (Juif et al., 2013).

OT signalling in the spinal cord has also been proposed to have an anti-inflammatory role which could be relevant in cases of chronic inflammatory pain disease, as suggested in a rat model of arthritis (Matsuura et al., 2016b).

- Supraspinal modulation

OT is not only involved in the peripheral and spinal level but can also modulate nociceptive responses in different central regions of the brain and brainstem. Indeed, pain stimulation induces changes in OT content in different brain areas, including HT, locus coeruleus, raphe magnus, caudate nucleus, PAG (Yang et al., 2007, 2011a; ZHANG et al., 2015). Intracerebroventricular (icv) injections of OT successfully reduce pain responses in rodents, including in inflammatory pain models (Russo et al., 2012), and in a model of incision-induced mechanical allodynia (ZHANG et al., 2015). In the last model, i.t OT injection had no effect on pain responses, suggesting a supraspinal mode of action rather than a spinal one. In accordance with these observations, icv injection of OT anti-serum reduces pain threshold (Yang et al., 2007) and supresses copulation-induced hypoalgesia (Futagami et al., 2016). A study by Condes-Lara and colleagues showed that stimulation of the PVN induces c-fos activation in PAG, raphe magnus and locus coeruleus, indicating a possible involvement of these structures in OT analgesia (Condés-Lara et al., 2015).

***PAG and RVM***. The PAG is a key area in pain modulation, since it is at the origin of a descending control of pain and highly connected with RVM and central amygdala (Rizvi et al., 1991; Martins and Tavares, 2017). Nociceptive stimulation induces an increase in intra-PAG OT levels (Yang et al., 2011a). Intra-PAG OT injections can increase pain threshold, but this effect is attenuated when mu or kappa opioid receptors are blocked in the PAG (Ge et al., 2002). Later a study showed that OT can change L-enkephaline, Met-enkephaline and beta-endorphin levels in the PAG (Yang et al., 2011b). This is in accordance with other studies showing that the antinociceptive icv action of OT is decreased when injected simultaneously with mu or kappa opioid receptor antagonists (Gao and Yu, 2004; Russo et al., 2012)

***Nucleus accumbens (NAc)***. Intra-NAc injections of OT can also induce analgesia in rodents (Gu and Yu, 2007). Once again, this analgesia is decreased by the blockage of mu and kappa opioid receptors, suggesting that the supraspinal action of OT involves the opioidergic system at different levels.

***Raphe Magnus***. Similarly, intra-raphe magnus injection of OT has an analgesic effect. It is partially blocked by the concomitant injection of mu receptor antagonist but not kappa or delta opioid receptor antagonists (Wang et al., 2003). This suggests that the serotoninergic system might be involved in the anti-nociceptive effect of OT. In accordance with that, the antinociceptive effect of PVN electrical stimulation is still present but reduced when raphe magnus is lesioned (Condés-Lara et al., 2012). Moreover, when infused intrathecally, serotonin can mimic the effect of OT and even potentiates OT-induced analgesia (Godínez-Chaparro et al., 2016)

***Amygdala***. The amygdala plays a key role in emotional responses, and is an important area for pain processing in its emotional dimension (Neugebauer, 2015). Within the amygdala, neurons located in the lateral part of the central amygdala express OTR (Huber et al., 2005). Injections of OT in the central amygdala dose dependently increases pain threshold (Han and Yu, 2009)

### *(d) Peripheral OT modulation of pain*

Via its neurohormonal release, OT can reach the general blood circulation and act on different targets. An increase in plasmatic OT has been described after several nociceptive stimulations, being either formalin injection in the hindpaw, peripheral inflammation or neuropathic pain (Yu et al., 2003; Martínez-Lorenzana et al., 2008; Chow et al., 2013; Matsuura et al., 2016a). After a protocol of forced swim stress (FSS), plasmatic levels of OT are also transiently increased from 15pg/mL to 45 pg/mL in rats (Wotjak et al., 1998). Moreover, exogenous peripheral application of OT can modulate nociceptive responses. In that case, OT could act by an action on OTR or V1aR, since OT analgesia is still present in OTR KO mice after systemic injection but lost in V1aR KO (Schorscher-Petcu et al., 2010). Another study also demonstrates that OT-induced activation of V1aR in the trigeminal ganglion increases rheobase (i.e. minimal intensity for an excitatory current to trigger action potential) and hyperpolarizes neurons in a model of infraorbital nerve injury (Kubo et al., 2017). Contrary to OTergic spinal analgesia, peripheral OT analgesia is apparently not blocked by naloxone (Kang and Park, 2000). Since OT is not able to cross easily the blood brain barrier (BBB), peripheral OT (including blood OT) does not seem to exert its action by targeting CNS structures, but obviously, acts on other peripheral targets which may possibly be OTRs expressed by skin cells or axonal peripheral afferent fibers of DRG neurons (Ermisch et al., 1985). Indeed DRG non peptidergic C-fibers as well as trigeminal ganglia neurons express OTR (Wrobel et al., 2011b; Moreno-López et al., 2013). OT applied on dissociated and cultured DRG rat cells inhibits the depolarization-induced increase in intracellular calcium in response to KCl application. This effect seems specific of nociceptive neurons since they also respond to capsaicin, an active principle of chili activating TRPV1 channels specifically expressed by a subtype of nociceptive C-type sensory neuron (Hobo et al., 2012). OT also suppresses ATP evoked currents in freshly-isolated DRG neurons, via cAMP-PKA and calcium intracellular pathway

(Yang et al., 2002). Protein kinase C is also suggested to be implicated in the increase in intracellular calcium (Ayar et al., 2014) Later, another study showed that OT induces membrane hyperpolarization of cultured rat DRG neurons, through an intracellular calcium increase and the activation of NO signalling pathway and KATP channels, as seen in figure 25 (Gong et al., 2015).

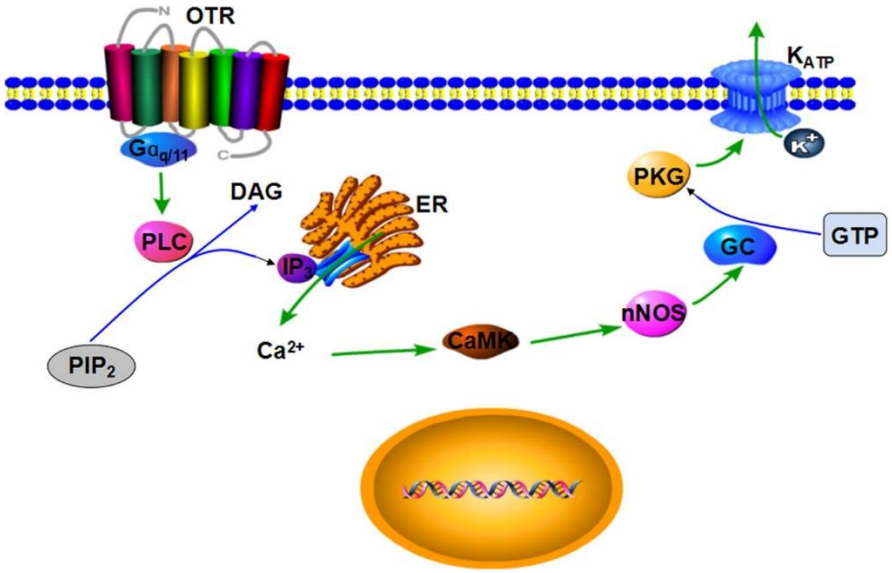


*Figure 25: Intracellular pathway involved in OT antinociceptive action in rat DRG neurons.*

Adapted from (Gong et al., 2015).

Peripheral OT can also inhibit the function of ASICs channels expressed by DRG neurons and activated by acidic stimuli. In that case, OT effect is similar to that of VP and is mediated by V1aR (Qiu et al., 2014). Interestingly, if OT acts mostly through OTR, or sometimes V1aR, a recent study showed that it can directly activate the TRPV1 receptor-channels, originally characterized as responding to noxious heat and capsaicin (Nersesyan et al., 2017). Using heterologous expressing systems and patch clamp experiments, they showed that OT induces calcium influx through TRPV1 receptor-channels and rapidly promotes desensitization of the channel. This lead to the hypothesis that OT analgesic action might rely on the transient activation and rapid desensitization of TRPV1 channel in nociceptors, as suggested in an in vivo model using co-applications of OT and capsaicin.

An important issue in this context of peripheral OT analgesia is that the final analgesic effect may depend on the actual dose of OT reaching the target tissues. Our laboratory characterized the effect of intravenous low and high doses of OTR and V1aR agonists on nociceptive pain processing by spinal WDR neurons. Low doses of TGOT (inf 5µg), as well as low VP doses (inf 500pg) have antinociceptive effects. The antinociceptive effects were blocked by application of OTR antagonist. However, higher doses of the peptides induced a pro-nociceptive effect, attributed to an action on V1aR (Juif and Poisbeau, 2013).

On top of that, direct application of OT in the peripheral site of lesion can also modulate lesion-induced pain symptoms. In a carrageenan model, subcutaneous injection of OT in the hindpaw reduces the oedema, neutrophil recruitment and partially decreases hyperalgesic symptoms (Petersson et al., 2001). This effect might rely on the capacity of subcutaneous OT to inhibit Aδ and C fibers activation since OTR immunoreactivity has been detected in nociceptive specific terminals of

the skin (González-Hernández et al., 2017). OT subcutaneous application can indeed reduce the activity of WDR neurons due to the activation of Aδ and C fiber. In recent years, the skin component of OT analgesia has raised numerous questions, especially since the observation that keratinocytes can release OT (Denda et al., 2012). The touch-induced analgesia measured after massage, acupuncture or even kangaroo mother care in preterm babies could indeed relay on skin OT release, among other mechanisms (Liu et al., 1990; Uvnäs-Moberg et al., 1993; Agren et al., 1995; Matthiesen et al., 2001; Vittner et al., 2017) .

### *(e) OT and visceral pain*

OT antinociceptive action has also been investigated in the context of visceral pain in rodents and humans. The most common visceral pain in human is irritable bowel syndrome (IBS). The mean prevalence for this disease is about 5-10 % in the United states, Europe and china (Enck et al., 2016). These patients suffer from abdominal pain and altered bowel function, which is often incapacitating for social or professional life (Dapoigny et al., 2004; Patel et al., 2016). As will be discussed later in this introduction, early life stress is also a risk factor to develop IBS, as a great amount of patient were submitted to physical, emotional or sexual abuse or other early stress during infancy (Drossman et al., 1990; Salmon et al., 2003; Ross, 2005). OT can modulate GI functions and is a good candidate to possibly explain some IBS related dysfunctions. It can also be used as a therapeutic agent to treat IBS symptoms.

Animal studies showed that OT can modulate visceral sensitivity. This idea is supported by the observation that lactating rats are less sensitive to visceral pain, as measured in response to urinary bladder distension (UBD) (Black et al., 2009). Exogenous i.p injection of OT decreases EMG response to UBD, both at baseline, and after chronic stress (Black et al., 2009). Intrathecal OT has also been tested as an anti-nociceptive agent in response to UBD in rats, decreasing visceromotor responses and the activity of UBD-responding dorsal horn neurons (Engle et al., 2012). In a model of visceral hypersensitivity induced by maternal separation, OT treatment reduces enteric glial cells activation and TLR4 signalling. In this model, animals display baseline and post-stress visceral hypersensitivity measured by the visceromotor response to colorectal distension. In this context, OT treatment also inhibits the visceral hypersensitivity induced by a water avoidance stress (Xu et al., 2018). OT antinociceptive function was further described in another model of visceral hypersensitivity induced by trinitrobenzene sulfonic acid application, a model in which colonic OTR are up-regulated (de Araujo et al., 2014).

In human, patients suffering from IBS show decreased pain thresholds in response to colonic distention after local OT infusion, at doses starting at 20mUI/min (Louvel et al., 1996). After a 2-week nasal infusion, OT treatment had a small effect on abdominal pain in patients with chronic constipation, but didn't improved GI function (ohlsson et al., 2005)

OT mechanisms of action have been investigated by in vitro recording of mesenteric afferent discharge in the rat. OT alone does not change the firing rate of primary afferents but modulates the effects of exogenous bradykinin and distention-induced increases in action potential discharge. This effect seems to rely on NO synthesis and $K_{ATP}$ channel activity (Li et al., 2015). The antinociceptive effect of OT along the GI tract might also be due to its anti-inflammatory properties. OTRs have been detected in colonic mast cells and can indeed inhibit mast cell degranulation and histamine release after trinitrobenzene sulfonic acid application, via a calcium-NO synthestase pathway (Gong et al., 2016).

### *(f) Specific case of OT and newborn analgesia*

In the newborn, the first nociceptive event is often delivery itself. The mechanical compression of the foetus during natural delivery, as well as forceps extraction is indeed a huge stressful event for the newborn (Lagercrantz, 2008). A study by Bergqvist suggested that during vaginal delivery, biological processes are recruited to protect the foetus and to reduce stress and pain reactivity (Bergqvist et al., 2009). Newborn analgesia during delivery has been suggested to rely on OT release in the foetus. Indeed, in a rat animal model Mazzuca and colleagues showed that newborn rats display higher pain thresholds in the tail flick test as compared with 2 days-old rats. Blocking OTR receptors in newborn rats suppressed this analgesia, confirming that it relies on endogenous OT increase (Mazzuca et al., 2011). To understand the mechanism of action of OT in the newborn, one must remember that in the neonates, chloride homeostasis is different from the adult SNC. NKCC1 membrane chloride transporters are highly expressed in the newborn neuronal cells, resulting in a depolarizing action of GABA which might even be excitatory in some case. In that context, OT modulates GABA signalling in primary nociceptive neurons and reduces GABA evoked calcium transient, an effect mimicked by the NKCC1 blocker Bumetanide. Similar protective actions of OT have been described in other foetal brain structures (Tyzio et al., 2006). Since the OT system is not functional at birth in the rat, the authors suggested that during delivery OT may be provided by the mother. In humans, the OT system is more mature at birth and OT analgesia may rely on foetal OT. This neuroprotective function of OT during delivery may be critical for the proper function of the brain, since the absence of OT-induced GABAergic inhibition induces autistic-like behaviours in rodent models (Tyzio et al., 2014).

### *(g) Efficiency of OT treatment in human*

In human, chronic pain conditions have been associated with decreased OT levels in plasma. For example, children with recurrent abdominal pain have lower OT plasmatic levels compared to control children (24 versus 63pmol/L) (Alfvén et al., 1994). Different pain sensitivity and pain tolerance in healthy people was also linked to differences in OT functions (Grewen et al., 2008). Clinical studies concerning the efficiency of OT treatment by different routes of administration have been led using double blind placebo controlled trials and experimental studies, but the results are still conflicting, as reviewed recently by Boll and colleagues (Boll et al., 2017). Intranasal OT seems the more promising since other administration routes, such as sublingual, show a too small bioavailability (< 0.07 %) to be of any therapeutic use (De Groot et al., 1995). Oral administration of the peptide is made uneasy because of the rapid degradation along the GI tract (Fjellestad-Paulsen et al., 1995). Intranasal OT might exert its effect via different pathways, as illustrated in figure 26. These pathways include passage through the nasal vasculature into systemic circulation, passage through the olfactory nerve into the olfactory bulb and surrounding lymphatic fluid and a trigeminal pathway to the brainstem (Quintana et al., 2015).

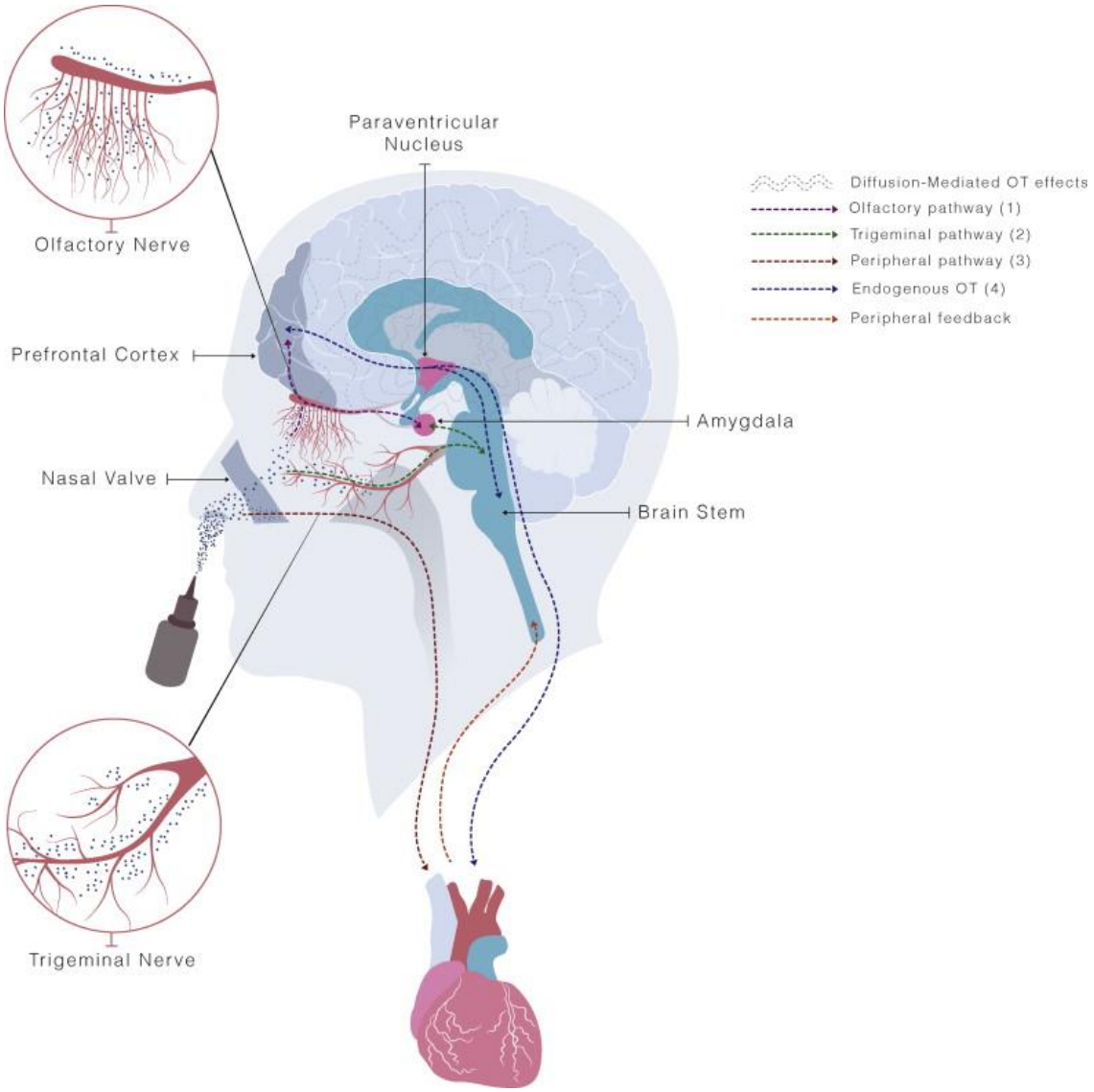


*Figure 26: Possible modes of action of intranasal oxytocin.*

Adapted from (Quintana et al., 2015)

Intranasal OT treatment seems efficient to decrease pain intensity ratings and pain unpleasantness in response to acute cold pressor pain, in response to radiant heat pulses activating Aδ/C-type fibers and to alleviate headache (Wang et al., 2013; Rash and Campbell, 2014; Paloyelis et al., 2016). In the latter study on headache, OT analgesia is associated with a decrease in the amplitude of N1 and N2 component of laser-evoked potentials measured by EEG. Several other studies did not show any analgesic effect of intranasal OT, in the modulation of ischemic pain sensitivity, for example, or in fibromyalgia pain syndromes (Goodin et al., 2014; Mameli et al., 2014). Intranasal OT even increased pain ratings of heat stimulation in women suffering from chronic neck and shoulder pain (Tracy et al., 2017). Interestingly, a clinical trial shows that, even if nasal OT does not change pain ratings and related brain activity, it enhances the influence of picture valence of unpleasantness ratings of noxious heat, when images with an emotional valence are showed simultaneously to the stimulation (Zunhammer et al., 2016). OT nasal treatment can also increase endogenous pain inhibition via conditioned pain modulation (Goodin et al., 2014). Apart from the intranasal route of administration, a dose dependant analgesic effect could be recorded in patients suffering from low back pain after i.t OT application, and in patients suffering from irritable bowel syndrome after local colonic OT

application (Yang, 1994; Louvel et al., 1996). Last but not least, a phase I study on intrathecal OT did not show any changes in pain scores in response to noxious heat stimuli (Eisenach et al., 2015).

*(ii) Interaction with the HPA axis*

The OT system is in close interaction with the HPA axis, involved in stress responses (Engelmann et al., 2004). Hypothalamic neurons release CRH and VP that stimulate the release of ACTH from the anterior pituitary gland. This leads to an increase in the production of glucocorticoids from the adrenal gland (cortisol in human and corticosterone in rodents). In response to stress, the hypothalamic OT system is activated, as showed by the increase in plasmatic OT levels in rodents detected after variety of stressors, including restraint, foot shock of forced swim (Carter and Lightman, 1086; Wotjak et al., 1998; Danevova et al., 2013; Minhas et al., 2016). In humans, lower salivary or plasmatic OT levels are also correlated with increased anxiety levels (Scantamburlo et al., 2007; Weisman et al., 2013; Lebowitz et al., 2015). Actually, OT has anxiolytic properties. In situation where OT levels are high, such as lactation and maternal-infant interactions, the reactivity of the HPA axis is indeed blunted (Neumann et al., 1999; Heinrichs et al., 2001). At the behavioural level, OT release induced by mating induces a decrease in anxiety-like activity in the elevated plus maze and in the black-white box in rodents (Waldherr and Neumann, 2007). Moreover, the OT KO mice exhibit increased anxiety behaviours in the elevated plus maze, which can be restored with exogenous OT icv injections (Mantella et al., 2003). In wild type animals, exogenous OT applications also display anxiolytic effects, as showed with intrahypothalamic infusion or icv injections or rodents in response to acute (Windle et al., 1997; Blume et al., 2008) or chronic stress (Zheng et al., 2010). I.p injection of carbetocin also modulates anxiety behaviour in the open field and elevated plus maze after chronic restraint stress (Klenerova et al., 2010). Injection into the CeA of ovariectomized females also reduces anxiety behaviour in the open field (Bale et al., 2001).

However, the effects of intranasal OT application on stress and anxiety are more conflicting. In rodents it does not change plasmatic corticosterone levels (Kent et al., 2016). It is also not efficient to counteract the effects of stress-induced social withdrawal in female mice (Steinman et al., 2016). In sharp contrast, chronic intranasal OT in monkeys can indeed reduce ACTH levels after acute social isolation (Parker et al., 2005). In humans the anxiolytic effect of OT treatment have been demonstrated in many studies, for example to ameliorate social anxiety in patients suffering from fragile X syndrome (Hall et al., 2012) or to prevent conflict-induced cortisol levels in couples (Ditzen et al., 2009)

Within hypothalamic nuclei, OT and CRH neurons are close to each other, possibly allowing cell-to-cell crosstalk (Jamieson et al., 2017). It is also possible that OT might exerts its anxiolytic effects through other indirect pathways, as demonstrated on serotoninergic neurons in the raphe nucleus (Yoshida et al., 2009) or on neurons in the amygdala (Bale et al., 2001).

*(iii) Social behaviour*

*(a) Pair bonding*

If OT can regulate sexual and reproductive behaviour, it is also implicated in partner choice and pair bonding. In particular, pair bonding has been widely studies in prairies and montane voles. Prairie voles are part of the 3 % of mammals displaying monogamous behaviour, a selective affiliation with a specific partner associated with aggression behaviour towards unfamiliar conspecifics (Carter et al., 1995). This is dependent on OT and VP system. Indeed, giving a central antagonist of OTR to a prairie vole females leads to an inhibition of partner preference after mating, and injecting OT itself can induce partner preference in non-mating females (Insel and Hulihan, 1995). Peripheral OT treatment also increases partner preference in female prairie voles (Cushing and Carter, 2000). In males, VP

rather than OT would be responsible for partner preference (Winslow et al., 1993a). These differences between monogamous and polygamous species have been associated with different OT and VP receptors distribution in the brain (Shapiro and Insel, 1989). OTR expression is higher in the prelimbic cortex, BNST, nucleus accumbens, midline nuclei of the thalamus and lateral amygdala in prairie voles compared to montane voles. Ross and colleagues used viral vector gene transfer to overexpress OTR in the nuclei accumbens of monogamous or polygamous voles, and showed that OTR over expression facilitates partner preference in monogamous animals but has no effect in polygamous animals (Ross et al., 2009b). However, the knockdown of OTR in the same region using RNAi from juvenile age to adulthood alters partner preference (Keebaugh et al., 2015). OTR in the nucleus accumbens hence participates in partner preference but are not sufficient to induce it.

An important observation to consider here while dealing with early life events is that neonatal exposure to OT can influence later pair-bonding and parental behaviour, but the effects seem to be highly dependent on the dose (Bales and Carter, 2003a; Bales et al., 2007; Jia et al., 2008a). For example, a treatment with 2mg/kg does not alter later partner preference, whereas a treatment with 4 mg/kg suppresses partner preference (Bales et al., 2007). Similar observation has been made with intranasal OT treatment between weaning and sexual maturity in male prairie voles; treatment with low or medium doses of OT (0.08IU/kg, or a medium dosage of 0.8IU/kg which is equivalent to a 40IU dosage given to a 110 lb human subject) leading to a deficit in partner preference at adulthood (Bales et al., 2013).

### *(b) Social recognition and memory*

Social recognition is crucial for proper interactions with conspecifics. In rodents, it relies mostly on the ability to recognize olfactory cues, whereas primates also use visual information. In particular, olfactory cues are used in some species for offspring recognition (Lévy et al., 2004). In rodents, there is however no recognition of individual members of the litters, except in the spiny mouse. In "precocial" species such as the goat or the sheep, mothers can discriminate between each young. OT action on the olfactory bulb (OB) and olfactory cortex has hence been studied. A recent optogenetic study lead in female rats showed that OT can modulate the activity of the olfactory cortex to promote social recognition (Oettl et al., 2016). In the OB, Infusion of OT helps to preserve social memory, but the infusion of the antagonist has no effect in males (Dluzen et al., 1998a). The effect of OT is dependent on noradrenaline release in the OB (Dluzen et al., 1998b, 2000). In the context of offspring recognition, OT release in the OB possibly facilitates young discrimination in ewes (Lévy et al., 1995). However, OT can facilitate social recognition through on action on other brain regions, such as the mPOA (Popik and van Ree, 1991) or the amygdala (Choleris et al., 2007; Dumais et al., 2016) in males and females. In OT KO male mice, social recognition is impaired, but not the memory of non-social information (Winslow et al., 2000a). Treatment with OT but not VP restored normal social recognition in these mice. Similarly, blocking OT signalling impairs social memory in wild type animals. In females, oestrogens are important to regulate OT effects on social recognition, and ER-α and β KO animals display similar impairment in social recognition as for OT KO and OTR KO mice. Treatment with OT but not VP restored normal social recognition in these mice. Similarly, blocking OT signalling impairs social memory in wild type animals. In females, oestrogens are important to regulate OT effects on social recognition, and ER-α and β KO display similar impairment in social recognition as for OT KO and OTR KO mice (Choleris et al., 2003)

### *(c) Aggression*

In males, aggressive behaviour is suggested to be mostly regulated by VP rather than OT (Caldwell et al., 2008). However, studies are conflicting. In one study, OT deletion decreased aggressive behaviour in male mice against an intruder in the home cage and in a neutral arena (DeVries et al., 1997). In

another study, OT deletion induced an increase in aggressive behaviour against an intruder (Winslow et al., 2000b). OT could regulate aggression to an unknown conspecific in the context of pair bonding in monogamous species, as demonstrated in the prairie voles (Winslow et al., 1993b). It has been proposed that the perinatal OT environment might influence later aggressive behaviour in males (Takayanagi et al., 2005), but neonatal treatment with OT did not change aggression levels in male mandarin voles or prairie voles (Bales and Carter, 2003b; Jia et al., 2008b).

In females, aggressive behaviour is increased in the post-partum period to protect the pups from potential danger. At the same time, OTR expression in the lateral septum increases suggesting a possible role in maternal aggression. C-fos induction in the BNST, mPOA, PVN and amygdala has also been described after aggression toward a male intruder in lactating females (Gammie and Nelson, 2001). However, a few studies suggest that OT may not play such a critical role in maternal aggression. Indeed icv injection of OTR antagonist prior to intruder exposure does not change aggressive behaviour, but only decreases maternal behaviour during maternal defence (Neumann et al., 2001). Although electrolytic lesion of the PVN on the 5$^{th}$ post-partum day decreases maternal aggression in rats (Consiglio and Lucion, 1996), kainic acid lesions on day 2 after parturition does not alter this behaviour (Olazabal and Ferreira, 1997). Giovenardi and colleagues used the antisense technique to lesion OT neurons in the PVN on the 5$^{th}$ postpartum or on the 8$^{th}$ day. Lesion on the 5$^{th}$ day increased maternal aggression but had no effect on day 8 (Giovenardi et al., 1998). This highlights the fact that a critical period for the effect of OT on aggression may exist. In contrast with males studies, neonatal OT treatment of mandarin or prairie voles pups 24H after birth shows long term effect on aggressive behaviour, which is increased after exposure to a male (Bales and Carter, 2003b; Jia et al., 2008b).

*(iv) Maternal behaviour*

During pregnancy and after birth, OT and OTR expression change in the mother's brain (Caldwell et al., 1987). On top of its roles in parturition and lactation, OT can also facilitate the onset and maintenance of maternal behaviour. In rodents, maternal behaviours correspond to nest building, pup retrieval, licking and grooming pups or crouching over them. As depicted earlier, some species display maternal behaviour to any pups without recognizing its own litter, like the rats, but others discriminate each pup like the sheep. In rats, virgin females avoid the contact with newborn pups and do not display any maternal behaviour towards foster rats. One of the first studies showing the involvement of OT in the induction of social behaviour was done in female virgin rats. An icv administration of OT induced the onset of maternal behaviour in these females. However, this is efficient only in oestrogen-primed females (Pedersen and Prange, 1979; Pedersen et al., 1982). Blocking OT function produces an alteration of maternal behaviour, that is either delayed or totally prevented (Fahrbach et al., 1985; Pedersen et al., 1985; van Leengoed et al., 1987; Insel and Harbaugh, 1989). However, this effect is dependant of the timing of the treatment. A treatment, several days after parturition, does not supress maternal behaviour (Fahrbach et al., 1985) but seems to decrease pups grooming, to increase self-grooming, as well as to decrease upright posturing over pups (Pedersen and Boccia, 2003).

In many species, the amount of maternal behaviour can vary between females. Some mothers display higher levels of pups licking and grooming and arched back nursing, whereas others show low levels of these maternal behaviours. Researchers tried to understand the mechanisms underlying these natural variations in maternal behaviour and, once again, identified the implication of the oxytocinergic system. Female rats with high maternal behaviour have higher OTR levels in the mPOA, lateral septum, central nucleus of the amygdala, PVN and BNST (Francis et al., 2000). Behavioural differences were suppressed by injection an OTR antagonist icv to high grooming mothers

(Champagne et al., 2001), confirming the role of OT in maternal behaviour modulation. Interestingly, these individual differences are transferred to the descendance (Francis et al., 1999). Epigenetic mechanisms are proposed to be responsible for this, as changes in DNA methylation or HDAC expressions have been identified in genes involved in HPA axis function in low vs high grooming mothers (Weaver et al., 2004). In particular, change in DNA methylation in the ER alpha promoter in the mPOA has been detected (Champagne et al., 2006). Knowing that oestrogens can modulate the expression of OTR, this supports the idea that natural variations in maternal behaviour may influence the proper function of OT system in the offspring. This is even more possible with regards to the ontogenesis of the OT system after birth, which is described in paragraph IV.

It has also been identified that stress reactivity in the offspring might be related to the levels of maternal behaviour provided by dams in the early post-natal period (Caldji et al., 2000; FISH et al., 2006). In humans also, negative events in early life, including those linked to parent-infant relationship, can affect behavioural and mental health at later age (Pechtel and Pizzagalli, 2011; Chen and Baram, 2016). This issue will be further discussed in part V of this introduction.

### III) Regulation of the activity of OT neurons

The activity of parvOT and magnOT neurons is controlled by a variety of neuronal intra and extra-hypothalamic inputs. Due to its various peripheral and central roles the OT system is regulated by afferent inputs that convey information on the osmotic, cardiovascular, inflammatory, nociceptive, or reproductive status of the body. The major modulatory pathways will be described in this part of the introduction. In a recent review, Brown and colleagues summarized the major inputs to magnocellular cells, as presented in figure 27.

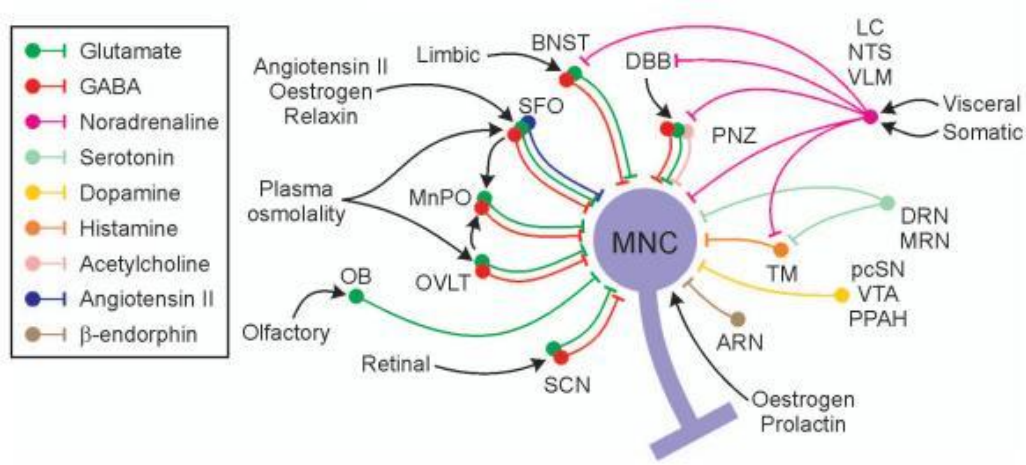


*Figure 27: Peripheral and afferent inputs to magnocellular cells.*

*ARN: arcuate nucleus; BNST: bed nucleus of the stria terminalis; DBB: diagonal band of Broca; DRN: dorsal raphe nucleus; LC: locus coeruleus; MnPO: median preoptic nucleus; MRN: median raphe nucleus; NTS: nucleus tractus solitarius; OB: olfactory bulb; OVLT: organum vasculosum of the lamina terminalis; pcSN: pars compacta of the substantia nigra; PNZ: perinuclear zone; PPAH: preoptic periventricular /anterior hypothalamic region; SCN: suprachiasmatic nucleus; SFO: subfornical organ; TM: tuberomammillary nucleus; VLM: ventrolateral medulla; VTA: ventral tegmental area. Adapted from* (Brown et al., 2013)

1) Activation inputs

***Noradrenergic inputs***. At parturition and during lactation, the activity of magnOT neurons is changed. They start to exhibit bursting activity occurring every 5-10 minutes and reaching firing rates of 50-100 spikes/s. This bursting activity allows the transient release of important doses of OT, crucial for uterine contraction of milk ejection (Russell et al., 2003). Afferent inputs implicated in this specific activity include projections originating mostly from A2 noradrenergic neurons in the NTS (Cunningham and Sawchenko, 1988; Raby and Renaud, 1989). NA release in OT nuclei is increased during lactation, as well as the number of NA synapses onto OT neurons (Michaloudi et al., 1997; Bealer and Crowley, 1998; Lipschitz et al., 2004).

Moreover, the NTS is highly implicated in the regulation of cardiovascular activity and relays information from baroreceptors and chemoreceptors (Ciriello, 1983). Noradrenergic inputs onto OT and VP neurons hence relay information about the osmotic status of the body (Bourque, 2008). Gastrointestinal and somatosensory information can also be relayed by these pathways (Leng et al., 1999). Noradrenaline acts on α1 receptors and induces the activation of magnocellular neurons (Yamashita et al., 1987). Injections of the antagonist of α1 receptors can indeed alter the response of OT neurons in the SON (Brown et al., 1998).

***Serotoninergic inputs*** PVN and SON neurons seem to be contacted by serotoninergic cells from the raphe nuclei (Sawchenko et al., 1983). However, retrograde and anterograde study both show that the surrounding regions of PVN and SON are more densely contacted by serotoninergic neurons. It is then possible that serotonin action on PVN and SON neurons might be indirect, since icv injections of serotonine modulated plasmatic OT levels (Jørgensen et al., 2003).

***Dopaminergic inputs***. Dopaminergic neurons from the A9 group of the substantia nigra, from the A10 cells of the ventral tegmental area and from the A14 and A15 cell groups of the hypothalamus project to magnocellular neurons in the SON (Cheung et al., 1998; van Vulpen et al., 1999). Dopamine increases the activity of magnocellular neurons by two pathways (Bridges et al., 1976). The first one is a direct depolarizing action via D2 receptors and the second is mediated by presynaptic D4 receptors inhibiting GABA release on magnocellular neurons (Yang et al., 1991; Azdad et al., 2003; Baimoukhametova et al., 2004). However, an inhibiting action of glutamatergic inputs via D4 receptors has also been described, suggesting a more complex regulation on magnocellular neurons (Price and Pittman, 2001). Altogether, these inputs play a role in role in lactation and parturition, and possibly on other behaviour such as pain modulation (Moos and Richard, 1982; Herbison et al., 1997; Gamal-Eltrabily and Manzano-García, 2017).

***Kisspeptinergic inputs***. At the end of pregnancy, the activity of OT neurons is increased to allow efficient uterine contraction and prevent haemorrhage. At that time, an increased kisspeptin fiber density, originating from the periventricular nucleus of the HT, has been detected around SON magnOT neurons in rats, possibly controlling neuronal activity (Seymour et al., 2017). Indeed, iv injections of kisspeptin-10 increase the firing rate of magnOT neurons in the SON. Similar effect is obtained using icv, but only in late pregnancy and lactation (Scott and Brown, 2011, 2013). The connections between kisspeptine and magnOT neurons have also been demonstrated in other species, including the teleost fish (Kanda et al., 2013)

***Substance P (SP) inputs*.** SON and PVN are contacted by SP fibers, originating from A1 neurons in the ventral medulla, among which 15-25% express SP (Bittencourt et al., 1991). This pathway may be involved in the regulation of blood pressure, and the prevention of post-partum haemorrhage (Feuerstein et al., 1984). SP or NK1 receptor agonist indeed stimulates the release of OT (Kapoor and Sladek, 2001; Juszczak and Stempniak, 2003).

***Histaminergic inputs*.** The histaminergic neurons from tuberomammillary nucleus send projections to magnOT and play a role during parturition. Because the injection of histamine receptor antagonist induces a delay in parturition and blocks suckling-induced OT release, the effect of histamine on OT neurons has been proposed to be excitatory and mediated by the activation of H1 or H2 receptors (Schagen et al., 1996; Luckman and Larsen, 1997). However, an H2 receptor-mediated inhibitory effect of histamine has also been demonstrated using patch clamp recording of rat HT neurons in brain slices (Yang and Hatton, 1994). Further studies are then needed to decipher the effects on histaminergic inputs on OT neurons.

***Olfactory inputs.*** The main and accessory olfactory bulb send glutamatergic excitatory projections to magnocellular cells (Smithson et al., 1992; Bader et al., 2012). Due to the importance of olfactory clues in reproductive, social and maternal behaviour in rodents, this connection might be important to modulate some aspects of these behaviours.

2) Inhibitory inputs

Local GABAergic neurons are present in the perinuclear zone and project to the SON and to the PVN (Roland and Sawchenko, 1993). PVN neurons also receive GABAergic inputs from the anterior HT, BNST, medial preoptic area, prifornical nucleus and dorsomedial HT (Boudaba et al., 1996). At parturition however, magnOT neurons display a switch in GABAa receptor subunits (Brussaard et al., 1997). During pregnancy, the activity of OT neurons is maintained at a low level to avoid premature uterine contraction and premature birth. An inhibition of OT neurons by opioidergic fibers originating from the arcuate nucleus has been described in this context. Opioidergic fibers can act directly on OT neurons or indirectly through the inhibition of excitatory noradrenergic inputs (Douglas et al., 1995; Leng et al., 1995). Allopregnanolone also participates to keep OT neurons activity low, by enhancing GABAergic inhibition on OT neurons (Russell and Brunton, 2006)

3) When OT itself modulate the activity of OT neurons and the question of internuclei connections

The question of the facilitation of OT neurons activity by OT itself has been raised in the context of the typical bursting activity that they display during lactation and suckling. One of the properties of this activity is that the burst activity is coordinated among OT neurons, as measured using in vivo paired recording of PVN and SON OT neurons (Belin and Moos, 1986). This activity starts at the beginning of suckling activity and a correlation between the number of nipples sucked and the number of action potentials in each burst has been demonstrated (Lincoln and Wakerley, 1975). Sensory stimulus is hence required to start bursting activity in OT cells. However, the injection of an OTR antagonist within HT nuclei decreased the burst activity during milk ejection. It also completely suppresses the milk ejection reflex induced by icv injection of OT (Lambert et al., 1993). Moreover, in vitro studies showed that infusion of OT in hypothalamic slices induces a dose dependant increase in OT release from PVN and SON in males or in lactating rats (Moos et al., 1984). In these experiments, changes in VP release have not been observed. OT autocrine and paracrine action, possibly via dendritic release, then appears to be a key mechanism to explain the synchronous bursting activity of OT cells during suckling (Freund-Mercier et al., 1988a; Ludwig and Leng, 2006). The somato-dendritic released OT has been proposed to act presynaptically on terminals contacting OT neurons to induce depression on evoked EPSC on OT neurons (Kombian et al., 1997).

Inter-nuclei connections between OT cells could also be implicated in the synchronization of PVN and SON neurons. Several years ago, Silvermann and colleagues described that the PVN receives input from contralateral PVN as well as ipsilateral SON (Silverman et al., 1981). In the SON, OTergic synapses have also been described on pre- and postsynaptic elements (Theodosis, 1985), suggesting

a connection between the two nuclei. Lamberts and colleagues also highlighted the facts that the application of OT or OTR agonists in the SON did modify the activity of OT neurons in the other nuclei (Lambert et al., 1993). More recently, we found a population of parvocellular PVN neurons that makes synaptic contact onto magnocellular OT neurons in the SON, which is presented in ARTICLE 1. We show that this connection is functionally active in the context of inflammatory pain and participates in the regulation of pain behaviour in rats.

## IV) Development of the OT system

In rodents, immunohistochemical studies were first performed to assess the question of the development of the OT and VP system. Altogether, all studies agree that SON and PVN appear early in embryonic life, possibly as soon as E13 in mice and rats, from the proliferative neuroepithelium of the 3rd ventricule (Altman and Bayer, 1978; Nakai et al., 1995). VP synthesis starts first during embryonic life, whereas mature OT is only detected after birth, with small disagreements on the exact embryonic or post-natal date (reviewed in (Grinevich et al., 2014).

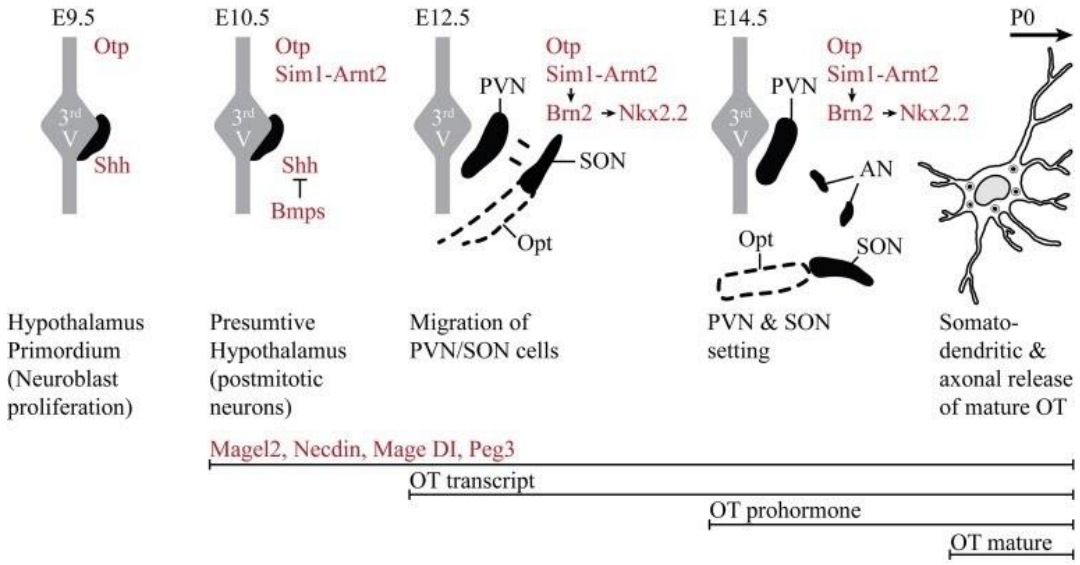


*Figure 28: Ontogenesis of OT neurons in the mouse.*

*Developmental stages of the hypothalamic nucleis are indicated, as well as the transcription factors known to influence PVN and SON neurons development. At the bottom is indicated what form of OT can be detected for each developmental stage. Adapted from* (Grinevich et al., 2014).

One early study by Choy and Watkins used the immunoperoxidase technique associated with purified antisera against OT or VP in rats (Choy and Watkins, 1979). They show that PVN and SON appear on the 18th day of intrauterine life (E18) but suggest that PVN appears to be less developed than SON, whereas in another studies the development of SON was suggested to start as soon as E13 (Altman and Bayer, 1978). Choy and Watkins detected VP starting at E19 in the hypothalamus and pituitary and started to detect it in the eminence median after birth. However, OT was not detected before post-natal day (P) 1 in the pituitary and P4 in the hypothalamus. At P7, PVN and SON cells are of

adult proportions but more closely packed compared to the adult hypothalamus and some of them are empty of any immunoreactive material. It is considered that the system is almost fully mature at P20 (Choy and Watkins, 1979). A few years later, similar conclusions were drawn by Lazcano and colleagues, who also detected no OT during foetal life. However, these authors detected VP only at E21 and later on. The neuronal topography of the SON is initiated at P15. During embryonic life, VP neurons are heterogeneously distributed throughout the SON, but start to concentrate in the ventral zone at P15, whereas OT neurons are found mostly in the dorsal zone. At adulthood, VP neurons concentrate in the ventral, caudal and intermediate parts of the SON and OT neurons in the dorsal, rostral and intermediate parts (Lazcano et al., 1990). More recently, Lipari and colleagues combined immunohistochemistry techniques with RT-PCR studies and observed that PVN and SON appear on the 18$^{th}$ and 16$^{th}$ day of intra-uterine life, respectively (Lipari et al., 2001). Even if the slower development of the PVN has been proposed by Choy and Watkins, Lipari and colleagues clearly distinguish two different time courses of development. Alstein and colleagues also demonstrated that the maturation of OT is incomplete during embryonic life, since pro-OT cleavage is low and results in the accumulation of intermediate forms of the peptide (Alstein et al., 1988). After birth, OT neurons also undergo changes in their electrophysiological properties, especially during the 2$^{nd}$ post-natal week when action potential properties changes and OT autocontrol decreases (Widmer et al., 1997; Chevaleyre et al., 2000).

The ontogeny of OT projections throughout the brain has been less studied, but one study shows that SON neurons send projections to the posterior lobe of the pituitary as soon as at E15, and to the PVN neurons at E17 (Makarenko et al., 2000). On another hand connections between the accessory nuclei and the posterior lobe of the pituitary only occur after birth (Makarenko et al., 2002).

Concerning the expression of OTR in the brain, receptor autoradiography and in situ hybridization studies reveal that OTR binding is first detected at E13-14 in regions corresponding to the dorsal motor nucleus of the vagus nerve, and is progressively detected in other areas of the brain as showed in figure 29 (Tribollet et al., 1989; Yoshimura et al., 1996). Tribollet and colleagues highlighted the fact that the transition from the infant to the adult pattern of expression occurs at 2 stages, at the pre-weaning period and after puberty. The onset of OTR receptor hence happens way before the beginning of OT synthesis in the foetal brain. This suggests that the foetal brain could still be receptive to maternal OT.

In human, the evaluation of OT and VP content in umbilical venous samples during labour suggests that the foetus is able to release OT and VP at birth (Chard et al., 1971). The development of the OT and VP system occurs sooner during foetal life in humans compared to rodents (Swaab, 1995). At 15 weeks of gestation, both peptides are detected in the foetal brain (Schubert et al., 1981). At 26 weeks of gestation, adult VP and OT adult cell numbers are detected in the PVN and the SON, even if their morphological features are still immature since nuclear volume is still increasing during pregnancy (RINNE et al., 1962). As for rodents, VP is detected sooner than OT in the foetal brain and both levels increase with foetal age (Burford and Robinson, 1982).

Due to its immature state during the early post-natal period, the OT system might be highly vulnerable to the alterations in the environment of the newborn. Early experience is indeed known to affect the function of the OT system, and alter OT-related behaviours such as anxiety and social abilities (Bales and Perkeybile, 2012; Hammock, 2015). For example, natural variations in maternal care influence the development of stress reactivity, maternal care and the expression of OTR (Caldji et al., 2000; Champagne and Meaney, 2007; Kundakovic and Champagne, 2015).

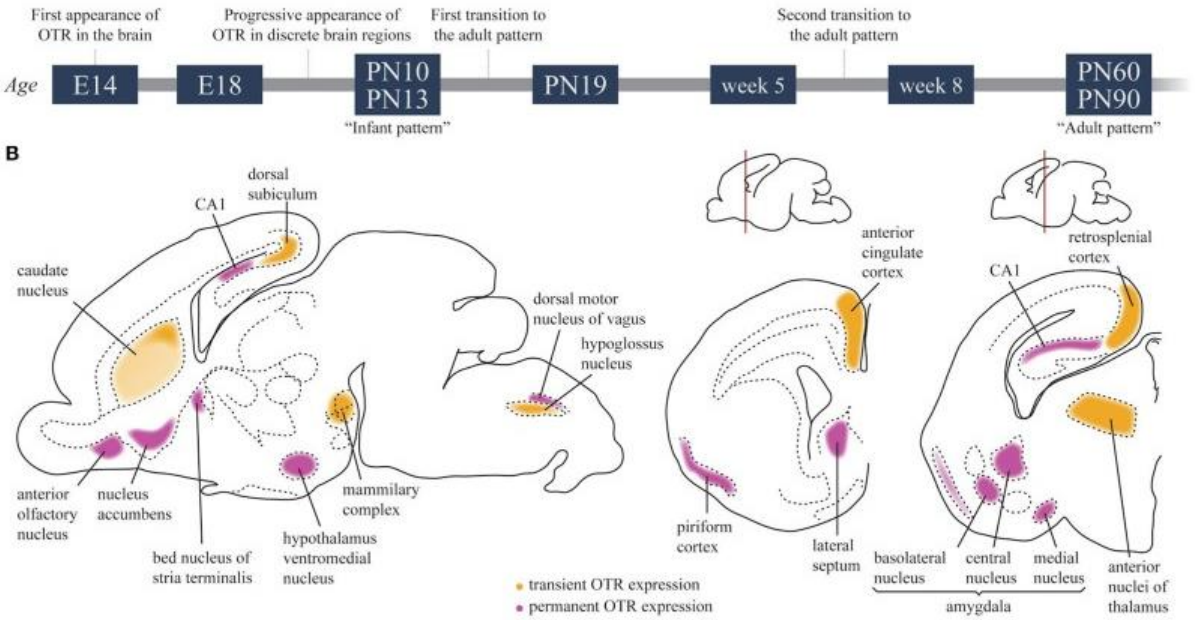


*Figure 29: Expression profile of OTR in the developing rodent brain.*

The picture shows OTR expression in the brain at P10-13; regions with transient OTR expression are in yellow and regions where OTR expression is maintained throughout adult life in purple. Adapted from (Grinevich et al., 2014)

## V) Early life stress and its long-term effect on brain functions

Today, the idea that early life events, such as stress or excess of nociception in the neonatal period and infancy, can have negative long-term consequences on the development of the nervous system is well accepted. Especially, the consequences on the development of the nociceptive system have been of great interest, since the recent acceptation that the infant is able to feel pain. Before the end of the 80's, common belief was that, due to the immaturity of its nervous system, the neonate is not able to feel pain. Dr KJ Anand was among the first to contradict this idea and to suggest that early neonatal pain could alter later brain and nociceptive functions (Anand and Hickey, 1987). Since then, awareness has risen about the potential deleterious effects of other early life interventions. In particular, newborn babies and, even more preterm babies, constitute a highly sensitive population. At birth, they are submitted to a high number of stressful procedures, most of them being extremely painful. This adds to the other stressors which include excessive visual or auditory stimulation, over-use of antiseptic solutions on the skin, lack of sensory clues from the parents… The long-term effects of neonatal pain have been well studied and will be presented in paragraph V.3. Several studies are also related to other aspects of the early sensory environment of the newborn. Acoustic stimulation can have short term effect on different physiologic responses (Brown, 2009), including a disrupt in sleep in the preterm baby (Kuhn et al., 2013). Excessive salient odor stimulation in juvenile rats (Hadas et al., 2016) or excessive auditory and visual stimulation (Christakis et al., 2012) in P10 mice consequently alters cognitive functions and vulnerability for drug abuse (Ravinder et al., 2016) at adulthood. The long-term effects on pain or somatosensory processing have however not been investigated yet.

During my PhD, I focused my work on a model of ELS called neonatal maternal separation (MS) and studied its long-term effects on pain responses and on the function of the descending controls of pain. ARTICLE 2 presents the long-term consequences of MS on OT analgesic system. However, the

MS model is not the only ELS model altering mother-pups interaction. Thus, a shorter overview of the other models and their effect on pain processing will be given in the next part of the introduction.

### 1) Notions of sensitive period and stress hyporesponsive period

Early life events occur during a critical period of brain development and possibly alter normal developmental processes, resulting in alterations of adult brain functions. Concerning the long-term effects of ELS on pain sensitivity, the critical period in rodents goes along the first 2-3 post-natal weeks (illustrated in figure 30), when most of developmental processes happen, as described before in this introduction. Physical injuries or stress occurring at older age can also have deleterious effects but possibly via different mechanisms than those observed during early life. During this period, neurons are highly sensitive to environmental changes. Changes in neuronal activity, hyperactivation or deprivation, might induce long-term plasticity in the stimulated pathways and in other associated ones.

Another important issue when studying ELS deals with the notion of stress hyporesponsive period (SHRP). The HPA axis is actually hyporesponsive to external stressors until around P14 in the rat (Levine et al., 1991). This is apparently due to several factors: an insensibility of the adrenal cortex to ACTH, a lower synthesis of CRF and ACTH, a lower vasopressin synthesis or an increased feedback signal at the pituitary level. Maternal influence has also been proposed as a regulator of the newborn's HPA axis, since it becomes responsive to different stressors after a period of maternal separation. Long-term effects of ELS might depend on the early activation of the HPA axis, which could be deleterious for further development and function of the nervous system.

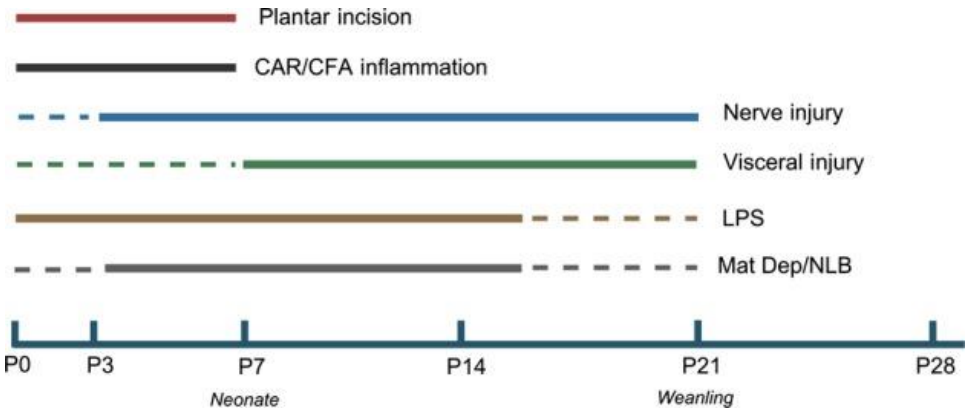


*Figure 30: Critical period for ELS to induce long term changes in pain responses in rodents.*

*Solid lines indicate periods defined in controlled studies. Dotted lines indicate periods where data are incomplete. Postnatal age (P) is in days. CAR, carageenan; CFA, complete Freund's adjuvant; LPS, lipopolysaccharide; Mat Dep, maternal deprivation; NLB, neonatal limited bedding. Adapted from* (Schwaller and Fitzgerald, 2014).

### 2) Early life stress in human

In humans, early life stress (ELS) includes painful interventions, parental deprivation with the neonate, physical abuse or violence, excess of sensory information or neglect during early infancy. These early adversities are known to have huge deleterious consequences on the development of the brain and on later adult behaviour, including cognitive and emotional disorders and blunted

responses to pain (Pechtel and Pizzagalli, 2011; Chen and Baram, 2016). A meta-analysis by Davis and colleagues in 2005 linked early childhood abuse to chronic pain at adulthood (Davis et al., 2005). Since then, other studies tend to the same observations linking childhood trauma to increased pain rating and catastrophizing at adulthood (Walsh et al., 2007; Paras et al., 2009; Sansone et al., 2013; Yamada et al., 2017). For example, a great amount of patients suffering from migraines reports previous childhood maltreatment (Tietjen et al., 2015; Tietjen, 2016), which is also observed with patients suffering from irritable bowel syndrome (IBS).

Events occurring in the early postnatal period are even more prone to alter later nociceptive function since the nociceptive system of newborn babies undergoes a huge maturation state during the 3rd semester of pregnancy and after birth. As mentioned above, prematurity can be considered as a huge traumatic event and is associated with a high number of painful and stressful procedures (Simons et al., 2003; Carbajal et al., 2008).

- **Specific case of irritable bowel syndrome**

IBS is a common visceral disorder which is often incapacitating for social of professional life. IBS patients suffer from chronic abdominal pain and altered bowel function, often associated with disturbed sleep (Dapoigny et al., 2004; Patel et al., 2016). Its prevalence rates range from 1.1% to 45% worldwide, with a mean of 5-10% in the united states, Europe and China (Enck et al., 2016). Among the IBS population, clinical studies reveal that a great amount a patient previously experienced physical, emotional or sexual abuse during infancy (Drossman et al., 1990; Salmon et al., 2003; Ross, 2005). In line, abused patients report more pelvic pain and other multiple somatic symptoms (Drossman et al., 1990). A study reported that among 333 IBS patients, 31% had lost a parent during childhood, 19% had an alcoholic parent, and 61% reported unsatisfactory relationship with their parents (Hislop, 1979), suggesting that early parent-infant interactions might be involved in the development of visceral function. In a more recent study, the authors observed that parental punishment, over-interference or rejection was a risk factor for IBS in an adolescent population, whereas emotional warmth seems to be a protective factor (Xing et al., 2014). Neonatal pain stress might also be a risk factor for the development of IBS. Gastric suction in the neonatal period has indeed been associated with a greater risk for functional intestinal symptoms including visceral hyperalgesia (Anand et al., 2004a). This has been mimicked in the animal using daily orogastric suction from P2 to P12, which leads to thermal hot and visceral hyperalgesia (smith et al., 2007). Neuroanatomical studies show that IBS patients are likely to display a thinning of the subgenual cingulate cortex, which has also been detected in patients with ELS history (Gupta et al., 2016).

### 3) The issue of early life pain

Awareness of the newborns pain susceptibility was accompanied some years later by the concern of possible detrimental consequences of early life pain on the development of the nociceptive system. The current hypotheses suggest that excess of sensory input to the newborn is sufficient to "imprint" the nociceptive system and to cause long term alteration in its function.

According to clinical studies lead by Simons and colleagues in 2003 and Carbajal and colleagues in 2008, infants admitted in neonatal intensive care units (NICU) after birth are submitted to a surprisingly high number of painful procedures (Simons et al., 2003; Carbajal et al., 2008). The number of painful procedures is estimated to a mean of 16 per day. It is important to note here that around 2/3 of them are not associated with a proper pre-emptive analgesia (Simons et al., 2003; Carbajal et al., 2008; Courtois et al., 2016a). On an average of 14 days of intensive care, preterm

babies are submitted to around 50 to 200 painful procedures for very preterm babies. The likelihood of triggering long term deleterious consequences in the newborn has led to reduce the number of painful procedures and to use pharmacological and non-pharmacological strategies in order to reduce stress and pain in many NICU. 8 years after Simons's publication, Roofthooft and colleagues re-evaluated the amount of painful procedures and analgesic use in newborns (Roofthooft et al., 2014). They observed a mean of 11.4 painful procedures per neonate and per day. This number was smaller than in the previous study for babies born at 30-32.6 weeks and 33-36.6 weeks, but not for more premature babies (<30 weeks). This still shows an improvement compared to the earlier studies. The most frequent painful procedures are endotracheal, nasopharyngeal and nasal suction followed by heel prick, as described in the earlier studies (Simons et al., 2003; Carbajal et al., 2008; Courtois et al., 2016a, 2016b). A decrease in the use of analgesics (60.3% of neonate in the first study vs 36.6 in this one) is also observed. Decreased use of morphine in the newborn is due to its poor beneficial effect on pain in ventilated neonates (Anand et al., 2004b; Carbajal et al., 2005; Bellu et al., 2010) and to recent studies indicating about a possible deleterious long term effect of early morphine administration on cognitive function (de Graaf et al., 2011). However, a recent study by Courtois and colleagues shows that 76% of venepunctures, one of the most frequent painful procedures in the newborn, are performed with pre-procedural analgesia (Courtois et al., 2016b), showing a real improvement in neonatal pain care.

*(i) Short and long-term effects in humans*

One of the first publication on the subject is the early work by Anna Taddio who shows that circumcision during the neonatal period significantly increases pain scores of these same children in response to vaccination at 4 or 5 months (Taddio et al., 1997). This publication also enlightened the need of effective anaesthetics during painful procedures in the newborn, since the use of a local anaesthetic EMLA during circumcision significantly reduces pain score measured with visual analogue scale during vaccination. In accordance with this hypothesis, major surgeries during the neonatal period do not necessarily alter pain responses during vaccination at 14 or 45 month when they are performed with an efficient preemptive analgesia (Peters et al., 2003).

These considerations are even more alarming in the case of preterm babies which constitute as much as 10% of new-born babies and are known to develop several health problems and disabilities at adulthood. Prematurity increases the risk for cerebral palsy and for poor cognitive function, and is associated with increased stress and anxiety and a higher risk to develop depression (Grunau et al., 2006; Moster et al., 2008). As stated in the previous paragraph, these babies undergo a huge number of painful procedures, which have been associated with short and long-term alterations on the nociceptive system. For example, repeated painful heel lances are known to induce, if untreated, an hypersensitivity to pain measured by a decreased threshold of response to Von Frey filaments (Fitzgerald et al., 1989). A major surgery during the first 3 months of life increases pain behaviour of the children for a later surgery on the same area. These newborns require more intraoperative and post-operative anaesthetics and analgesics (fentanyl and morphine) than infants who were not exposed to prior surgeries. They also display higher pain score in COMFORT and VAS measures, as well as increased norepinephrine plasma concentration (Peters et al., 2005). This is also true if the later surgeries occur in areas of the body that had not been previously subjected to early surgery. This suggests that not only peripheral mechanisms are involved but that a possible reorganization of spinal and supraspinal areas may occur following neonatal surgery. Another observation supporting this hypothesis is that, at the same postmenstrual age, infants born preterm and integrated at least

40 days in NICU display increased noxious-evoked neuronal activity in the brain as compared with children born at term, suggesting an alteration of pain processing in higher brain centers (Slater et al., 2010a).

Concerning the long term effects, early life pain is associated with increased perceptual sensitization to tonic heat and increased heat pain thresholds, both in preterm and full-term children that have been integrated in NICU for a long period of time (Hermann et al., 2006). Also at school age, former preterm display more pain catastrophizing compared to full-term children with no NICU experience (Hohmeister et al., 2009a). Mechanical sensibility is also altered in children aged 9-12 years who were previously submitted to cardiac surgery as newborns, as they are less sensitive to von Frey filament stimulation (and to cooling and warming) both in the area of the scar and in a non-injured area (Schmelzle-Lubiecki et al., 2007). Another study reports a generalized decrease in thermal sensibility at 11 year-old in former extremely preterm children (Walker et al., 2009a). It seems that former preterm are also more susceptible to develop chronic pain at older age (Grunau et al., 2006), including migraines that occur very early in age and require a stronger pharmacological treatment (Maneyapanda and Venkatasubramanian, 2005). At adulthood, individuals with an history of prematurity display a decreased pain tolerance (Vederhus et al., 2012). Other injuries, such as burn injuries in infancy (between 6 to 24 months) also have long term effects on pain sensitivity at school age, depending on the severity of the burn injury (Wollgarten-Hadamek et al., 2009). Children who suffered from moderate burn display lower mechanical plain threshold and an increased perceptual sensitization to repetitive mechanical stimulations but no alteration of thermal sensitivity. On the other hand, children who suffered from severe burn injury display elevated thermal pain threshold and an increased perceptual sensitization. This has been associated with a dysfunction of stress-induced activation of descending inhibitory controls of pain in children who suffered from severe burn injury (Wollgarten-Hadamekl et al., 2011). In line, another study shows no differences in nociceptive threshold between full-term and preterm children, but an alteration of diffuse noxious inhibitory controls (DNIC) in preterm infants (Goffaux et al., 2008). In imaging studies using functional magnetic resonance lead at 11 to 16 years, preterm children display an activation of specific brain regions that were not activated in full-term children (such as the thalamus, anterior cingulate cortex, cerebellum, basal ganglia, and PAG), and an increase in the activation in primary somatosensory cortex, anterior cingulate cortex and insula in response to thermal pain stimulation (Hohmeister et al., 2010). These latter observations are important to start deciphering the mechanisms underlying specific ‘‘pain phenotypes’’ resulting from ELS, which have been more specifically investigated in animal models. It is important to consider here that the early social environment may be a key regulator of later behaviour. For example, a study shows that the mothers of preterm children have a stronger tendency to act in a pain-promoting manner regarding their children (Hohmeister et al., 2009b). This could shape their later relationship with pain and play a role in pain catastrophizing behaviours.

Even beyond any long term pain concern, infants submitted to neonatal pain display altered HPA axis function and response to stress and an alteration of different brain function as reviewed here (Eckstein Grunau, 2013). Recent imaging studies suggest that painful procedures in the neonatal period are associated with poorer neurodevelopment and can lead to a decreased head circumference, a decreased volume of white matter in the brain and to an altered maturation of subcortical grey matter in preterm babies and thinner cortex in frontal and parietal lobes (Brummelte et al., 2012; Vinall et al., 2012; Ranger et al., 2013). Concerning the long term effects, an alteration in

cortical oscillatory activity have been observed in very preterm children at school age (Doesburg et al., 2013).

*(ii) In animals*

To further understand how neonatal pain induces such heavy long-term effects, different animal models have been developed. Many great and extend reviews on the subject have been published in the past few years (Schwaller and Fitzgerald, 2014; Baccei, 2016; Victoria and Murphy, 2016a, 2016b). Different types of painful procedures and different timing have been used in the literature. Depending on the type of nociceptive stimulation, long term hyposensitivity and hypersensitivity have both been described. Most of the time, a mild neonatal insult lead to a general hyposensitivity associated with a hypersensitivity to pain in the area that has been injured in the neonatal period. Anyway, on both cases neonatal insult deeply alters the development and function of the nociceptive system. A summary of the consequences on pain behaviour of the different models of early life injuries are presented in Table 1 and figure 31.

These long term alterations of nociceptive sensitivity seem to be linked to multiple factors, being either a change in the function of the descending control of pain, a change in peripheral inputs on the spinal cord, a change in spinal cord synaptic integration, plasticity and signalling, or a change in spinal inflammatory mediators and microglia (LaPrairie and Murphy, 2009; Schwaller and Fitzgerald, 2014; Baccei, 2016).

In addition to the effects on pain sensitivity, other behavioural alterations have been demonstrated. Once again, the results are somehow dependant on the timing and the type of nociceptive stimulation. Anand described other behavioural alterations after repeated needle prick, such as a preference for alcohol, an increased anxiety and defensive behaviour (Anand et al., 1999). However, adult rats subjected to neonatal formalin injection showed a decreased ethanol preference, a decreased locomotor activity, and greater anxiety-like behaviour (Bhutta et al., 2001; Roizenblatt et al., 2010; Mohamad et al., 2011; Negrigo et al., 2011). A reduction of exploratory activity in open field at adulthood is also observed in rats subjected to neonatal visceral distension (Wang et al., 2008). But some studies observed a decrease in anxiety-like behaviour after neonatal pain (Anseloni et al., 2005; Victoria et al., 2013; Chen et al., 2016). A change in motivational orexinergic pathway has also been demonstrated after neonatal pain (Low and Fitzgerald, 2012), a change in sleep architecture (Roizenblatt et al., 2010) and in spatial learning (Chen et al., 2016).

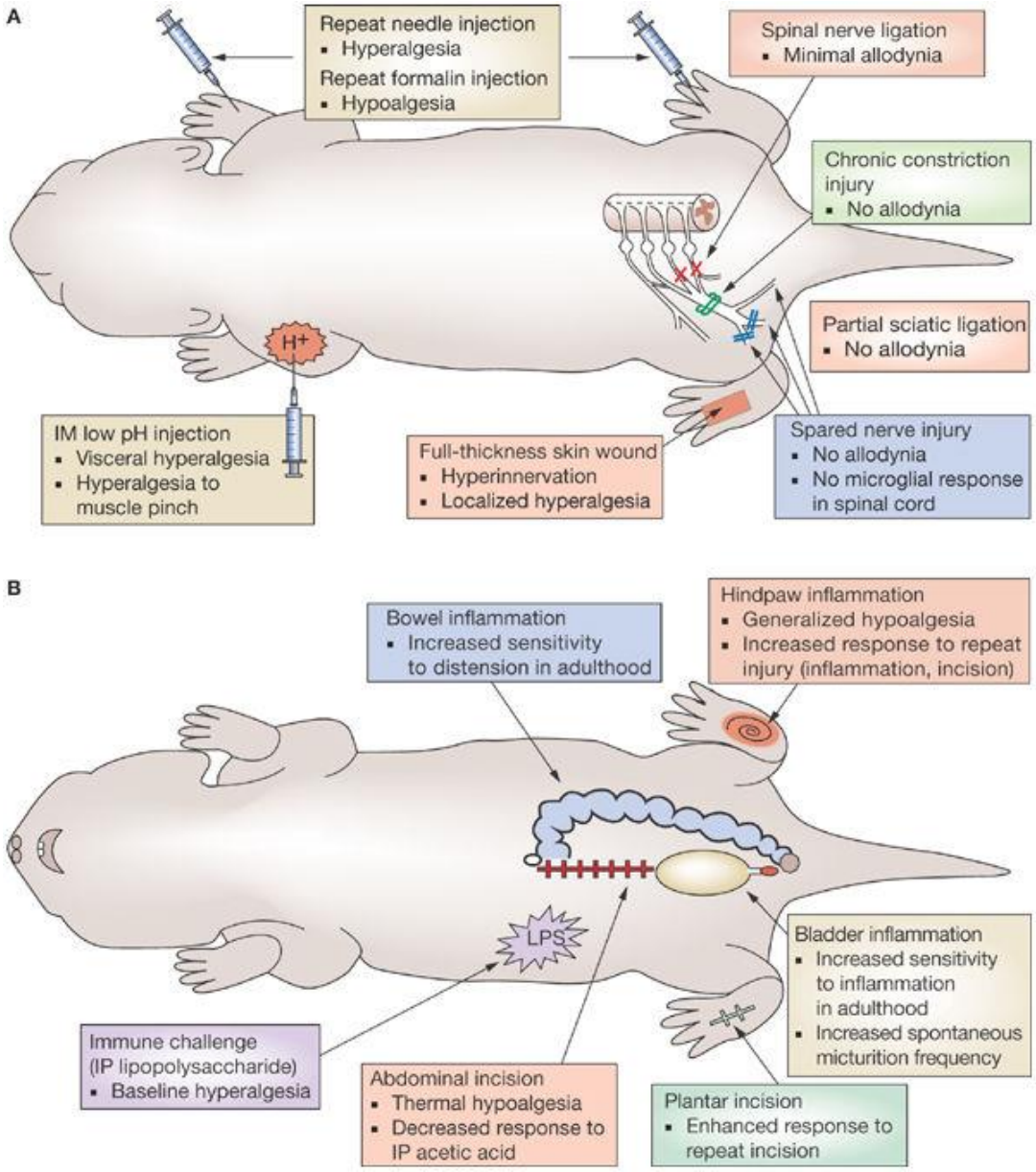


*Figure 31: Overview of animal models of early life pain.*

*The top panel presents a dorsal view of a rat pup and the bottom panel presents a ventral view of a rat pup. IM, intramuscular; IP, intraperitoneal. Adapted by* (Fitzgerald and Walker, 2009)

| Model of EL injury | Age | Animal | LT effect on baseline sensibility | LT effect on response to another injury | Sex diff. | ref |
|---|---|---|---|---|---|---|
| **Peripheral Infl.** | P0 or P1 | Rat Sprague Dawley | Mechanical and thermal hypoalgesia | ↗ hyperalgesia after re-inflammation or hindpaw incision | Hyposensitivity more pronounced in ♀ | (Ren et al., 2004; Chu et al., 2007; LaPrairie and Murphy, 2009) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | P0 | Rat Sprague Dawley | Mechanical and thermal hyperalgesia | ↗ hyperalgesia after re inflammation | Study in ♂ only | (Lidow et al., 2001) |
| | P0, P1, p3 or P14 | Rat Sprague Dawley | No difference in mechanical and thermal sensitivity | ↗ sensibility to formalin, CFA or capsaicin injection. But not in sciatic nerve ligation model or tail nerve injury model | N.D or study in ♂ only | (Ruda et al., 2000; Walker et al., 2003; Hohmann et al., 2005; Lim et al., 2009) |
| | P1 or P14 | Mice CD1 and B6C3F1 | Thermal hyperalgesia in CD1 mice and in B6C3F1(for P14 group only) | Similar CFA induced hypersensitivity | Study in ♂ only | (Benatti et al., 2009) |
| **Hindpaw Formalin** | P1 only | Rats Wistar | No difference in thermal latency but increased paw licking in females | ND | Increased paw licking only in ♀ | (Negrigo et al., 2011) |
| | P1 + P2 ± MS 60 min | | No difference in thermal sensibility in formalin group. Hypoalgesia in formalin + MS group | ↗ response to formalin | Study in ♂ only | (Butkevich et al., 2016a) |
| | P1 to P7 | Rats Long - Evans | Thermal hypoalgesia in HP. No difference on TF test | N.D | In both ♂ and ♀ | (Bhutta et al., 2001) |
| **Hindpaw incision or lesion** | P3 or P6 | Rats Sprague Dawley | N.D | ↗ hyperalgesia after P17/P20 or adult hindpaw incision | None or study in ♂ only | (Walker et al., 2009b; Beggs et al., 2012) |
| **Repeated needle pricks** | P0 to P7 | Rats Sprague Dawley | Hyperalgesia at P22 but not at adulthood | Hypersensitivity to plantar incision | N.D | (Anand et al., 1999; Knaepen et al., 2013; van den Hoogen et al., 2016) |
| | P0 to P7 | | Hyperalgesia at 14-23 weeks in VF test but not HP | ↗ response to formalin | Basal hypersensitivity in ♂ only and formalin hypersensitivity in ♀ only | (Page et al., 2013) |
| **Neuropathy** | P10 | Rats Sprague Dawley | Mechanical hypersensitivity after P30 | N.D | Study in ♂ only | (Vega-Avelaira et al., 2012) |
| **pH4 saline injection in gastrocnemius muscle** | P8 to P19 | Rats Sprague Dawley | No change in hindpaw sensitivity. Hypersensitivity to muscle compression and to CRD | N.D | Study in ♂ only | (Miranda et al., 2006) |
| **Laparotomy** | P0 | Mice CD1 | Thermal hyposensitivity and decreased abdominal | N.D | N.D | (Sternberg et al., 2005) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | constriction in response to 9% acetic acid | | | |
| **Bladder infl.** | P14 or P28 or P14 to P16 | Rats Sprague Dawley | No differences in baseline sensitivity | ↗ EMG response to bladder distension after re-inflammation | Study in ♀ only | (Randich et al., 2006; DeBerry et al., 2007) |
| **Colonic infl.** | P8 to P21 | Rats Sprague Dawley | ↗ sensibility to CRD | N.D | Study in ♂ only | (Al-Chaer et al., 2000) |
| | P10 | | No difference in VMR at baseline | ↗ response to colonic inflammation at adulthood | Study in ♂ only | (Aguirre et al., 2017) |
| **CRD** | From P8, P10 or P14 to P21 | Rats Sprague Dawley | No change in VF but thermal hyperalgesia in the abdominal region and hindpaw. ↗ EMG response to CRD | N.D | Study in ♂ only | (Al-Chaer et al., 2000; Wang et al., 2008) |

*Table 1: Summary of neonatal pain models and their main consequences on nociceptive behavior.*

*CRD : colorectal distension, EMG : electromyography, HP = hot plate, infl. = inflammation ND = non determines, P = post natal day, TF = tail flick, VF = von frey, VMR = visceromotor response ↗ = increase*

## 4) The model of neonatal maternal separation

### *(i) History and presentation of the model*

In relation with clinical research, neonatal maternal separation (MS) can occur in the case of sick newborn babies or preterm babies. When they need to be kept in an incubator during their stay in neonatal intensive care units (NICU) it impacts the early interactions between the parents and the child. One study evaluated that the duration of parental visits tends to decrease along the days of stay in NICU, possibly inducing a lack of interactions with their child, even if the holding frequencies of the newborn by the parents increased (Reynolds et al., 2013). But it can also occur when the mother is in the impossibility to deal with motherhood after birth, due to health problems for example. An American study indeed shows that the mother has to be integrated in intensive care unit with a prevalence of 0.9 for 1000 birth (Ray et al., 2012).

Mother-child interactions are critical for the good development of the child, and the absence or alteration of in maternal behaviour could induce strong cognitive, social or autonomic deficits. Indeed, rodents studies clearly demonstrate behavioural differences in rodents that have been raised by low grooming or high grooming mother, in reward directed behaviours (Peña et al., 2014), fear responses (Menard et al., 2004), reactivity to threat (Menard and Hakvoort, 2007), sexual behaviour (Cameron et al., 2011), or pain response (Walker et al., 2008). This raises the question of the importance of sensory stimulation in the newborn, being maternal touch, maternal voice or maternal odor, in the development of sensory and nociceptive systems. Early studies focused on the effects of MS in monkeys and shaped our actual knowledge about the importance of maternal-infant bond in early life. More specifically, John Bowlby proposed the theory of attachment in 1978, which describes the social link between mother and child as an innate biological response necessary for the survival of species (Harlow and Zimmermann, 1959; Harlow and Suomi, 1971). Actual studies aim to go further in the mechanisms underlying the detrimental effects of MS which is why different rodent

models of MS have been developed. In fundamental studies, the most common models of MS used in the rat consist either of a daily separation during P2 to P12 or P21, during a long (3H), or a short (15 minutes, also called handling procedure) time, as described by Plotsky and Meaney (Plotsky and Meaney, 1993). In this introduction, I will focus mainly on the long and repeated MS protocol, which has been widely studied and causes many deleterious long-term alterations of the nervous system. One of the goals of my PhD was indeed to determinate the consequences of a repeated MS from P2 to P12 during 3H/day on pain responses, and on the function of the OT descending control of pain. Other rodent models of non-painful ELS altering mother-child interaction are also used in the literature and will be described more briefly here.

Overall, all MS models induce sensory deprivation from the mother and sometimes from the littermates. This is inevitably associated with food deprivation, and possibly a change in body temperature, which has immediate effect on neuroendocrine and growth functions and could affect the development of the nervous and nociceptive systems (Kuhn and Schanberg, 1998). Even after the period of separation, maternal behaviour towards the pups is altered in most MS models, being potentially increased just after reunion but described as fragmented and inconsistent (Lovic et al., 2001; Boccia et al., 2007; Aguggia et al., 2013; Couto-Pereira et al., 2016).

### *(ii) Consequences of MS on brain functions*

This paragraph will review most of the long term deleterious effects of MS on the nervous system, with an extended focus on the nociceptive system and on pain responses. An overview of MS long-term effects on pain behaviours is presented in table 2 and 3.

#### *(a) Brain anatomy and myelination*

MS occurs in a critical period of development concerning the processes of myelination of the brain. It is then easy to consider that MS could affect the proper myelination of cerebral pathways, which could lead to further cognitive or sensitive dysfunctions. For now, only a few studies exist but they suggest that MS in the rat lead to a precocious and badly regulated myelination of the brain (Miki et al., 2014). In a mouse model of 4h separation from P2 to P5 followed by 8h of separation between P6 to P16, Bordner and colleagues describe at P75 a modification in the expression of the proteins composing myelin, such as a decrease in MBP, OMG, MAG and RTN4. They also describe a change in the expression of genes that control the differentiation of oligodendrocytes, suggesting an altered maturation of these cells (Bordner et al., 2011). In a rat model of MS, impaired medial prefrontal cortex myelination has been described in both males and females, and a lack of mature oligodendrocyte. This was linked to an increased Wnt activation due to HDAC inhibition induced by MS (Yang et al., 2017). By modulating Wnt signaling pathway, the authors were able to rescue myelination and associated behaviour (Yang et al., 2017).

#### *(b) Stress/ anxiety and depression*

Many of the first studies on MS focused on the effects on the HPA axis, as MS animals display at adulthood a strong anxiety behaviour and an increase in the release of ACTH and corticosterone in response to an external stressor (Plotsky and Meaney, 1993; Liu et al., 2000; O'Mahony et al., 2009a). It is also correlated with an increase in CRF and its receptor CRF1mRNA in the amygdala, LC and HTs in rats (Plotsky et al., 2005; O'Malley et al., 2011a), and an alteration of feedback mechanisms of the HPA axis, as a decrease in GR and MR receptors is detected in the hippocampus. MS animals are also more sensitive to the additional exposition to another stressor, which is

correlated to increased expression of CRFR1 in hypothalamus, prefrontal cortex and hippocampus, and of CRFR2 in the amygdala (O'Malley et al., 2011a). The implication of OT or VP signaling have also been proposed in rodents and rhesus macaques (Zhang et al., 2012; Baker et al., 2017; Lesse et al., 2017). At adulthood, a consequence of MS would be a higher susceptibility to develop "depressive like" behaviour, measured in the swim test by an increase immobility and an alteration of sucrose preference (Aisa et al., 2007; Shu et al., 2015). An alteration of the serotoninergic system both in the newborn (Ohta et al., 2014) and later at adulthood (Bravo et al., 2014; Ohta et al., 2014) might explain this behaviour.

*(c) Social interaction*

The early interactions with the mother in early life are crucial for the modelling of the social brain. The quality and amount of grooming in rodents can indeed affect the offsprings and shape their behaviour at adulthood (Champagne, 2008). In rats, decreased time spent in nose-to-nose contact with a conspecific and an increased latency for the first contact was described after MS, in an age and sex-specific manner (Holland et al., 2014). In another protocol, sex-differences in social play behaviour have also been described, with males displaying an increased amount of spinning in the social play test compared to male separated 15 minutes (Lundberg et al., 2017). In addition, early social odor preference is impaired by MS in CD1 mice, as pups in the MS group display a longer preference for the home-cage nest odor compared to a clean familiar shaving odor than control animals (Thomas et al., 2010). Once again, different mechanisms could explain social dysfunctions following MS, including alterations among the OT and VP systems (Veenema, 2012).

*(d) Cognition*

As the hippocampus seems to be a preferential target for structural and functional alteration by MS, different research suggested that memory would be impaired by MS. Object recognition as well as behaviour in Morris Water maze is impaired in both MS rats and mice at adulthood (Aisa et al., 2007; Wang et al., 2011). Juvenile mice display a reduced cognitive flexibility which is no longer detected at adulthood (Thomas et al., 2016). These observations have been associated with morphological changes in the hippocampus, as well as a decrease in NGF levels and in the density of mossy fibers in striatum oriens (Huot et al., 2002; McNamara et al., 2002).

*(e) Somato-sensory responses*

As detailed previously, the sensory system and in particular the nociceptive system is submitted to a huge re-organization after birth, through an activity-dependent maturation, both in the spinal cord and in cortical areas (Beggs et al., 2002; Zuo et al., 2005). This makes it particularly sensible to any environmental changes during early life. In the context of MS, it is tempting to hypothesize that the lack of sensory information from the mother during the repeated separation time would disturb the normal activity-dependent maturation of somato-sensory systems and lead to altered sensory responses at adulthood. Using a 3H MS model in C57BL6 mice from P2 to P14, Takatsuru and colleagues indeed demonstrated that at 4 weeks, MS mice have decreased scores in the tape test, where they have to detect of small piece of adhesive tape on the hindpaw (Takatsuru et al., 2009). However, this was not observed at 7-8 weeks or 11-13 weeks. This has been associated with a decreased number of spines in the somatosensory cortex, and an increased loss rate of mushroom spines (Takatsuru et al., 2009). Interestingly, with a different MS protocol of 1H during 3 days at either P1-3, P5-7 or P14-16, Bock and colleagues described an increase in spine density in the somatosensory cortex in male Wistar rats (Bock et al., 2005). Structural changes have also been described in other structures implicated in sensory and pain responses. An hypertrophy and increase

in spine density was detected in the basolateral amygdala (Koe et al., 2016), and a decrease in dendritic spine in the prefrontal cortex(Monroy et al., 2010; Chocyk et al., 2013).

*(iii) Consequences of MS on pain responses*

*(a) Visceral sensitivity*

Clinical studies made researchers and medical teams suspect that ELS could induce long term alterations in the visceral system, including changes in visceral sensitivity and states of chronic visceral pain. In animal models of ELS, visceral pain is investigated by measuring the EMG response to colorectal distension (CRD). As reported in Table 2, adult animals previously subjected to MS often display an increased visceral sensibility, being either the visceromotor response (VMR) to CRD at baseline (Coutinho et al., 2002; Moloney et al., 2012a), or after a protocol of water avoidance test (WA). However, a few studies showed no differences in baseline sensitivity, and only revealed visceral hypersensitivity after the re-exposure to an additional stressor at adulthood. In mice, these observations have been extended to urinary bladder sensitivity both at baseline and after WA (Pierce et al., 2016), and to vaginal sensitivity (Pierce et al., 2014). Interestingly, visceral hypersensitivity following MS seems to follow an age dependent pattern. Yi and colleagues demonstrated that the proportion of hypersensitive rats decreases with age, from 87.5% on P21 from 70% on P56 in females and from 90.0% to 66.7% in males (Yi et al., 2017). MS has also been superimposed to the genetic Kyoto rat model, which displays a susceptibility to anxiety and depression-like behaviour. In that case, MS didn't increase anxiety and depression symptoms but still increased visceral hypersensitivity, underlying the importance of genetic background in the consequences of MS (Hyland et al., 2015).

Face to these multiple observations, MS has even been proposed as an animal model for IBS (Sengupta, 2009). However, an important aspect of IBS in humans is also that it has a higher prevalence in women (see our recent review (Melchior et al., 2016)). In animal studies, the sex-specificities of MS consequences on visceral pain are unfortunately not often considered since many studies are lead on males only. Among the existing studies, MS has been showed to induce visceral hypersensitivity in males only when the entire litter is submitted to the MS protocol, whereas it develops in females even when only half of the little is submitted to MS (Rosztóczy et al., 2003).

| Model | Timing | Animal | Nociceptive test | Age at testing | Observations | Sex-differences | Reference |
|---|---|---|---|---|---|---|---|
| **MS** | 3H/day P1/2 to P14/15 | Mice C57BL/6 | VMR to CRD ± WA test | adult Adult | No differences at baseline but ↗ sensibility after WA | Study in ♂ only | (Fuentes et al., 2016) |
| | | | VMR to vaginal balloon distension | | No difference | Study in ♀ only | (Pierce et al., 2014) |
| | | Rats Sprague Dawley | VMR and AWR to CRD | adult | ↘ CRD threshold and ↗VMR and AWR | Study in ♂ only | (Tsang et al., 2012; Hu et al., 2013; Xiao et al., 2016; Zhou et al., 2016b) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | Increased sensibility in ♀ at P56 | (Yi et al., 2017) |
| | Rats Long Evans | VMR to CRD ± Water avoidance | adult | ↗ VMR to CRD and hyperalgesia after WA | Study in ♂ only<br><br>Or no effect in ♀ | (Coutinho et al., 2002; Schwetz et al., 2005; van den wijngaard et al., 2009; van den Wijngaard et al., 2012)<br><br>(Prusator and Greenwood-Van Meerveld, 2016a) |
| | | | | No difference at baseline but hypersensitivity 6 and 24H after WA | Study in ♂ only | (Welting et al., 2005) |
| | Rats Wistar | VMR to CRD<br><br>± Water avoidance | adult | ↘ CRD threshold and ↗VMR | Study in ♂ only | (Barreau et al., 2004) |
| | | | | Hyperalgesia after WA with higher VMR in MS females compared to CTRL females | Hyperalgesia in ♀ when MS whole or half litter. In ♂ only when MS whole litter. No change after WA in ♂ | (Rosztóczy et al., 2003) |
| 3H/day from P1-3 to P21 | Rats Sprague Dawley | VMR to CRD | adult | ↗ EMG response | Study in ♂ only | (Chen et al., 2017) |
| | Mice C57Bl/6 | VMR to vaginal balloon distension | adult | ↗ EMG response | Study in ♀only | (Pierce et al., 2014) |
| | | VMR to CRD and urinary bladder distension ± Water avoidance | | ↗response to UBD and ↘ to CRD. No SIA after WA for CRD but SIH for UBD 1H after WA and SIA 8 days after WA | Study in ♀ only | (Pierce et al., 2016) |
| | | Perigenital Von Frey testing | | ↘ threshold | Study in ♂ only | (Fuentes et al., 2015a) |
| 3H/day from P2 to P11/12 | Rats Sprague dawley | VMR to CRD | adult | ↘ threshold and ↗ total pain | Study in ♀ only | (Moloney et al., 2016) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | behaviour | | |
| | | | | | ↘ threshold and ↗ pain response | Study in ♂ only | (Hyland et al., 2009; O'Mahony et al., 2009b; Felice et al., 2014) |
| | | Rats Kyoto | VMR to CRD | adult | ↘ threshold and ↗ pain behaviour | Study in ♂ only | (Hyland et al., 2015) |
| | 6H/ day from P2 to P15 | mice C57BL/10JNju (Tlr4+/+) and C57BL10/ScNJ Nju (Tlr4-/-) | Abdominal withdrawal response to CRD | adult | ↘ threshold and ↗ AWR | N.D | (Tang et al., 2017) |
| **MS + mat. stress** | 3H from P1 to P14 | Mice balb/c | VMR to CRD | adult | ↗ response to CRD | Study in ♂ only | (Moloney et al., 2012b) |

*Table 2: Summary of MS consequences on visceral sensitivity.*

*AWR = abdominal withdrawal response, CRD = colorectal distension, MS= maternal separation, N.D = non determined, SIA = stress induces analgesia, UBD = urinary bladder distension, VMR = visceromotor response, WA = water avoidance*

As the numerous consequences of ELS are being identified, another question has been raised concerning the possible transgenerational transmission of ELS-induced behavioural alterations (Cowan et al., 2016). On this subject a study investigated if this stress-induced visceral hypersensitivity detected in MS rats could be transferred to the descendance, even if the second generation (F2) pups are not submitted to MS. They studied pain sensitivity in the offspring of MS dams and showed that 80% of them display hypersensitivity to colorectal distension after water avoidance test whereas it is detected in only 19% of the offspring of control F2 pups. The underlying mechanisms were investigated by cross-fostering the rat pups. MS pups were fostered by control non-handled mothers and control pups fostered with MS dams. Surprisingly, non-handled pups nursed by MS dams displayed stress induced visceral hypersensitivity at adulthood, suggesting that the phenotype was closely linked with maternal behaviour. (van den Wijngaard et al., 2013). Epigenetic programming, which will be discussed in a later paragraph, is also proposed as a possible explanation for the transgenerational transmission of ELS induced behaviours.

Furthermore, numerous other hypotheses have been proposed to explain the development of visceral hypersensitivity in MS animals. The origin seems to be multiple, going from peripheral, spinal and supraspinal anatomical and functional neuronal alterations in the pups, to a change in the composition of the microbiota or in immunological agents which can interact with the nervous system.

- Change in colonic function and peripheral inflammatory processes

Changes in colonic morphology and function are likely to be involved in the phenomenon of MS-induced visceral hypersensitivity. Many morphological changes have been described, in the mucosal or muscular layer or in the permeability of the intestinal barrier, which is linked to an increased cholinergic activity among enteric nerves (Söderholm et al., 2002; Barreau et al., 2004; Gareau et al.,

2007b; O'Malley et al., 2010). Moreover inflammatory processes are also affected by MS. Visceral hypersensitivity after MS has been linked to increased mast cell degranulation due to the mediator NGF or by CRF (Barreau et al., 2004; Hyland et al., 2009; van den wijngaard et al., 2009; van den Wijngaard et al., 2012; Pierce et al., 2016), and to increased stress induced colonic motility (Schwetz et al., 2005). An increased level of different pro-inflammatory cytokines has been detected in the colons of MS rats (Barreau et al., 2004), including IL-6 which increases the activity of submucosal neurons (O'Malley et al., 2011b).

In the past few years, many studies have highlighted the complex interaction between the gut and the brain, suggesting that the composition of the gut microbiome could regulate brain related processes and behaviours, including HPA axis function, cytokine production, microglial activation…(Rea et al., 2016; Sharon et al., 2016; Kelly et al., 2017). Different environmental components of early life are able to modulate the function of the so called brain-gut axis, such as early stress, nutrition or maternal care (O'Mahony et al., 2009a; Farshim et al., 2016). Besides, the composition of the microbiota is closely linked to the emergence of visceral pain, as summarized in a recent review (Chichlowski and Rudolph, 2015). Recently, Zhou and colleagues compared microbiota alterations in IBS patients and in the rat MS model and showed that they both display dysbiosis (Zhou et al., 2016c). In the rat MS model, it is suggested that the concentration of Fusobacterium, which is also increased in IBS patients (Jeffery et al., 2012), is linked to the intensity of visceral pain. To study the role of the microbiota, experiments using germfree animals and microbiota transfer have been developed. Transferring IBS patients microbiota into germ free animals resulted in the development of visceral hypersensitivity in these animals (Crouzet et al., 2013). On top of that, treatments with probiotics comforted the idea that gut microbiota is implicated in the long-term consequences of ELS. It allowed to alleviate behavioural symptoms (Desbonnet et al., 2010), gut morphological and functional dysfunction (Gareau et al., 2007a), and even visceral pain symptoms (Distrutti et al., 2013).

Of course, alterations in the function of higher brain centers and spinal and peripheral sensory pathways are also implicated in ELS-induced visceral symptoms. However, some of these alterations might also explain other pain related symptoms and will hence be discussed later in a dedicated paragraph.

*(b) Somatic sensitivity*

The effects of MS on somatic sensibility are somehow more conflicting, depending on the separation protocol and the nociceptive test, as shown in Table 3. Indeed, Coutinho and colleagues described an hyposensitivity to thermal stimulation in the tail flick test, as the withdrawal latency is longer in MS males vs control (Coutinho et al., 2002) This has also been demonstrated in female rats using hot plate test (Weaver et al., 2007). On another hand, an increased mechanical and thermal sensitivity has been described in the MS rat model (Juif et al., 2016; Prusator and Greenwood-Van Meerveld, 2016a). Similar observations were obtained with MS in mice (Takatsuru et al., 2009; Pierce et al., 2014; Fuentes et al., 2015b). However, in other studies no difference have been described for thermal or mechanical sensitivities in rats (Kalinichev et al., 2001; Lariviere et al., 2006; Uhelski and Fuchs, 2010; Vilela et al., 2017) or mice (Pierce et al., 2014; Nishinaka et al., 2015a; Gracia-Rubio et al., 2016; Amini-Khoei et al., 2017). One study also studied the impact of MS on orofacial sensitivity and observed MS-induced mechanical allodynia at adulthood (Yasuda et al., 2016). Fuentes and colleagues investigated perigenital sensitivity in a mice MS model, and measured mechanical threshold by Von Frey testing on the side of the scrotum. They observed a decreased mechanical

threshold in adult MS mice, associated with increased mast cells activation in the prostate (Fuentes et al., 2015b), suggesting that MS could be used as a preclinical model for chronic prostatitis/chronic pelvic pain syndrome.

- ***Response to inflammation or neuropathy***

On top of possible increased baseline sensitivity to pain, animal studies suggest that MS induces a long-term sensibility to other pain-triggering stimulation at adulthood. For example, MS animals are more sensitive to inflammatory pain, as showed by an increased behavioural responses after formalin or CFA injection in the hindpaw (Uhelski and Fuchs, 2010; Vilela et al., 2017). However, this is in conflict with other studies showing no differences in formalin or CFA or carrageenan response (Lariviere et al., 2006; Uhelski and Fuchs, 2010). In a mouse model of MS, associated with social isolation after weaning, Nishinaka and colleagues demonstrated an increased thermal and mechanical hypersensitivity and mice subjected to partial sciatic nerve ligation at 9 weeks (Nishinaka et al., 2015b).

| Model | Timing of MS | Animal | Nociceptive test | Age at testing | Observations | Sex differences | Reference |
|---|---|---|---|---|---|---|---|
| **MS Only** | 3H/day from P2 to P12 | Rat Sprague Dawley | Calibrated forceps and Hargreaves method | P24, P55 and P100 | ↘ mechanical and thermal threshold | N.D | (Juif et al., 2016) |
| | 3H/day from P1 or P2 to P14 | Mice NMRI | TF and HP | P60 | No baseline diff. Lack of SIA after restraint stress | Study in ♂ only | (Amini-Khoei et al., 2016) |
| | | Mice C57Bl/6 | VF and analgesiometer | adult | ↘ mechanical and thermal threshold | Study in ♂ only or N.D | (Takatsuru et al., 2009; Fuentes et al., 2016) |
| | | | | | No difference at baseline but hypersensitivity following vaginal distension | ♀ only | (Pierce et al., 2014) |
| | | Rat Sprague Dawley | VF on whisker pad skin | adult | ↘ threshold | Study in ♂ only | (Yasuda et al., 2016) |
| | | Rat Long Evans | TF | adult | ↗ latency of response. ↘ SIA after WA test | Study in ♂ only | (Coutinho et al., 2002) |
| | | | TF and HP | | No diff. at baseline | Baseline latency higher in ♂ | (Kalinichev et al., 2001) |
| | | | HP | | ↗ latency of response | Study in ♀ only | (Weaver et al., 2007) |
| | | | VF | | ↘ threshold in males | No change in ♀ | (Prusator and Greenwood-Van Meerveld, 2016b) |
| | | Rats Wistar | HP, VFand CFA and formalin | adult | No diff. at baseline but ↗ response to CFA and formalin | Increased response at 4H after CFA and 7H in ♂ | (Vilela et al., 2017) |
| | 3H/day | Mice | VF and | adult | ↘ mechanical and | Study in ♂ only | (Fuentes et |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | from P1 or P2 to P21 or P22 | C57Bl/ 6a | analgesio meter | | thermal threshold<br><br>+ no further change after vaginal distension in females | Study in ♀ only | al., 2016)<br><br>(Pierce et al., 2014) |
| | | Rats lewis and Fisher | TF and formaline | | No difference | Lower tail flick latencies in fischer ♀ | (Lariviere et al., 2006) |
| | 1H/day from P2 to P9 | Rats Sprague - Dawley | HP | adult | ↘ latency | Higher latencies in ♂ | (Imanaka et al., 2008) |
| | 6H/day from P2 to P15 | Rats Wistar | HP, VF and Formalin | adult | No diff at baseline but ↗ phase 2 in formalin test | Study in ♂ only | (Uhelski and Fuchs, 2010) |
| **MS and early weaning** | 4H P2 to P5 + 8h from P6 to P16 + weaning at P17 | Mice CD-1 | Electrical nociceptive threshold | P30 | No difference | No sex-differences | (Gracia-Rubio et al., 2016) |
| **MS and social isolation** | 6H from P15 to P21 and isolation after weaning | Mice ddY | Plantar, VF and partial sciatic ligation model | adult | No diff. at baseline but ↗ response to neuropathy | No sex-differences | (Nishinaka et al., 2015b) |

*Table 3: Summary of MS consequences on somatic sensitivity.*

*CFA = complete freund adjuvant, HP= hot plate, ND = non determined, SIA = stress induced analgesia, TF = tail flick, VF = von frey*

### *(iv) Proposed mechanisms for MS-induced alteration of pain responses*

#### *(a) Supraspinal alterations*

***Changes in pain-related areas of the brain***. To understand the origins of visceral and somatic hypersensitivity following ELS, brain imaging has been used both in clinical and fundamental studies. Using $H_2{}^{15}O$ microPet scan in MS rats, Wouters and colleagues showed that specific supraspinal areas are activated or deactivated after colorectal distention before or after water avoidance test. At baseline, colorectal distension increases blood flow in cerebellum and PAG, and activates PAG, somatosensory cortex and the hippocampus, and deactivates the frontal cortex (Wouters et al., 2012). Hippocampus implication in visceral pain was further investigated in a rat MS model. Hippocampal GluR2 expression is increased in these rats as well as LPS intensity at SC-CA1 synapses induced by high frequency stimulation (Chen et al., 2017). NBQX injections in the hippocampus alleviated the visceral hypersensitivity, confirming the implication of hippocampal glutamatergic transmission in the development of MS-induced pain hypersensitivity (Chen et al., 2017). In MS adult rats, an increased activity in the insular cortex is also suspected to contribute to the visceral hypersensitivity (Chung et al., 2007a; Ren et al., 2007; Zhang et al., 2017).

Pathological and chronic pain conditions are sometimes associated with a dysfunction of descending controls of pain. These controls are carried by different areas of the brain and lead to an inhibition or a facilitation of the nociceptive message in the spinal cord. In particular, a dysfunction in amygdala,

RVM and/or PAG, important relays for descending pain controls, could have important consequences on pain behaviours. Increased activity and synaptic transmission of basolateral amygdala neurons have been recorded in MS rats, and associated with an increased TRPV1 expression which acts in a presynaptic mechanism to increase amygdala neurons activity (Xiao et al., 2016). In that study, blocking TRPV1 activity was successful to alleviate MS-induced visceral hypersensitivity in MS animals (Xiao et al., 2016). An increase in BNDF expression and its receptor TrkB has been detected in the amygdala, and also in the RVM in MS rats after colorectal distension (Chung et al., 2009). Moreover, an increased p-ERK expression, a marker of neuronal activation, has been observed after the onset of neuropathic pain in the PVN and amygdala in MS animals, but not in control animals, but only in females (Nishinaka et al., 2016). Astrocytic function in the locus coeruleus has also been suggested to be implicated in females in this hyper-response following nerve injury (Nakamoto et al., 2017). Taken together, these results are in favor of an increased descending facilitation from the amygdala through the RVM in MS rats, contributing to the measured hyperalgesia.

**Central inflammation.** In the past few years, microglial activity and inflammatory factors have been considered as potentially responsible for the development of chronic pain syndromes (Grace et al., 2014). In this context the question of a neuroimmunological origin of sensory and nociceptive dysfunction after MS has risen. Tang and colleagues showed that TLR4 KO mice don't develop visceral hypersensitivity after MS (Tang et al., 2017). Moreover, hypothalamic TRL4 expression, carried mostly by microglial cells, is increased in MS mice, as well as other inflammatory factors, IL-1$\beta$, TNF-$\alpha$ but not IL-6 (Tang et al., 2017). In the rat, similar increases in hypothalamic cytokines have described for TNF-$\alpha$ and IL-6 but not IL-1$\beta$ (Roque et al., 2015). An increase in microglial activation after MS has been demonstrated in different brain structures including prefrontal cortex and hippocampus (Gracia-Rubio et al., 2016)

### *(b) Spinal changes*

In the spinal level an increased activation of superficial and deep laminae of the spinal cord has been demonstrated in MS rats in a c-fos study after CRD (Chung et al., 2007b). Among other targets, neurotrophic factors participate in the maintenance and plasticity of spinal pain circuits and have been proposed as factors responsible for MS-induced nociceptive alterations. During the early post-natal period, our laboratory showed that NGF and GNDF expressions are different in MS and control animals (Juif et al., 2016). The expression of NGF transcripts is lower at P7 and peaks at P18 in MS animals, whereas it decreases along the post-natal period in control animals. GDNF transcripts levels are very low at P7 in MS animals but reach control levels at P14. These early differences could have important impacts on the development of spinal cord circuits and on their function at adulthood. At adulthood, other studies showed that NGF expression is still different in MS rats compared to control, associated with an increase in TrkA immunoreactive fibers in the lumbosacral spinal cord (Chung et al., 2007a). A pharmacological attempt to rescue MS pain phenotype was performed using NGF antagonist K252a and allowed to restore normal pain sensitivity in MS animals (Tsang et al., 2012). BDNF has also been proposed as having a key role in MS-induced nociceptive symptoms, since it is over-expressed in the SC of MS rats (Wu et al., 2016).

Protein Kinase M zeta, a specific sort of protein kinase C that is implicated in long term synaptic potentiation and involved in inflammatory or neuropathic pain, has been showed to participate to MS symptoms (Tang et al., 2016). First of all, it is overexpressed in thoracolumbar and lumbosacral spinal cord in MS animals. And secondary, an i.t treatment with zeta inhibitory peptide was successful to relieve visceral hypersensitivity in MS rats. Finally, the overall hyperactivity of the spinal

cord might be due to changes in activating neurotransmitters. This seems to be possible since a decrease in glutamate reuptake has been identified after MS, due to a reduction in glial excitatory amino acid transporter 1 (Gosselin et al., 2010).

### *(c) Peripheral hypothesis*

At the DRG level, recent studies suggest an increase in the excitability of primary afferent neurons, as it has been showed using patch clamp recording on colon-specific DRG neurons (Luo et al., 2011; Hu et al., 2013). In agreement with this, an increased expression of Nav 1.8, but not Nav 1.9 has been detected in DRG neurons of adult MS animals (Hu et al., 2013; Juif et al., 2016). A decreased expression of kv1.2 expression in colonic DRG neurons after MS has been reported, associated with a suppression of I(K) current in these neurons (Luo et al., 2011). The orofacial hypersensitivity measured after MS has been associated with an increase in p2X3receptor expressing neurons in the trigeminal ganglion. The blocking the receptor indeed increased mechanical thresholds in MS rats (Yasuda et al., 2016) .

### *(d) Epigenetics*

During the past few years, the field of epigenetics has raised a great interest, both in the context of chronic pain development and in the context of ELS. Epigenetic concerns mechanisms capable of enhancing or suppressing the expression of specific genes, but without changing the initial DNA sequence. Such changes can occur in response to environmental modifications. Epigenetic mechanisms can act at different levels presented in detail in a recent review (Turner, 2001) and illustrated in figure 32. The first level of action is (1) Histone modification by the addition or suppression of an acetyl group. In that case the acetyl group is added to the histone by histone acetyltransferases (HAT) which weakens the interaction with DNA and allows a better access to transcription factors. This leads to an increase in the expression of the targeted gene. Deacetylation is promoted by histone deacetylases (HDAC) and has the opposite effect, decreasing the expression of the targeted gene. Histone modification can also include methylation, which final effect can either be an increase or a repression of gene expression depending on the site of methylation. The second level is (2) DNA modifications on CpG island sites, which leads to the compaction of DNA and limits access to transcription factors, hence suppressing gene expression. Specific proteins are able to bind methylated DNA, such as methyl-CpG-binding protein 2 (MeCP2) or methyl DNA-binding domain (MBD) proteins, leading to further suppression of gene expression. Phosphorylation of these proteins can remove them from their binding site, leading to promoted gene expression. They are also capable of recruiting histone modification enzymes such as HDAC. Another epigenetic mechanism is (3) based on the existence of small non-coding RNA called mi-RNA. They are composed of around 22 nucleotides and can bind protein-coding mRNAs, leading to the degradation of the targeted mRNA and the repression of protein expression. These epigenetic processes are closely linked together and interact to enhance or suppress gene expression.

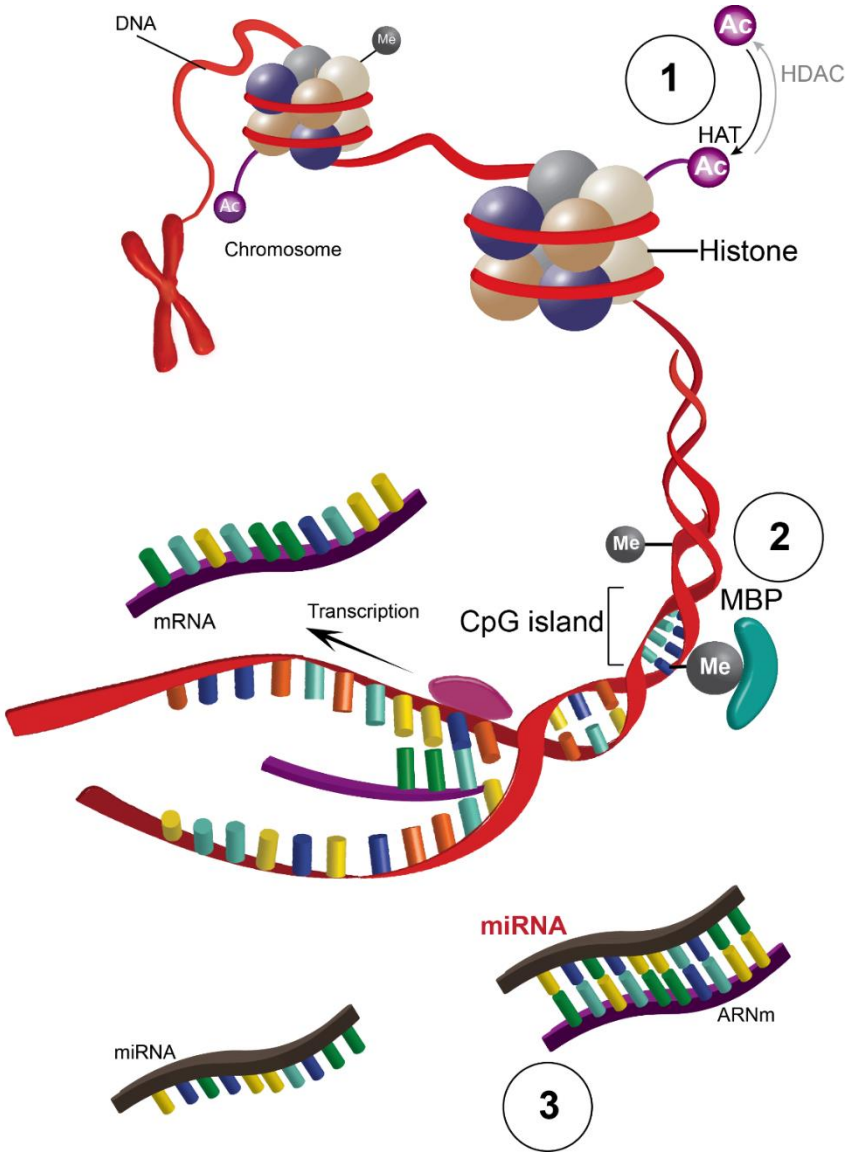


*Figure 32: Epigenetic regulation of gene expression.*

*1,2,3 = see in the text. Adapted from* (Melchior and Poisbeau, 2015)

Epigenetic seems to be highly implicated in the long-term alterations in pain pathways after ELS, and possibly in the transgenerational transmission of specific behaviours. In human studies, Chau and colleagues described that former very preterm children who suffered a great among of neonatal pain stress have higher methylation at 7/10 CpG sites in the serotonin transporter SLC6A4 promoter compared to term children at 7 years of age (Chau et al., 2014). In a rodent MS model, Moloney and colleagues described an alteration in histone acetylation in the spinal level, especially at the level of H4K12. In this study, an adult treatment with SAHA, an HDAC inhibitor, was efficient to reverse the MS-induced visceral hypersensitivity (Moloney et al., 2015). In a prenatal stress model, the subsequent visceral hypersensitivity has been linked to increased BDNF spinal expression and to increased histone H3 acetylation, and decrease in HDAC1 association with the promotor of BDNF gene (Winston et al., 2014). A similar epigenetic modification of spinal BDNF gene has also been found in an early life pain model of colonic inflammation (Aguirre et al., 2017). In that case an increased binding of pCREB on the cAMP response element is suggested, leading to the recruitment

of histone acetyl transferase. As demonstrated earlier, IBS is one of the most prevalent consequences of ELS. Clinical studies investigated if IBS was associated with epigenetic mechanisms, but most studies focused on miRNA expression in the peripheral level. IBS patients present significant changes in the expression of miR-29a and b, miR-103, miR-16 , miR-24 and miR-125 in colonic and bowel tissues, which is in close relation to the increased intestinal membrane permeability that they suffer from (Zhou et al., 2010, 2015; Liao et al., 2016). miR-199 is also suggested to have a key role in visceral pain symptoms in IBS patients. Its expression is indeed decreased in the gut and associated with an increased TRPV1 expression (Zhou et al., 2016a). Blocking miR-199 activity was successful to decrease TRPV1 signaling and pain symptoms in a rat IBS model of intracolonic infusion of 2,4,6-trinitrobenzene sulfonic acid (Zhou et al., 2016a). Similarly, miR-24 expression is up-regulated in IBS patients and in the same mouse model, and targets the serotonin transporter SERT (Liao et al., 2016). In the mouse model, inhibiting miR-24 activity was also successful to alleviate visceral pain symptoms (Liao et al., 2016). In the blood, miR-150 and miR-342-3p were elevated in IBS patients and are also associated to pain modulation (Fourie et al., 2014).

### 5) Other ELS models targeting mother-pups interactions and their effect on pain behaviour

#### *(i) Handling*

The long MS model of at least 3H of separation is often studied in parallel with a control group called the "handling" group, where the pups are subjected to a brief manipulation and separation with the mother (LEVINE et al., 1957). This parallel is interesting because the behavioural consequences are quite different between the long and the short MS protocol. For example, it leads to a greater ability to cope with stress at adulthood (Meaney et al., 1991). The HPA axis is altered by this ELS protocol, but not in the same direction as the long MS protocol. ACTH and corticosterone production are indeed decreased in stressful situation when compared to MS animals or control animals (Skripuletz et al., 2010), and the negative feedback on the HPA axis seems more efficient (Plotsky and Meaney, 1993). A possibility would be that maternal presence and sensory interactions with the pups, as well as the quality of maternal care would be essential for the proper development of the brain. In the handling procedure maternal care seems to be of greater quality, the dams displaying increased arched-back nursing and higher OT levels (Pryce et al., 2001; Couto-Pereira et al., 2016).

Concerning pain sensibility, handling protocol seems to lead to an overall hyposensitivity to pain at adulthood, as presented in table 4. Decreased hot plate latencies have indeed been measured in the rat F344 and Lewis strain (Stephan et al., 2002). However, sex specificities are been suggested, since another study showed an increased latency in hot plate only in females (Smythe et al., 1994). The hyposensitivity has also been described in a mouse handling model and on tail-flick and formalin test (Pieretti et al., 1991; d'Amore et al., 1995; Clausing et al., 1997; D'Amato et al., 1999; Sternberg and Ridgway, 2003). These changes could be linked to a differential activity of the opioidergic system, since the hyposensitivity in hot plate is prevented by naloxone pretreatment (Pieretti et al., 1991). However, a decreased latency of response to 50°c hot plate has been described in a rat handling model (Weaver et al., 2007). The intensity of morphine analgesia is also increased in handled animals in one study (Sternberg and Ridgway, 2003) and decreased in another one (D'Amato et al., 1999). Similar to the long MS model, stress-induced analgesia is impaired, but only in females mice (Sternberg and Ridgway, 2003). The decreased sensibility to pain concerns also visceral sensitivity, since the visceromotor response to colorectal distension is decreased both at baseline and after

stress (Schwetz et al., 2005). As for the long MS model, intestinal permeability seems to be affected by neonatal handling (Oines et al., 2012).

| Model of ELS | Timing | Animal | Age at testing | Nociceptive test | Observations | Sex-differences | Reference |
|---|---|---|---|---|---|---|---|
| **Handling** | 15 min from P1/2 to P13/14 | Mice NMRI | adult | TF and formalin | ↗ TF latencies and ↘ response to formalin | Study in ♂ only | (D'Amato et al., 1999) |
| | | Rats Long-Evans | adult | TF and HP | No difference | Baseline latency higher in ♂ | (Kalinichev et al., 2001) |
| | | | | HP | ↘ latency of response | Study in ♀ only | (Weaver et al., 2007) |
| | 15 min from P2 to P22 | Rats Lewis and Fischer | adult | TF and formaline | No difference | Higher baseline sensibility in Fischer ♀ | (Lariviere et al., 2006) |
| | 10 min P2 to P21 | Mice CD-1 | P30 | Tail flick | No difference | Study in ♂ only | (Loizzo et al., 2010) |
| | 5 min from P8 to P21 | Mice DBA/1 | adult | HP | ↘ latency to lick hindpaw | Study in ♂ only | (Clausing et al., 1997) |
| | 5 min from P2 to P28 | Rats Lewis and F344 | adult | HP | ↗ latencies in both strains | Lower latencies in ♀ | (Stephan et al., 2002) |
| **Handling + daily saline injection** | 12 min from P2 to P19 | Mice CD-1 | P30, 35 and P50 | TF and HP | ↗ TF latency at P30 but not P50 and ↗ HP latencies | Study in ♂ only | (D'Amore et al., 1993; d'Amore et al., 1995) |
| | 10 min P2 to P21 | | P30 | TF | ↗ threshold | Study in ♂ only | (Loizzo et al., 2010) |

*Table 4: Neonatal handling effects on pain responses.*

*HP = hot plate, TF = tail flick*

### *(ii) Maternal deprivation*

In the literature, MS and maternal deprivation (MD) are sometimes used as synonyms, yet maternal deprivation mostly refers to a single 24H separation with the mother, often occurring at P9. This induces a single but prolonged lack of sensory interaction with the mother, but also an important lack of nutritive behaviour. Like in the MS protocol, the temperature is controlled with a heating pad or heating lamp to avoid hypothermia. Compared with the MS model, the nutritional aspect might have a more important role in the consequences observed in later childhood and adulthood. Concerning maternal behaviour, a transient increase in maternal behaviour has been detected in MD model after reunion (Llorente-Berzal et al., 2011). This protocol also results in behavioural alterations at adulthood, being increased stress and anxiety, altered cognitive processes and emotional disorders (see review (Marco et al., 2015)).

The long-term effects on pain behaviour have been less studied than in the other MS models. However, Burke and colleagues demonstrated changes in baseline mechanical and thermal sensitivity at adulthood in Wistar rats subjected to MD. An increased latency to jump has been measured in MD

females using the hot plate test, as well as decreased Von Frey threshold (Burke et al., 2013). Adult male rats previously subjected to MD also display an increased sensibility to formalin (Butkevich et al., 2016b). No change has been detected in cold sensitivity, suggested a differential effect of MD depending on the modality (Burke et al., 2013). The same study also showed an increased vulnerability to neuropathy in MD females in the spinal nerve ligation model, associated with an altered inflammatory response.

*(iii) Artificial rearing*

In this model of ELS, rat or mice pups are housed without the mother in a post-natal controlled environment, where maternal stimulation and nest are carefully regulated, often called as "a pup in a cup" procedure. The protocol has first been described in rats (Dominguez and Thomas, 2008) and has also been developed in the mouse (Beierle et al., 2004; Zeng et al., 2012). Pups are housed in a plastic cub with corncob bedding in an environment at 34-37°c. They are fed using artificial milk distributed through a cannula (Messer et al., 1969). Maternal touch toward the pups is somehow mimicked using a paintbrush. The amount of sensory stimulation can hence be manipulated to study the consequences on brain development. Most of the time, 2 different AR protocols are used, one with small tactile stimulation called *AR min* and one high tactile stimulation called *AR max*. On most studies *AR min* pups have long term alterations on the function of brain functions, whereas mimicking maternal touch by paintbrush stimulation often reduces or supresses the detrimental effects of AR.

AR also seems to affect the sensory system, since it changes the properties of electrophysiological recording of auditory event-related potential, which is observed by a delayed N1 component (Kaneko et al., 1996). In the peripheral level, AR alters the electrophysiological (decrease in the amplitude of the compound action potential induced by electrical stimulation) and histological properties (lower thickness of myelin sheet and of axon diameter) of the sensory sural nerve (Segura et al., 2014). It has to be noted here that some of these alterations are also transmitted to the offsprings of AR mothers (Gonzalez et al., 2001), which once again highlight the possible role of epigenetic mechanisms. Only a few studies investigated the effects of AR on pain responses. However, a study by De Medeiros aimed to investigate if neonatal pain induced in rat pups reared with or without (AR) mother has the same effect at adulthood (de Medeiros et al., 2009). As a neonatal pain experience, they used repeated injection of formalin during the first 2 post-natal weeks. They showed that the pain response to early injection of formalin did not differ in pups between the 2 AR conditions, and that maternal presence (Control group) could decrease paw inflammation in comparison with the 2 AR groups. At adulthood, the AR animals display higher pain sensitivity both at baseline, as measured with the plantar test, and after formalin compared to CTRL reared animals. Concerning visceral pain nothing is known about a potential risk factor after AR. However, a study demonstrated that gut microbiota is modified by AR in the mouse, in a model using an intragastric cannula for milk delivery [695]. This would require further investigations in other AR models, to see if this observation is due to the mode of milk delivery, a change in milk composition or linked to other parameters.

*(iv) Limited bedding*

Neonatal limited bedding (LB) is a model of ELS where the mother and the pups are not spatially disturbed, but where only the minimum of nesting material is provided to the mother for one week (Molet et al., 2014). In this model, the limited access to nesting material induces strong anxiety in the dam, and strongly affects maternal behaviour, described as poor and fragmented (Ivy et al., 2008).

Concerning pain responses, LB leads to an increased nociceptive sensitivity at adulthood, as presented in table 5. LB during P2 to P9 induces an increase in visceromotor response to CRD in the adult rat, in both males and females (Guo et al., 2015; Holschneider et al., 2016). This has been associated with a differential brain activation in areas implicated in pain regulation (somatosensory, insular, cingulate and prefrontal cortices, locus coeruleus/lateral parabrachial nuclei, periaqueductal gray, sensory thalamus, amygdala, hypothalamus), and to an increase in functional connections between the pain areas (Holschneider et al., 2016). Another study described a mechanical hypersensitivity measured with Von Frey filament in the hindpaw, and a visceral hyperalgesia in the adult male rat following LB, but not in cycling females (Prusator and Greenwood-Van Meerveld, 2015a). A muscular hypersensitivity has also been described, as mechanical nociceptive thresholds (compression force induced on the gastrocnemius muscle to induce a withdrawal of the leg) was 22% lower in LB rats (Green et al., 2011). This muscle hyperalgesia was reversed by an intrathecal injection with antisense against IL-6 receptor subunit gp130, (Alvarez et al., 2013), suggesting a strong role of inflammatory mediators. The study by Green and colleagues also highlights an increased sensibility to inflammatory factors, as the animals are more sensitive to a muscular injection of prostaglandin E2. It also induces a cutaneous hyperalgesia that is also longer in the NLB group (Green et al., 2011).

| Model of ELS | Timing | Animal | Age at testing | Nociceptive test | Observations | Sex- differences | Reference |
|---|---|---|---|---|---|---|---|
| **Limited bedding** | P2-P9 | Rats Sprague Dawley | adult | VMR to CRD | ↗ visceral sensitivity | none | (Holschneider et al., 2016) |
| | | | | Von frey and VMR to CRD | ↗ visceral and somatic response | effect on ♂ only | (Prusator and Greenwood-Van Meerveld, 2015b) |
| | | | | Randall Sellito and muscle nociceptive threshold | ↘ muscular threshold but not cutaneous | Study in ♂ only | (Green et al., 2011; Alvarez et al., 2013) |
| | | Rats Long-Evans | Adult | Von frey and VMR to CRD | ↘ mechanical threshold and ↗ VMR | Hypersensitivity in males ♂. Oestrous cycle doesn't change results in ♀ | (Prusator and Greenwood-Van Meerveld, 2016b) |
| | | Rats wistar | adult | VMR to CRD | ↗VMR | Higher VMR in CTRL ♀ vs CTRL ♂ for 60mmHg | (Guo et al., 2015) |

*Table 5 : Consequences of the limited bedding model of ELS on pain responses.*

CRD = colorectal distension, VMR = viscero motor responses.

### *(v) Early weaning*

The model of early weaning (EW) alone is less used in animal studies. It mimics a precocious stop in lactation and a sudden loss of mother-pups interaction. Weaning usually occurs after the hyporesponsive period, when rat pups can control their body temperature and eat without being dependent on maternal milk. Most of the time, the model consists of weaning pups from the mother

by separating the mother before the supposed weaning date (around P16). Fraga and colleagues also developed a model of EW where the mother is allowed to stay with the pups, but is wrapped with bandages or injected with a prolactin inhibitor to block access to milk during the last 3 days of lactation (Fraga et al., 2014). As in other ELS models, EW has been described to induce many behavioural alterations. It decreases maternal care and disturbs the function of the HPA axis and of many brain centers as reviewed by Kikusui and Mori (Kikusui and Mori, 2009) .

Only a very few studies have focused on the consequences of EW on later pain responses. However, Kikusui and colleagues recently investigated the effects of early weaning in mice on empathy and on the social contagion of pain (Kikusui et al., 2016). The idea was that mice have different pain behaviours in presence of other familiar or non-familiar mice and that this socio-emotional behaviour is affected by early mother-infant interactions. They used normally weaned and early weaned mice which were subjected to visceral pain, modeled by intraperitoneal acetic acid injections. The animals were housed either alone, or with a partner which was naïve or also injected with acetic acid. They recorded the behavioural response to the injection (stretching and writhing) and tried to determine if the behaviour was changed by the presence of the partner in the two rearing groups. The presence of a naïve partner decreased pain behaviour in the injected mouse, but only in the normally weaned group. The presence of a naïve partner seems to have beneficial effect on pain behaviour in control mice, which can be referred to as “social buffering”, where the presence of a partner has calming effects, decreasing stress and anxiety. Moreover, in their study, when the 2 mice were injected with acetic acid, they displayed increased pain behaviour. This was also observed in the early weaned group but only for stretching behaviour. In control mice, there is a sort of “contagion” of pain, since the animals display increased pain behaviour when the 2 animals are injected, which is partially lost in EW animals. In conclusion, it indicates that EW may change the emotional component of pain, and especially the emotional transmission of pain, being the ability to be affected by a partner’s pain. In other word EW changes the ability to feel empathy for the pain of others. This social buffering is lost in EW animals, which, according to the authors, could be attributed to the early disrupted mother-infant interaction and an alteration of social brain circuits, especially the oxytocinergic system which is involved in social behaviour and empathy. As I presented earlier in this introduction, OT can modulate many aspect of nociceptive responses and, if affected, could induce a susceptibility to chronic pain conditions for example. This hypothesis has hence been investigated in ARTICLE 2, with the MS model of ELS.

# H) PhD objectives and working hypothesis

My PhD work has been divided in 2 major studies, both focusing on the mechanisms of action of OT in the modulation of pain. The first study aimed to go deeper in the understanding of hypothalamic circuits allowing a coordinated analgesic action of OT at the central and at the peripheral level. The second study aimed to determine if alterations occurring in the early post-natal period could affect the development and function of this analgesic system.

## I) Connectivity between OT hypothalamic nuclei and between the two OT subpopulation of neurons and its functional relevance in pain

The first part of my PhD work was born from a collaboration between our team and the laboratory of Prof. Valery Grinevich, from the DKFZ in Heidelberg (Germany). Their research group lead many studies focusing on OT function within the CNS.

As presented in the introduction, OT can produce significant analgesic effects, by acting both in the periphery through blood release, and by acting on CNS targets including the spinal cord. Up to now, these two components of OT analgesia have been studied separately and it is unknown if these two analgesic actions are coordinated. They seem to rely on different subpopulations of OT neurons, the parvocellular and magnocellular. But the anatomical and functional connections between the two populations, if existing, have not been unravelled yet.

In this first study, we aimed to determine if anatomical connections were present between the two populations of OT neurons and the two OT nuclei and if such connections might be functionally relevant in the context of pain modulation. We used a combination of optogenetic tools, paired with in vivo electrophysiological recordings and pain behaviour assessment to decipher the functional role of this circuit. This work is presented in ARTICLE 1, published in 2016 in the journal *Neuron*.

## II) MS consequences on pain responses and on the function of OT analgesia

On top of its function on pain modulation, OT has numerous functions around birth. In particular, it is implicated in the early interactions between mother and child and in the establishment and maintenance of several aspects of maternal behaviour. The early disruption of maternal behaviour has showed to be detrimental for brain function and behaviours, inducing high level of stress and maladaptive social behaviour. Importantly, these are functions that are controlled by the OT system. However, the consequences of early life negative events on the OT system itself have not been investigated, specifically in the context of pain modulation. Also, the developmental state of OT system after birth makes it particularly vulnerable to early changes in the newborn environment.

The aim of this study was hence to determine if early life stress, using the MS model, could have long-term detrimental effects on OT analgesia, and lead to altered nociceptive sensitivities at adulthood. We used a combination of in vivo electrophysiology and pain behaviour assessment to analyse MS consequences at adulthood. This work is presented in ARTICLE 2, submitted to *Pain* in December 2017. After a few modifications and complementary experiments, the revised version will be resubmitted in April 2018.

# I) Article 1

## I) Overview

**Introduction.** OT is synthesized by two distinct neuronal populations within HT nuclei: magnocellular and parvocellular neurons. If magnocellular neurons have been widely studied, little is known about the parvocellular population. Projections from these neurons to the brain stem and spinal cord have been well described. Apart from a role in nociceptive processing they are likely to play a role in the modulation of autonomic functions. The magnocellular population of OT neurons is responsible for the release of OT in the blood stream, possibly leading to a peripheral anti-nociceptive effect via an action on DRG neurons. However, it is unclear if and how these two populations of neurons act together and coordinate within the same nuclei and between PVN and SON. Here, we characterized the connections between parvocellular and magnocellular neurons in the rat hypothalamus and tested their role in the modulation of nociception.

**Main results.** An anatomical study was lead using viral constructs to express fluorescent molecule in PVN OT neurons. We found that PVN neurons projects to both ipsi- and contra-lateral SON, where they establish synaptic contacts with magnocellular neurons identified as oxytocinergic. The in vivo electrophysiological recording of SON neurons demonstrated that a specific optogenetic activation of PVN OT axons in the SON induces firing in SON OT neurons. This stimulation was associated with an increase in blood OT level. The identity of PVN neurons projecting to SON was further investigated by anatomical study and by studying their electrophysiological properties and capacity to reuptake systemically injected Fluorogold. It corresponds to a small population (≈30 neurons) of OT neurons, of the parvocellular type. We also showed that axon collaterals from these parvocellular neurons make synaptic contact with “wide dynamic range” (WDR) neurons, expressing OTR and the substance P receptor NK-1, in the deep layer of the spinal cord.

To determine if this sub-population of PVN parvOT neurons projecting to SON magnOT neurons and to the SC could modulate pain response, we used in vivo electrophysiological recording of SC neurons and performed a behavioural study on an inflammatory pain model. The stimulation of these parvOT neurons induced a fast inhibition of C-fiber-induced discharge of WDR neurons, when the axons are stimulated directly on the spinal cord level. A stimulation of axons within the SON induced a slower but longer-lasting decrease in WDR activity. At the behavioural level, activating this sub-population allowed to induce a significant OTR-dependant analgesia in an inflammatory pain model. Using an inhibitory DREADD construct, we also showed that the inhibition of these neurons decreased pain threshold, hence increasing CFA-induced hyperalgesia, showing that this population is endogenously activated in rats in inflammatory pain conditions.

**Author contribution.** This work was done in collaboration with numerous researchers. For my part, I performed the following experiments:

- Extracellular in vivo recordings of spinal cord neurons during stimulation of OT projections in the SON and in the spinal cord.
- Behavioural experiments on inflammatory CFA model with optogenetic stimulation and DREADD inhibition

Behavioural experiments with optogenetic stimulation and DREADD inhibition on the neuropathic cuff model.

Article

Neuron

# A New Population of Parvocellular Oxytocin Neurons Controlling Magnocellular Neuron Activity and Inflammatory Pain Processing

## Highlights

- Thirty parvocellular oxytocin neurons (ParvOT) alleviate acute pain
- ParvOT project to WDR sensory neurons in spinal cord (SC)
- ParvOT activate OT release from magnocellular OT neurons (magnOT)
- Dual pain suppression by peripheral magnOT and central SC OT

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## In Brief

Eliava, Melchior, Knobloch-Bollmann, Wahis et al. demonstrate that thirty specialized oxytocin neurons in the rat hypothalamus coordinate activity of oxytocin neurons and deep dorsal horn spinal processing, as revealed by the repression of nociceptive messages and the promotion of analgesia.

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Article

# A New Population of Parvocellular Oxytocin Neurons Controlling Magnocellular Neuron Activity and Inflammatory Pain Processing

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## SUMMARY

**Oxytocin (OT) is a neuropeptide elaborated by the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei. Magnocellular OT neurons of these nuclei innervate numerous forebrain regions and release OT into the blood from the posterior pituitary. The PVN also harbors parvocellular OT cells that project to the brainstem and spinal cord, but their function has not been directly assessed. Here, we identified a subset of approximately 30 parvocellular OT neurons, with collateral projections onto magnocellular OT neurons and neurons of deep layers of the spinal cord. Evoked OT release from these OT neurons suppresses nociception and promotes analgesia in an animal model of inflammatory pain. Our findings identify a new population of OT neurons that modulates nociception in a two tier process: (1) directly by release of OT from axons onto sensory spinal cord neurons and inhibiting their activity and (2) indirectly by stimulating OT release from SON neurons into the periphery.**

## INTRODUCTION

Oxytocin (OT), a neuropeptide that plays an important role in sociability, is produced in the brain exclusively in the hypothalamic paraventricular (PVN), supraoptic (SON), and intermediate accessory nuclei (Swanson and Sawchenko, 1983). OT neurons can be classified in magnocellular OT (magnOT) and parvocellular OT (parvOT) neurons, which are distinct in size and shape, subnuclear location, the amount of OT production, and involvement in distinct circuitries and functions (Armstrong et al., 1980; Swanson and Kuypers, 1980; Sofroniew, 1983; Swanson and Sawchenko, 1983).

According to a long-held dogma, magnOT neurons provide systemic OT supply by release into the blood via the posterior pituitary (Scharrer, 1928; Scharrer and Scharrer, 1940, Bargmann and Scharrer, 1951). Simultaneously, magnOT neurons innervate the forebrain, including the nucleus accumbens (Ross et al., 2009; Knobloch et al., 2012; Dölen et al., 2013) and the central nucleus of the amygdala (Knobloch et al., 2012). The forebrain fibers, as exemplarily studied in the central amygdala, allow for focal release and discrete, modulatory action of OT (Knobloch et al., 2012). These characteristics might account for the distinct impact of OT on numerous types of brain-region specific behaviors (Lee et al., 2010).

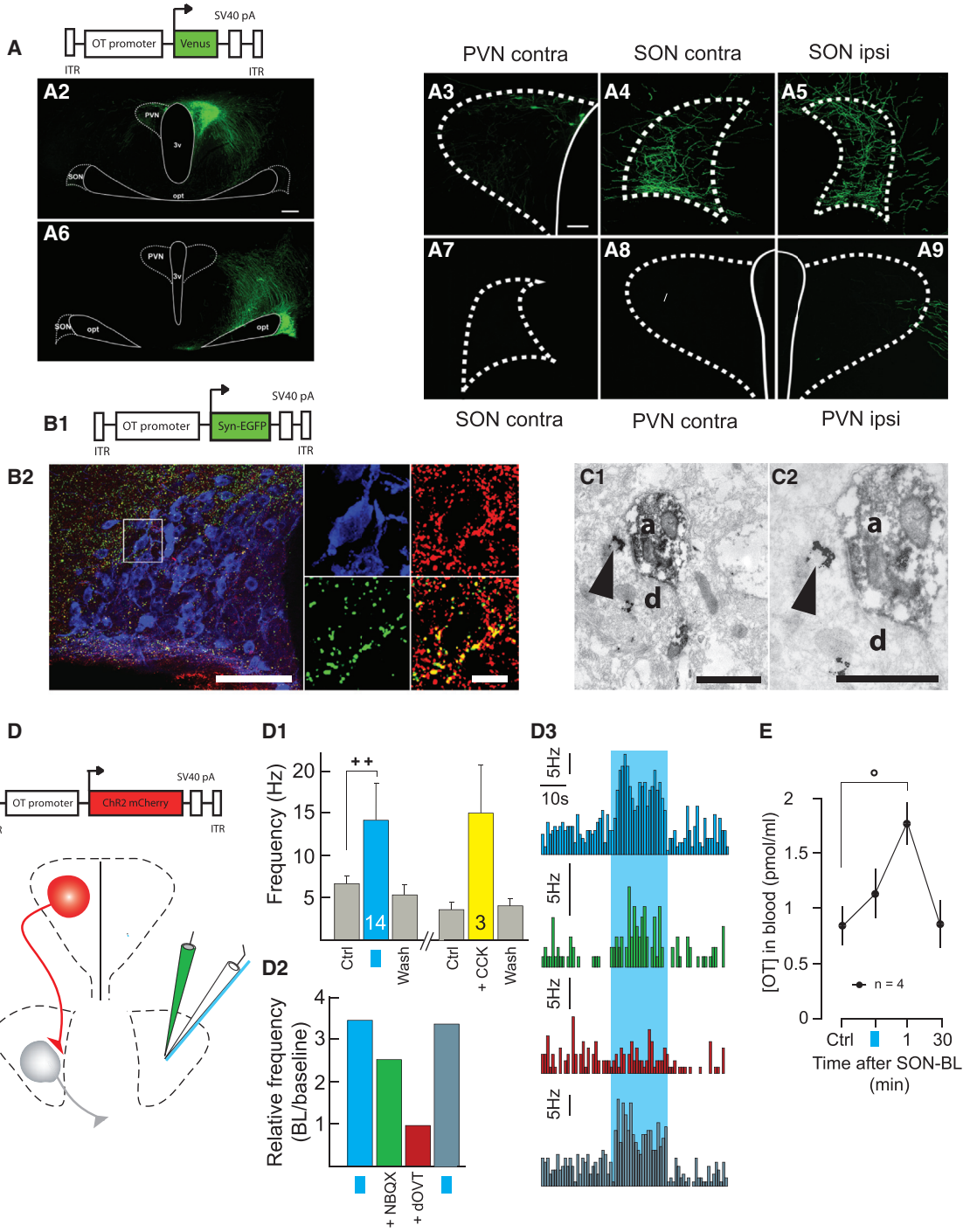


**Figure 1. Anatomical and Functional Connectivity between OT Neurons of the PVN and SON**

(A) OT projections from PVN to SON.

(A1) Scheme of the viral vector used to infect PVN neurons.

(A2–A9) PVN OT neurons infected with cell-type specific viral vector project Venus-positive axons to contralateral PVN (A3) and to contra- and ipsilateral SON (A4 and A5). OT neurons of the SON (A6) do not project Venus axons to contralateral SON (A7) or PVN (A8) and only marginally enter the external border of ipsilateral PVN (A9). The scale bars represent 200 μm (left) and 50 μm (right).

(B) OT axon terminals contain vGluT2.

(B1) Scheme of viral vector.

(B2) GFP-positive terminals in the area of the SON (left). In the magnified inset (right), the OT neuron (blue) is surrounded by GFP terminals, which also contain vGlut2 (red). Both of the immunosignals overlap (yellow) in virtually all of the terminals. The scale bars represent 100 μm (left) and 25 μm (right).

*(legend continued on next page)*

In contrast to magnOT neurons, parvOT neurons project to distinct brainstem nuclei and different regions of the spinal cord (SC) (Swanson and Sawchenko, 1983; Sawchenko and Swanson, 1982). Based on the location of parvOT axons and the effects of externally applied OT, it has been proposed that OT from parvOT axonal terminals contributes to modulation of cardiovascular functions, breathing, feeding behavior, and nociception (Mack et al., 2002; Petersson, 2002; Condés-Lara et al., 2003; Atasoy et al., 2012). However, no selective and specific genetic access to parvOT neurons has been available and, hence, there was no evidence for the capacity of parvOT axons to release endogenous OT and to selectively modulate the above-mentioned functions. Moreover, it has remained unknown how parvOT neurons are incorporated into the entire OT system and functionally interact with magnOT neurons.

Based on recent reports that OT-modulated nociception and pain response comprise a peripheral (Juif and Poisbeau, 2013) and a central component (Juif et al., 2013; González-Hernández et al., 2014), it is tempting to propose that these components are dependent on different OT cell types. The central component results from parvOT innervation of SC targets (Swanson and McKellar, 1979), whereas peripherally acting OT, in contrast, is provided to the blood stream by magnOT neurons and presumably targets C-type fibers in the dorsal root ganglion (DRG; Juif and Poisbeau, 2013). We therefore hypothesized that the complementary, analgesic OT action—at central and peripheral levels—depends on the communication between magnOT and parvOT neurons residing in spatially segregated OT nuclei. Our present results reveal that the modulation of pain signals by OT is triggered by only a handful of parvOT neurons that innervate simultaneously "sensory wide dynamic range" (WDR) neurons in the deep laminae of the SC, expressing neurokinin-1 (NK1R) and OT receptors (OTR), and SON neurons that secrete OT in the periphery. We show that these separate innervations underlie a two-tier modulation of pain by OT reaching the SC through fast, direct neuronal projections and a slower, indirect peripheral pathway.

## RESULTS

### Intrahypothalamic Axonal Trees of the OT System

To examine the intrahypothalamic OT system, we used recombinant adeno-associated virus (rAAV), allowing cell-type specific fluorescent labeling of OT neurons with 98%–100% cell-type specificity, as reported in Knobloch et al. (2012). To compare the OT system with the vasopressin (VP) system, we used AAV carrying different fluorescent markers driven by an evolutionarily conserved VP promoter (for specificity, see Table S1; Figure S1A).

After injection of rAAV expressing Venus, under the control of an OT promoter (Figure 1A), we observed that OT neurons of the PVN give rise to fibers connecting to the ipsi- and even contralateral SON and form a pronounced plexus (Figures 1A4 and 1A5). Interconnections within the intrahypothalamic VP system, in contrast, were absent (Figure S1C). The OT plexus might stem from PVN OT neurons projecting above the third ventricle to the contralateral PVN (Figure 1A3). OT connectivity from the PVN to SON was present in females and males (Figure S2A). The connection between the OT nuclei was one-way: the SON-arising OT fibers reached only marginally the ipsi- (Figure 1A9) and never the contralateral SON (Figure 1A7) or PVN (Figure 1A8).

The OT PVN-SON connection was reconstructed using light sheet microscopy. As presented in Figure S2B, descending fibers from the PVN mainly project rostro-ventrally, turn horizontally at the level of the SON, and enter the SON from the rostral position, to run caudally along the whole extent of the nucleus.

### PVN OT Neurons Innervate the SON and Control MagnOT Neuron Activity to Induce OT Release into Blood Circulation

At the light microscopic level, Venus-labeled OT axons that arose from the PVN formed tight appositions to dendrites and somata of magnOT SON neurons resembling synaptic contacts. To assess if synapses were present, we injected the PVN with rAAV that expresses the synaptic marker synaptophysin fused to the green fluorescent marker EGFP in PVN OT neurons (Figure 1B1). GFP-positive puncta were found in the SON. The vast majority of terminals with GFP signal overlapped with VGluT2 signal (red, Figure 1B2). GFP/VGluT2 terminals engulfed OT cell bodies and dendrites (blue, Figure 1B2). We found that EGFP signals overlapped with VGluT2 in 92.6% ± 8.3% of all terminals (Figure 1B2). These light microscopic observations suggested the presence of synaptic contacts, which we further confirmed at the electron microscopic level: EGFP-positive OT axons from the PVN (EGFP: greyish filling) formed asymmetric (presumably glutamatergic) synapses on OT dendrites of the SON (OT: dark aggregate in pre- and postsynaptic elements; Figures 1C1 and 1C2).

Based on the anatomical evidence for OT connections between PVN and SON neurons, we aimed for a functional characterization of these connections. We expressed the blue-light

(C–C2) Electron microscopy OT axon terminals (Venus visualized as diaminobenzidine [DAB] endproduct, OT, as a silver-gold-intensified DAB) form asymmetric synapses on OT-ir dendrite within the SON. The OT-immunoreactivity (clusters of silver particles, arrows) are shown in the presynaptic axon (a) terminal and postsynaptic dendrite (d) at lower (C1) and higher magnifications (C2). The scale bar represents 0.5 μm.

(D) Scheme of the viral vector and setup of in vivo electrophysiological recordings (white pipette) in SON, together with SON-BL stimulation (blue fiber) and drug infusion (green pipette).

(D1–D3) Functional connection between PVN and SON OT neurons. (D1) Average spike frequencies of SON OT neurons before (Ctrl), after either SON-BL (n = 14, blue bar) or systemic injection of CCK (n = 3, yellow bar), and after washout effect (Wash). (D2) Relative frequency increase induced by SON-BL in control condition (blue bar), after infusion of NBQX (1 μM, 0.5 μl; green bar), after additional infusion of dOVT (1 μM, 0.5 μl; red bar), and after 30 min washout of the drugs (dark blue bar). (D3) Histograms of the frequency rates recorded under conditions described in (D2).

(E) Effect of unilateral SON-BL effect on OT blood concentration at the end of SON-BL, 1 min and 30 min after (n = 4). All results are expressed as average ± SEM. The statistical significances: ++ $p < 0.01$ and Wilcoxon's test. (°$p < 0.05$, Friedman's test followed by Dunn post hoc test) The blue squares represent 20 s BL stimulation at 30 Hz with 10 ms pulses of BL stimulation.

(BL)-sensitive ChR2 protein (Nagel et al., 2003) fused to mCherry in PVN OT neurons (for construct validation, see Knobloch et al., 2012). In vivo extracellular recordings in anaesthetized animals revealed the expression of functional ChR2 in the PVN, as evident from BL-induced (PVN-BL, 20 s at 30 Hz with 10 ms pulses), reversible, and reproducible increases of spike frequencies in these PVN neurons (on average the frequency increased from 4.1 ± 0.7 to 7.8 ± 0.7 Hz; data not shown).

We then further tested in vivo whether exposure to BL of PVN-OT axons in the SON (SON-BL, scheme in Figure 1D) could also activate, ipsilaterally, SON neurons. SON-BL exposure evoked a reversible increase in spike frequencies of SON neurons, from 6.7 ± 1.5 to 14.1 ± 2.7 Hz, confirming that BL stimulation of parvOT PVN axon terminals could excite SON neurons (Figure 1D1). To verify that OT was the main transmitter involved, we recorded the response of a single neuron to the SON-BL in the absence of any drug, or after sequential infusion of AMPA and OTR antagonists (respectively, NBQX and dOVT) into the SON, and after their washout (Figures 1D2 and 1D3). Interestingly, while NBQX decreased the baseline frequency of SON neurons, SON-BL paired to NBQX application still efficiently increased the relative frequency of discharge of the recorded neuron. Subsequent dOVT infusion totally blocked the SON-BL response, with full recovery 30 min after washout (Figures 1D2 and 1D3). These results are in accordance with our previous observations in the central amygdala (Knobloch et al., 2012).

We aimed at providing functional evidence for the OT nature of the SON neurons that were contacted by the PVN. To this purpose, we first of all injected into the blood circulation cholecystokinin (CCK) a hormone inducing the activation of OT neurons (Verbalis et al., 1986). CCK induced a prominent increase in spike frequencies of SON-BL responding neurons from 3.6 ± 0.8 to 15.0 ± 5.9 Hz (Figure 1D1), establishing an indirect argument of the OT identity of the in vivo recorded SON neuron. Second, as magnOT neurons are known to release OT in the blood, we performed a time-dependent measurement of OT concentrations in plasma by mass-spectrometry after SON-BL. This revealed a significant increase of OT plasma concentrations at 60 s after SON-BL (from 0.84 ± 0.17 to 1.76 ± 0.22 pmol/ml; Figure 1E). Taken together, these findings provide evidence for an OT identity of the SON neurons that are activated by axonal terminals originating from OT neurons in the PVN.

## OT Neurons Projecting to SON MagnOT Neurons Are ParvOT Neurons Displaying Distinct Anatomical and Electrophysiological Characteristics

To identify PVN neurons projecting to the SON, we injected into the SON retrogradely transported and monosynaptically transmitted canine adenovirus 2 (CAV2). After counting of sections containing the entire PVN, we identified in total a very small population of GFP/OT-positive neurons residing bilaterally (Table S2; 31.5 ± 8.5 neurons). CAV spread occurs within 200 μm of the injection site (Schwarz et al., 2015), making unlikely the diffusion of the virus from the SON to PVN (the distance between these two nuclei is about 1.5 mm; see Paxinos and Watson, 1998).

To characterize the magno- versus parvocellular nature of back-labeled PVN cells, we combined CAV2 with systemic administration of Fluorogold (Figure S3A). Fluorogold, when injected intraperitoneal (i.p.), is taken up by neurons projecting beyond the blood brain barrier, for example, by magnOT neurons, thus allowing to distinguish them from the parvOT (Fluorogold-negative) neurons (Luther et al., 2002; Table S2). Notably, all magnOT neurons of the SON were Fluorogold-positive (data not shown). After neuron counting in sections containing the entire PVN, we established that the vast majority of the 31.5 ± 8.5 GFP/OT-positive neurons (90%) did not contain Fluorogold (Table S2). In addition to the detection of back-labeled GFP-positive neurons in the PVN, we observed GFP neurons in other structures typically known to innervate the SON, further confirming the specificity of our retrograde labeling (Miselis, 1981; Cunningham and Sawchenko, 1988; Figure S3B).

To characterize the parvOT neurons projecting to the SON, we next injected into the SON CAV2 expressing Cre recombinase and into the PVN rAAV carrying a double-floxed inverted open reading frame (ORF) (DIO) of GFP under the control of the OT promoter (Figure 2A1). By this combination, we limited GFP expression exclusively to SON-projecting parvOT neurons. In line with previous results, this revealed a unique position of back-labeled GFP neurons in the dorso-caudal PVN (Figures 2A2, 2A3, S2B, and S2C). Individual GFP neurons have bipolar spindle-like morphology (Figure 2A4) distinct from neighboring magnOT neurons (Figure 2A5). The number of back-labeled PVN GFP (exclusively OT) neurons was comparable (33.4 ± 9.1), with the estimation of non-selectively labeled PVN neurons identified by costaining with OT antibodies.

OT neurons similar in morphology and location were obtained in our initial study (data not shown) with the application of latex retrobeads (Katz and Iarovici, 1990) in the SON, which, however, labeled only few cells in the PVN (and other structures innervating the SON; Figure S3), precluding quantitative analysis.

We determined the electrophysiological characteristics of fluorescently labeled neurons in the PVN to assess their parvocellular nature. We conducted whole-cell patch clamp recordings in slices (Figure 2B) in current clamp applying a protocol of depolarizing current injections (Figure 2B1). This was aimed to determine the presence of a transient outward rectification, which is typically found in magnOT, but not in parvOT neurons (Luther et al., 2002). We recorded in a total of seven animals 11 fluorescent putative parvOT, and found that none, as expected, exhibited a hyperpolarizing notch. Conversely, all of the 13 non-fluorescent neurons from the same region (putative magnOT) showed the typical transient outward rectifying current, as known as (aka) ''notch''. Quantification of these differences was made by analyzing the time to spike (spike delay) and rise slope, which was the slope measured between beginning of the depolarization and the peak time of the first action potential. Both of these parameters showed highly significant differences between the two groups of neurons (Table S4; Figure S4). Differences in the spike frequency also showed a tendency, though with less significance than previously reported (Luther et al., 2000). The electrophysiological responses were in agreement with the morphology of the cells. Neurons classified electrophysiologically as parvOT had a small soma and a more elongated shape, while the ones classified as magnOT had a big soma and were more rounded (Figure 2B2).

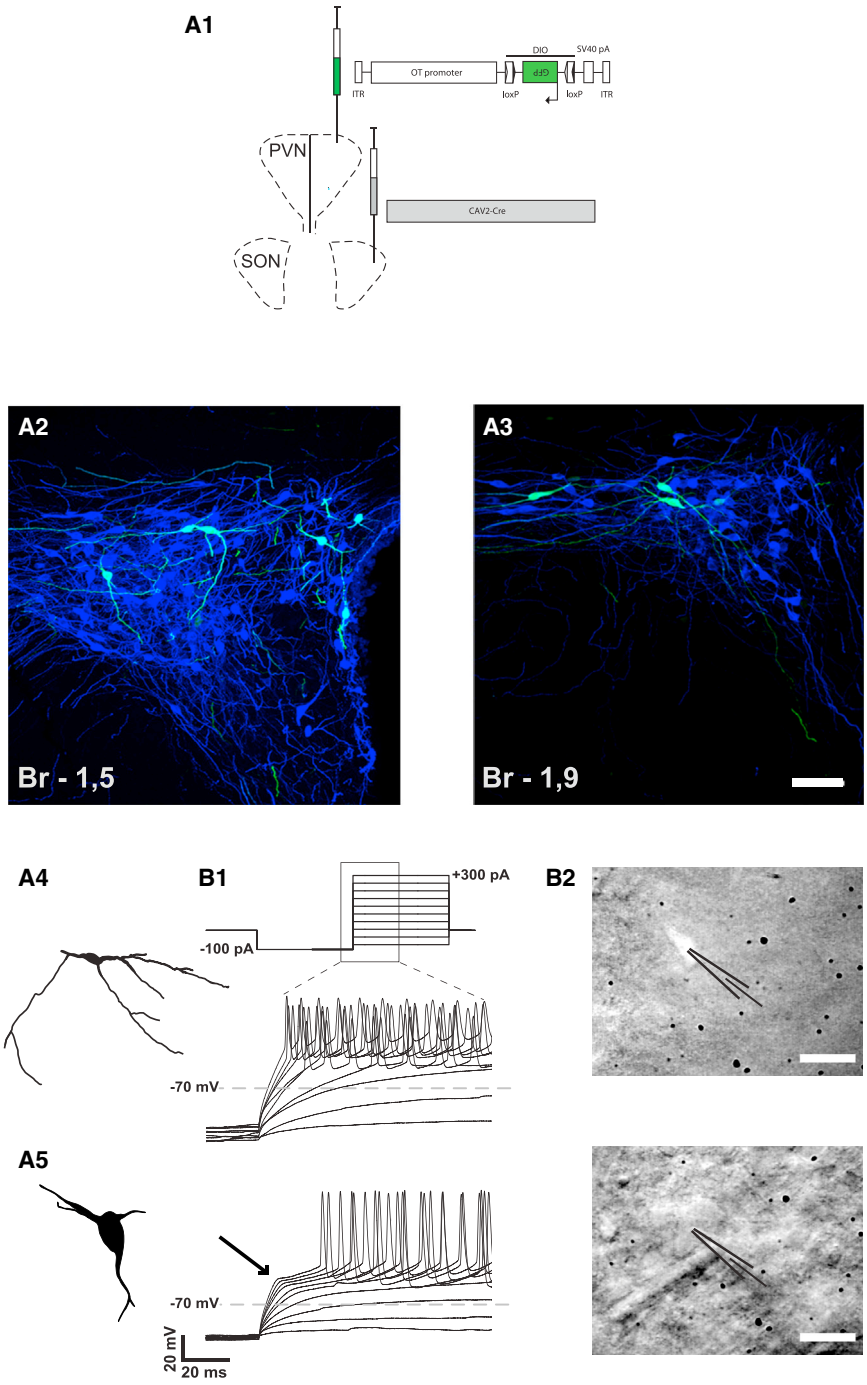


**Figure 2. Anatomical and Electrophysiological Characteristics of PVN OT Neurons Projecting to the SON**

(A) Identification of a subset of OT neurons projecting from PVN to SON.

(A1) Scheme showing the injection of viruses in the SON and PVN.

(A2 and A3) Defined subset of back-labeled OT neurons (green) in dorso-caudal PVN displays consistent morphology: small oval somas (12 to 20 μm in diameter) with predominantly longer horizontal axes. The scale bar represents 50 μm in (A2) and 50 μm in (A3).

(A4 and A5) The morphology of these cells is clearly distinct from the typical magnocellular neurons with large cell bodies and less branching processes (A5).

(B) Functional differentiation of this subset of PVN OT neurons.

(B1) Current steps protocol starting from a hyperpolarizing current chosen to reach −100 mV (here 100 pA) followed by progressively more depolarizing current injections (upper trace). The representative changes in membrane potential for the parvOT and magnOT PVN neurons during the part of the current steps as indicated by the zoomed area are shown (lower traces). The ParvOT neurons (middle trace) do not display the transient outward rectification specific for the magnOT neurons (lower trace, arrow).

(B2) Photographs of a GFP-fluorescent parvOT neuron (upper) in the PVN (labeled by injection of CAV2-Cre into the SON and OT-DIO-GFP AAV in the PVN) and in the same area a typical magnOT neuron (lower) as indicated by the patch pipettes. The scale bars represent 20 μm.

## ParvOT Neurons Innervating MagnOT Neurons Also Project Specifically to NK1R/OTR Positive WDR Neurons in the Deep Layers of the SC

The above established exclusive labeling of parvOT neurons and all their processes using a combination of CAV2-Cre with OT cell typed-specific Cre-dependent rAAV (Figures 3A1–3A3) allowed us to follow projections of this OT cell population up to the distal (L5) segments of the SC. After labeling presumably all PVN OT neurons, axons can be visualized in both superficial and deep SC layers (Figure S5A). In contrast, we found that synaptophysin-GFP-filled terminals from parvOT only were loosely and sparsely distributed in superficial laminae, but heavily innervate deep laminae, in close proximity to neurokinin 1 receptor (NK1R)-positive large cells (diameter 30–40 μm; Figure 3A2) identified as sensory WDR neurons (Ritz and Greenspan, 1985). Importantly, in an additional labeling (on separate slices because both OTR and NK1R antibodies had been raised in the same species), we found in this same region cells that expressed OTR (Figure 3A3). The specificity of the antibody was confirmed in transfected HEK cells (Figure S5B1) and brainstem sections of OTR knockout mice (Figure S5B2), in agreement with a previous study using these antibodies in mouse cortex (Marlin et al., 2015). To show the co-localization of NK1R and OTR in the same cells, we performed fluorescent in situ hybridization and found the presence of respective mRNAs in the same neurons of deep layers of the SC (Figures 3B1–3B4). As a next step, we wanted to demonstrate that NK1R-positive neurons of deep SC laminae could

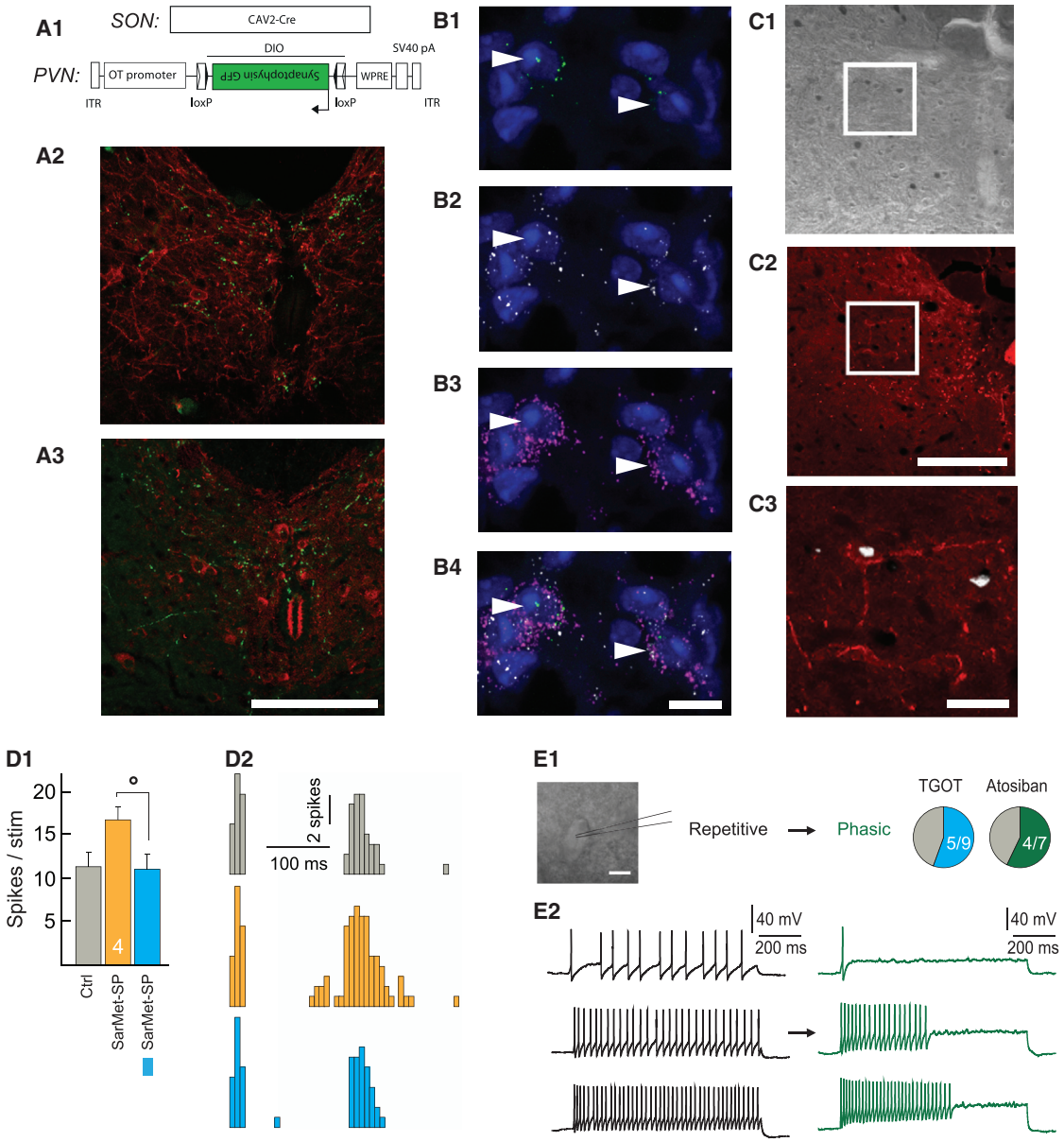


**Figure 3. ParvOT Neurons Project to SC and Innervate NK1R/OTR WDR Neurons in Deep Laminae**

(A) ParvOT projections to the SC.

(A1) Scheme of the viruses injected into the SON and PVN.

(A2) Detection of synaptophysin-GFP containing terminals (green) in close proximity to NK1R-positive neurons (red) in SC deep laminae.

(A3) Synaptophysin-GFP terminals locate close to OTR-positive neurons of deep laminae. The scale bars represents 500 μm in (A2) and 500 μm in (A3).

(B–B4) Colocalization of NK1R and OTR mRNAs in the same neurons of SC deep laminae. Immunofluorescent in situ hybridization revealed the presence of OTR mRNA (green dots; B1 and B4) and NK1R mRNA (white dots; B2 and B4) in the same neurons, which were visualized by detection of vGlut1/2/3 mRNAs in their somas (pink/violet dots; B3 and B4). The nuclei of cells were stained by DAPI. The arrow heads point NK1R/OTR double positive neurons. The scale bars represent 10 μm.

(C–C3) NK1R-positive SC neurons start to express c-Fos after intraplantar injection of capsaicin in the hindpaw. The c-Fos signal (DAB) was detected in deep laminae of SC (C1), where the NK1R (red) were located (C2). The digital overlay of the two signals demonstrates localization of c-Fos in the NK1R-postive neuron (C3). The scale bars represent 500 μm in (C1) and (C2) and 50 μm in (C3).

(D) WDR C-fiber evoked spikes in response to a series of isolated hindpaw stimulations in control condition (Ctrl), during application of the specific agonist of NK1R SarMet-SP (orange), and during SarMet-SP paired with BL (blue).

(D1) Average of C-fiber evoked spikes (n = 5).

(D2) Representative traces.

(E) Discharge profile of putative WDR recorded in current clamp applying a protocol of depolarizing current injections before (black) and after bath application of 1 μM TGOT (blue, n = 9) or 1 μM Atosiban (green, n = 7).

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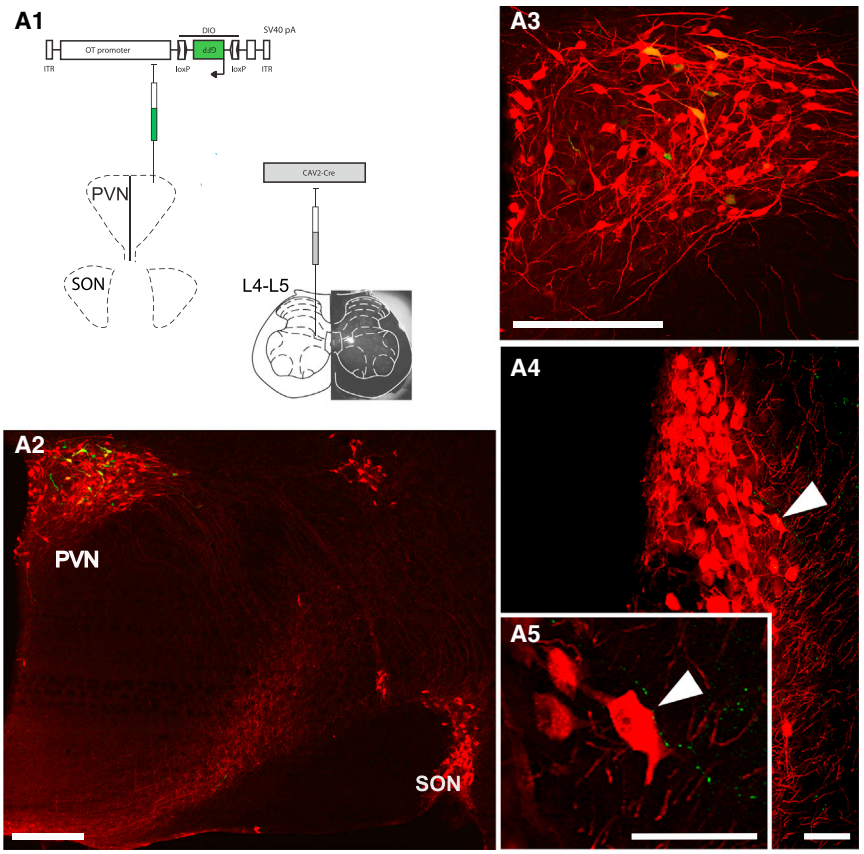


**Figure 4. ParvOT-MagnOT-SC Anatomical Unit**

(A) Scheme of viruses injected into the SC and PVN. The actual SC injection site (fluorescent latex bead accumulation) is shown as an insert underlying SC drawing.

(A2–A5) PVN parvocellular cells back-labeled from SC (green). The GFP-positive cell bodies were found in the caudal portion of the PVN and always colocalized OT (red) (A3, magnification from A2). Fibers, projecting from back-labeled PVN OT neurons to SON (arrow in A4, more caudal to A2) GFP-expressing varicouse axons in close proximity to cell bodies and dendrites of SON magnoOT neurons (high magnification in A5) are shown. The scale bars represent 500 μm in (A2) and (A3) and 75 μm in (A4) and (A5).

be activated by sensory/pain stimulation. Bilateral injection of capsaicin in the hindpaws indeed induced c-Fos expression in large NK1R neurons (Figures 3C1–3C3). Furthermore, back-labeled parvOT neurons were also activated by capsaicin (data not shown).

To show that NK1R WDR neurons are functionally modulated by both NK1R specific agonist (SarMet-SP) and parvOT-deriving OT, we measured in vivo the WDR C-fiber evoked spikes in response to a series of isolated hindpaw stimulations. We found that the C-fiber evoked spikes were increased in the presence of SarMet-SP, as expected (Budai and Larson, 1996). Interestingly, BL-activation of ChR2 expressing parvOT fibers in the SC (SC-BL; schemes in Figures 5A and 5B) upon SarMet-SP significantly reduced the number of C-fiber evoked spikes from 16.8 ± 1.1 to 11.2 ± 1.5 (Figures 3D1 and 3D2). These findings show that release of OT from parvOT axons can effectively inhibit the activity of WDR neurons potentiated by NK1R activation.

Then, we analyzed the inhibitory effect of OT on WDR neuron firing properties. To do so, we performed in vitro whole-cell patch clamp recordings in current clamp applying a protocol of depolarizing current injections (Figure 3E; Breton et al., 2009). Putative WDR neurons, identified by their large cell body and repetitive firing related to stimulation intensities (Figure 3E; Ritz and Greenspan, 1985), were located around the central canal (cc) and in deep layers V–VI. As expected, [Thr4,Gly7]-OT (TGOT) was able to change the firing properties of these neurons, from repetitive to phasic in 5/9 of recorded cells (Figure 3E), similar to what was described in superficial layers (Breton et al., 2009). Importantly, the application of a specific biased agonist for OTR linked to $G_i$ subunit, Atosiban (Busnelli et al., 2012), induced the exact same effects in 4/7 recorded cells (Figure 3E). This experiment demonstrates for the first time on living tissue that OTR can functionally bind a $G_i$ protein, thus elucidating the inhibitory mechanism of OT on the firing properties of WDR neurons.

Finally, to demonstrate that the population of identified parvOT neurons is a single anatomical unit and that the same cells project collaterals to both the SON and SC, we injected a CAV2-Cre virus in deep laminae of L5 and Cre-responder AAV expressing GFP under the OT promoter in the PVN (Figure 4A1). We detected back-labeled cell bodies of GFP/OT neurons in the PVN and their axonal projections in close proximity to somas and dendrites of magnOT neurons of the SON (Figures 4A2–4A5).

## ParvOT Neurons Projecting to MagnOT SON Neurons and NK1R/OTR Positive WDR Neurons in Deep Layers of the SC Control the Central Nociceptive Processing

To test whether the specific population of PVN-OT neurons projecting to both the SON and SC indeed acts on nociceptive input, we recorded SC neuronal responses in vivo during electrical stimulation of their hindpaw receptive field. The coding

(E1) Proportion of putative WDR neurons discharge pattern changed from repetitive to phasic after TGOT or Atosiban bath application.

(E2) Example response of putative WDR neuron to 20 pA (top), 40 pA (middle), and 60 pA (bottom) current injection before (black) and after (green) Atosiban bath application. The scale bar represents in (E1) 30 μm. All results are expressed as average ± SEM. The statistical significance: ° $p < 0.05$, Friedman's test followed by Dunn post hoc test.

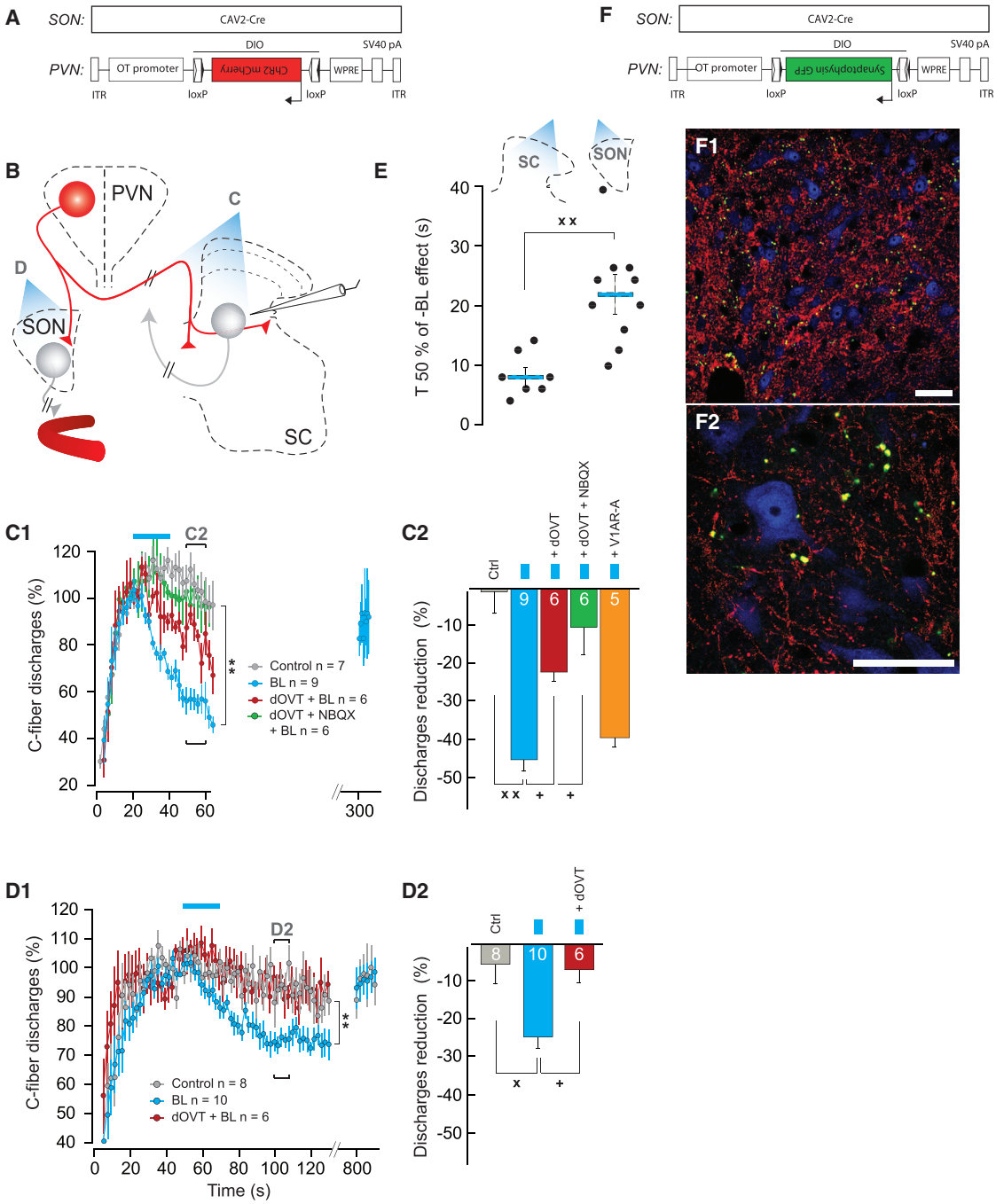


**Figure 5. Stimulation of ParvOT PVN Axons in SON and SC Modulates Responses of WDR Neurons**

(A) Viruses injected into the SON and PVN.

(B) Scheme of the experimental procedures.

(C) Effect of SC-BL on WDR-C discharges.

(C1) Time course of WDR-C in control condition (n = 7), when shining SC-BL alone (n = 9), after local dOVT application (n = 6), or local dOVT + NBQX application (n = 6).

(C2) Average discharge reduction of WDR-C on Ctrl (n = 7), when shining SC-BL alone (n = 9), after local dOVT application (n = 6), local dOVT + NBQX application (n = 6), or local V1AR-A application (n = 5). The statistical significance of drug modulation of the SON-BL effect was assessed by comparing the effect of SON-BL on the same neuron before and after drug injection.

(D) Effect of SON-BL on WDR-discharges.

(D1) Time course of WDR-C in control condition (n = 8), measured 30 s after shining SON-BL (as indicated in C1) alone (n = 10), or after systemic dOVT systemic injection (n = 6).

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properties and short-term potentiation (wind-up; WU) following repetitive receptive field stimulation were calculated from the response of WDR neurons in deep laminae. Recordings include the deep laminae, which integrate convergent peripheral sensory information from fast-conducting (A-type) and slow-conducting (C-type) primary afferent fibers (Figures 5 and S6).

We first tested the inhibitory action of OT released from parvOT-hypothalamo-spinal terminals by shining BL directly onto the dorsal surface of the SC (SC-BL). In this set of experiments, we used the same combination of viruses (CAV2-Cre and rAAV carrying OT promoter-DIO-ChR2-mCherry) to elicit OT release from parvocellular PVN fibers. SC-BL efficiently reduced the WDR discharges from C- (−44.6% ± 3.7%; Figures 5C1 and 5C2) and Aδ- (−36.3% ± 4.5%), but not from Aβ- fibers (−0.3% ± 2.8%; Figure S6G2). The half-efficacy of SC-BL inhibition was 8.3 ± 1.3 s (Figure 5E). The WU returned to control values ∼300 s after SC-BL (Figure 5C1). SC-BL had no effect on superficial layer neuron activity in the same recording condition as for WDR neurons (Figure S6F). The OTR antagonist dOVT, directly applied to the surface of the SC, significantly, but not entirely, reduced Aδ- and C-fiber mediated discharges (Figures 5C1 and 5C2). In contrast, the VP receptor type 1A antagonist applied on SC failed to change the SC-BL inhibition of WU intensity (Figure 5C2), whereas it could efficiently block the effect of exogenously applied AVP (data not shown). Since VGluT2 was detected in synaptophysin-GFP-containing (Figures 5F1 and 5F2; overlap of GFP and vGluT2 signals was found in 89% ± 7.4% GFP terminals) axonal terminals of parvOT neurons near cell bodies of WDR-like neurons (Figure 5F2), we assessed the effect of NBQX in vivo. Coapplication of both dOVT and NBQX entirely blocked the SC-BL effects (Figures 5C1 and 5C2). Thus, stimulation of parvOT axons in SC deep layers leads to a fast, short-lasting decrease in nociceptive processing which is mediated by central OTR, and to a lesser extent by ionotropic Glut receptors.

We then assessed the efficiency of OT release from parvOT neurons onto magnOT SON neurons in modulating nociception (Figures 5B and S6A). Eliciting OT release from parvOT fibers in SON by BL (SON-BL) significantly reduced the WDR discharges evoked by slow-conducting C-type fibers (−24.9% ± 3.1%; Figures 5D1 and 5D2) and fast-conducting fibers Aδ- (−30.0% ± 6.8%), but not by non-nociceptive, fast-conducting Aβ- fibers (−6.4% ± 3.5%; Figure S6G1). The half-efficacy of SON-BL induced inhibition of WU was 22.2 ± 3 s (T 50%; Figure 5E), a value which was significantly higher than the SON-BL effect (Figure 5E). The WU intensity returned to control values only 800 s after SON-BL (Figure 5D1). Moreover, to further confirm that the reduction in WU intensity was related to the elevated level of blood OT (see Figure 1E), we injected the OTR antagonist dOVT intravenously before applying SON-BL. As expected, this abolished the SON-BL inhibition of WDR discharges that were evoked by both Aδ- and C-fibers (Figures 5D1, 5D2, and S6G1). Thus, the central release of OT from parvOT axons targeting magnOT SON neurons leads to a systemic release of OT, which reduces nociceptive processing by WDR neurons. This effect was slow to appear and long-lasting.

In summary, the subpopulation of PVN OT parvOT neurons projecting both to magnOT SON neurons and to NK1R/OTR WDR neurons from deep layers of SC exerts an inhibition of spinal nociceptive processing by fast action on SC neurons and a relatively slower effect on peripheral targets by stimulation of SON neurons and subsequent induction of OT release into blood.

## Activation of ParvOT Neurons Results in Analgesia

In the last part of our work, we analyzed the functional importance of these parvOT neurons in the processing of inflammatory compared to nerve injury-induced neuropathic pain. To this purpose, we measured both the effects of stimulation or inhibition of parvOT neurons on the symptoms of either a peripheral painful inflammatory sensitization triggered by a single unilateral intraplantar injection of complete Freund adjuvant (CFA) or a nerve injury-induced neuropathy induced by the cuffing of the sciatic nerve (Cuff; Pitcher et al., 1999; Figure S7C1). To this purpose, we used rats that expressed either ChR2 or hM4Di (Zhu and Roth, 2014) restricted to parvOT PVN neurons synapsing on magnOT SON neurons (Figure 6A). The efficiency of ChR2-mediated activation and hM4Di-mediated inhibition of OT neurons was assessed respectively by targeting unilaterally the PVN by BL or by i.p. administration of CNO and was confirmed both in vitro (Figures S7A1 and S7A2) and in vivo (Figures S7B1–S7B4).

PVN-BL stimulation significantly, but not entirely, alleviated the CFA-mediated hyperalgesia by raising the threshold of response to both the mechanical (from 56.7 ± 7.6 g to 116.6 ± 16.4 g) and thermal hot stimulation (from 2.8 ± 0.2 to 4.8 ± 0.7 s; Figures 6C1 and 6C2). In contrast, PVN-BL failed to mitigate the mechanical hyperalgesia measured in condition of the Cuff peripheral neuropathy (Figures S7B2 and S7B3). Furthermore, return of the pain symptoms occurred after PVN-BL was fully blocked by i.p. injection of the blood brain barrier (BBB)-permeable OTR antagonist L-368,899 (Figures 6C1 and 6C2).

Conversely, CNO-induced inhibition of parvOT neurons significantly increased the CFA-mediated hyperalgesia by lowering

(D2) Average discharge reduction of WDR-C on Ctrl (n = 8), when shining SON-BL alone (n = 10), or after systemic dOVT injection (n = 6). The statistical significance of dOVT modulation of the SON-BL effect was assessed by comparing the effect of SON-BL on the same neuron before and after dOVT injection (n = 6).
(E) Comparison between individual (black dots) and average T 50% (blue bar) effect of SON-BL (n = 10) and SC-BL (n = 7) on recorded WDR.
(F) Viruses injected into the SON and PVN.
(F1 and F2) Axonal terminals containing synaptophysin-GFP fusion protein in proximity to SC L5 neurons.
(F1) Overview of fiber distribution within SC: VGluT2 (red), synaptophysin-GFP (green), and NeuN (blue).
(F2) A zoom-in shows the green signal (green) largely overlaps with the VGluT2 signal (red) in terminals surrounding cell bodies. The scale bars represent 50 μm in (F1) and (F2). All results are expressed as average ± SEM. The statistical significance: °$p < 0.05$, Friedman with Dunn post hoc test; + $p < 0.05$, ++ $p < 0.01$, and Wilcoxon's test; **xx** $p < 0.01$ and Kruskal and Wallis test; and ** $p < 0.01$ BL versus Control, two-way ANOVA.

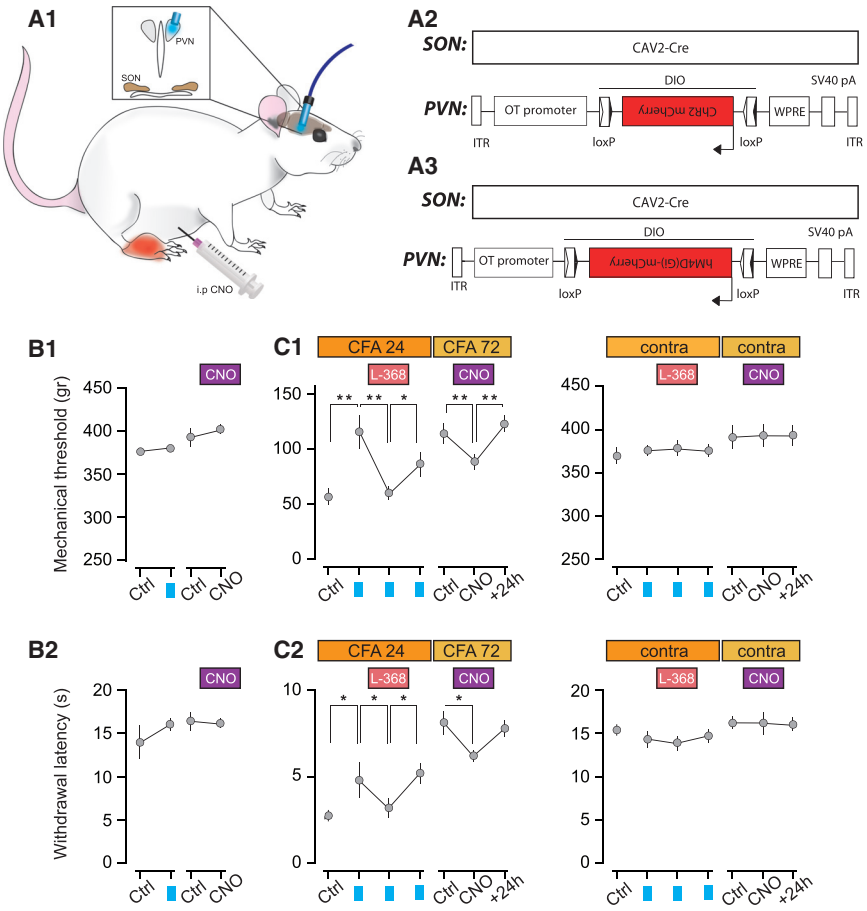


**Figure 6. Activation/Inhibition of ParvOT PVN Neurons Modulates Mechanical Threshold and Thermal Hot Latency in Animals Subjected to Complete Adjuvant Injection**

(A–A3) Scheme of the experimental procedure. The CAV2-Cre was injected in the SON and Cre-responding virus driving either (A2) ChR2 or (A3) hM4Di to achieve the expression of respective proteins in OT neurons of the PVN.

(B–B2) Mechanical thresholds and (B2) thermal hot latencies of naive animals before and after PVN-BL (ChR2, n = 6 and CNO, n = 10).

(C–C2) Mechanical thresholds and (C2) thermal hot latencies of the CFA-injected hindpaw (left graphs) and the contralateral hindpaw (right graphs). The effect of PVN-BL was assessed before, right after i.p. injection of OTR antagonist L-368,899 (1 mg/kg), and after its washout (n = 6). The effect of CNO (3 mg/kg) was measured 1 hr after i.p. injection and its 24 hr washout (n = 10). All results are expressed as average ± SEM. The statistical significance: * $p < 0.05$, ** $p < 0.01$, and one-way ANOVA followed by Tukey's multiple comparison post hoc test.

the threshold of response to both the mechanical (from 115 ± 12.1 g to 88 ± 9.8 g) and thermal hot stimulation (from 8.1 ± 0.9 s to 6.3 ± 0.3 s; Figures 6C1 and 6C2). CNO had no effect in rats with the Cuff (Figures S7C2 and S7C3). These results from gain- and loss-of-function approaches highlight the role of parvOT control of peripheral painful sensitization, supported by our in vivo electrophysiological data.

In the course of our study, we observed that both PVN-BL and CNO failed to modify mechanical and thermal hot sensitivity in the absence of any peripheral sensitization, for example, in the contralateral paw or in naive animals (Figures 6B1, 6B2, 6C1, 6C2, S7C2, and S7C3).

Taken together, these findings provide evidence that 30 parvOT neurons are able to strongly promote analgesia in a pathological condition of inflammatory, but not nerve injury-induced neuropathic pain, presumably by both central (SC-mediated) and peripheral (SON-mediated) mechanisms.

## DISCUSSION

Here, we identified, by a combination of latest state of the art viral-vector based (Grinevich et al., 2016a), anatomical, optogenetic, electrophysiological, and behavioral approaches, a small (n ~30) subpopulation of parvOT neurons in the PVN, which projects to magnOT neurons in the SON and to NK1R or OTR-positive WDR neurons in the deep layers of the SC. Functionally, we demonstrated that this network can inhibit spinal pain processing in a dual manner with two distinct time courses. Thus, nociceptive transmission from Aδ- and C-type primary afferents to WDR neurons is efficiently repressed by OT release from parvOT in the deep layers of the SC and from SON magnOT in the blood. Release in the SC is directly triggered from parvOT-spinal projections and follows a fast mode of action; release in the blood is indirectly triggered from SON magnOT neurons that are activated by parvOT projections and follows a slower time course. The functional role of this subpopulation of parvOT neurons was further confirmed in two rat models of peripheral painful sensitization, indicating that activation of parvOT neurons can decrease mechanical and thermal sensitivities in inflammatory, but not nerve injury-induced neuropathic pain.

### Synaptic Crosstalk between OT Neurons

The question of how OT neurons in different nuclei within the hypothalamus interact with each other is a recurrent theme in past literature, but has not been elucidated experimentally. Belin and colleagues recorded pairs of OT neurons from SON and PVN and proposed an internuclear connection serving as a basis for synchronous firing during lactation (Belin et al., 1984, Belin and Moos, 1986). The hypothesis of an OT-mediated communication was stated already in the early 80's (Silverman et al., 1981), following observations that application of OT (or dOVT) into the third ventricle or in the SON synchronized (respectively, desynchronized) activity of OT neurons in PVN and SON (Freund-Mercier and Richard, 1984; Lambert et al., 1993). Furthermore,

the presence of synapses containing OT-immunoreactivity was demonstrated in the SON (Theodosis, 1985). Although we did not examine internuclear connectivity that underlies synchronized burst firing, our anatomical and functional data demonstrate that PVN-SON interconnectivity plays an important role in inhibiting spinal nociceptive processing and alleviation of inflammatory pain.

In an early study, lesion of the SON did not cause any loss of magnOT neurons in the PVN (Olivecrona, 1957), providing a first indication that parvOT PVN neurons might be at the basis of internuclear connection to the SON. However, as of today, the parvOT neurons in the PVN have remained much less studied than the magnOT neurons, mostly because of technical difficulties, specifically in labeling and modulating the activity of parvOT neurons. To our knowledge, the possibility to study a direct parvOT innervation of the SON by retrograde tracing techniques has seldom been discussed (e.g., Lambert et al., 1993) and any potentially involved parvocellular neurons have never been identified.

At the SON level, Bruni and Perumal (1984) have described an extensive network of small-diameter, beaded, unmyelinated fibers with no particular organizational pattern and of unknown origin that establishes functional axo-somatic and axo-dendritic contacts with magnOT neurons. At 30 years later, we reveal here a monosynaptic connection between parvOT PVN and magnOT SON neurons as respective pre- and postsynaptic components. The detection of a postsynaptic SON component was further confirmed by their stimulation through application of CCK (Renaud et al., 1987) and an increase in peripheral OT levels.

In contrast to the OT system, direct connectivity between VP-ergic neurons in rats has not been convincingly demonstrated and, accordingly, we were unable to find VP/Venus positive fibers descending the PVN in the SON and vice versa.

### ParvOT Neurons Modulate NK1R Positive WDR Neurons

In addition to the control of magnOT activity, this newly described subpopulation of parvOT neurons densely projects exclusively to the deep layers (V, VI, and X) of the SC. Axonal terminals from parvOT were found in close appositions with NK1R positive WDR neurons, some of which are likely OTR-positives. However, we are not excluding projections of these parvOT neurons to non-WDR deep neurons. Nevertheless, their functional and selective inhibition of C- and Aδ- mediated discharges in WDR suggest that nociceptive C-fiber project to deep layers, accordingly with models of dorsal horn circuits that include projections to the lamina V (Cervero and Connell, 1984; Ribeiro-da-Silva and De Koninck, 2008). Functionally, this fits with our results suggesting that OT modulates the excitability of WDR shown as an inhibition of discharges mediated by fibers containing substance P.

### ParvOT Neurons Coordinate Neuroendocrine and Hardwired Inhibitory Pain Control

In accordance with our anatomical data, WDR action potential discharges in response to noxious peripheral stimulation are reduced by optogenetic manipulation of the subpopulation of OT neurons in the PVN and its subsequent stimulation at the level of the SON. This reduction was selective to sensory information transmitted by Aδ- and C-fibers, which are, in their majority, nociceptive-specific.

Regarding peripherally mediated OT effects, it has recently been shown that OTR could be expressed by non-peptidergic C-type sensory neurons in DRG (Moreno-López et al., 2013) and the in vitro application of OT suppresses their activity (Gong et al., 2015). Furthermore, intravenous administration of a selective OTR agonist induces an inhibition of discharges mediated by nociceptive-specific primary afferents (Juif et al., 2013). Our present work provides an additional support for this idea by selectively activating a circuit leading to release of OT to the blood (Figure 7). The effect was fully peripheral, since inhibition of nociceptive messages was completely abolished by the addition of the OTR selective antagonist dOVT in the blood flow.

Identification of a subpopulation of parvOT neurons projecting collaterals to both the SON and deep layers of the SC gave rise to the idea that these neurons may exert both a peripheral and central control by OT which we found to take place with a dual time course. This was confirmed by optogenetically stimulating parvOT PVN axons located either in the SON or in the SC. This stimulation led to a reduction of WDR discharges in response to a peripheral noxious stimulation, which was selective for Aδ- and C-type nociceptive fibers. The effects in deep layers of the SC seemed to be mediated by the OTR, as we did not find any effects of VP V1a receptor similar to what has been reported (Qiu et al., 2014). As OT terminals on WDR-like neurons contained VGluT2, we assessed glutamate (Glu) and OT contribution to the SC-BL effect on WU intensity. This revealed that both OT and Glu participated to the inhibition of WU. These results are in accordance with our in vitro patch-clamp experiment and can be interpreted by a network effect as OT axons are likely to form en passant synapses (Knobloch et al., 2012), allowing local (micro)volume-transmission from release sites (Knobloch and Grinevich, 2014; Grinevich et al., 2016b). The combination of two processes can then explain the observed effects: (1) OT acts on OTR in WDR neurons to inhibit them via $G_i$ intracellular pathway (Figure 3E) and (2) coreleased Glu either activates local GABA-interneurons in layers V–VI and around the cc (Schneider and Lopez, 2002; Deuchars et al., 2005), which, in turn, inhibits WDR neurons, or binds a mGluR leading to the direct inhibition of WDR neurons by a $G_{i/o}$ pathway (Gerber et al., 2000; Niswender and Conn, 2010).

Surprisingly, evoked spinal OT release by this subpopulation of parvOT did not modify nociceptive processing by neurons in superficial layers. This suggested that the OT inhibition of WDR firing was not induced by OTR activation in superficial layers, but only in deep dorsal horn layers. This was in agreement with our anatomical data describing the vast majority of parvOT neurons projecting to the deep layers. We failed to reveal any functional contribution of this subpopulation of parvOT projecting to SON and SC in a nerve-induced neuropathic pain, which may be modulated by OT projections to superficial layers of the dorsal horn. In contrast, they exerted a tonic inhibitory control on WU and pain symptoms in the peripheral inflammation. We speculate that the inflammatory component in pain state

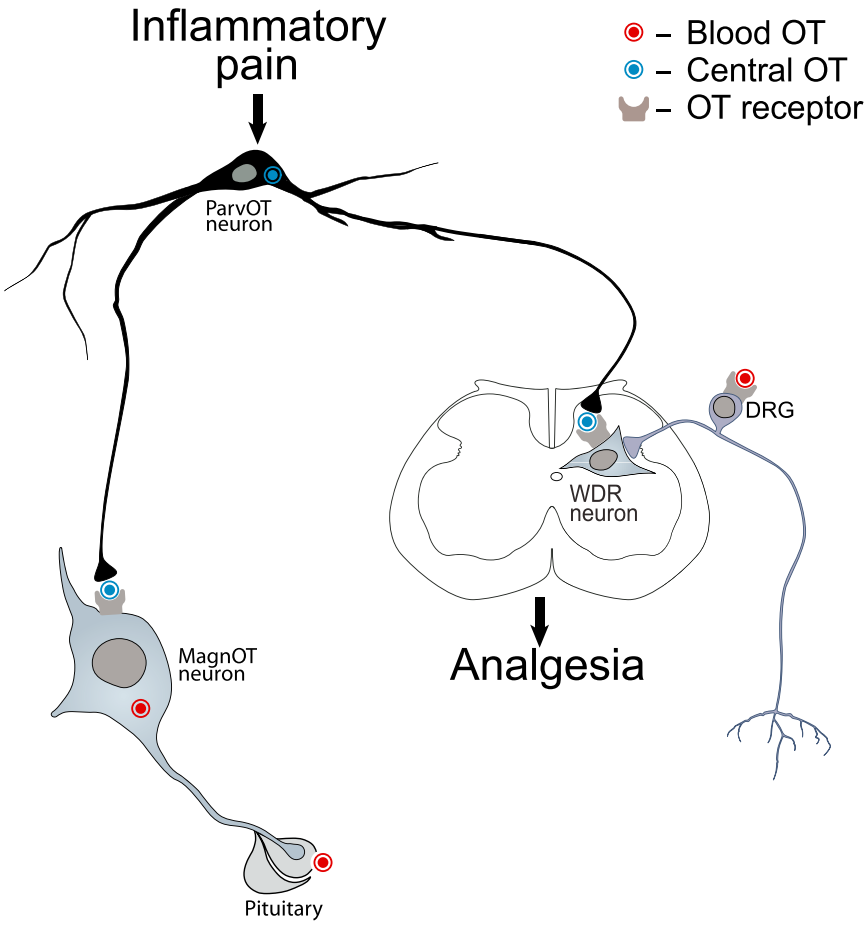


**Figure 7. The Role of the Novel Type of ParvOT Neurons in Coordinating Central and Peripheral OT Release to Promote Analgesia**

We hypothesize that pain stimulates the identified subset of parvOT PVN neurons, which simultaneously release OT in the SON and SC, exerting respectively delayed and longer lasting and immediate and shorter lasting analgesia. The peripheral analgesic effect of OT is likely mediated by its action on BBB-free sensory neurons of the DRG.

regulates the excitability of this subset of parvOT neurons, as observed by c-Fos induction in parvOT neurons (data not shown). Our results indicate that the described subpopulation of parvOT neurons specifically targets NK1R and OTR positive neurons located in the deep layers of the SC to exert antinociceptive action on WDR to promote analgesia.

## Described ParvOT Neurons as a New OT-Ergic Cell Type

Following pioneering works of Swanson and Kuypers (1980), Sawchenko and Swanson (1982), and Swanson and Sawchenko (1983), parvOT neurons have been considered as heterogeneous cell populations with descending projections to brainstem and/or SC regions. However, in the present study, we found a small group of parvOT neurons that forms a pathway distinct from the classical hypothalamo-neurohypophyseal axonal tract through the hypothalamus. Although we did not analyze in detail axonal collaterals of these parvOT cells in other forebrain regions, their existence in the hypothalamus adds a new feature to parvOT cells. In addition, these neurons simultaneously project collaterals to deep layers of the SC, representing a unique group of specifically located cells, which is likely distinct from the OT-immunoreactive neurons described by Jójárt et al. (2009) that massively project axons to the superficial layers of the SC. Based on the unique connectivity of the identified parvOT neurons, we speculate that they represent a new type of OT neurons, which coordinate central and peripheral inhibition of nociception and pain perception, and hence, play a role in promoting analgesia (Figure 7).

## EXPERIMENTAL PROCEDURES

### Animals

Anatomical, electrophysiological, optogenetic, and behavioral studies were performed with Wistar rats (for details of the experiment see the respective figure legend). If not mentioned, rats were housed under standard conditions with food and water available ad libitum. All experiments were conducted under licenses and in accordance with EU regulations.

### Viruses

rAAVs (serotype 1/2) carrying conserved regions of OT and VP promoters and genes of interest in direct or "floxed" orientations were cloned and produced as reported previously (Knobloch et al., 2012). CAV2 equipped with GFP or Cre recombinase was purchased from the Institute of Molecular Genetics in Montpellier CNRS, France (Bru et al., 2010)

### Neuroanatomy

To trace internuclear connections, rAAVs expressing Venus were injected into the PVN or SON to follow their axonal projections within the hypothalamus. Alternatively, CAV2-Cre was injected into the SON, while Cre-dependent floxed rAAV was injected into the PVN to identify OT PVN neurons synapsing onto SON neurons.

To trace hypothalamus-SC connections, a CAV2-Cre virus was injected into the SON and floxed rAAV, into the PVN or CAV2-Cre virus was injected in the SC, while Cre-dependent rAAV, into the PVN. This allowed us to visualize OT axon pattern in the SC and to identify projecting PVN OT neurons, respectively. After transcardial perfusion with 4% paraformaldehyde (PFA), brains and/or SC were sectioned and stained with antibodies against OT, VP, vGluT2, GFP, NeuN, NK1R, and OTR. Images for qualitative and quantitative analyses were taken on confocal microscopes Leica SP2 and SP5.

### Electrophysiology Experiments

For in vitro patch-clamp recordings, 4 to 8 weeks after injection of virus in adult rats, brains were removed, the hypothalamus or lumbar SC was isolated, cut into 400 μm coronal slices, and kept in artificial cerebrospinal fluid (ACSF: 118 mM NaCl, 25 mM NaHCO3, 10 mM glucose, 2 mM KCl, 2 mM MgCl2*6H2O, 2 mM CaCl2*2H2O, and 1.2 mM NaH2PO4) saturated 95% dioxygen (O2), 5% carbon dioxide (CO2). Visualized neurons were patched with borosilicate glass pipette (4–9 MΩ) filled with 140 mM KMeSO4, 10 mM HEPES, 2 mM MgCl2, 0.1 mM CaCl2, 0.1 mM (1,2-bis(o-aminophenoxy)

ethane-N,N,N',N'-tetraacetic acid) (BAPTA), 2 mM ATP Na salt, and 0.3 mM guanosine triphosphate (GTP) Na salt (pH 7.3), adjusted to 300 mOsm, and voltage-clamped at −60 mV.

For in vivo extracellular recordings, 4 to 8 weeks after injection of virus, adult animals were anaesthetized with 4% isoflurane and placed in stereotaxic frame. Extracellular neuronal activity was recorded using a stainless electrode with 10 MΩ impedance (FHC; UE(FK1)).

### Behavioral Experiments

Mechanical allodynia was measured using a calibrated forceps (Bioseb). Thermal allodynia/hyperalgesia was measured using the Plantar test using Hargeaves method (Ugo Basile). Peripheral painful inflammatory sensitization was obtained by a single unilateral intraplantar injection of CFA (Sigma-Aldrich, 100 μl in the right paw). Nerve injury-induced neuropathy was induced using the cuffing method.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2016.01.041.

## AUTHOR CONTRIBUTIONS

Conceptualization, A.C. and V.G.; Methodology, P.P., P.H.S., R.Stoop, A.C., and V.G.; Formal Analysis, M.E., M.Melchior, H.S.K.-B., J.W., and A.C.; Investigation—Neuroanatomy, M.E., H.S.K.-B., M.d.S.G., L.C.R., F.A., T.G., M.B., M.Mitre, and G.G.; Investigation—In Vivo Electrophysiology, M.Melchior, J.W., Y.T., and N.P.-D.; Investigation—In Vitro Electrophysiology J.W., A.C.C., and R.T.d.R.; Investigation—Behavior, M.Melchior, J.W., M.d.S.G., N.P.-D., and L.L.T.; Investigation—Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), V.C. and Y.G.; Resources, B.C., R.C.F., M.V.C., R.Sprengel, and R.K.; Writing—Original Draft, M.Melchior, H.S.K.-B., J.W., P.P., P.H.S., R.Stoop, A.C., and V.G.; Writing—Review & Editing, M.E., M.Melchior, H.S.K.-B., J.W., P.P., P.H.S., R.Stoop, A.C., and V.G.; Visualization, M.E., M.Melchior, H.S.K.-B., J.W., A.C., and V.G.; Supervision, A.C. and V.G.; Project Administration, R.Stoop, A.C., and V.G.; and Funding Acquisition, A.C. and V.G.

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## Supplemental Information

# A New Population of Parvocellular Oxytocin Neurons Controlling Magnocellular Neuron Activity and Inflammatory Pain Processing

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# SUPPLEMENTAL MATERIALS

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**A new population of parvocellular oxytocin neurons controlling magnocellular neuron activity and inflammatory pain processing**

## SUPPLEMENTAL TABLES

**Table S1 (corresponds to Figure 1 and S1):**

**Specific Expression of VP Promoter, Virally Introduced to Hypothalamic Nuclei of Naïve and Water Deprived Rats**

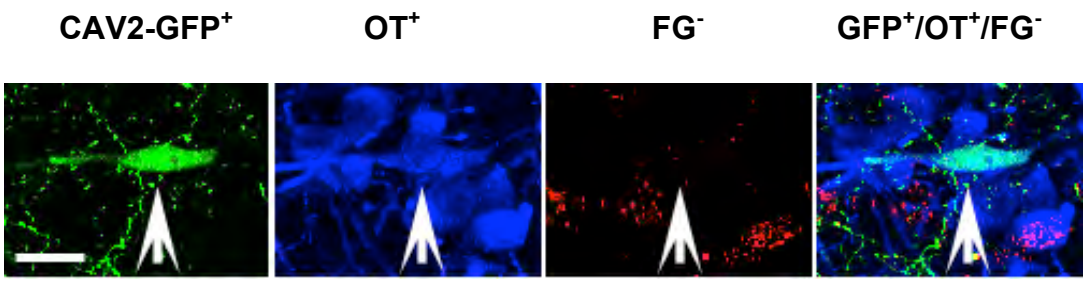
| Animals | Comparison | SON | PVN |
|---|---|---|---|
| Naïve Rats | VP *vs* Venus | 95.46% ± 5.53% | 98.9% ± 1.74% |
| | | n=552 | n=858 |
| | Venus *vs* VP | 94.44% ± 3.29% | 94.98% ± 3.26% |
| | | n=552 | n=858 |
| Water deprived Rats | VP *vs* Venus | 100% ± 0% | 100% ± 0% |
| | | n=1115 | n=1169 |
| | Venus *vs* VP | 99.81% ± 0.56% | 99.59% ± 0.52% |
| | | n=1115 | n=1169 |

Quantification of VP-immunopositive neurons infected with rAAV carrying VP promoter and Venus. n, absolute number of identified neurons per structure and condition. Results are presented as percentage ± SEM.

**Table S2 (correspond to Figure 1 and S1):**

**Quantification of Back-labeled PVN ParvOT Neurons Projecting to the SON**

| Cell phenotype | $GFP^+$ | $GFP^+/OT^+$ | $GFP^+/OT^+/FG^+$ | $GFP^+/OT^+/FG^-$ |
|---|---|---|---|---|
| n | 52.2 ± 18.09 | 31.5 ± 8.5 | 3 ± 1 | 28.5 ± 6.5 |




The table shows the number of fluorogold (FG)-containing back-labeled PVN parvOT neurons projecting to the SON. n, absolute number of identified neurons per structure (6 sections per PVN of each rat, 3 rats). Results are presented as Mean ± SEM.

Panel provides an example of a GFP positive ($GFP^+$) back-labeled PVN cell, which contains OT immune-signal ($OT^+$), but not Fluorogold ($FG^-$), indicating that this is a parvOT neuron. Scale bar represents 10 μm.

**Table S3 (corresponds to Figures 1-6):**

**Experimental Procedures, Number of Animals, Target Structures,**

**Viral vectors, Expression time, and Corresponding Figure.**

| Field | Name of experiment | Number of rats used | Viral vectors | Target structures | Expression time | Fig. |
|---|---|---|---|---|---|---|
| Anatomy | Intra-hypothalamic fiber trees of OT Neurons | 10 5/nucleus | rAAV Otpr_Venus | PVN or SON unilateral | 4 weeks | 1 |
| Anatomy | Intra-hypothalamic fiber trees of AVP neurons | 10 5/nucleus | rAAV_Vppr_Venus | PVN or SON unilateral | 4 weeks | S1 |
| Anatomy | Cell type specific expression of AVP promoter rAAV | 5 | rAAV_Vppr_tdTomato and rAAV_Otpr_Venus | PVN and SON bilateral | 2 weeks | S1 |
| Anatomy | Physiological responsiveness of AVP promoter rAAV | 6 3 naïve, 3 water deprived | rAAV_Vppr_Venus | PVN and SON bilateral | 2 weeks | S1 |
| Anatomy | Light microscopic characterization of PVN OT contacts in the SON | 10 5/viral approach | rAAV_Otpr_Venus or rAAV_Otpr_Syn-EGFP | PVN bilateral | 4 weeks | 1 |
| Anatomy | Electron microscopic characterization of PVN OT contacts in the SON | 3 | rAAV_Otpr_Venus | PVN bilateral | 4 weeks | 2 |
| Anatomy | Retrograde tracing with retrobead injection into SON and systemic fluorogold application | 6 | Rhodamin retrobeads and Fluorogold | SON (angled) unilateral and i.p. | 10 days | 3 |
| Anatomy | Retrograde CAV-GFP virus application for visualization of PVN cells projecting to the SON | 3 | CAV2-GFP | SON unilateral | 4-5 weeks | Table S2 |
| Anatomy | Retrograde CAV-Cre virus application for Cre-dependent marker expression in PVN-SON connecting OT neurons to trace fibers into the SC | 4 | CAV2-Cre and Otpr_DIO_Venus | SON unilateral and PVN bilateral | 4-5 weeks | S4 |
| Anatomy | Translucent brain with unilaterally labeled PVN OT neurons and their fiber path | 2 | rAAV_OTpr_Venus | PVN unilateral | 4 weeks | S2 |
| Anatomy | Injection site size verification and virus spread evaluation in SON | 10 | CAV2mCherry and LV Ef1A | SON bilateral | 2 weeks | 3 |
| Anatomy | Immunohistochemical identification of SC cells, found in close proximity to spinally projecting OT fibers | 9 | CAV-Cre and OTpr_DIO_GFP | SON bilateral and PVN bilateral | 4 weeks | 4 |
| Anatomy | Identification, quantification and anatomical description of spinally projecting OT PVN cells, back-labelled after Cav-Cre virus delivery into lumbar segment of spinal cord | 5 | CAV-Cre and OTpr_DIO_GFP | L4-L5 unilateral and PVN bilateral | 4 weeks | 1 & 3 |
| Anatomy | Immunhistochemical confirmation of glutamatergic nature of PVN–SON, and PVN-SC mutual synapses | 3 | OTp_iCreW and EF1a p_DIO_Synaptophysin-GFP | PVN bilateral | 2 weeks | 3 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Anatomy | Detection of c-Fos in NK1R and OTR neurons in parvOT neurons after capsaicin application | 9 | CAV-Cre and OTpr_DIO_GFP | SON bilateral and PVN bilateral | 4 weeks | 1 |
| Anatomy | Detection of NK1R and OTR mRNA by in situ hybridization | 4 | - | - | 3 weeks | 3 |
| Electrophy. (in vivo) | SON-BL and SC/SON recording | 9 | Otpr_ChR2-mCherry | PVN bilateral | 4 weeks | 1 |
| Electrophy. (in vivo) | PVN stimulation and recording | 8 | Otpr_ChR2-mCherry | PVN bilateral | > 8 weeks | - |
| Electrophy. (in vivo) | SC-BL with dOVT treatment | 11 | CAV2-Cre and rAAV- CAG-FLEX-ChR2-mCherry | SON bilateral and PVN bilateral | 8-10 weeks | 5 |
| Electrophy. (in vivo) | SC-BL with V1aR antagonist treatment | 2 | CAV2-Cre and rAAV- CAG-FLEX-ChR2-mCherry | SON bilateral and PVN bilateral | > 8 weeks | S5 |
| Electrophy (in vivo) | SC-BL with NBQX + dOVT and NBQX treatments | 10 | Cav-Cre and CAG-DIO-ChR2-mCherry | SON bilateral and PVN bilateral | > 8 weeks | 5 |
| Electrophy. (in vivo) | SON-BL and SON recording with NBQX and dOVT infusion in SON | 5 | Cav-Cre and CAG-DIO-ChR2-mCherry | SON bilateral and PVN bilateral | 4 weeks | S5 |
| Electrophy (in vivo) | SC-BL and SP treatments | 5 | Cav-Cre and CAG-DIO-ChR2-mCherry | SON bilateral and PVN bilateral | > 8 weeks | 3 |
| Electrophy (in vitro) | Validation of ChR2 approach and electrical differenciation of parvOT vs magnOT | 8 | OTp- hM4D(Gi)-mCherry | PVN bilateral | > 8 weeks | S6 |
| Electrophy (in vitro) | Validation of DREADD approach | 8 | CAV2-Cre and OTp-DIO-hM4D(Gi)-mCherry | SON bilateral and PVN bilateral | > 8 weeks | S6 |
| Electrophy (in vitro) | Direct action of OT on WDR neurons | 14 | - | - | > 8 weeks | 3 |
| Behavior | BL in CFA model | 8 | CAV2-Cre and rAAV- CAG-FLEX-ChR2-mCherry | SON bilateral and PVN bilateral | 4 weeks | 6 |
| Behavior | DREADD in CFA model | 10 | CAV2-Cre and OTp-DIO-hM4D(Gi)-mCherry | SON bilateral and PVN bilateral | 4 weeks | 6 |
| Behavior | DREADD in cuff model | 12 | CAV2-Cre and OTp-DIO-hM4D(Gi)-mCherry | SON bilateral and PVN bilateral | 4 weeks | 6 |
| Behavior | BL in cuff model | 10 | CAV2-Cre and OTp-DIO-CHR2-mCherry | SON bilateral and PVN bilateral | 4 weeks | 6 |
| Neuroendo-crinology | OT content in plasma | 5 | CAV2-Cre and OTp-DIO-CHR2-mCherry | SON bilateral and PVN bilateral | 4 weeks | 1 |
| **TOTAL number of rats injected with viruses** | | | | **224** | | |

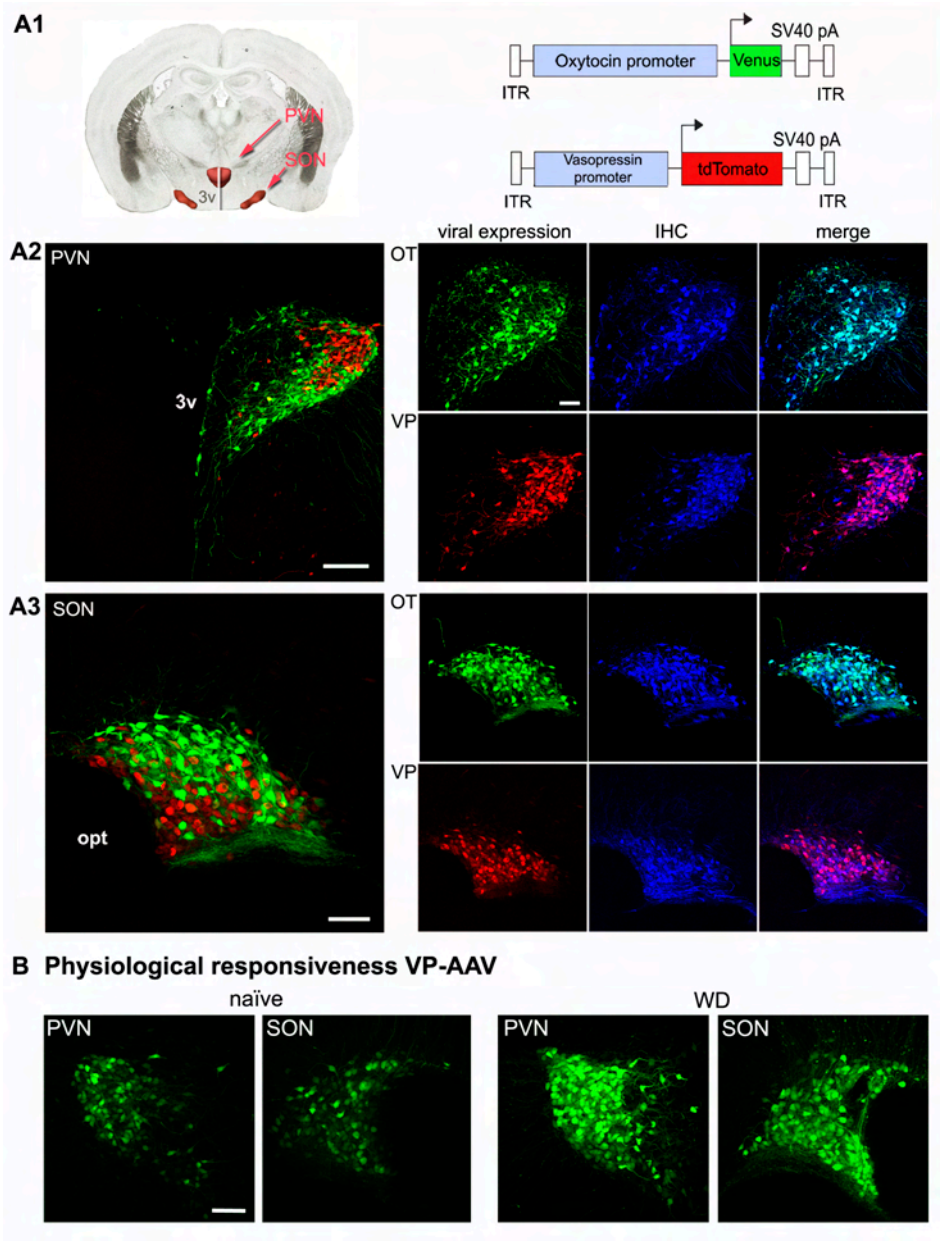
**Table S4 (corresponds to Figure 2 and S2):**

**Passive and Active Properties of Parvocellular and Magnocellular Neurons, as Distinguished by the Presence or Absence of Fluorescent Labeling**

| Parameter analyzed | Parvocellular (n=11) | Magnocellular (n=13) | P value (unpaired) |
|---|---|---|---|
| Membrane potential (mV) | -56 ± 3 | -59 ± 4 | 0.27 |
| Input resistance (MOhm) | 420 ± 65 | 390 ± 40 | 0.74 |
| AP half width (ms) | 0.49 ± 0.02 | 0.55 ± 0.06 | 0.40 |
| Spike frequency (Hz) | 20.4 ± 4.0 | 11 + 4 | 0.12 |
| Spike delay (ms) | 33 ± 4 ms | 85 ± 19 | 0.04* |
| Rise slope (mV/ms) | 1.2 ± 0.2 | 0.5 ± 0.2 | 0.02* |

Quantified electrophysiological characteristics of parvocellular and magnocellular OT neurons in the PVN.

## SUPPLEMENTAL FIGURES



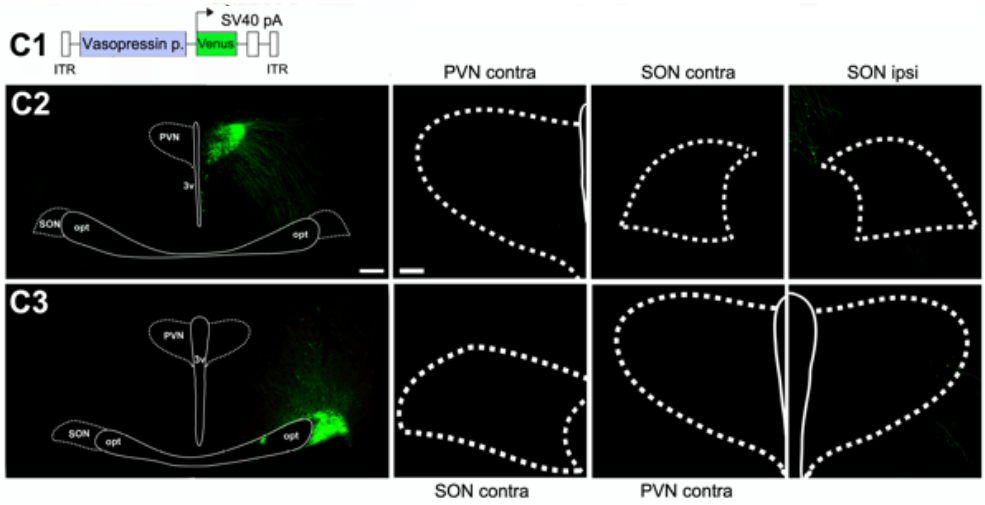



**Figure S1 (corresponds to Figure 1). Cell Type Specific Expression and Physiological Responsiveness of VP Promoter rAAV, Projections of VP neurons.**

(**A**) rAAV expressing Venus under the control of a 2.6 kb mouse OT promoter and tdTomato under the control of a 1.9 kb mouse VP promoter were injected concomitantly into PVN and SON (**A1**). Counterstaining for either OT or VP revealed a striking co-localization of the Venus (green) and OT signal (blue; **A2** and **A3**, upper rows) as well as tdTomate (red) and VP signal (blue; **A2** and **A3**, lower rows), indicating cell specific expression from both rAAVs in PVN and SON (further characterization of OT promoter rAAV in Knobloch et al., 2012). Scale bars represent 200 μm in A2 left pannel, 100 μm in A3 left pannel and 75 μm in right pannels.

(**B**) After two days of water deprivation, the intensity of intrinsic Venus fluorescence increased compared to naïve conditions and indicates a preserved physiological responsiveness of the short virally delivered VP promoter (pictures taken with equal confocal settings). Scale bar represent 75 μm.

(**C**). Injection of AAV, expressing Venus under the control of VP promoter (**C1**) unilaterally in the PVN (**C2**) or SON (**C3**). No GFP-containing fibers were detected within the SON after PVN injection and vice versa as well as fibers were absent in contralateral hemisphere. Scale bars represent 200 μm in overview images (left panels), 50 μm in enlarged images of individual nuclei. 3v – third ventricle; opt – optic tract, SON - supraoptic nucleus.

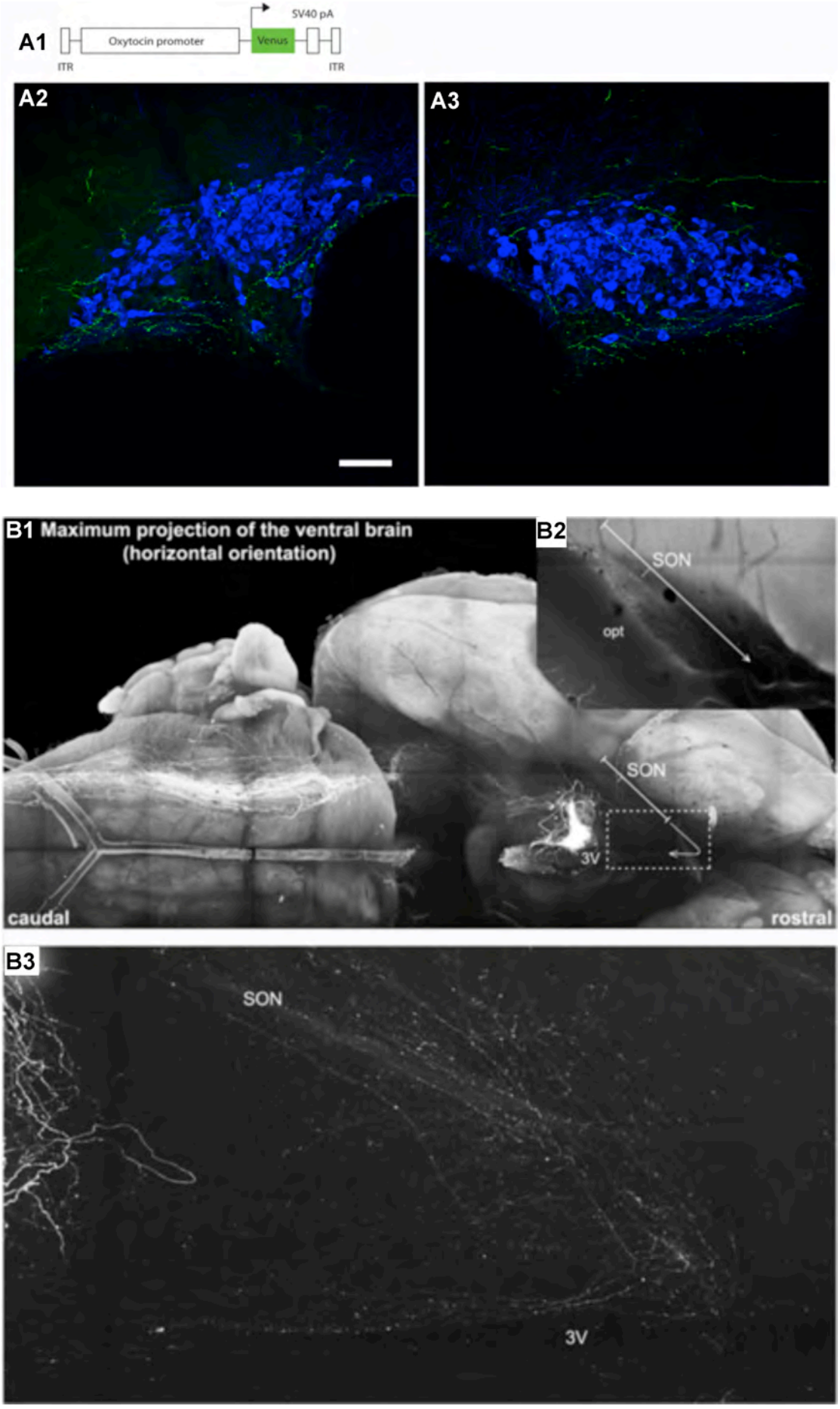

A1
5V40 pA
Oxytocin promoter
Venus
ITR
ITR
A2
A3
B1 Maximum projection of the ventral brain
(horizontal orientation)
B2
SON
opt
SON
3V
caudal
rostral
B3
SON
3V

**Figure S2 (corresponds to Figure 1). Reconstruction of OT Connections Between the PVN and SON.**

(**A**) Injection of OT promoter-Venus AAV (**A1**) into the PVN of female (**A2**) and male (**A3**) rats resulted in the appearance of Venus-positive axonal terminals within the SON of rats of both genders. Scale bar: 50 μm.

(**B**) The cleared rat brain (female) contains virally-mediated Venus labeling unilaterally in OT neurons of the PVN. Depicted are maximum projections of horizontal light sheet recordings of the ventral brain (**B1**: comprising the hypothalamus, **B2**: comprising the SON, **B3**: comprising the hypothalamus without the PVN). Fiber streaming towards the brainstem and SC are prominently labeled. Fine OT fibers, descending from the PVN, are recognizable in the SON, a 'banana-shaped' area bordering the optic tract (indicated in **B1** and **B2**). (**B2**) Magnification of the dashed region of B1, revealing OT fibers stream longitudinal within the SON. (**B3**) Further magnification reveals that OT fibers stream beyond the rostral end of the SON to the brain midline (not crossing). There they kink and run ascending and caudally along the third ventricle to the PVN (compare to bent arrow in **B1**). The dashed square in **B1** indicates the location of magnification **B2**.

3v – third ventricle; opt – optic tract, SON - supraoptic nucleus.

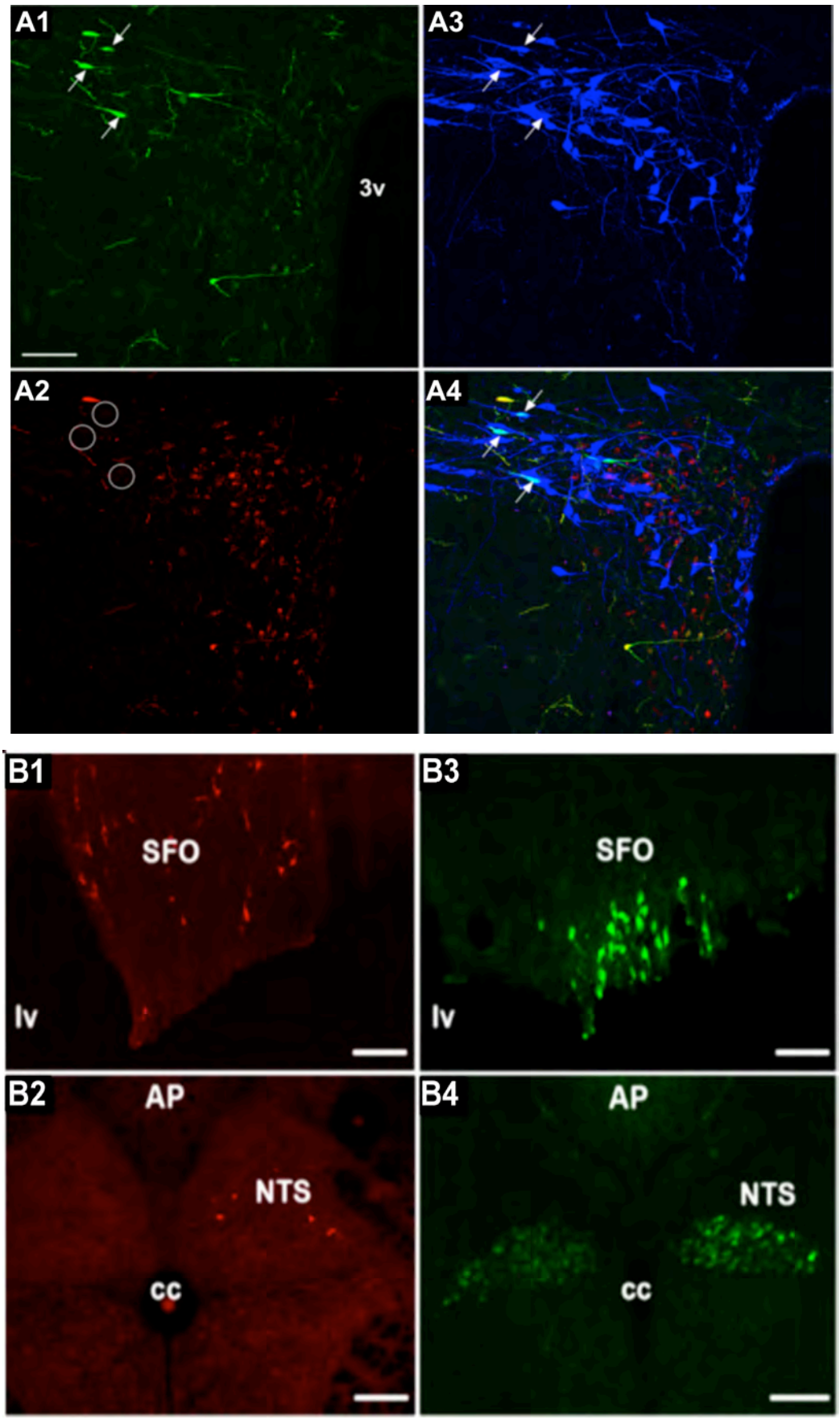

A1
3v
A3
A2
A4
B1
SFO
lv
B3
SFO
lv
B2
AP
NTS
cc
B4
AP
NTS
cc

**Figure S3 (corresponds to Figure 2). CAV2-based Labeling of PVN Neurons and Efficiency of CAV2 vs. Retrobeads.**

(**A**) Overview of the PVN with GFP-positive back-labeled neurons after injection of CAV2-GFP into the SON. The cells positive for GFP (green, arrows, **A1**) and oxytocin (blue, arrows, **A3**) are negative for fluorogold (red, **A2**; respective areas are encircled). Overlay of the three channels is in **A4**. 3v – third ventricle, scale bar = 100 μm.

(**B**) As proof of the method for uptake and traveling of Rhodamine-labeled Retrobeads (red), back-labeled neurons in the subfornical organ (**B1**) and nucleus of the solitary tract (**B2**), known to project to the SON, were detected in rats injected into the SON. (**B3, B4**) GFP-expressing neurons in the same brain regions after the injection of CAV2-GFP virus into the SON. Scale bars represent 100 μm.

AP – *area postrema*, cc – central canal, lv – lateral ventricle, NTS – nucleus of the solitary tract, SFO – subfornical organ.

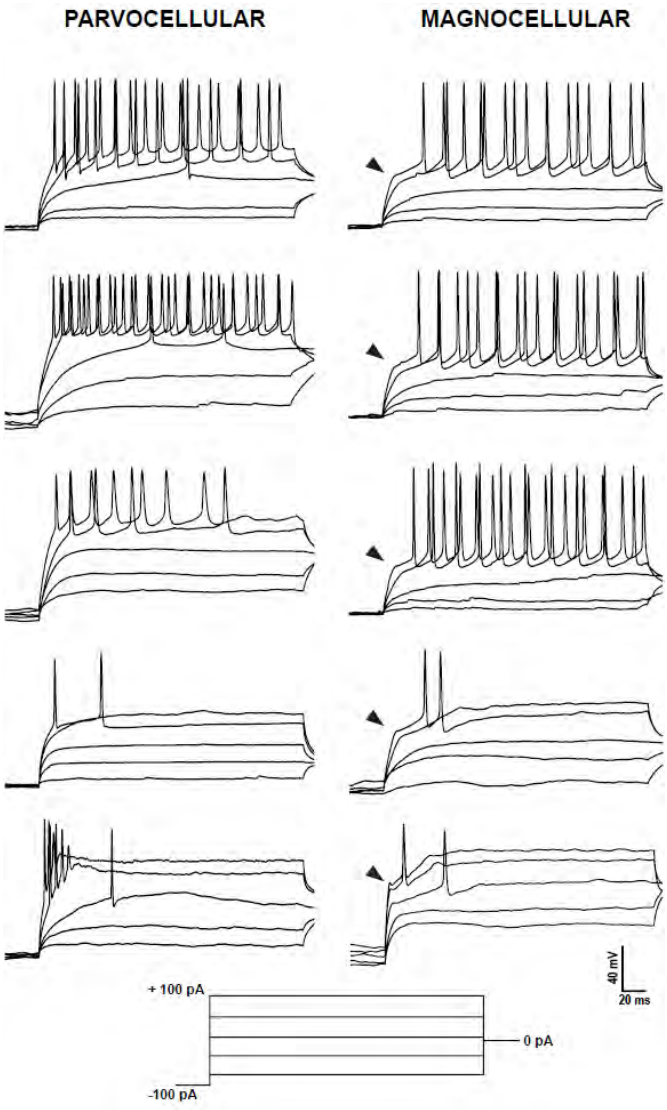



**Figure S4 (corresponds to Figure 2). Electrophysiological Properties of ParvOT vs. MagnOT Neurons.**

Example traces of spiking patterns of five putative parvo, (left column)- and magnocellular (right column) neurons following an initial hyperpolarizing step followed by depolarizing steps caused by current injections of different magnitudes (as shown below the traces). All putative magnocellular neurons (characterized by the absence of fluorescence) exhibited the typical "hyperpolarizing notch" as indicated by the arrows.

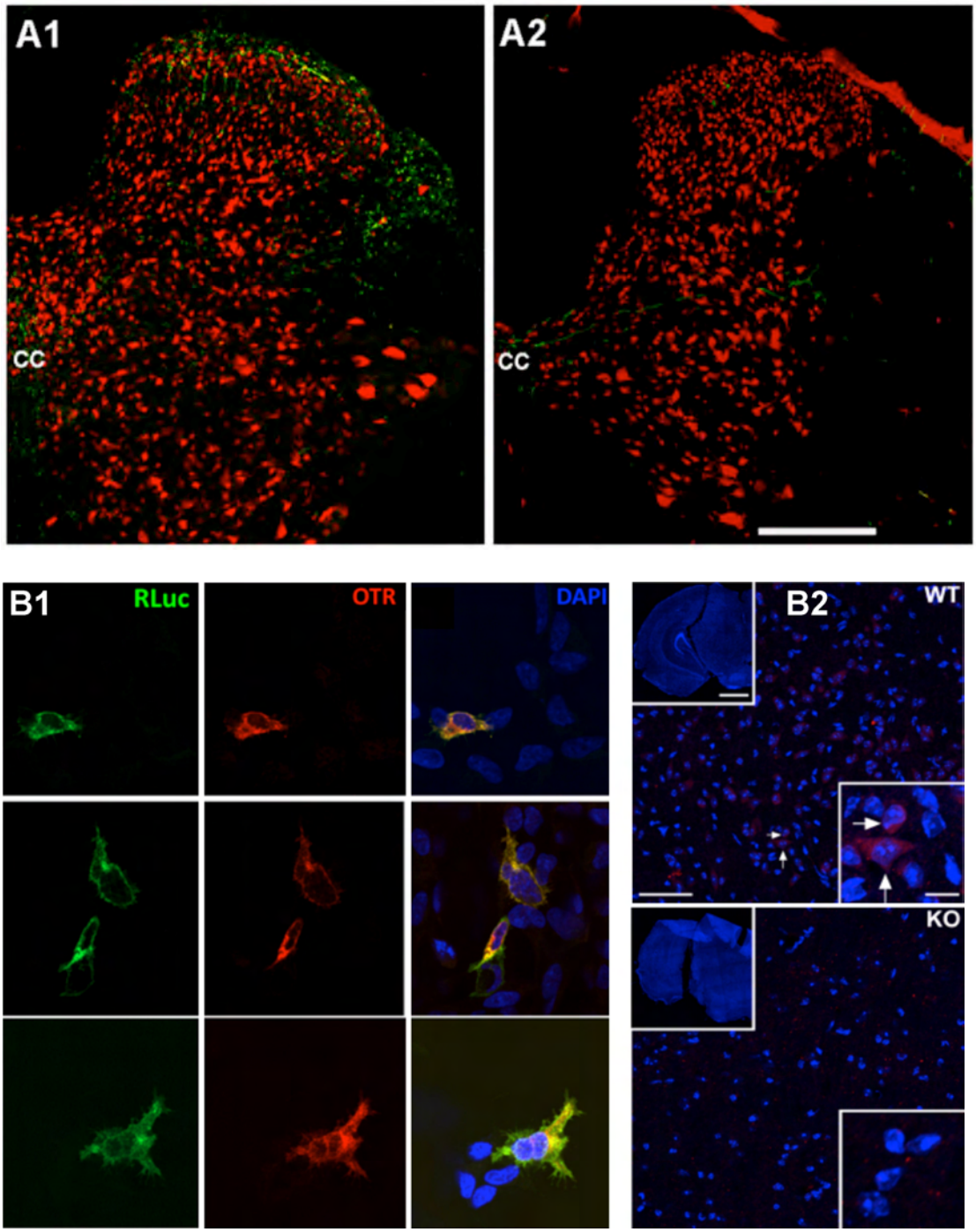



**Figure S5 (corresponds to Figure 3). Distribution of GFP fibers on Coronal Spinal Cord Sections and Verification of Specificity of OTR antibodies.**

(**A**) GFP fibers. (**A1**) Labeling all OT neurons (including all parvocellular neurons) resulted in appearance of OT/GFP axons in both superficial and deep layers. However, labeling only a fraction of parvOT neurons (also

projecting to the SON; **A2**) resulted in the appearance of GFP fibers only in deep layers**.** Cell bodies were visualized by NeuN immunostaining (red). Scale bar: 250 μm. cc: central canal.

(**B**) Specificity of OTR antibodies. (**B1**) Transfected HEK cells: Mouse OTR fused to RLuc was stained with anti-Rluc antibody (Millipore mAb 4400) (green; RLuc) and the oxtr-2 antibody (red; OTR); nuclei were stained with DAPI (blue). Double staining was observed only in OTR-Rluc expressing cells in which plasma mebrane and intracellular compartments were labeled. (**B2**) Brain sections of OTR KO mice: While in pons of WT mice (right panel) a population of neurons contains OTR immunosignal (red), in KO mice OTR immunoreactivity has not been detected. Cell nuclei were stained by DAPI (blue). Scale bars for left up inserts (overview of section) is 1000 μm, for right low – 10 μm, and for the central panel – 50 μm.

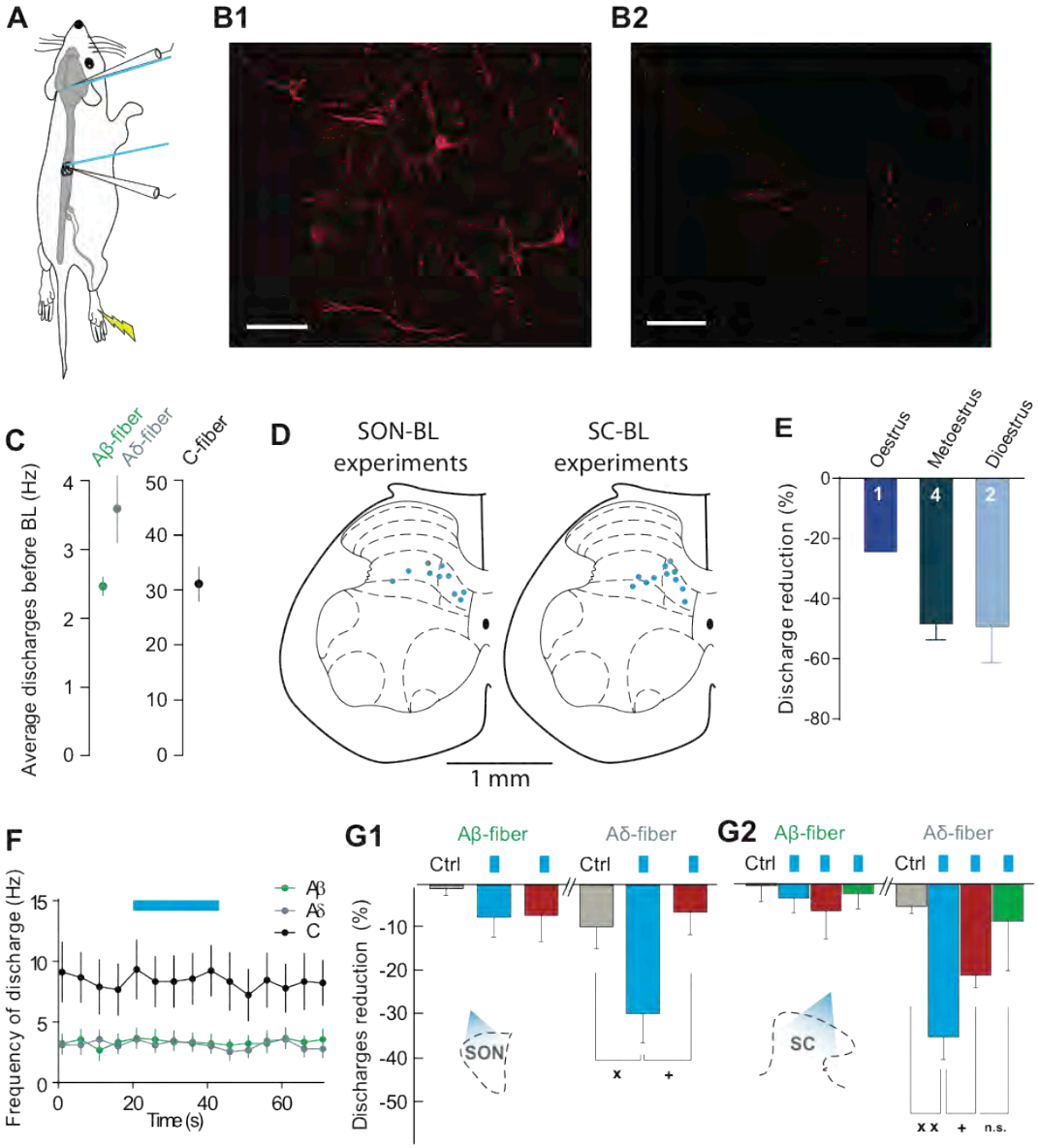



**Figure S6 (corresponds to Figure 5). Responses of SC Neurons are Independent of Estrous Cycle and VP Receptors.**

(**A**) Scheme of the experimental setup.

(**B1**) ChR2-mCherry expressing axons in the SON. (**B2**) Presence of the ChR2 expressing axons in the deep layers of lumbar segments of the spinal cord. Scale bars in **B1** and **B2** is 100 µm.

(**C**) Basal frequencies of Aβ- (WDR-Aβ), Aδ- (WDR-Aδ) and C- (WDR-C) fibers per paw stimulation (1/s) upon stabilized wind-up (n = 31).

(**D**) Localization of the WDR neurons recorded for SON-BL and SC-BL experiments.

(**E**) Comparison of the SC-BL effect on WDR-C discharges regarding the estrous cycle (oestrus n = 1, metoestrus n = 4, dioestrus n = 2).

(**F**) Effect of SC-BL on superficial layer neurons (n=9).

(**G1**) Effect of SON-BL in WDR-Ab and WDR-Ad in non responding WDR neuron (gray, n=8), responding WDR neuron (blue, n=10) and in responding WDR neuron in presence of dOVT (red; n=6). (**G2**) Effect of SC-BL in WDR-Ab and WDR-Ad in non responding WDR neuron (gray, n=7), responding WDR neuron (blue, n=9) and in responding WDR neuron in presence of dOVT (red; n=6) or dOVT + NBQX (green; n=6).

BL – blue light, SON – supraoptic nucleus, SC – spinal cord, WDR – wide dynamic range.

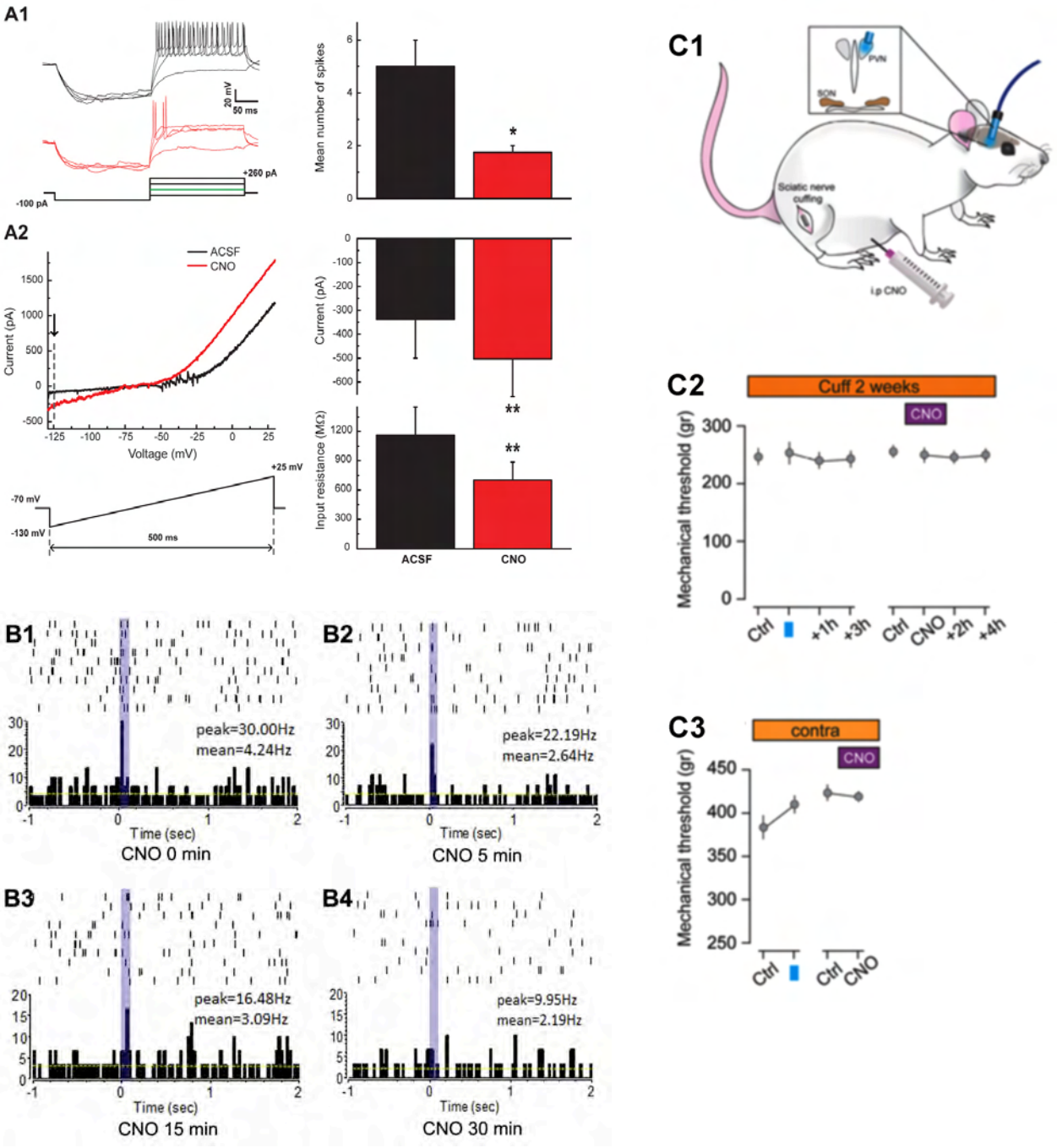



**Figure S7 (corresponds to Figure 6). CNO Inhibits OT Neuron Activity *in Vitro* and *In Vivo* and Manipulation with ParvOT Neurons After Sciatic Nerve Cuffing.**

(**A1**) Left panel: examples of voltage responses induced by current injections in the current clamp configuration, before (black) and during (red) CNO (5 min at 20 $\mu$M). The protocol used is shown below the traces. Right

panel: CNO reduced the mean frequency of spikes induced by current injections (+40 pA), green line in protocol on the left, n=8. (**A2)** Left panel: example of current-voltage curve obtained from a 500 ms voltage ramp (shown below) from -130 to +25 mV, before (black) and during CNO (5 min at 20 $\mu$M, red). Right panel: CNO increased the inward current (upper graph) measured at -125 mV (dashed line) during the voltage ramps and decreased the input resistance (lower graph) calculated from responses to hyperpolarizing voltage steps; n = 16, * $p < 0.05$, ** $p < 0.01$, Student's paired t-test. Data represented as mean ± SEM.

(**B1)** BL-induced OT neuron firing increases from basal rate of 1-3 Hz to 20 Hz *in vivo*. CNO (i.p. 3 mg/kg) gradually decreased basal and BL-evoked activity of the same OT neuron at 5 (**B2**), 10 (**B3**) and 30 (**B4**) min after the injection.

(**C1**) Scheme of the experimental procedure for behavioral assessment of parvOT role in neuropathic pain model (cuff). (**C2**) Mechanical thresholds of the cuffed hindpaw two weeks after surgery. Effect of PVN-BL was measured 5 min, 1h and 3h after PVN-BL (n = 10; left pannel). Effect of CNO (3 i.p. mg/kg) was measured 45 min, 2h and 4h after i.p. injection and its 24 h washout (n = 12; right pannel). (**C3**) Mechanical thresholds of the contralateral hindpaw two weeks after surgery. Blue squares represent 20 s at 30 Hz with 10 ms pulses of BL stimulation.

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

### Animals

Anatomical, electrophysiological, optogenetic and behavioral studies were performed with adult female Wistar rats except for the adult male Wistar rats in Figure S1 (for details of the experiment see the respective figure legend). Rats were housed under standard conditions with food and water available *ad libitum* (except for the water deprivation paradigm in Figure 1B; S1B and S1C) and maintained on a 12-hour light/dark cycle in accordance with EU rules. In total, we used 224 rats as described in Table S3.

### Cloning of rAAV Vectors

The generation of rAAVs with specific expression in OT-cells is described in our previous work (Knobloch et al., 2012). Briefly, the conserved promoter region of 2.6 kb was chosen using the software BLAT from UCSC (http://genome.ucsc.edu/cgi-bin/hgBlat), was amplified from BAC clone RP24-388N9 (RPCI-24 Mouse, BACPAC Resources, CHORI, California, USA) and was subcloned into an rAAV2 backbone carrying an Ampicillin-resistance. Genes of interest introduced to the plasmid in floxed and unfloxed version are Venus, Channelrhodopsin2-mCherry, Synaptophysin-EGFP, and hM4Di. The rAAV vector that carries a conserved VP promoter sequence was designed in a similar way. It comprises a 1.9 kb sequence stretch (revealed by BLAT) that allows for cell-specific expression in hypothalamic VP neurons. As a gene of interest, Venus or tdTomato were introduced to the plasmid.

**Production of Viruses**

Production of chimeric virions (recombinant Adeno-associated virus 1/2; rAAV 1/2) was described in Knobloch et al., 2012. Briefly, human embryonic kidney cells 293 (AAV293; Agilent #240073) were calcium phosphate-transfected with the recombinant AAV2 plasmid and a 3-helper system (During et al., 2003). rAAV genomic titers were determined with QuickTiter AAV Quantitation Kit (Cell Biolabs, Inc., San Diego, California, USA) and are ~$10^{13}$ genomic copies per ml for all rAAV vectors used in this study. Canine Adeno Virus (CAV) serotype 2 (Bru et al., 2010; titer 2.5x$10^{12}$ particles /ml) carrying the gene for Cre-recombinase (CAV2-Cre) was purchased from Institut de Génétique Moléculaire de Montpellier CNRS UMR 5535.

**Stereotaxic Injections**

*Intrahypothalamic stereotaxic injections* were performed as described (Grinevich et al., 2015) and coordinates were chosen in accordance to the rat brain atlas (Paxinos and Watson, 1998) for PVN (X axis: +/-0.4 mm; Y axis: -1.8 mm; Z axis: -8.0 mm) and SON (X: +/- 1.6 mm; Y: -1.4 mm; Z; -9.0 mm). Conducting the 15°-angled beads injections into the SON under rotation of the stereotax arm changed the coordinates accordingly (right hemisphere: X -4.44 mm; Y -1.3 mm; Z -8.48 mm). The injected volume for all viruses (rAAV and CAV2) was 300 nl (undiluted). Rhodamine conjugated Retrobeads (Lumafluor Inc., Durham, NC, USA; Katz et al., 1984) were diluted 1:1 with 1x PBS and injected in a 75 nl volume.

*Injection into the spinal cord.* Animals have been ainjected unilaterally by Cav2-Cre virus into SC lumbar segment (L4 or L5 spines). The same animal received injection of Cre-dependent EGFP expressing virus was bilaterally to the rat PVN. The tip of injection pipette for SC injection was lowered at the stereotaxic coordinates used in our vivo recording experiments. To localize the Cav2-Cre injection site in SC we co-injected virus with a low volume of latex retrobeads, which formed a dense fluorescent beads in the injection site.

**Fluorescence Immunohistochemistry**

After transcardial perfusion with 1x PBS and 4% PFA, rat brains and SC were vibratome sectioned (50 μm) and stained with combinations of various primary antibodies against GFP (detects Venus; Abcam; ab 13970 chicken; 1:10.000), OT- and VP-Neurophysin (1:300; mouse; provided by Harold Gainer; Ben-Barak et al., 1985), NeuN (Chemicon; MAB377 mouse; 1:1000), VGluT2 (Synaptic Systems; 135403 rabbit. 1:5000), dsRed (detects tdTomato; Clontech; 632496 rabbit; 1:1000); Fluorogold (Millipore; AB153 rabbit; 1:3000), Cre (1:1000; rabbit; provided by Prof. Günther Schütz, DKFZ), and c-Fos (Santa-Cruz, 1:1000; rabbit). To label spinal cord neurons for OT or NK1 receptors rabbit polyclonal antibodies against oxytocin receptor (OTR, 1:1000; from Dr. Robert C. Froemke lab) and NK1R (ThermoFisher, Temecula, 1: 5000) were used.

The signals were visualized with the following secondary antibodies, FITC-conjugated, CY3-conjugated or CY5-conjugated antibodies (Jackson Immuno-

Research Laboratories, Inc.; 1:500) or Alexa 350 and 594 (Invitrogen; 1:500 or 1:200, respectively). All images were acquired on a Zeiss Axio Imager M1 light or a Leica TCS SP5 confocal laser scanning microscope. Digitized images were analyzed using Fiji (NIMH, Bethesda, MD, USA) and Adobe Photoshop CS5 (Adobe, Mountain View, CA).

Degree of VGluT2 and EGFP-colocalization in the varicosities of spinally projecting PVN axons was assessed by counting EGFP-immunoreactive (ir) varicosities within the area of 400 square µm ca. 500 µm lateral to the central canal of SC (3 rats, 3 planes for each, 680±72 puncta per animal). Vast majority of EGFP-ir punctae (89 ± 7,4 %) were positive for VGluT2.

**Specificity of immunostaining with antibodies against OXTR**

***Cell cultures, cDNA constructs, transfections and immunofluorescent staining***

HEK293T cells were maintained in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum, 200 U/ml penicillin, 200 mg/ml streptomycin, 2 mM L-glutamine (all purchased from Sigma). Cells were transiently transfected with cDNAs encoding for the mouse WT-OTR tagged at its N-terminus with RLuc (cloned into the pRLuc Perkin Elmer vector) and with the human WT-OTR tagged at its C-terminus with EGFP (cloned into the pEGFP Clonetech vector). Cells were seeded in 6-weel plates on glass coverslips (6 x $10^5$ cells/cover slip), allowed to attach for 24 hours and then transfected with Turbofect (ThermoScientific). 48 hours after transfection cells were fixed for 20 minutes at room temperature

with 4% (w/v) paraformaldehyde and immediately processed for immunofluorescence.

For immunofluorescence, transfected cells were incubated with the 29300 antibody (Oxtr2; Froemle lab) diluted 1:100 in GDB buffer (20 mM sodium phosphate buffer, pH 7.4, 450 mM NaCl, 2% (w/v) gelatine, 10% (v/v) Triton X-100) + 0.2% (w/v) BSA, for 2.30 h at room temperature. The samples were then incubated for 1 h with secondary antibodies in GDB buffer (Alexa 488 goat anti-mouse and Alexa 555 goat anti rabbit from Molecular Probes). Glass cover slips were mounted on glass slides with Mowiol and analyzed under a confocal microscope LSM 510 Meta (Zeiss).

***Staining with OTR in the brainstem of WT and OTR KO mice***

Anesthetized animal with 1.5:0.5 mixture of ketamine-xylazine, were perfused with heparin-PBS until blood is flushed and perfuse for 10 min at room temperature (about 45 ml per mouse) with 4% paraformaldehyde (PFA) – PBS. After extractions of brains, they were postfixed for 2 hours-overnight at 4°C in 4% PFA and dehydrated in 30% sucrose for 18 – 24 hours at 4°C. Cryosections (thickness is 16 µm ) were collected on SuperFrost Plus glass slides and stored at -80$^0$C until immunostaining sprocedure. Briefly, sections were incubated PBS containing 0.2% v/v Triton X-100 and 5% v/v normal donkey serum and incubated with oxtr2 antibodies (dilution is 1:200) in 1% BSA on PBS for 24-48 h at 4C. Then sections were washed with PBS and incubated 1-2 h at RT with secondary Alexa–conjugated antibodies (dilution is 1:500). Finally, after washing in

PBS sections were stained with DAPI/Hoechst solution and coverslipped with Fluoromount.

**Alignment of antibodies sequences with sequences of mouse and rat OXTR**

Due to the fact that antibodies were generated against mouse OTR and the subject of our study was a rat, we compared sequences of two antibodies, oxtr1 (an N-terminal portion of the OTR; used in cell cultures) and oxtr2 (part of the 3rd intracellular loop of the OTR; used for staining in brain sections of mice and rats) with the rat OTR. Generation and initial testing of the antibodies has been described in Marlin et al., 2015.


The alignment of the OTR mouse and rat sequences and labelled in bold our target sequence for the antibodies against oxtr1 and oxtr 2 in the mouse sequence. Aminoacids with a * underneath are the same between the mouse and rat oxtr.

As can be seen, oxtr 1 has 22/25 aminoacids overlap (88%) between mouse and rat, and oxtr 2 has 27/28 overlap (96%). The proteins seem overall very similar with only 9 different aminoacids out of 388, and most of the changes conserve the main characteristic (polar/charged vs. hydrophobic).

### *In situ* Hybridization

*In situ* hybridization (ISH) was performed on 25-µm cryostat-cut coronal sections prepared from fresh-frozen mouse lumbar spinal cord L4/5 (male C57BL/6J, 19-20 weeks). Spinal cords were flushed out with PBS using a 23G needle and a 30 ml syringe, immediately frozen in Tissue-Tek O.C.T. compound and stored at -80 degrees Celsius. ISH was performed according to the manufacturer's instructions (Advanced Cell Diagnostics) for Fresh Frozen RNAscope Multiplex Fluorescent Assay. Spinal cord sections were (treated with positive and negative controls) and then examined for coexpression of OTR and NK1R using ACD designed target probes for OTR, NK1R and vGluT1/2/3, as well as the nuclear stain DAPI. *Z*-stacks were collected across a depth of 2 µm at 1 µm steps with an upright laser scanning microscope (LSM-710, Carl Zeiss) using a 63x-objective and keeping acquisition parameters constant.

### Characterization of Cell Type Specificity and Physiological Responsiveness of the Virally-Delivered VP Promoter

The specificity of marker expression in VP neurons infected by VP promoter rAAVs was analyzed for PVN and SON separately. Injection of

300 nl rAAV mix per hypothalamic nucleus (titer VP virus: 5.8 x $10^{13}$ genomic copies/ml) resulted after two week expression in a confined appearance of tdTomato signal (red) exclusively in Vasopressin neurons (elaborated by counterstaining for VP in blue; merged color turquoise; Figure S1A) and Venus signal (green) exclusively in OT neurons (blue; merged color magenta; Figure S1A). A subsequent quantification of the expression specificity (performed with the brighter marker Venus; viral titer 2.7 x $10^{13}$ genomic copies/ml) was performed in three naïve and three physiologically stimulated (two days water deprived) male rats (Table S1). Per rat and nucleus, Venus and VP positive neurons were counted (after immunohistochemical staining) on three sections of different rostro-caudal localization (Bregma levels PVN: -1.5, -1.8, -2.0 mm; SON: -1.1, -1.4, -1.7 mm). Results were calculated as percentage ± SEM. Preserved physiological responsiveness was assessed after two days water deprivation at the end of the two-week expression period. Expression differences are visualized as intensity differences of the intrinsic fluorescence at equal Bregma levels taken as single layer confocal microscopy pictures with identical microscope settings (Figure S1B).

**Translucent Brain Sample Preparation and Imaging**

Rats were injected unilaterally into the PVN with rAAV OT promoter-Venus and perfused four weeks later with 1x PBS and 4% PFA, followed by 15 hours postfixation in 4% PFA/PBS. Dehydration and optical clearing was performed at 30°C with increasing concentrations of *tert*-butanol in water (30%, 50%, 70%, 80%, 96%, 100%,100%; v/v, two days each step)

followed by incubation in clearing solution (BABB: 1 vol. benzyl alcohol + 2 vol. benzyl benzoate; cf. (Dodt et al., 2007)), for one week, with the pH of dehydration and clearing solutions adjusted to 9.5 with triethylamine (Schwarz et al., 2015). Venus fluorescence (excitation: 514 nm; detection: 520-550 nm) was recorded from the uncut translucent brain on a custom-built light sheet microscope controlled by custom acquisition software based on Labview 8.6 (Schwarz et al., manuscript submitted) as xy mosaics of z stacks (xyz voxel size: 1.613 x 1.613 x 3.226 microns). Individual image planes were saved as 16bit TIFF files. Mosaic tiles were stitched with a plugin written for ImageJ/Fiji (Niedworok et al., 2012; www.fiji.sc). For analysis of PVN-SON connectivity, a gamma function (gamma=0.125) was applied to the resulting data stacks to enhance dim signals, followed by conversion to 8bit TIFF format: After another conversion to Knossos cube format, axons were traced with Knossos software (Helmstaedter et al., 2011).

**Electron Microscopy**

Rats were perfused transcardially with 4% PFA in phosphate buffer containing 0.05% glutaraldehyde at pH7. The 50 µm coronal brain sections containing the SON were incubated with rabbit polyclonal anti-GFP antibodies (Molecular Probes; 1:5000). The GFP signals were visualized using the standard avidin-biotin complex (ABC) protocol and DAB chromogen. Sections were further intensified by silver-gold in accordance with Liposits and colleagues (1986).

**Fluorogold**

Rats received an i.p. injection of 4% Fluorogold in 0.9% NaCl (20 mg/kg body weight) to label all neuroendocrine cells protruding beyond the blood-brain-barrier (most importantly, magnOT neurons). Ten days later, rat brains were transcardially perfused, sectioned and stained as described above. The Fluorogold (own emission: 530-600 nm upon UV excitation) was visualized with Alexa 350 (Invitrogen; 1:500) to shift the detectable emission to ~440 nm (microscope requirement).

**Counting of ParvOT Neurons Projecting to the SON**

Three weeks after injection of CAV2-GFP virus into the SON, the total number of GFP-positive neurons within the PVN was counted in six sections (section thickness 50 µm), containing the entire PVN in three rats. Another three rats received i.p. injection of Fluorogold (FG, see above) three weeks after injection of CAV2-GFP virus and were killed three weeks later. Three sections containing rostral, middle and caudal part of the PVN were stained for GFP, OT and FG and the total number of $GFP^+/OT^-$, $GFP^+/OT^+$, $GFP^+/OT^+/FG^-$ and $GFP^+/OT^+/FG^-$ was counted (see Table S2). In similar manner we counted the number of back labeled OT neurons, using CAV2-Cre virus injection in the SON and Cre-dependent rAAV, expressing GFP under the control of OT promoter in the PVN. Using combination of two viruses we achieve expression of GFP exclusively in OT PVN neurons.

**Surgical Procedure for Catheter and Optic Fiber Implantations**

Implantation of catheters in the jugular vein was performed under isoflurane anesthesia (2% in pure oxygen at a flow rate of 1 l/min). The jugular vein was exposed and a small incision was made in the vessel with iridectomy scissors. The catheter (PE50, Warner Instruments, Hamden, USA), filled with saline and soaked at the tip in heparin (Heparin-Natrium Braun, 10.000 IU/ml), was inserted into the vein and ligated to the vessel. The wounds were loosely closed with a suture (Silkam 4-0, Unodis, Haguenau, France).

For *in vivo* behavioral experiments, we used a blue laser (λ 473nm, output of 100 mW/mm², DreamLasers, Shanghai, China) coupled with optical fibers (BFL37-200-CUSTOM, EndA=FC/PC, and EndB=Ceramic Ferrule; ThorLabs, Newton, New Jersey; final light intensity ~ 10 mW/mm², 30 Hz, 10 ms pulses, 20 s duration), which were connected to a chronically implanted optic fiber to target the PVN (CFMC12L10, Thorlabs). Optic fibers were chronically implanted under isoflurane anesthesia (4% induction, 2% maintenance) at stereotaxic positions of -1.8 mm antero-posterior and 3 mm lateral from Bregma with a 21° angle to avoid previous injection scars and were stabilized with dental cement. This should lead to a specific stimulation of the PVN, as prevalent measurements with blue laser stimulations in rodent brain have shown that the blue light of the laser does not penetrate the tissue further than 500 µm (Yizhar et al., 2011).

**OT blood concentration**

For measurement of OT concentrations, blood samples were obtained using a catheter in the femoral artery implanted under isoflurane anesthesia. The femoral artery was exposed, and a small incision was made with iridectomy scissors. The catheter (PE50, Warner Instruments, Hamden, USA) filled with saline and soaked at the tip with heparin was inserted into the artery and ligated to the vessel. A first sample of around 500 µl of blood was made before the BL stimulation. Then BL was applied for 20 seconds. Three other blood samples were made: just after BL stimulation, 2 min and 30 min after BL stimulation. Blood samples were centrifugated at 4°C, 3000 rpm for 15 min, the plasma was collected and stored at -80°C.

*Plasma preparation for LC-MS/MS analysis*

5,76 pmole of D5-oxytocine internal standard was added to 50 µl of lithium heparin plasma. Plasma was acidified up to 1 % $H_3PO_4$ (v/v) in a final volum of 400 µ l and was centrifuged (14,000 x g, 5min). The resulting supernatant was collected prior to solid phase extraction (SPE). SPE procedure was performed with a positive pressure manifold (Thermo Electron). OASIS HLB SPE-cartridges (1 cc, 30 mg, Waters, Guyancourt France) first activated with 1 ml of acetonitrile (ACN) and then washed with 1 ml of $H_2O$ 99 % / $H_3PO_4$ 1 % (v/v). The sample was loaded and the SPE-cartridge was washed with 1 ml of $H_2O$ 99 % / $H_3PO_4$ 1 %. After a 2 ml wash with $H_2O$/formic acid 0.1 % (v/v) and with 1 ml of ACN 5 % / $H_2O$ 94.1 % / formic acid 0.1 % (v/v/v), elution was performed with 500 µl of

acetonitrile 60% / $H_2O$ 40% (v/v). Eluates were collected and dried under vacuum prior to mass-spectrometry (MS) analysis (see below).

*LC-MS/MS instrumentation and analytical conditions* - LC-analyses were used to determine the presence of oxytocin in the selected reaction monitoring mode (SRM). Analyses were performed on a Dionex Ultimate 3000 HPLC system (Thermo Scientific, San Jose, CA, USA) coupled with a triple quadrupole Endura (Thermo Scientific). The system was controlled by Xcalibur v. 2.0 software (Thermo Scientific).

Extracted plasma samples were solubilised in 100 µl of $H_2O$/formic acid 0.1 % (v/v) and 20 µl of the solution were loaded into an Accucore RP-MS column (ref 17626-102130; 100 x 2.1 mm 2.6 µm, Thermo Electron) heated at 35 °C. Oxytocin and D5-oxytocin elutions were performed by applying linear gradient of buffers A/B. Buffer A corresponded to $H_2O$ 99.9 % / formic acid 0.1 % (v/v), whereas buffer B was ACN 99.9 % / formic acid 0.1 % (v/v). A linear gradient of 20 % - 95 % of solvent B at 400 µL / min over 2.5 min was applied followed by a washing step (0.5 min at 85 % of solvent B) and an equilibration step (1 min of 20 % of buffer B).

Qualitative analysis and quantification were performed in SRM using an Endura triple quadrupole mass spectrometer and deuterated internal standards. For ionization, 3500 V of liquid junction voltage and 292 °C capillary temperature were applied. The selectivity for both Q1 and Q3 was set to 0.7 Da (FWHM). The collision gas pressure of Q2 was set at 2 mTorr of argon. For oxytocin and D5-oxytocin, the selection of the monitored

transitions and the optimization of the collision energy were preliminary and manually determined. The transitions and the corresponding collision energies (CE) used for SRM were the following: m/z 504.3 → m/z 86.3 (CE = 39 eV), m/z 504.3 → m/z 285.2 (CE = 30 eV) for oxytocin with 2 charges, and m/z 506.7 → m/z 86.4 (CE= 32 eV), m/z 506.7 → m/z 290.3 (CE= 26 eV) for D5-oxytocin (with 2 charges). Identification of the compounds was based on precursor ion (with 2 charges), selective fragment ions and retention times obtained for oxytocin and D5-oxytocin internal standard. Quantification of oxytocin was done using the ratio of daughter ions response areas of the D5-oxytocin.

**Determination of the Phase of Estrous Cycle**

To test if the estrous cycle could modify the answer of WDR neurons to BL, we analyzed the estrous phase by using vaginal smear cytology (Marcondes et al., 2002). At the end of the *in vivo* recording experiment, a micropipette filled with 100 µL of saline solution (NaCl 0.9%) was placed in the rat vagina. Cells were removed and dissociated by pipetting up and down for at least three times. A drop of the smear was placed on a glass slide and observed by a light microscope with a 40x or a 100x objective lens. Estrous phase was determined based on the proportion of leukocytes, nucleated epithelial cells and anucleate cornified cells.

**Pharmacological inactivation of OT neurons: Verification of the DREADD technique**

*In vitro electrophysiological recording*

To test effects of CNO *in vitro*, we used rats that had been infected with a combination of CAV2-Cre (SON) and Cre-dependent virus, expressing hM4Di under the control of OT promoter. 6-8 weeks after infection coronal slices were prepared from the paraventricular nucleus and fluorescent neurons (indicative of the expression of the virus) were selected for whole-cell patch-clamp recordings. After establishing the clamp, neurons were either recorded in current or voltage clamp mode.

In current clamp mode, cells were subjected to a hyperpolarizing current injection (bringing the membrane potential close to -100 mV), followed by progressively more depolarizing current injections (see Figure 2B1) which triggered changes in membrane potential characteristic of parvocellular neurons (as shown previously in Figure 2) and causing progressively more action potentials. Application of CNO led to a decrease in input resistance as a result of the opening of inward rectifying potassium channel leading to a decrease in number of action potentials (see example traces in left part figure S7A1, before CNO in black, during CNO in red). To quantify the effects of CNO, the number of action potentials were counted at one specific current injection (shown in green) and averaged over several neurons, plotted in the right part of figure S7.

In voltage clamp mode, cells were subjected to a 500 ms voltage ramps starting from -125 going up to +25 mV. The voltage ramp and an example of the resultant current are plotted in figure S7A2 (left part, resp lower and

upper panels). As can be observed, application of CNO caused an increase in the ramp evoked current (in red) both at negative and positive potential, indicating the opening of inward rectifying potassium channels. The currents and changes in membrane resistance averaged over several neurons are plotted in the right parts of figure S7A2 (resp. upper and lower panels).

Taken together, these experiments show the inhibitory effects of CNO application on the excitability of virus-infected neurons as a result of the activation of inward rectifying potassium channels.

*In vivo optoelectrode recording*

Animals were anesthetized in stereotaxic frame by 1.5% isofluran mixed with oxygen and $NO_2$. A mixture of AAVs, carrying either ChR2 or hM4Di driven by the OT promoter was unilaterally injected into the PVN. (Begma -1.8 Lateral -0.3 Deep 8.0). Six screws (two with ground wires) were drilled into skull around the injection site together with dental cement (Tetric Evoflow) were used to ankle the base for electrode implantation. After this procedure, electrodes with assembled optic fiber implanted 0.5 mm above the PVN. After 2 week recovery the tetrodes were gradually lowered into the PVN under laser stimulation (100 Hz, 5 ms, 10 sys, 40 mW) until an OT neuron was identified by PSTH. CNO (1mg/kg) was injected i.p. and recording of OT cells was continued during 30 min.

Eight custome made nickel tetrodes were arranged in order, surrounded by the optic fiber (Thor Lab, Φ200um) and assembled in the 32-channel microdrive. Tetrodes impedance was measured between 0.3mΩ-0.5mΩ before the surgery. Data acquire system was built on OpenEphys Acquisition board (v2.2), head stage connected with 32-channel preamplifier and Ultra thin SPI cable (Intan Tech) with sampling rate at 30k Hz, low-pass filter set to 600 Hz, high-pass filter 6000 Hz in OpenEphys GUI. Laser stimulation was triggered by waveform generator 33220A (Keysight Tech) and Laser generator BL473T5-200FC (Shanghai Laser & Optics Century Co., Ltd); wave length 473nm, output power 245mW, which together generate 450 nm blue light stimulation at programed pattern. Additional I/O board allows synchronized digital signal of optogenetic stimulation in data acquire board. Data analysis: OpenEphys GUI 0.3.5 is used in online data analyse. Matlab, Simpleclust 0.5, and Neuroexplorer 3.2 are used in offline data sorting and analyses.

### *In vitro* Electrophysiology

*Slice Preparation*. Four-to-eight weeks after injection of virus in adult rats, brains were removed, the hypothalamus or lumbar spinal cord was isolated, cut into 400 μm coronal slices, and kept in artificial cerebrospinal fluid (ACSF, consisting of 118 mM NaCl, 25 mM NaHCO3, 10 mM glucose, 2 mM KCl, 2 mM MgCl2*6H2O, 2 mM CaCl2*2H2O, 1.2 mM NaH2PO4) saturated with oxycarbon gas (95%O2, 5%CO2). Fluorescent cells were identified by fluorescence microscopy in combination with oblique illuminated infrared video microscopy.

*Electrophysiological Recordings.* Whole-cell patch-clamp recordings were visually guided by infrared oblique light videomicroscopy (DM-LFS; Leica), using 4–9 MΩ borosilicate pipettes filled with 140 mM KMeSO4, 10 mM HEPES, 2 mM MgCl2, 0.1 mM CaCl2, 0.1 mM BAPTA, 2 mM ATP Na salt, 0.3 mM GTP Na salt (pH 7.3), adjusted to 300 mOsm, and amplified with an Axopatch 200B (Axon Instruments). Data were lowpass filtered at 5 kHz with the amplifier and sampled at 10 kHz using pClamp 10 data-acquisition and analysis software (Axon Instruments). The liquid junction potential (12 mV) was corrected for according to Neher (1992).

*Spinal cord recording and WDR depolarization protocol.* Lamina V-VI and around the central canal putative WDR neurons were recorded based on the visualization of their large body size (~30µm) and further electrophysiologically identified through their increasing firing rate with the intensity of stimulation. In current-clamp mode, the membrane potential was adjusted at -60mV. Firing profiles were then characterized using current step injections (from -80 to 200pA, 10pA increments for 900ms, repeated every 20s). This characterization was performed 5-10min after the beginning of the recording in order to ensure stable recordings and optimal dialysis of the intracellular compartment. To investigate the possible role of washout on cell firing properties, some neurons were recorded for more than an hour and no significant changes in the firing profiles (spike frequency and spike amplitude), passive membrane properties and resting membrane potential were noted indicating that the intracellular dialysis with our experimental intracellular solution had no effect on the passive and active membrane properties of deep

laminae neurons (not shown). Neurons were kept for analysis only if they displayed stable resting membrane potentials more negative than -50mV and proper spike overshoots (>15mV).

*Hypothalamus recording and electorphysiological identification of parvOT vs magnOT.* In order to distinguish between the parvocellular and the magnocellular neurons from the PVN we used a current step protocol which has been previously shown to reliably allow identification of this type of cells. Initially described by Tasker and Dudek (Tasker and Dudek, 1991), this method has been used in several other studies in order to allow discrimination between parvocellular and the magnocellular neurons (Brisson et al., 2013; Chu et al., 2013; Luther and Tasker, 2000; Yuill et al., 2007). Passive and active electrophysiological parameters of neurons were analyzed using Clampfit 10.0 (Axon Instruments, USA). The "rise slope" was analyzed using the same fraction of the trace as for the spike delay, that is, from the start of the depolarizing step until the beginning of the first action potential.

## ***In vivo*** **Electrophysiology**

*Single-unit Extracellular Recording of Dorsal Horn Spinal Neurons in vivo*

Adult Wistar rats were anesthetized with 4% isoflurane in pure oxygen and a laminectomy was performed to expose the L4-L5 SC segments. During the procedure, the isoflurane level was reduced to 2%. The animal was then placed in a stereotaxic frame with the L4-L5 region being held by two clamps

placed on the apophysis of the rostral and caudal intact vertebras. The dura mater was removed and the SC covered with a thin oil layer. To record wide-dynamic-range neurons (WDR), a stainless electrode (FHC, USA; UE(FK1)) was lowered into the medial part of the dorsal horn of the SC, at a depth of around 500 -1100 µm from the dorsal surface (see Figure S8 for localization of recorded WDR). We recorded WDR neurons of lamina V, receiving both non-noxious and noxious information from the ipsilateral hind paw. A few superficial layers (I-II) neurons were also recorded (Figure S8F). Voltage changes were amplified and filtered (low 300 Hz, High 3000 Hz) by a DAM-80 differential amplifier (WPI, Aston, UK). Recordings were analyzed using the spike 2 software, collecting data through a CED 1404 analog-to-digital interface (CED, Cambridge, UK). The stimulating thresholds to evoke action potential, resulting from the stimulation of the peripheral receptive fields on the rat hindpaw, were attributed to the recruitment of A- and C- type sensory fibers based on their latency from the stimulus artifact as follows: Aβ- 20 ms, Aδ- 20-90 ms, C- 90-300 ms and post discharge 300-800 ms.

*Single-unit Extracellular Recording of PVN Neurons in vivo*

Adult Wistar rats were anaesthetized with 4% isoflurane and the animal was placed in a stereotaxic frame. During the procedure, the isoflurane level was reduced to 2%. Then a stainless steel electrode coupled with an optical fiber was inserted into the PVN in order to stimulate parvOT neurons expressing ChR2 and record their activity. Hardware and software were the same as previously mentioned. To confirm that the activation of SON neurons was OTR

related, we stereotaxically infused 0.5 µl / 2 min of dOVT 1 µM, or NBQX 1 µM directly in the SON and repeated the recording session.

*Wind-up Protocol*

We measured the WDR action potential firing responses induced by a stimulation of the hindpaw inducing a short-term plasticity named Wind-up (1 ms pulse duration, frequency 1 Hz, intensity corresponding to 3 times the C-fiber threshold). Wind-up (WU) is a property of WDR neurons, which answer by an increased firing when they are stimulated repeatedly at specific intensities and frequencies (Mendell and Wall, 1965; Schouenborg, 1984). As WU is dependent on C-fiber activation, it can be used as a tool to assess nociceptive information in the SC and OT antinociceptive properties.

**Nociceptive Behavioral Tests**

*Mechanical Allodynia*

In all experiments, we used a calibrated forceps (Bioseb, Chaville, France) previously developed in our laboratory to test the animal mechanical sensitivity (Luis-Delgado et al. 2006). Briefly, the habituated rat was loosely restrained with a towel masking the eyes in order to limit stress by environmental stimulations. The tips of the forceps were placed at each side of the paw and a graduate force is applied. The pressure producing a withdrawal of the paw, or in some rare cases vocalization, corresponded to the nociceptive threshold value. This manipulation was performed three times for each hind paw and the values were averaged.

*Thermal Allodynia/Hyperalgesia*

To reveal the animals' heat sensitivity, we used the Plantar test using the Hargreaves method (Ugo Basile, Comerio, Italy). We compared the response of each hindpaw (Hargreaves et al. 1988) when testing healthy animals and animals having received unilateral intraplantar CFA injection (Sigma-Aldrich, 100 µl in the right hindpaw). Exposed to a radiant heat, the latency time of paw withdrawal was measured three times per hind paw and the values were averaged.

**Optic Stimulation of OT Neurons**

For optic stimulation of OT release at fibers descending parvOT PVN cells, ChR2 was expressed from Cre-dependent rAAV (CAG-DIO-ChR2-mCherry; obtained from Deisseroth Lab). Cre was retrogradely transported to respective PVN neurons from the CAV2-Cre injected SON site. Optical stimulation was provided using a blue laser (λ 473 nm, output of 100 mW/mm², DreamLasers, Shanghai, China) coupled with optical fibers (BFL37-200-CUSTOM, EndA = FC/PC, and EndB = Flat cleave; ThorLabs, Newton, New Jersey) to activate ChR2-containing neurons at the level of SON, SC or the PVN as described below.

For the SON-BL experiment, the optical fiber was lowered in the SON together with a stainless steel electrode (FHC, USA; UE(FK1) to activate the axons of PVN-OT neurons projecting to the SON. We recorded WDR neurons in the SC during the following protocol: 50 s WU, 20 s SON-BL (10 ms pulses at 30 Hz, ~ 10 mW/mm²) plus WU, 50 s WU, for a total of 130s recording session.

Recover of the control WU was tested every five min after the end of the first recording session. In order to confirm that the reduction in WU intensity was related to an OT release in the blood circulation and acting on peripheral OTR, we injected intravenously 100 µl of 1 µM dOVT (d(CH2)5-Tyr(Me)-[Orn8]-vasotocine; Bachem, Weil am Rhein, Germany), and repeated the stimulation protocol after 15 min.

For the SC-BL experiment, the optical fiber was placed on the surface of the SC in order to activate the fibers of the parvOT PVN neurons that express ChR2 (hence projecting both to the SON and the SC), a few millimeters over the tungsten electrode. BL was applied in 10 ms pulses at 30 Hz. We recorded WDR neurons in the SC during the following protocol: 20 s WU, 20 s SC-BL (10 ms pulses at 30 Hz, ~ 10mW / mm²) plus WU, 20 s WU, for a total of 60 s recording session. Recovery of the control WU was tested every five min after the end of the first recording session. To confirm that the reduction in WU intensity was OTR related, we infused 500 µl of dOVT 1 µM, $AV_{1A}R$ 1 µM (Phenylacetyl$^{1}$,O-Me-d-Tyr$^{2}$,Arg$^{6,8}$,Lys$^{9}$]-vasopressin amide; $AV_{1A}R$: Sigma Aldrich, Saint-Louis, France) or NBQX 1 µM directly on the SC for 20 minutes and repeated the recording session. For the superficial neurons, which do not express WU, we used repeated hindpaw stimulation (1 ms pulse duration) at a frequency of 0.5 Hz and an intensity corresponding to three times the threshold for C-type sensory fiber activation. We used the following protocol: five hindpaw stimulations alone, five stimulations under BL, and five stimulations without BL.

**Statistical Analysis**

Friedman test with Dunn post-hoc test was used to compare the average level of blood OT in function of time after SON-BL. Two-way repeated ANOVA was used for the assessment of BL effects on WU efficacy. Wilcoxon's test was used to compare the average variation of spikes frequencies measured for the same neurons in control, BL, and antagonists + BL conditions. Kruskal and Wallis test was used to compare the average variation of spikes frequencies measured for different neurons in control and BL conditions. One-way ANOVA, followed by multiple comparison post-hoc Tukey test, was used to analyze behavioral data. Differences were considered significant for $p < 0.05$.

**RRIDs**

| Compound | Compagny | Proper citation |
|---|---|---|
| GFP chicken | Abcam; ab 13970 | (Abcam Cat# ab13970, RRID:AB_300798) |
| VP-Neurophysin mouse | Harold Gainer | |
| NeuN mouse | Chemicon; MAB377 | (Millipore Cat# A60, RRID:AB_2314889) |
| VGluT2 rabbit | Synaptic Systems | (Synaptic Systems Cat# 135 103, RRID:AB_2315570) |
| dsRed rabbit | Clontech; 632496 | (Clontech Laboratories, Inc. Cat# 632496, RRID:AB_10015246) |
| Fluorogold rabbit | Millipore AB153 | (Millipore Cat# AB153, RRID:AB_90738) |
| Cre rabbit | Schutz | |
| c-Fos rabbit | Santa-Cruz AB_2106765 | (Santa Cruz Biotechnology Cat# sc-7202, RRID:AB_2106765) |
| NK1R rabbit | Thermo Fisher AB_1958926 | (Thermo Fisher Scientific Cat# PA1-32236, RRID:AB_1958926) |
| Wistar rats | | RGD:7241799 |

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# J) Article 2

## I) Overview

**Introduction.** OT is a hypothalamic peptide with multiple functions within the CNS and in the periphery. It is well known for its roles around birth, during parturition, lactation, but also in the modulation of maternal behaviour and attachment between mother and child. Moreover, OT has strong analgesic effects, by a direct action on spinal cord neurons and a peripheral release, targeting preferentially DRG C-type sensory neurons. OT analgesia occurs under inflammatory or neuropathic insults, as well as in response to a strong stressor. In the newborn, OT analgesia during parturition was suggested to be protective for brain function. A deficit in OT signalling has recently been proposed to explain neurodevelopmental brain pathologies such as autism spectrum disorders. In the context of pain, the development of the nociceptive system starts before birth but continue to mature during the post-natal period, both in human and rodents. This includes the descending controls of pain that are functional 3 weeks after birth in the rat. These developmental processes make the nociceptive and OT system vulnerable to early life adversity. Maternal separation is a well-established model of ELS, known to impact the HPA axis, social and cognitive behaviours and sensory systems, associated with epigenetic changes among different brain circuits. We tested if MS induced a dysfunction in pain responses and OT analgesia, and if neonatal OT treatment could be efficient to counteract the deleterious effects of MS.

**Main results.** Assessment of nociceptive sensitivities using the calibrated forceps and Hargreaves method reveals that MS induces mechanical and thermal hypersensitivity at adulthood. The efficiency of OT analgesia was assessed using two models, the first one is a model of OT-related SIA induced by a forced swim stress procedure (FSS), and the second one is an inflammatory pain model. MS induces a loss of efficient OT analgesia after FSS. Carrageenan inflammation induces both a mechanical and a thermal hypersensitivity in both MS and control rats. However, compared to control animals, MS rats display a longer lasting thermal hyperalgesia after carrageenan inflammation. This model is known to recruit the OT system and induce a release of OT in the spinal cord in control animals. We confirmed that using an antagonist of OTR to reveal the existence of an endogenous OT analgesic tone at the spinal level. OTR i.t injection induced a rebound of hyperalgesia in control animals but had no effect in MS animals. We concluded that OT analgesia in inflammatory conditions is altered by MS, which was further confirmed using in vivo electrophysiological recordings of spinal cord neurons. Then we wanted to identify if the alteration of OT analgesia arises from a spinal or a supraspinal dysfunction. In that purpose we assessed OTR expression in the spinal cord, which was similar between MS and control animals. We demonstrated that these receptors are functional and that their activation can induce an efficient analgesia. This lead us to the hypothesis that OT analgesia deficiency might rely on supraspinal alterations. On the second part of the study, we tried to counteract the effect of MS on pain responses by a repeated neonatal treatment by OT, allopregnanolone or an HDAC inhibitor SAHA during the separation procedure. We successfully restored normal baseline sensitivities and efficient OT-related SIA with all treatments. Only SAHA treatment restored OT anti-hyperalgesia after carrageenan inflammation.

In conclusion, MS has long-term negative effects on nociceptive responses, inducing an overall nociceptive hypersensitivity, associated with dysfunction in the OT descending control of pain. Mother-pups interactions and a fine regulation of OT levels during the neonatal period seem to be critical for the proper development of nociceptive circuits and subsequent behaviours.

**Author contribution.** I conducted all the experiments and analysis in this study, except for PCR analysis.

PAIN

# Pharmacological rescue of nociceptive hypersensitivity and oxytocin analgesia impairment in a rat model of neonatal maternal separation

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| **Abstract:** | Oxytocin (OT), known for its neurohormonal effects around birth, has recently been suggested for being a critical determinant in neurodevelopmental disorders. This hypothalamic neuropeptide exerts a potent analgesic effect through an action on the nociceptive system. This endogenous control of pain has an important adaptive value but might be altered by early life stress (ELS), possibly contributing to its long-term consequences on pain responses and associated comorbidities. We tested this hypothesis using a rat model of neonatal maternal separation (MS) known to induce long term consequences on several brain functions including chronic stress, anxiety, altered social behavior and visceral hypersensitivity. We found that adult rats with a history of MS were hypersensitive to noxious mechanical/thermal hot stimuli and to inflammatory pain. We failed to observe OT receptor-mediated stress-induced analgesia as well as OT anti-hyperalgesia after carrageenan inflammation. These alterations were partially rescued if MS pups were treated by intraperitoneal daily injection during MS with OT or its downstream second messenger allopregnanolone. The involvement of epigenetic changes in these alterations was confirmed since neonatal treatment with the HDAC inhibitor SAHA, not only normalized nociceptive sensitivities, but also restored OT receptor-mediated stress-induced analgesia as well as the endogenous anti-hyperalgesia in inflamed MS rats. There is growing evidence in the literature that ELS might impair the nociceptive system ontogeny and function. This study suggests that these alterations might be restored while stimulating OT receptor signaling or HDAC inhibitors, using molecules that are currently available or part of clinical trials for other pathologies. |

# Pharmacological rescue of nociceptive hypersensitivity and oxytocin analgesia impairment in a rat model of neonatal maternal separation

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**ABSTRACT**

Oxytocin (OT), known for its neurohormonal effects around birth, has recently been suggested for being a critical determinant in neurodevelopmental disorders. This hypothalamic neuropeptide exerts a potent analgesic effect through an action on the nociceptive system. This endogenous control of pain has an important adaptive value but might be altered by early life stress (ELS), possibly contributing to its long-term consequences on pain responses and associated comorbidities. We tested this hypothesis using a rat model of neonatal maternal separation (MS) known to induce long term consequences on several brain functions including chronic stress, anxiety, altered social behavior and visceral hypersensitivity. We found that adult rats with a history of MS were hypersensitive to noxious mechanical/thermal hot stimuli and to inflammatory pain. We failed to observe OT receptor-mediated stress-induced analgesia as well as OT anti-hyperalgesia after carrageenan inflammation. These alterations were partially rescued if MS pups were treated by intraperitoneal daily injection during MS with OT or its downstream second messenger allopregnanolone. The involvement of epigenetic changes in these alterations was confirmed since neonatal treatment with the HDAC inhibitor SAHA, not only normalized nociceptive sensitivities, but also restored OT receptor-mediated stress-induced analgesia as well as the endogenous anti-hyperalgesia in inflamed MS rats. There is growing evidence in the literature that ELS might impair the nociceptive system ontogeny and function. This study suggests that these alterations might be restored while stimulating OT receptor signaling or HDAC inhibitors, using molecules that are currently available or part of clinical trials for other pathologies.

## INTRODUCTION

Oxytocin (OT) is a nonapeptide synthesized by hypothalamic neurons mainly located in the paraventricular (PVN) and supraoptic (SON) nuclei [48]. OT acts both as a neurohormone when released into the blood via the posterior pituitary or as a neurotransmitter when synaptically released into spinal cord (SC) and supraspinal regions. Recently, our laboratory identified a small population of 30 parvocellular neurons in the PVN which coordinate the peripheral and spinal release of OT to limit inflammatory pain symptoms [14]. Indeed parvocellular OT neurons of the PVN trigger the OT secretion in superficial and autonomic layers of the SC where OT receptors are found [48]. OT displays anti-nociceptive properties by increasing GABAergic inhibition in the SC thus decreasing excitability in superficial and deep SC neurons [48] In addition to these central-related effects, a peripheral component of OT-mediated analgesia via a direct effect at the dorsal root ganglion (DRG) level has been documented [42].

Activation of the OT analgesic system occurs under different conditions such as inflammatory sensitization [14], neuropathic pain [35], or forced swim stress (FSS) [64]. In the carrageenan model of inflammatory sensitization, spinal OT levels are increased for at least 24 h and OT receptor-mediated local production of the neurosteroid allopregnanolone (AP) leads to a sustained GABAergic activity to maintain spinal antinociceptive control [25]. After a non-noxious FSS, OT is also responsible for significant and transient stress-induced analgesia (SIA) [64]. In this context, a deficit in OT signaling might be related to neurodevelopmental brain pathologies such as autism spectrum disorders [39,66].

The development of the nociceptive system starts before birth and follows a similar pattern in the rat and in the human fetus [55,57]. It is well accepted that the developmental stage of the rat just after birth is similar to that of the human fetus at the beginning of the 3$^{rd}$ trimester of gestation. Descending controls of pain mature during the first 3 weeks after birth in the rat[17] and begin to mature during the end of the 3$^{rd}$ semester in humans [20]. At the SC level, spinal inputs and chloride homeostasis undergo re-organization steps after birth to be fully matured [3,4] making them possibly vulnerable to early adverse events. Neonatal maternal separation (MS) is a well-established model of early life stress (ELS) that induces long term neuroendocrine and behavioral changes in rodents, with impact on the HPA axis, social and cognitive behaviors, and sensory and nociceptive systems [27,45]. Long-term consequences of MS are indeed visceral hypersensitivity

[56], epigenetic changes, and associated sensory dysfunctions [41,59]. Recently, a chronic adult treatment with histone deacetylase (HDAC) inhibitor was shown to be efficient to counteract MS-induced visceral hypersensitivity [41].

In this study, we used behavioral measures and *in vivo* electrophysiological recordings from SC dorsal horn neurons to characterize the long-term consequences of MS on the oxytocinergic descending control of pain. Then, we aimed to prevent the development of MS pain phenotype using pharmacological tools, targeting the OT system or epigenetic factors early during the postnatal period.

## MATERIALS AND METHODS

### Animal care and neonatal maternal deprivation procedure

Female Wistar rats with litters were housed in a temperature-controlled room (22°C) under a 12h light-dark cycle (lights on at 07:00 AM), with *ad libitum* access to food and water. All experiments were conducted in conformity with the recommendations of the European Committee Council Direction of September 22, 2010 (2010/63/EU), with an authorization for animal experimentation from the French Department of Agriculture (License 67-116 to PP) and with a procedure agreement evaluated by the regional ethical committee (CREMEAS authorization number APAFIS#7678-20 16112223027528 v2).

At birth, litters were randomly assigned to two groups: Neonatal MS and non-separated control litters. The litters allocated to the MS group were removed from the nest cages 3 h per day from postnatal days 2 (P2) to 12 (P12) and placed on a heating pad (37°C) in a separate cage. The litters allocated to the control group remained with their mother in their home cages during the entire MS interval and received no special handling other than that necessary to change the bedding of their cage. Pups were weaned at P21 and housed four rats per cage before being randomly chosen at 8 weeks or more for behavioral analysis or electrophysiological recordings.

### Measure of basal mechanical and thermal nociception

Mechanical nociception was measured on adult rats with a calibrated forceps (Bioseb, Vitrolles, France) as previously described [33]. Briefly, the habituated rat was loosely restrained with a towel masking the eyes in order to limit stress by environmental stimulations. The tips of the forceps were placed at each side of the paw and a gradually increasing force was applied. The pressure producing withdrawal of the paw corresponded to the nociceptive threshold value. Thermal nociception was measured by a blind experimenter with the Hargreaves method, as previously described [19]. Briefly, rats were placed in plexiglas boxes (20cm x 25cm x 45cm) for a 15-20 minutes habituation period and then hindpaws were exposed to a radiant heat and the latency time required to induce paw withdrawal was recorded and considered as the nociceptive heat threshold. For both tests, measurements were performed three times for each hindpaw and values were averaged.

Forced swim stress

At adulthood, rats were exposed to a forced swim stress procedure (FSS) in a Plexiglas® cylinder (diameter: 30 cm; wall height: 60 cm), filled with water (height: 40 cm) at a temperature of 20°C for 10 minutes. After FSS exposure, animals were gently dried. Mechanical thresholds were measured before (baseline) and 15 min after FSS in both MS and control animals. This protocol has been described to be associated with a plasmatic and spinal release of oxytocin [64]. The oxytocinergic contribution of FSS to SIA was confirmed in a subset of rats receiving an intrathecal (i.t) injection of 10µL of OTR antagonist dOVT ((d($CH_2$)$_5^1$,Tyr(Me)$^2$,Thr$^4$,Orn$^8$,des-Gly-$NH_2^9$)-Vasotocin, Bachem, Germany) at 200µM or 10µL saline 30 minutes before FSS.

Carrageenan Inflammatory pain model

To induce a painful inflammatory sensitization in the rat, 150µL of carrageenan (3% in NaCl 0.9%, Sigma Aldrich, Germany) were injected in the plantar surface of the right hindpaw for behavioral experiments, whereas injections in both hindpaws were performed for electrophysiological. Control animals received either similar injections of NaCl 0.9% or no injection.

In vivo recording of spinal cord neurons and analysis

Male and female rats were anaesthetized with isoflurane (2 %; pushed by pressurized air at a flow rate of 0.5 L.min$^{-1}$) and body temperature was regulated using a thermostatically controlled heating blanket (Harvard Apparatus Ltd, France) maintaining the body temperature at 37°C. Animals were set up in a stereotaxic frame (La Précision Cinématographique, Eaubonne, France), with the cervical and sacral vertebrae firmly held. The lumbar SC (L4-L5) was exposed by laminectomy. After removal of the meninges, the SC surface was covered with a thin isolating layer of mineral oil. This opening allowed us to record SC neurons and apply several drugs directly on the SC. At the end of the experiment, animals were killed with an overdose of isoflurane.

Single unit extracellular in vivo recordings were made from wide dynamic range (WDR) neurons, located in lamina V and receiving peripheral sensory messages mediated by Aβ, Aδ and C-type primary afferent fibers. In the lumbar enlargement of the SC, we monitored voltage changes through stainless microelectrodes (FHC, Bowdoin, USA) connected to an extracellular differential amplifier (DAM-80, WPI, Aston, UK). Electrical signals were then digitized (Power 1401 CED,

Cambridge, UK), processed with the Spike 2 software (CED Cambridge, UK). Neurons were characterized by their responses to transcutaneous electrical stimulation of the hindpaw receptive field as previously described [26]. Electrical stimulation was applied through two thin cutaneous pin electrodes placed in the center of the receptive field on the hindpaw. Increasing intensities were applied from 1 V to C fiber activation threshold (duration of 1 ms). Trials consisted of a train of 90 electrical stimuli (3 x C-type fiber threshold) applied repetitively (1 Hz) to the excitatory receptive field. This method has been usually used to obtain a stable and reproducible "wind up" [37], a property of C-type nociceptive fibers. The electrically-evoked response of the neuron was captured and displayed as a post-stimulus histogram, for A-type and C-type discharge. Action potentials appearing early after the stimulus artifact were classified as A-type (0-50 ms) whereas the delayed response corresponded to C-type (50-800 ms).

Spinal Cord sampling and PCR analysis

Lumbar spinal cord samples were collected, reconstituted in a guanidine thiocyanate/β-mercaptoethanol preparation using ultraturax and stored at -80°c. Total RNA was extracted according to an adaptation of the phenol-chloroform method [11], followed by DNaseI treatment (TURBO DNaseTM, Ambion, Life technologies, Saint Aubin, France). 800ng RNA were used for reverse transcription with the RT iScript kit (Bio-Rad, Marnes-la-Coquette, France). Quantitative PCR was performed using SYBR Green Supermix (Bio-Rad), on the iQ5 Real Time PCR System (Bio-Rad). Amplifications were carried out in 42 cycles (20s at 95°C, 20s at 60°C and 20s at 72°C). Serial dilutions of samples were used to create a standard curve, and amplification data is shown as relative gene expression, calculated as the ratio between cDNA concentration of the gene of interest and that of the housekeeping gene. Preliminary experiments showed that hypoxanthin-guanine phosphoribosyltransferase (HPRT) transcripts were very stable between samples. Therefore, HPRT was selected as the housekeeping gene and amplified using the following primer set: 5'-GGTGAAAAGGACCTCTCGAA-3' (forward) and 5'-TCAAGGGCATATCCAACAACA-3' (reverse). Primer sequences were the following for OTR: 5'-TCGCCGTCTACATTGTACCG-3' (forward) and 5'-GCTGCCGTCTTGAGTCTCAG-3' (reverse). Samples were accurately dispensed in duplicates using a robotic workstation (Freedom EVO100, Tecan, Lyon, France), and amplification specificity was assessed by a melting curve study.

Neonatal rescue procedure

To reverse MS long term consequences on pain behavior, a pharmacological neonatal treatment was performed in MS pups. During MS protocol, all pups in the litter were injected with the same molecule and then tested at adulthood to investigate the long-term efficiency of neonatal treatment. OT (Bachem, Weil am Rhein, Germany), the HDAC inhibitor SAHA (Suberoylanilide hydroxamic acid, Vorinostat, ApexBio Technology, Houston, USA), or vehicle (NaCl, oil or 10% DMSO respectively) were injected intraperitoneally (i.p. 10µL) at 1mg/kg and 5mg/kg respectively once daily (o.d) during the MS procedure (*i.e* from P2 to P12). In another subset of animals, allopregnanolone (AP, Santa Cruz biotechnology, Dallas, USA) was injected subcutaneously at 10mg/kg once every two days during the MS procedure. To avoid unnecessary supplementary handling of the pups, injections were performed at the beginning of the separation process. Pups were then normally weaned at P21, and no special handling was performed until P45, when the efficiency of neonatal treatment was assessed for each neonatal group using behavioral testing. First, mechanical thresholds were measured prior (baseline) and after FSS (SIA measurement). Then one week later, the efficacy of the OT descending control of pain after carrageenan inflammation of the hindpaw was measured with the same protocol as the one previously described (using spinal dOVT injection 24H after inflammation).

Drugs and Treatments

OT receptor (OTR) agonists and antagonist were prepared as a concentrated solution in water (1mM) and diluted to the needed concentration in NaCl (0.9%) before experiment. The selective OTR antagonist dOVT (d(CH2)5-Tyr(Me)-[Orn8]-vasotocine) was used at a final concentration of 1 µM in Saline (NaCl 0.9%). dOVT was injected i.t. (volume 20 µL) or directly on the spinal cord in electrophysiological recordings (volume 500 µL). OT and TGOT ((Thr$^4$,Gly$^7$)-Oxytocin) (Bachem, Weil am Rhein, Germany) were used as an agonists for OTRs. OT was dissolved in saline and injected i.t or i.p. at a concentration of 10 µM or 1mg/kg, respectively. TGOT was also dissolved in NaCl 0.9% and injected at a concentration of 1 µM (i.t.) or 0.5mg/kg (i.p., rescue experiment). Allopregnanolone and SAHA were freshly prepared before the experiment. Allopregnanolone was used at 10mg/kg and dissolved in mineral oil by sonication at 4°C. The non-selective HDAC inhibitor SAHA was dissolved in 10% DMSO and injected i.p. at a concentration of 5mg/kg.

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6 software (LaJolla, CA, USA). For each experiment, parametric analysis was used only after verifying the normal distribution of the values. Unpaired Student t-test was used to compare baseline nociceptive sensitivity and OTR expression in MS and CTRL rats. Repeated Two-Way ANOVA was used to analyze inflammatory symptoms, followed by Sidak post hoc test. One way ANOVA and Tukey post-hoc tests were used to compare the effects of spinal application of dOVT, TGOT and OT in control or MS rats, to evaluate the efficiency of SIA in both groups and to analyze the success of the rescue experiments on baseline nociceptive sensitivities. Two Way ANOVA followed by Sidak and Kruskal Wallis (KW) followed by Dunn's post hoc test was used to analyze wind-up efficiency. Differences were considered to be statistically significant for $p < 0.05$.

## RESULTS

### Consequences of MS on the nociceptive sensitivity and on the efficacy of oxytocinergic SIA

We first characterized the long-term effects of MS on baseline mechanical and thermal nociceptive thresholds (Figure 1A), as well as the intensity of stress-induced mechanical analgesia (SIA) measured after FSS (Figure 1B). Compared to the control group, MS animals exhibited hypersensitivity to both mechanical and thermal stimulation at adulthood (P>100). MS rats displayed significantly lower mean pressure thresholds (control: 445.5 ± 6.1 g, N = 12; MS: 268.5 ± 7.1 g, N = 12; $p < 0.001$, unpaired Student t-test, t = 18.83, df = 22) and paw withdrawal latencies to noxious heat (control: 21.1 ± 0.8 s, N = 12; MS: 12.6 ± 0.4 s, N = 12; $p < 0.001$, unpaired Student t-test, t = 9, df = 22).

**Figure 1 near here**

Previous studies showed that both central and peripheral release of OT contributed to SIA after a FSS [26,50]. Because MS is known to alter the normal reactivity to stress and lead to a dysfunction of the HPA axis, we investigated the efficacy of the oxytocinergic component of SIA in MS animals (Figure 1B). As expected, the FSS paradigm induced a significant analgesia 20 minutes after swim in the control group (Figure 1B). Mean baseline-corrected mechanical thresholds were increased by 239 ± 12.3 g in the control group (N=8) but only by 10.7 ± 23.4 g in the MS group (N=8). An injection of dOVT prior to FSS decreased SIA intensity to 56.9 ± 20.1 g in the control group (N=8) and had no effect in the MS group (N=8) where it varied of 20.75 ± 20.7 g ($p < 0.001$, One-Way ANOVA, F = 28.9). These results suggest a blunted adaptation to stressful conditions in MS rats associated with possible dysfunction of the oxytocinergic descending control of pain.

### Behavioral response to a painful inflammatory sensitization by carrageenan

We next compared the intensity and time course of inflammatory pain symptoms triggered by an intraplantar hindpaw injection of carrageenan to possibly highlight differences in the expression of mechanical/thermal hyperalgesia in control and MS rats (Figure 2). In both groups, carrageenan injection induced a sustained mechanical and thermal hyperalgesia which peaked 7 h after injection, as previously described [25]. Although basal mechanical nociceptive thresholds (i.e., before carrageenan injection) were significantly lower in MS rats, the time course of mechanical hyperalgesia was not significantly different between both groups (Figure 2A1-B1). In the control group, mean pressure threshold dropped from 445.8 ± 18.7 g to 30.1 ± 5.1 g, 7 h after injection (N = 6; repeated measures two-way ANOVA, treatment x time, $F_{(12,120)} = 63.81$, $p < 0.001$) and mechanical hyperalgesia lasted for at least 6 days for control. As mentioned above, at baseline, MS rats exhibited a lower mean pressure threshold of 295.5 ± 4.8 g (N = 6),

which further decreased to 70.8 ± 12.6 g, 7 h after carrageenan injection. As illustrated in Figure 2B1, hyperalgesia was detected at a significant level for 6 days (N = 6; repeated measures two-way ANOVA, treatment x time, $F_{(12,120)} = 30.78$, $p < 0.001$). Concerning thermal hot sensitivity, 7 h after carrageenan injection mean latencies decreased to 3 .3 ± 0.6 s and 4.2 ± 0.8 s in control and MS rats (N=6 per group) respectively. Interestingly, thermal hot hyperalgesia persisted for at least 1 days for control rats and up to 4 days in MS rats (Figure 2B2) (repeated measures two-way ANOVA, treatment x time, $F_{(8,80)} = 13.67$, $p < 0.001$ for CTRL and $F_{(10,100)} = 10.69$, $p < 0.001$ for MS).

**Figure 2 near here**

**Oxytocinergic contribution to the intensity of carrageenan-induced pain symptoms and to the spinal cord processing of nociceptive messages**

In the carrageenan model of inflammatory sensitization, spinal concentrations of OT are elevated during the early phase of inflammation and have been shown to limit pain sensitivity [26]. To investigate whether MS affects OTergic anti-hyperalgesia, i.t application of the selective OTR antagonist dOVT was performed 24 hours after carrageenan injection.

In the control group and as previously shown [25], a single i.t injection of dOVT (1µM, 20µL), induced a rebound of mechanical and thermal hyperalgesia. Anti-hyperalgesia mediated by OTR could be seen in the CTRL group as a decrease in the mean baseline-corrected mechanical threshold 4 h (*i.e*. 28h after carrageenan) after dOVT injection by 75.6 ± 8.4 g (nN= 10 Figure 3A). This decrease was absent in the saline-treated group (2 ± 8.4 g, N=10) (One-way ANOVA F = 17.5, $p < 0.001$). For thermal withdrawal latency (Figure 3B), OTR anti-hyperalgesia could also be seen as a significant decrease in the mean values after dOVT compared to saline (N = 10; One-way ANOVA, F = 9.3, $p < 0.001$). This corresponded to a change of 3.9 ± 0.6 s (N=10) and of -1.1 ± 0.8 s (N=10), for the dOVT and saline groups, respectively.

Both results confirmed OTR activation to be responsible for a limitation of pain symptoms early after carrageenan injection in control animals. Interestingly, there were no changes in the mean mechanical (dOVT: delta of -5.8 ± 10.8 g, N=8; saline: delta of 27.2 ± 9 g, N=7; One-way ANOVA F=17.5 $p<0.001$) and thermal hyperalgesia values (dOVT: delta of 1 ± 0.7 s, N=8; saline: delta of 0.1 ± 0.7 s, N=7; One-way ANOVA F=9.3 $p<0.001$). Together, this suggests that MS has long-term consequences on the efficacy of OTR-mediated spinal analgesia, at least in short-term inflammatory painful sensitization.

**Figure 3 near here**

To further characterize this MS-altered OTR-mediated analgesia in MS adult rats, we recorded WDR neurons in the SC of anesthetized rats and analyzed the spinal processing of nociceptive messages through the expression of windup, a nociceptive-specific short-term plasticity [22]. As illustrated in figure 4, windup recorded 24 h after carrageenan injection was significantly amplified after the spinal application of dOVT (1µM). In control animals (N=6, figure 4A), windup protocol led to an average maximum frequency of 23.6 ± 1.5 spikes/s. This value significantly increased to a maximum frequency of 44.1 ± 4.8 spikes/s after dOVT application (N = 6; Kruskall-Wallis test, p = 0.03 KW =8.9). As shown in figure 4B and C, we did not see any change in windup frequencies in MS animals (N=6) after dOVT application (before application: 26.8 ± 4.3 spikes/s; after application: 25 ± 4.1 spikes/s; Kruskall-Wallis test p = 0.03 KW =8.9).

**Figure 4 near here**

Altogether, electrophysiological and behavioral experiments suggested that the OT descending inhibitory control of pain is inefficient in MS rats.

**OTR expression and OTR-induced analgesia**

In order to better understand the possible molecular and cellular alterations induced by MS, we first analyzed the expression of OTR and a possible dysfunction of its downstream signaling, focusing on the SC as it is the first relay for nociceptive messages. There were no difference in the spinal expression of OTR gene transcripts between control (N=7; 3.1 ± 0.9 spinal relative expression) and MS (N=8; 2.4 ± 0.2 spinal relative expression) rats (unpaired Student's t-test, p = 0.44, t = 0.78, df = 13), as illustrated in figure 5A.

**Figure 5 near here**

To further confirm that OTR were not only expressed at similar levels in both groups but were equally functional, we measured the analgesic behavioral response to i.t injection of OT (1µM 20µL) or to the selective agonist TGOT (1µM 20µL) in MS rats (Figure 5B). Both OT (N = 7) or TGOT (N = 8) significantly increased mechanical thresholds 20 minutes after the injection in a reversible manner (OT: from 288.4 ± 5.0 g to 533.9 ± 13.1 g; TGOT: from 246.7 ± 11.2 g to 460.1 ± 17.3 g; Two-way ANOVA, treatment x time, $F_{(11,38)} = 66.94$; $p < 0.001$). This result confirmed that spinal OTR are functional in MS rats and capable of producing efficient analgesia.

**Neonatal treatments with OT and allopregnanolone**

Many studies provided evidence that OT in the neonatal period could be protective against early life adverse events or lesions [2,38]. Moreover, MS is known to reduce maternal behavior towards the pups and a subsequent altered development of OT systems, possibly leading to blunted OT levels [31]. This

includes molecular mechanisms affecting chloride homeostasis and the production of neuroprotective neurosteroids such as allopregnanolone [25].

In a first set of experiments, we treated MS rat pups o.d. from P2 to P12 with OT (1mg/kg in 0.9% NaCl, i.p., Figure 6). As shown in figure 6A, neonatal OT treatment fully restored normal mechanical nociceptive thresholds when measured at P50 (control: 320.7 ± 10.9 g, N = 12; MS: 235.1 ± 6.9 g, N = 8; OT-treated MS: 320.4 ± 6.3 g, N = 10; One-way ANOVA, $F = 23.39$, $p < 0.001$). OT neonatal treatment also restored an efficient analgesia after FSS (Figure 6B; delta for Control group: 261.8 ± 18.6 g, N = 9; MS: 17.8 ± 11.6 g, N = 8; OT-treated MS: 168.9 ± 10.4 g, N = 9; One-way ANOVA, $F = 30.08$, $p < 0.001$). In contrast to SIA after FSS, neonatal OT treatment failed to restore OT anti-hyperalgesia 24 hours after carrageenan-induced inflammation, measured as before using i.t dOVT injection (Figure 6C). The dOVT injection did not significantly amplified mechanical hyperalgesia (delta for MS: -5.8 ± 10.9 g, N = 8; OT-pretreated MS: -45.6 ± 19.7 g, N = 8; One-Way ANOVA, $F = 14.28$, $p < 0.001$).

**Figure 6 near here**

Recently, an original long-lasting molecular mode of action of OT in SC neurons has been proposed. It leads to the stimulation of AP synthesis, a neurosteroid well-known for its analgesic and neuroprotective roles [49]. We thus tried to restore OT function in MS rats by submitting newborn pups to AP during the MS period. As illustrated in the different panels of figure 6, a neonatal treatment with s.c AP (10mg/kg) between P2 and P12 was associated with a mean mechanical threshold at adulthood which was higher (i.e., hyposensitivity) than those measured in control animals (Figure 6A; Control: 320.7 ± 10.9 g, N = 12; AP-treated MS: 394.1 ± 21.9 g, N = 10; One way ANOVA, $F = 23.39$, $p < 0.001$). As for neonatal OT treatment, SIA could be restored by AP treatment (Figure 6B; baseline corrected mean mechanical threshold in AP-treated MS: 351 ± 47 g, N = 8, One Way ANOVA, $F = 30.08$, $p<0.001$). As previously reported, endogenous OT anti-hyperalgesia could not be revealed after dOVT i.t. injection (Figure 6C; AP-treated MS: 23 ± 16 g, N = 8; One Way ANOVA, $F = 14.28$, $p < 0.001$). Taken together, our results likely suggested MS to be associated with a deficit in OT signaling.

**Rescue of MS pain phenotype by targeting histone deacetylases**

The long-term consequences of MS on pain sensitivity and on the rat ability to cope with stress/pain potentially rely on epigenetic mechanisms. Indeed, several reports highlight the effects of MS on the HPA axis and on maternal behavior [7,9]. We hence performed a neonatal chronic treatment from P2 to P12 with SAHA (10 mg/kg i.p.), a non-selective HDAC I and II inhibitor. Neonatal treatment of newborn pups with SAHA during MS was associated with mean mechanical nociceptive thresholds at adulthood (P50) which were similar to the control group (Figure 6D; Control: 320.7 ± 10.9 g, N = 8; SAHA-treated MS:

303.8 ± 9.4 g, N = 10; One way ANOVA, F = 14.28, $p < 0.001$). This treatment also partially restored SIA after FSS (Figure 6E; SAHA-treated: 130 ± 26.7 g, n = 7; One Way ANOVA, F = 34.73, $p < 0.001$). Interestingly, SAHA treatment partially restored OT anti-hyperalgesia after carrageenan inflammation (Figure 6F; Control: -75.6 ± 8.4 g; SAHA-treated: 41.7 ± 12.3 g; One-Way ANOVA, F = 15.75, $p < 0.0001$).

## DISCUSSION

In summary, we provide functional evidence indicating that MS strongly affects adult nociception and the efficacy of the oxytocinergic descending inhibitory control. MS is associated with (i) hypersensitivity to mechanical and thermal hot stimulation at adulthood, (ii) increased vulnerability to inflammatory pain, and (iii) dysfunction of the OT descending control of pain after non-painful FSS or painful inflammatory sensitization. We further succeeded in rescuing these abnormalities in the nociceptive phenotype by repeated neonatal injections of OT, AP, or SAHA. Altogether, our data suggest that MS has a strong impact on nociceptive integration, which can blunt the efficacy of descending controls of pain such as the oxytocinergic system which targets the spinal GABAergic inhibitory system. It also suggests that the OT or epigenetic systems can be a good target to further develop early treatments to counteract the negative effects of early life stress, at least in this model.

### Early life events promote nociceptive hypersensitivity and alter OTR-mediated analgesia

Early life stress includes a wide range of adverse events generated by MS, pain, abuse, stress or neglect during early infancy. These events have long-term negative effects on brain function in both human and rodent studies. In human, early life stress has been associated with an increased risk of developing chronic pain states at adulthood [13], increased pain ratings and catastrophizing [53,61]. Clinical studies also revealed early life stress could be a risk factor for the development of irritable bowel syndrome [51,52]. In animal studies, the visceral hypersensitivity following MS has been widely demonstrated, and correlated with differential activation of supraspinal regions, including the periaqueductal gray, which is a key area for descending pain controls. In the present study, we confirmed that nociceptive hypersensitivity extends to somatic mechanical and thermal hot stimuli, as previously published by our laboratory [28] and in other ELS models [1,47,58,65]. Our observation of an increased thermal pain symptom with the carrageenan model is consistent with studies showing an increased response after formalin injection [60] or after partial sciatic ligation [44] in MS animals. Nociceptive hypersensitivity could be easily explained by a long-lasting up-regulation of voltage-gated channels Nav1.8 and Nav1.9 in DRG neurons as recently published [28].

In this study, impairment of the oxytocinergic pain control has been revealed after recruitment by a non-painful (FSS) and painful stress (inflammatory sensitization by carrageenan). The hypothalamus, where OT producing neurons are located, seems particularly vulnerable to early life stress and a dysfunction of the oxytocinergic system may contribute to these alterations as OT plays a key role in the modulation of stress responses, anxiety, social interactions and pain responses. Recently, MS in rodents was shown to be associated with changes in OTR and vasopressin V1aR binding sites in specific brain regions [34]. Similar changes were also demonstrated in adolescent rat pups after ELS [23] [46] suggesting a vulnerability of this

system to the early environment. In humans, early life stress has been associated with low oxytocin concentrations in CSF [21], altered OT levels after social interaction [63] and an impaired inhibition of intranasal OT on salivary cortisol concentrations [36], and on limbic deactivation during stress [18]. So far, only very few evidence of an altered OT descending control of pain were available in the literature. Here, we show that OT analgesia is impaired after MS, both in painful and non-painful conditions. To exert its analgesic effects at the spinal level, OT relays on three main spinal mechanisms: (i) activation of a spinal microcircuit leading to an increased GABAergic inhibitory tone [5], (ii) decrease of the excitability of spinal cord neurons in the superficial layers [6] and (iii) stimulation of allopregnanolone synthesis which further amplifies GABAergic inhibition by allosteric modulation and leads to a long-term nociceptive effect [25]. In our study, we showed that spinal OTR are functional in MS rats and that OTR activation can induce an efficient and transient analgesia, suggesting that the lack of OT-analgesia could hence result supraspinal alterations. Exploring hypothalamic OT neurons pharmacological and electrophysiological characteristics might help us to have a deeper understanding of the mechanisms leading to pain-related ELS consequences. Moreover, a lack of AP synthesis following OTR activation could also explain the lack of OT anti-nociception following carrageenan inflammation in MS rats. Further experiments may be needed to investigate the enzymatic activity of type I 5α-reductase and 3α-Hydroxysteroid dehydrogenase, responsible for spinal AP synthesis.

**Rescuing MS long-term alterations of the nociceptive system**

In our working hypothesis, we asked whether the lack of physical interaction between the mother and the pups could be a critical determinant to explain the deleterious plasticity of the nociceptive system and the deficit in oxytocinergic analgesia. Indeed, mother presence has already been showed to be protective against pain. Kangaroo mother care, in particular, is more frequently recommended and has been shown to have immediate analgesic effect on pain responses in the newborn [24,32] and to be protective against long term alterations usually following preterm birth [10,16]. As OT has a key role in the modulation of parental behavior and attachment [8,15], we performed a rescue experiment by repeated neonatal OT treatment during MS, which reversed some consequences of MS on pain behavior. In the literature, OT has been proposed as a therapeutic agent for different neurodevelopmental pathologies such as autism spectrum disorders, since it decreases anxiety and optimizes social behavior [39,66]. In animal models, postnatal intraperitoneal or subcutaneous injections of OT have been shown to prevent feeding, social and memory deficit in the Prader-Willi model [38,54]. Here, we only studied the beneficial consequences of an early treatment with OT on pain behaviors but further experiments would be helpful to confirm if this treatment could also counteract the other detrimental effects of MS on pain-related comorbidities such as stress, anxiety, cognitive, or social impairments. These observations might have great clinical relevance in

neonatal intensive care units since it confirms, once again, the crucial role of physical interactions between the parents and the newborn to optimize newborn development and install proper homeostatic controls to cope with stress (including pain).

A close reciprocal relationship exists between the oxytocinergic system and neurosteroids. Allopregnanolone is not only a downstream second messenger of OTR activation [25], it has well-known anxiolytic, analgesic and neuroprotective properties [49]. In this study, a rescue experiment with neonatal allopregnanolone treatment appeared successful to restore normal pain sensitivity and a functional OT analgesia after FSS. Neonatal allopregnanolone level alterations can indeed lead to changes in behavior at adulthood, including anxiety behavior, novelty response, aversive learning, prepulse inhibition and changes in the anxiolytic effect of intrahippocampal allopregnanolone [12]. Our result is also in good agreement with other studies using AP treatment to prevent neonatal anxiety after a short-term MS (24 hours) [30] or, before birth, to protect the fetus against prenatal stress [67]. The mechanisms by which AP could be protective against MS consequences on pain controls are still unknown. However, neonatal AP level alterations could interact with chloride homeostasis development, since AP has also been shown to change KCC2 expression in the hippocampus [40].

Eventually, it is not surprising that HDAC inhibitor and epigenetic changes are involved in the long-term consequences of MS on nociception and OT analgesia. It has long been known that neonatal stress or trauma have long lasting effects through epigenetic mechanisms [7,9,43]. Epigenetic changes in histone acetylation, methyl binding protein or miRNA expression has been demonstrated after MS in different brain areas [29,41,43]. In the pain context, histone acetylation can modulate nociceptive response in inflammatory neuropathic and visceral pain models [62]. Moloney and colleagues showed that MS is associated with an alteration in histone acetylation at the spinal level, especially at the level of H4K12 [41]. They were the first to show that adult SAHA treatment can reverse MS-induced visceral hypersensitivity. In our study, we confirm that the beneficial effects of adult SAHA treatment extend also to somatic nociceptive sensitivities, and that a preventive neonatal treatment with the same agent is also effective to prevent mechanical and thermal hypersensitivity at adulthood. At this stage, the underlying mechanisms are still to be identified as well as the molecular targets by which SAHA exerts its protective effects against MS.

In conclusion, MS induced both a basal hypersensitivity to pain and a dysfunction in OT descending control of pain, in painful and non-painful conditions. Some of these alterations can be rescued by neonatal HDAC or OT/AP treatment, but other alternatives need to be explored to be able to recover completely the OT antinociceptive action under inflammatory conditions. By studying the mechanisms of action of these

pharmacological treatments, we might be able to identify new therapeutic targets to avoid MS induced reprogrammation of pain circuits.

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Conflict of interest statement

The authors have no conflict of interest.

**FIGURES AND LEGENDS**

**Figure 1**. **Altered baseline nociceptive sensitivity and SIA efficacy in MS rats. A**. Baseline nociceptive thresholds for mechanical (left histogram) and thermal hot stimulation (right histogram) between control (black bars) and MS rats (white bars). Statistical code for: Unpaired Student t-test ***p<0.001 N=12/group. **B**. Stress induced-analgesia detected 20 minutes after a forced swim stress procedure aimed at stimulating OT endogenous controls (10 minutes, water at 20°C) for control (N=8 black bar), MS (N=8 white bar), control pre-injected with dOVT (N=8 red bar) and MS rats pre-injected with dOVT (N=8 grey bar). The variation of mechanical threshold in grams have been calculated as: Delta = mechanical threshold post FSS - mechanical threshold pre-FSS. Statistical code for One Way ANOVA followed by Tukey post hoc test: ***p<0.001.

**Figure 2**. **Increased sensibility to inflammatory pain in MS rats.** Time course of mechanical (top panels) and thermal hot (bottom panels) nociceptive thresholds for control (A) and MS rats (B). Carrageenan injection was done at day 0 just after the daily measure. Note that the duration of thermal hyperalgesia was longer for MS rats. Statistical code for Two Way ANOVA treatment X time followed by Sidak post hoc test to compare ipsilateral and contralateral hindpaws for each time point: **p<0.01, ***p<0.001 N=12 per groups.

**Figure 3**. **Lack of efficiency of OT anti-hyperalgesic control on behavioral symptoms of inflammatory pain in MS rats.** Changes in the mean mechanical (A) and thermal hot (B) thresholds, 24 h after a carrageenan hindpaw injection, in control (N=10) and MS rats (N=8) submitted to a single injection of the selective OTR antagonist dOVT (1µM, 20µL) or saline (NaCl 0.9%, 20µL), measured as: Delta = threshold pre dOVT injection- threshold post dOVT injection. Statistical code for One Way ANOVA and Tukey post hoc test: * p<0.05, **p<0.01, ***p<0.001. CTRL: control, MS: maternal separation, s: saline.

**Figure 4**. **Lack of efficiency of OT anti-nociceptive control on spinal cord wide dynamic range neurons. A and B**. Number of action potentials measured during windup when recording from wide dynamic range neurons *in vivo*. Results are given for anesthetized control (CTRL, **A**) and MS rats (**B**), displaying an hindpaw inflammation for 24 h due to carrageenan injection, and submitted to a repetitive stimulation of the corresponding receptive field on the hindpaw (1Hz, intensity: 3x threshold for C fibers). Windup characteristics are provided before (black symbols) and 20 minutes after local (spinal) application of the selective antagonist dOVT (1µM, in red). Statistical code for Two Way ANOVA Treatment X Time and Sidak post hoc test: * p<0.05, ***p<0.001. **C**. Mean frequencies of action potential discharge at wind-up plateau phase for all groups. Statistical code for Kruskal Wallis test and Sidak post hoc test: * p<0.05 N=6 per group.

**Figure 5**. **Function of OTR in MS rats. A**. Expression of transcripts coding for spinal OTR in control and MS rats. No significant difference between groups has been detected using Unpaired Student t-test, N=7 control and N=8 MS. **B**. Changes in mechanical nociceptive threshold of MS rats after i.t injection of OT (1µM, 20µL, purple circles N=7), TGOT (1µM, 20µL, blue triangles N=8) or vehicle (NaCl, 20µL white squares N=7). Statistical code for Two-way ANOVA treatment X Time followed by Sidak post hoc test: ***p<0.001.

**Figure 6. Neonatal treatment with OT, AP or SAHA can partially suppress MS consequences**. **A to C**. Effect of a neonatal repeated treatment with oxytocin (OT, 1mg/kg o.d. i.p. injections between P2 and P12, purple bars) or allopregnanolone (AP, 10mg/kg i.p. injections between P2 and P12 every 2days, red bars), on mechanical nociceptive thresholds (A, N=12 control and N=8 MS), stress induced analgesia (B, N=9 control and N=8 MS), and OTR-dependent inhibitory control 24 h after carrageenan inflammation (C, n=8 per group). Results are shown for control (CTRL) and MS rats. **D to F**. Effect of a neonatal repeated treatment with SAHA (5mg/kg, daily i.p. injection between P2 and P12: dark green), a non-selective HDAC inhibitor, on mechanical nociceptive thresholds (A, N=8 control and MS, N=10 SAHA treated MS), stress-induced analgesia (B, N=9 CTRL, N=8 MS and N=7 SAHA treated MS), OTR-dependent inhibitory control 24 hours after carrageenan inflammation (C, N=10 ctrl, N=8 MS N=8 SAHA treated MS). Results are shown for control (CTRL) and MS rats. Statistical code for One WAY ANOVA followed by Tukey post hoc test for panels A to E and Kruskal Wallis for panel F: *p<0.05; **p<0.01; *** p<0.001.

Figure 1

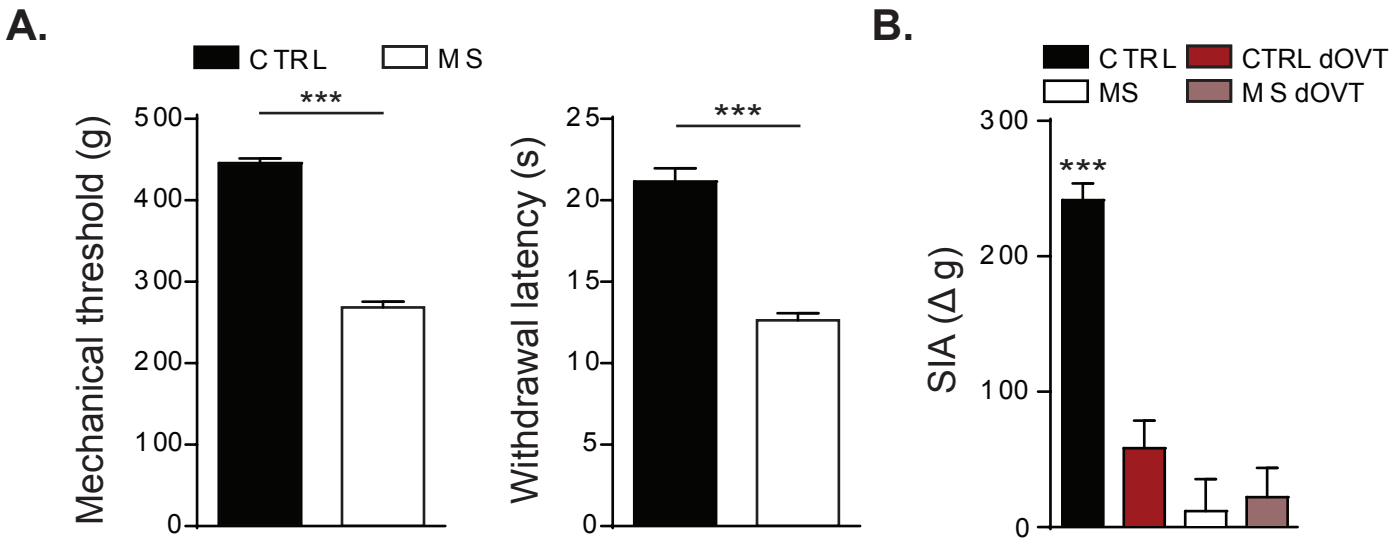
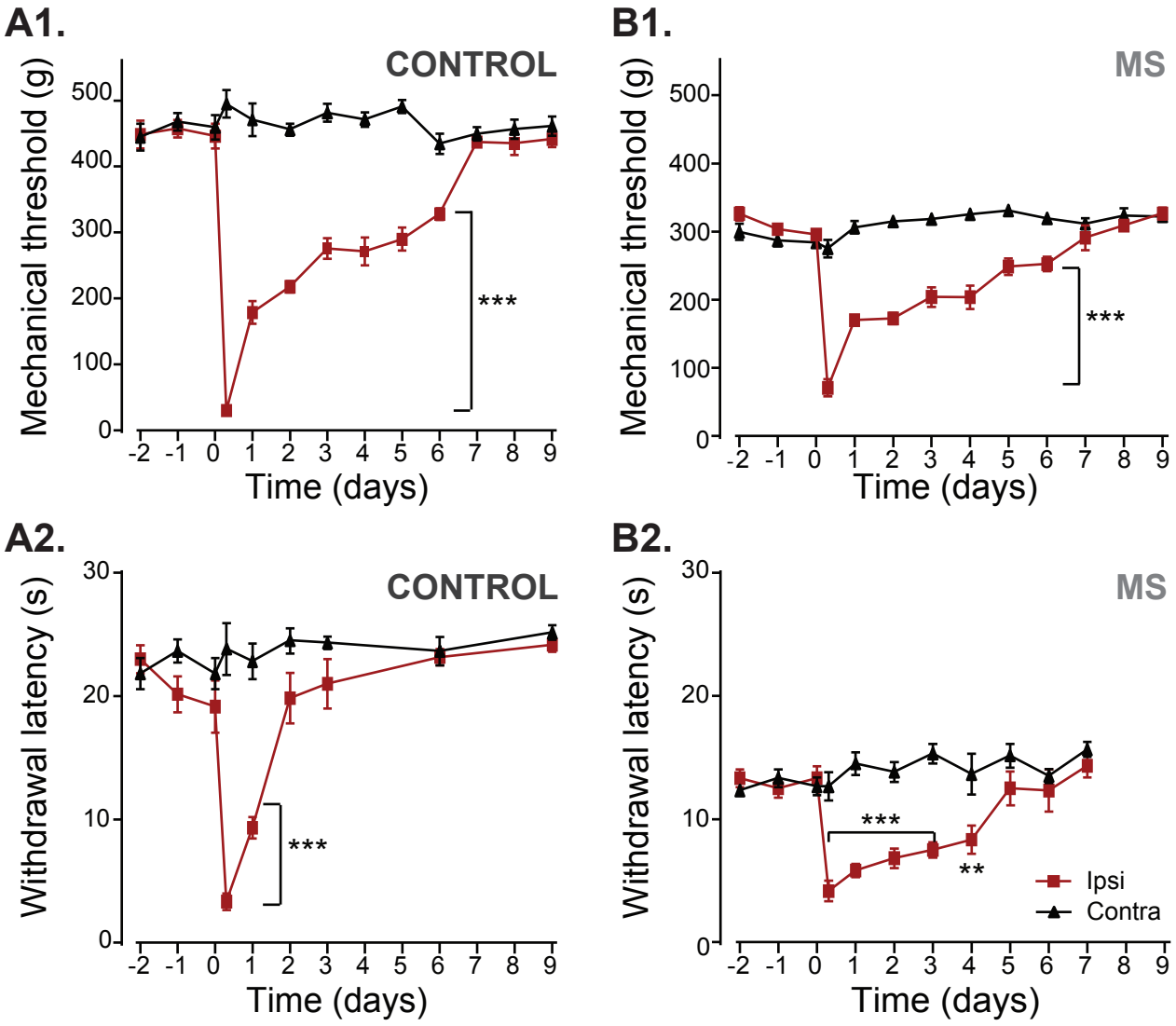

A.
CTRL
MS
Mechanical threshold (g)
500
400
300
200
100
0
***
Withdrawal latency (s)
25
20
15
10
5
0
***
B.
CTRL
CTRL dOVT
MS
MS dOVT
SIA (Δ g)
300
200
100
0
***

Figure 2


A1.
CONTROL
Mechanical threshold (g)
500
400
300
200
100
0
***
-2 -1 0 1 2 3 4 5 6 7 8 9
Time (days)
B1.
MS
Mechanical threshold (g)
500
400
300
200
100
0
***
-2 -1 0 1 2 3 4 5 6 7 8 9
Time (days)
A2.
CONTROL
Withdrawal latency (s)
30
20
10
0
***
-2 -1 0 1 2 3 4 5 6 7 8 9
Time (days)
B2.
MS
Withdrawal latency (s)
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-2 -1 0 1 2 3 4 5 6 7 8 9
Time (days)

Figure 3

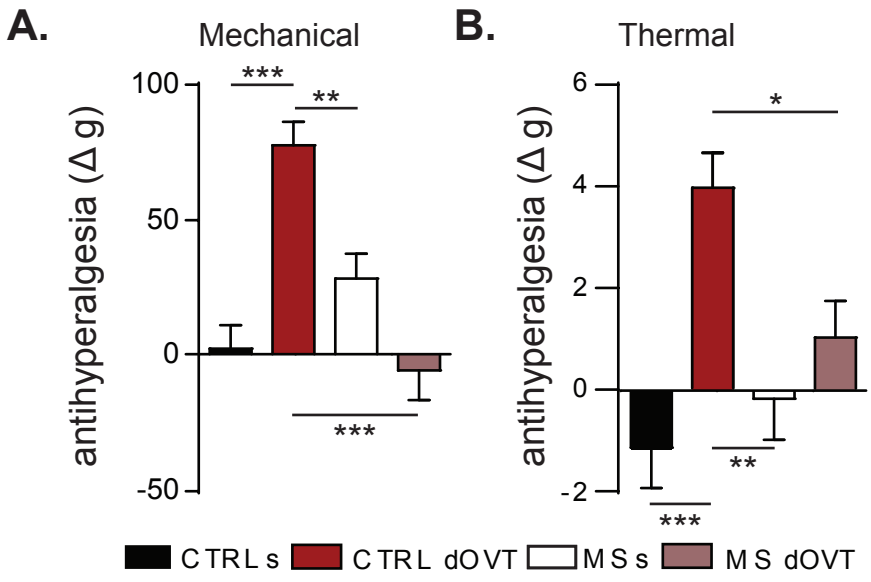

A.
Mechanical
B.
Thermal
antihyperalgesia (Δ g)
100
50
0
-50
***
**
***
antihyperalgesia (Δ g)
6
4
2
0
-2
*
**
***
CTRL s
CTRL dOVT
MS s
MS dOVT

Figure 4

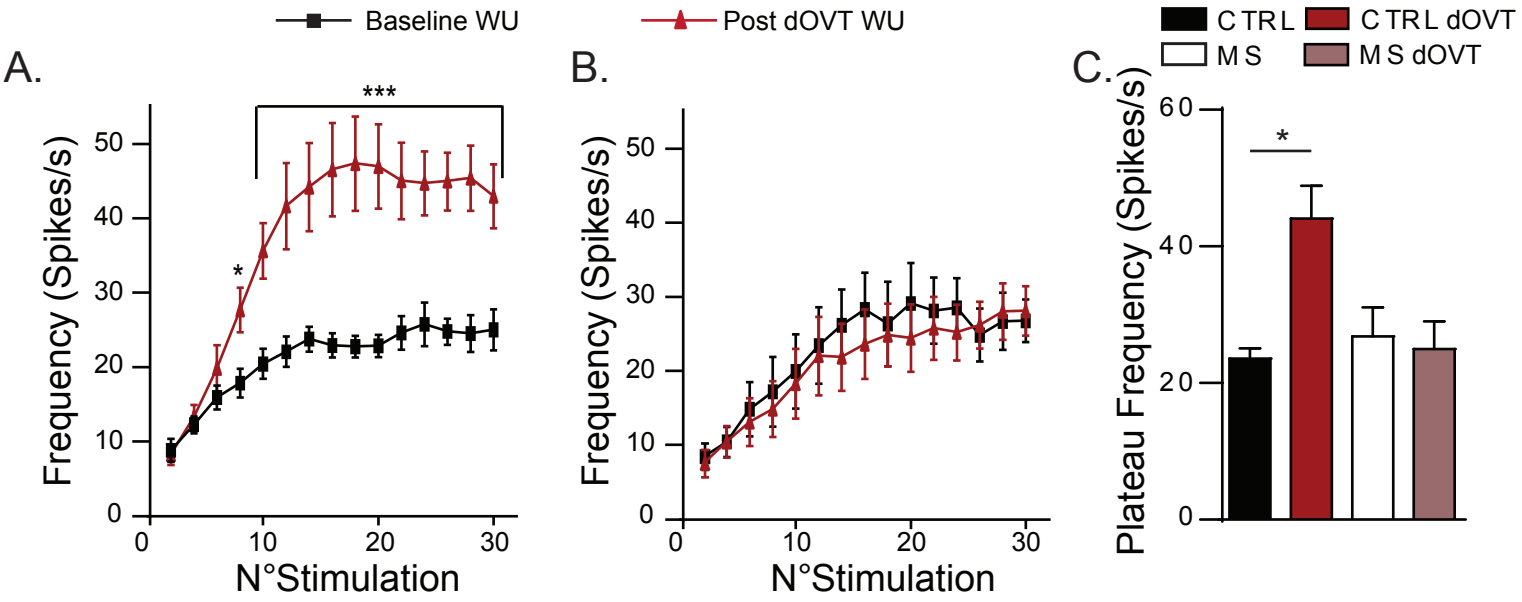

A.
Baseline WU
Post dOVT WU
***
*
Frequency (Spikes/s)
N°Stimulation
B.
Frequency (Spikes/s)
N°Stimulation
C.
CTRL
CTRL dOVT
MS
MS dOVT
Plateau Frequency (Spikes/s)
*

Figure 5

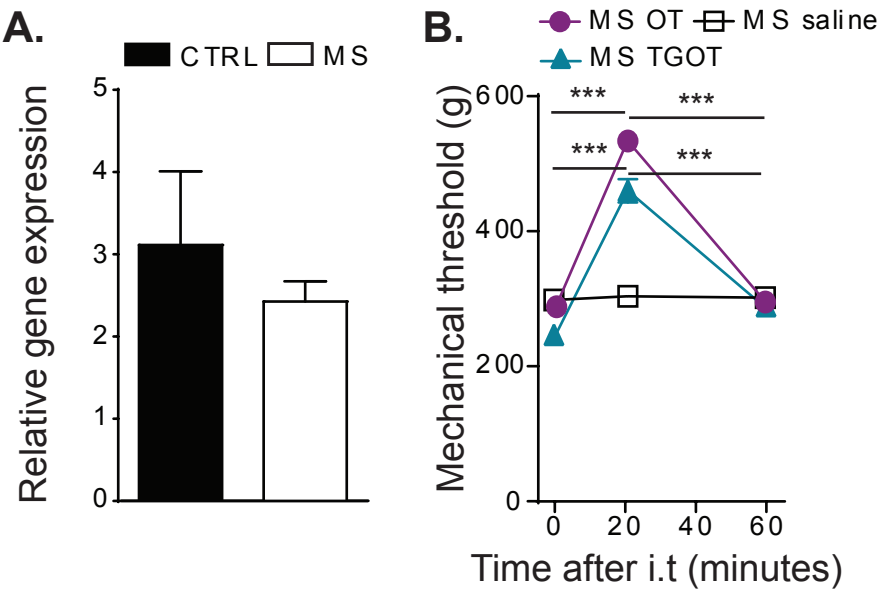
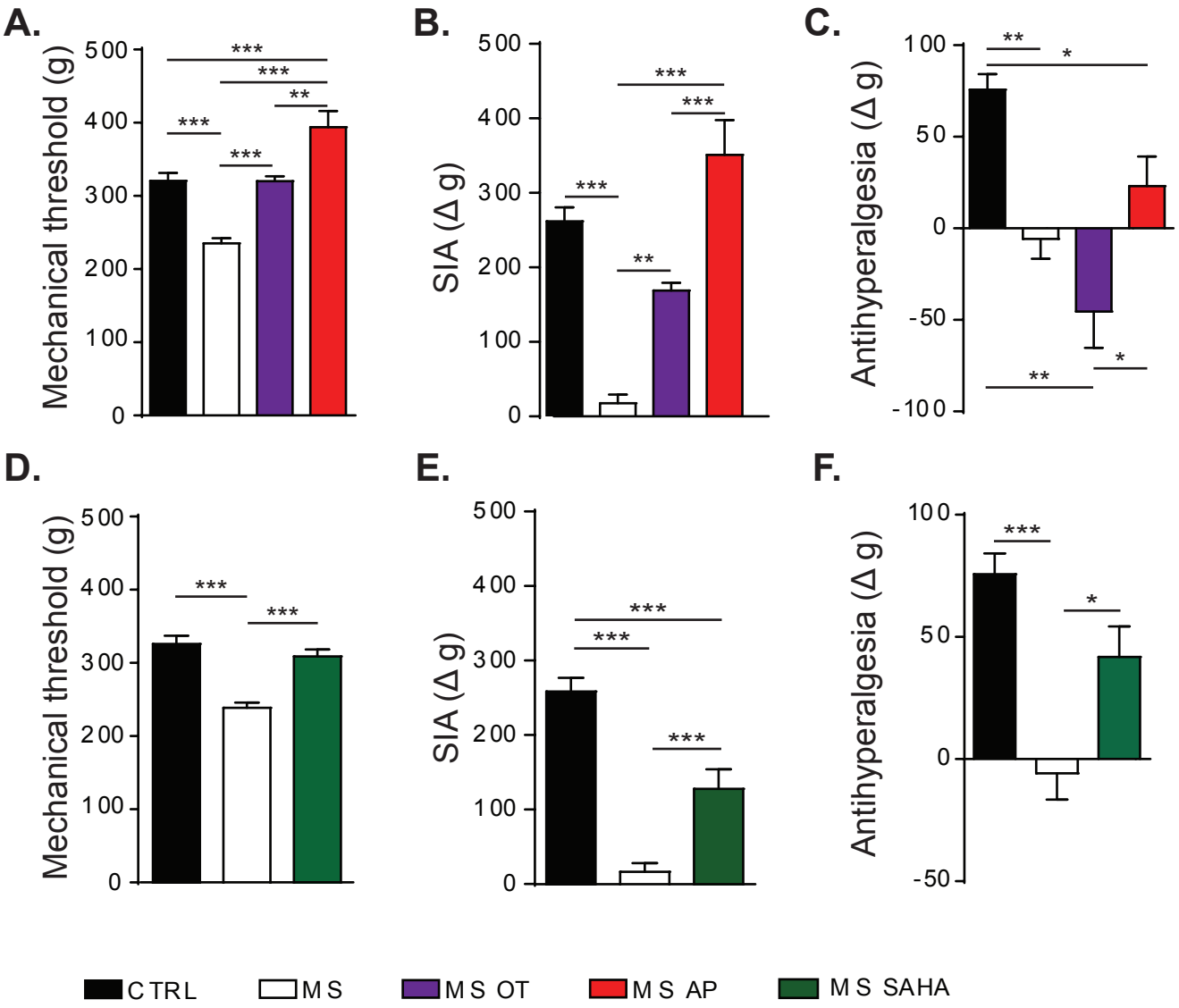

A.
CTRL
MS
Relative gene expression
0
1
2
3
4
5
B.
MS OT
MS saline
MS TGOT
Mechanical threshold (g)
0
200
400
600
***
***
***
***
0
20
40
60
Time after i.t (minutes)

Figure 6


A.
Mechanical threshold (g)
500
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100
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B.
SIA (Δ g)
500
400
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100
0
***
**
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C.
Antihyperalgesia (Δ g)
100
50
0
-50
-100
**
*
**
*
D.
Mechanical threshold (g)
500
400
300
200
100
0
***
***
E.
SIA (Δ g)
500
400
300
200
100
0
***
***
***
F.
Antihyperalgesia (Δ g)
100
50
0
-50
***
*
CTRL
MS
MS OT
MS AP
MS SAHA

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Maternal separation renders adult rats hypersensitive to noxious stimuli and incapable to recruit oxytocin analgesic controls. Rescue is achieved by neonatal treatments with oxytocin, allopregnanolone or HDAC inhibitors.

# K) Discussion

During this PhD work, I studied the oxytocinergic descending inhibitory control of pain in physiological and pathological states. At first, we tried to go deeper in the understanding of the mechanisms allowing a dual antinociceptive action of OT, via a peripheral and a central mode of action. Then, we tried to determine if the development of the OT system and the anti-nociceptive action of OT could be altered in a situation of ELS, where the early OT-linked environment of the newborn is affected by neonatal maternal separation.

**Part I. PVN and SON coordination**

In this study, we identified a small population of around 30 parvOT neurons who is responsible for a dual anti-nociceptive action. This population can produce an efficient analgesia in inflammatory conditions by two pathways. The first is by a direct projection on spinal cord neurons and the second is by the stimulation of OT blood release by magnOT neurons. The spinal projection allows a fast antinociceptive action of OT, and the further release of OT in blood allows a secondary slower action. Interestingly, this anti-nociceptive action is specific of inflammatory pain, and not efficient in our model of nerve injury-induced neuropathic situation. The specificity to inflammatory pain might rely on physiological needs. Inflammatory peripheral insults might require both the antinociceptive and peripheral anti-inflammatory action of OT to resolve the injury. OT has been shown to be involved in wound healing (Detillion et al., 2004) and could be implicated in the disappearance of local inflammation in the hindpaw. The inflammation is also associated with a local increase in temperature and with thermal hyperalgesia. In that context, the dual release of OT also allows a modulatory action on TRPV1 channels (Nersesyan et al., 2017). However, the ability of OT to modulate neuropathic pain has been demonstrated in the literature in other models (Condés-Lara et al., 2005; Martínez-Lorenzana et al., 2008), suggesting that one or multiple other sub-population of OT neurons might be in charge of this modality. This support the idea that OT cells may be specialized for a give function of sub-function (ie. pain modality) and are able to discriminate between nociceptive inputs. Then is there any indication of the existence specific subpopulations of parvOT neurons? Anatomical data strongly support this hypothesis, since specific groups of parvOT have been showed to modulate different brainstem and spinal cord nuclei. For example, two distinct populations of ParvOT neurons have been identified recently, projecting to the ventral tegmental area and substantia nigra. OT neurons projecting to the VTA enhance the activity of dopaminergic neurons whereas the population projecting to the substantia nigra decrease the activity of dopaminergic neurons. However, specific markers for these subpopulations and the pathways leading to the specific activation of one or multiple subpopulations of OT neurons still need to be investigated.

Anatomically, the connections between the two OT nuclei have been previously proposed but not demonstrated. This question has especially been raised in the context of the synchronized bursting activity during lactation (Belin et al., 1984). One study indeed described OT synapses onto SON neurons, but the functional relevance of such connections had not been investigated (Theodosis, 1985). Here, we show that this connection is functionally relevant in the context of pain, but this double projection might also be a possible supplementary mechanism to explain the specific activation pattern of OT neurons during lactation. Moreover, we demonstrated no such interconnection for VP neurons and no burst synchronization has been observed for VP cells. It is then possible that parvOT neurons participate in driving the synchronized activity of magnOT neurons between nuclei, on top of the other proposed mechanisms. Anyway, this double projection could be relevant beyond pain or reproductive functions. MagnOT indeed send projections to the

periphery but also to forebrain regions where they exert a modulatory activity on various behaviours. The connection between the two populations of neurons could then allow a subtle and simultaneous modulation of OT action in different brain areas.

At the spinal level, OT projections from PVN neurons had previously been identified, but mostly on superficial laminae and autonomic areas (Swanson and McKellar, 1979; Jójárt et al., 2009). Here we showed that our small population of neurons project directly onto wide dynamic range neurons (WDR) located in deep layers of the SC. However, it would be interesting to see how the activities of the different parvOT populations are coordinated, and if they act in synergy or if they are activated by different inputs. Moreover, WDR are known to response to cutaneous, muscular as well as visceral inputs (Le Bars, 2002), and evidences show a modulatory role of OT in visceral function and visceral pain. It would then be interesting to see if the specific stimulation of the subpopulation of neurons we identified could also modulate visceral nociception through its action on WDR neurons. Coupling the optogenetic activation of these fibers in the SC with the recording of VMR to CRD for example, would be interesting in this context. In our study, we showed that OT anti-antinociceptive effect on WDR neurons is mediated by OTR and not V1aR. However, its effects are not totally blocked by the spinal blockade of OTR. We showed that OT is probably co-released with glutamate, which participate in the anti-nociceptive effects, as previously demonstrated for OT action in the central amygdala (Knobloch et al., 2012). OT effects were indeed totally blocked by the concomitant application of dOVT and NBQX, an antagonist of AMPA/Kainate receptors.

In conclusion, these results highlight the fact that specialized OT circuits seem to exist within PVN neurons. Further studies of the specialized neuronal circuits of the HT are needed to identify specific markers of these populations. This would help to develop genetic or molecular tools to drive their activity. Here, we described this small sub-population in the rat. We still must determine if similar circuits are present in other species and in human. If so, it could constitute a promising therapeutic target to treat some pain-related diseases.

**Part II : Maternal separation consequences on the oxytocinergic control of pain**

- Altered nociceptive sensitivities and impaired OT analgesia

In the second part of my PhD work, we show that neonatal maternal separation alters adult nociceptive behaviours and the efficacy of the oxytocinergic descending inhibitory control. MS rats display lower mechanical and thermal threshold at baseline, as well as a longer carrageenan-induced thermal hyperalgesia. The baseline mechanical and thermal hypersensitivity that we detected is consistent with other studies using this model, but also with the human literature. ELS in human has, for example, been associated with increased pain ratings at adulthood and is proposed as a risk factor for the development of IBS (Walsh et al., 2007; Paras et al., 2009; Sansone et al., 2013; Yamada et al., 2017). The mechanisms underlying MS-induced alterations might be complex, affecting several actors controlling neuronal excitability in the nociceptive system.

<u>Are MS-induced pain-related changes due to sensory or spinal alterations?</u>

It has recently been published that MS induces an up-regulation of voltage gated channels Nav1.8 and Nav1.9 in DRG neurons, resulting in increased excitability of nociceptive neurons (Hu et al., 2013; Juif et al., 2016). Moreover, contrary to the baseline hypersensitivity concerning both mechanical and thermal modalities, the carrageenan-induced hypersensitivity is specific to thermal hot modality. Interestingly, a recent paper showed that OT can directly modulate TRPV1, a receptor activated by nociceptive thermal stimuli (Nersesyan et al., 2017). Since we showed that OT analgesia is impaired

after MS, a possibility might be that the increased duration of thermal inflammatory hypersensitivity would be due to an impaired OT action on TRPV1 receptor.

In line, our laboratory recently investigated the expression of chloride transporter in the spinal cord of MS rats, with the hypothesis that a disruption in chloride homeostasis would lead to a decreased chloride-mediated inhibition. These results have not been published yet, but it appears that MS rats have an increased NKCC1 expression in the spinal cord. The i.t treatment of these rats with Bumetanide, an NKCC1 inhibitor successfully restored normal mechanical threshold. Increases in NKCC1 have been proposed to promote the development of chronic pain, which suggest that it may play a role in the deleterious effects of MS (Price et al., 2005). In early life, chloride homeostasis is submitted to critical developmental changes, controlling the switch from depolarizing to hyperpolarizing (inhibitory) GABAergic currents (Wang et al., 2002; Watanabe and Fukuda, 2015). Interestingly, chloride homeostasis can, among other mechanisms, be regulated by OT and OTR activity, mainly by optimizing the activity of the KCC2 chloride exporter (Leonzino et al., 2016). OT effects on chloride homeostasis and on GABAergic inhibitory activity during delivery has also been proposed to involve inhibition of NKCC1 activity, since bumetanide exerts the same effects as OT (KHAZIPOV et al., 2008; Mazzuca et al., 2011). Accordingly, OT deficit in early life is likely to affect chloride homeostasis and inhibitory function mediated by chloride permeable channels, such as GABAA receptors. This idea fits well with our current demonstration that neonatal OT treatment during MS restores normal baseline nociceptive thresholds as well as stress-induced analgesia (but only partially here).

Other mechanisms could explain OT analgesia dysfunction seen in MS animals. We failed to reveal any OTR spinal expression changes and we, therefore, made the hypothesis that the dysfunction could preferentially come from a supraspinal alteration. However, the absence of long-term anti-hyperalgesia provided by OT after inflammation could still be explained, at least partially, by a blunted synthesis of allopregnanolone in the SC. Indeed, OT exerts a long term anti-nociceptive effect via the stimulation of AP synthesis in the spinal cord (Juif et al., 2013). AP acts as a positive modulator of GABAA receptors in the spinal cord and can induce a potent analgesia, including in inflammatory pain states (Charlet et al., 2008). Moreover, AP seems to regulate preferentially thermal nociception after inflammation. Indeed, blocking spinal AP synthesis with Finasteride was without effect on mechanical hyperalgesia but increased thermal hyperalgesia and delayed recovery of normal heat sensitivity (Poisbeau et al., 2005). The similarity of the time-course of thermal hyperalgesia in Finasteride treated animals and the one observed in MS animals also suggests that AP levels might be impaired after MS. To answer this hypothesis, we plan to further dissect steroid system in the spinal cord and measure the expression of enzymes involved in AP synthesis.

Are MS-induced pain-related changes due to supraspinal alterations?

Behavioural studies, not only on pain but also on other OT-related behavior, suggest that the supraspinal OT system is affected by ELS. Different OT and OTR expressions have indeed been detected in the brain of MS or handled animals (Veenema, 2012). However, the anatomical and functional properties of OT neurons in MS animals have not been investigated in detail. Further experiments will include measurement of OT mRNA levels in the hypothalamus and the expression of OTR, to determine if OT is normally synthesized in OT neurons. An interesting perspective would be to take advantage of the optogenetic tools we used in the first study of this PhD. This would allow us to activate parvOT neurons projecting to the spinal cord to check if the exogenous activation of these neurons can produce an efficient analgesia. Although the pathways leading to OT activation in response to stress or in response to inflammatory input are still not precisely determined, there is a possibility that the recruitment of OT neurons is impaired in MS animals. The optogenetic stimulation of these neurons would bypass the endogenous activation of OT neurons, and possibly restore functional OT analgesia.

Then regarding the first study presented in this work, another question can be raised. Are the deep inter-nuclei connections of OT neurons altered by MS, and could it play a role in the altered pain responses that we observed? We have no answer for that question so far, and it would need further investigations. Besides, pain is not the only related OT function that is altered by MS. These animals display deficits in social and maternal behaviour for example, as well as increased stress reactivity (Veenema, 2012). However, their ability to give birth and to lactate pups is not impaired. The lactation process relies on huge amount of OT release by magnocellular neurons and on the characteristic bursting activity of OT neurons, facilitated by the autocrine action of OT (Freund-Mercier et al., 1988a; Ludwig and Leng, 2006). This suggests that OT-related alterations could target specific populations of OT neurons. A deeper understanding of HT microcircuits is then needed to dig this hypothesis completely. A possibility here might be that MS preferentially impairs the function of the parvocellular population of OT neurons, more than the magnocellular population that seems to be functioning, at least according to the behavioural studies.

- Developmental processes underlying MS consequences – hypothesis following rescue experiments

We performed a rescue experiment aiming to counteract the consequences of MS on pain responses. OT, AP as well as SAHA treatment were efficient to restore normal baseline sensitivities and OT analgesia after FSS. This led us to emit a few hypotheses about the developmental factors regulating the maturation of pain circuits

<u>OT as a developmental regulator of neuronal maturation ?</u>

Neonatal treatment with OT has previously proven to be effective to prevent some effects of neonatal LPS injections in the rats. LPS indeed induces a decrease in mRNA levels for BDNF and NGF in the hippocampus, which is prevented by chronic neonatal OT treatment (1mg/mL, P2 to P6, 50µL per pups) (Bakos et al., 2014). In magel2 null mice, a model of Prader-Willi syndrome, the repeated treatment of OT during the first 7 post-natal days restores normal adult social behaviour and spatial learning skills (Meziane et al., 2015). Manipulations of OT levels during early life are known to alter OT-related behaviour in rodents at later age (Bales and Carter, 2003a; Kramer et al., 2003; Mogi et al., 2014). In that context we could imagine that the maturation of the OT system occurs via an activity-dependent process, as it is the case in other sensory neuronal pathways. The action of OT through OTR in various areas of the brain could shapes the experience-dependent plasticity occurring during development (Zheng et al., 2014). Our hypothesis would be that an early decrease of maternal interaction could lead to a decrease of OT levels in the pups, resulting in altered development of OT circuits. Measuring peripheral and brain OT levels in the pups during the separation period would hence be interesting to support this hypothesis. A recent study shows that early sensory deprivation induced by whisker deprivation in neonatal mice decreases OTergic neuronal firing rates as well as OT levels in the HT and cortex (Zheng et al., 2014). The early deprivation is associated with reduced excitatory synaptic transmission in primary somatosensory cortex and primary visual and auditory cortex, which is rescued by the exogenous application of OT. Blocking OT action resulted in the same effects as early deprivation. They hence identified OT as a key factor for experience-dependant cortical development.

However, a possible source of neonatal OT is breast milk (Takeda et al., 1986). In that case OT might act on the recently observed OTRs in the oral cavity, which is close to the nasal cavity where OTRs are also present in the neonatal period in mice, and produce effects on the CNS (Greenwood and Hammock, 2017). Measuring OT levels in breast milk of MS mothers would be an interesting perspective. We could indeed make the hypothesis that the separation stress alters milk levels of OT and have deleterious consequences in the pups. To further confirm this hypothesis, a similar rescue experiment with oral OT should be considered.

Allopregnanolone as a key regulator of inhibitory function ?

In addition to OT treatment, we performed a treatment with the neurosteroid AP which prevented the deleterious effects of MS. AP is one of the neuroactive steroid that plays an essential role in the development of the CNS. During foetal life AP levels are high and deficits in AP lead to cell death and delayed myelination (Kelleher et al., 2011). After birth, AP levels in the brain drop rapidly in rodents, with a time course overlapping the switch to an hyperpolarizing GABA signal (Kelleher et al., 2013). The fine regulation of AP levels is critical for proper brain development in many brain structures, including the thalamus and the cortex (Belelli et al., 2017). Then at adulthood, brain AP levels are subjected to variations in response to an acute stressor (Purdy et al., 1991; Park et al., 2017). Evidence show that modifications of AP levels during the early postnatal life can affect brain development and behaviour at older age. For example, injections of finasteride, an inhibitor of one of the enzymes responsible for AP synthesis from cholesterol, from P5 to P9 in newborn rodents impaired the developmental upregulation of KCC2 chloride transporter in the hippocampus (Mòdol et al., 2014).

At the behavioural level, it induces an anxiogenic-like behaviour in the elevated plus maze and impairs aversive learning capacity (Martín-García et al., 2008). Moreover, in a model of maternal deprivation at P9 the administration of AP prior to the separation (10mg/kg from P5 to P9) prevented the effects of MD on exploratory behaviour (Llidó et al., 2013). Altogether, these observations suggest that neonatal injection of AP could be protective against early life adversities.

Are MS-induced alterations linked to epigenetic programming?

Then, the SAHA treatment highlights the involvement of epigenetic mechanisms underlying MS effects. This has previously been confirmed at adulthood with a 5 days SAHA treatment (Moloney et al., 2015), but this is the first study assessing the effect of a neonatal treatment on MS animals. Several epigenetic alterations have been identified after MS, especially among the HPA axis, as it has been stated in the introduction of this manuscript. In our study, SAHA treatment is used as a proof of concept that epigenetic mechanisms can modulate the development and function of nociceptive circuits in response to ELS. However, the specific targets of SAHA treatment, which allow the rescue of normal nociceptive responses and OT functions, remain unknown. Concerning the OT system, a study showed that the OTR promotor gene is subjected to increased association of acH3K14 after MS in rats, at least in the BNST and CeA which are involved in emotion and stress regulation (Litvin et al., 2016). Moreover, OTR expression is altered in many other brain regions after MS, which also could rely on epigenetic mechanisms (Lukas et al., 2010). The lab is currently investigating changes in HDAC or other epigenetic factors in the spinal cord of MS animals, which will be helpful to improve our therapeutic strategy and identify specific targets. SAHA, also called Vorinostat, is indeed a non-selective inhibitor of HDAC class I and II. A selective treatment would be better to decrease the risk of possible side effects induced by wide range molecules such as SAHA. However, SAHA is interesting because it is already used in human as an anti-tumoral agent and several clinical trials are ongoing to assess its tolerability and efficacy in the paediatric population (Saletta et al., 2014).

Of course, rescue experiments were restricted to the evaluation of mechanical nociceptive responses and should be extended to the analysis of thermal and visceral responses. Other behavioural analysis such as anxiety and social behaviour would also give us interesting output, since these functions are impacted by early OT manipulations (Bales and Carter, 2003a; Carter, 2003; Kramer et al., 2003) .

- Global perspectives and comments on the MS model

Our study focused on the effects of MS on the descendance. However, one should not forget that the stress of the separation also affects the dams. They display increased plasmatic corticosterone levels,

a strong anxiety state and depressive-like behaviour, that last even after weaning (Boccia et al., 2007; Eklund et al., 2009; Aguggia et al., 2013). When tested for nociceptive sensitivity 4 to 6 weeks after weaning using the tail-flick test, they also display a decreased sensibility to the anti-nociceptive effects of morphine (Kalinichev et al., 2000). This suggests that nociceptive circuits are not only altered in the descendance after MS, but that the mother's nociceptive circuits are also impaired.

Altogether, ELS models where mother-pups interaction is disturbed highlight the importance of the mother's presence in the early stages of development of the newborn. In human, great efforts are made to restore and preserve mother-child interactions in NICU. In particular a procedure called kanagaroo mother care (KMC) is more and more used, especially in preterm babies. This strategy aims to restore the physical interactions between the parents and the newborn (Ruiz-Pelaez et al., 2004). The child is carried on the chest of the parent, allowing him to benefit from the parent's scent and warmness, and to follow the movement of the parent's breathing. A recent meta-analysis suggested that kangaroo care is efficient to improve the outcome of preterm birth, decreasing mortality and risks of neonatal sepsis, but also improving autonomic function and brain maturation and decreasing later hospital readmission (Boundy et al., 2016). On top of that, many studies showed that KMC in the newborn is beneficial against pain. Indeed, it decreases pain behaviours and the autonomic responses to heel stick or intramuscular injections in the preterm newborn (Johnston et al., 2003; Ludington-Hoe et al., 2005; Castral et al., 2008; de Sousa Freire et al., 2008; Kashaninia et al., 2008; Cong et al., 2009). It is also effective in the very preterm newborn, decreasing PIPP scores after heel lance procedure and decreasing the recovery time after the procedure (Johnston et al., 2008). Kangaroo care can also be provided by fathers; however Johnston and colleagues showed that maternal kangaroo care is more effective to decrease pain during heel lance procedures (Johnston et al., 2011). Interesting studies by Johnston and colleagues tried to decipher if maternal voice, alone or associated with maternal touch was sufficient to induce an effective analgesia during pain procedures. They recorded maternal voice and used it during heel-lance procedure, which was not sufficient to decrease pain behaviour in the baby (Johnston et al., 2007). In another study, they showed that maternal voice associated with maternal contact was also not enough to change the autonomic response to an invasive procedure, but that is has beneficial effect on recovery time, which was faster in the group that benefitted from maternal presence (Johnston et al., 2012). Mother-infant interactions induce a release of oxytocin in the newborn (Matthiesen et al., 2001), which could at least partially explain the analgesic effects of kangaroo care. Besides, on top of the analgesic effect, KMC is an effective strategy to reduce stress in the newborn, decreasing corticosterone levels and cardiac frequency, but also to decrease stress in the mother (Cho et al., 2016). A study even showed that skin to skin contact increases the amount of maternal milk (Hurst et al., 1997), which could be linked to the beneficial effects of KMC on preterm weight intake (Bera et al., 2014).

***Long term effects of KMC***. If it is well accepted that KMC can induce an efficient analgesia in the newborn, the possibility of long lasting protective effects on pain responses still has to be explored. However, a few medical teams are starting to publish follow up studies of KMC children. Schneider and colleagues used transcranial magnetic stimulation to record the function of motor circuits in the primary motor cortex in adolescent born preterm which benefited from KMC, a control preterm group and adolescents born at term (Schneider et al., 2012). They showed that KMC has positive effects on the function of motor circuits since this group presented faster conduction and hemispheric transfer time, but also more frequent inhibitory processing. This shows a better

synchronization and connectivity of motor circuits in adolescent infant which benefitted from KMC, as compared with control preterm infants. Feldman and colleagues followed ancient preterm babies at up to 10 years, and measured cognitive development, executive functions, stress response and autonomic function (Feldman et al., 2014). They showed that KMC has protective effects on stress response and lead to better cognitive and executive functions, as well as a better organized sleep. One research team recently compared health status, cognitive and social functions and behaviour at 20 year-old between KMC group and a control group (Charpak et al., 2017). Among other results, the overall frequency of chronic conditions was the same in the two groups, but the motor functional deficit was higher in the control group than in the KMC group. Also, on their neuroimaging study, KMC group had larger volume of grey matter, cerebral cortex and left caudate nucleus than the control group, suggesting a protective effect of kangaroo care on brain development.

Finally, if the health protective effect is not that clear, KMC seems to be protective against the deleterious behavioural and anatomical consequences of prematurity on the development of the brain. For now, we don't know if kangaroo care can erase some deleterious consequences of early pain on later pain responses, but other follow up studies might help to answer this question in the next few years. Besides, fundamental studies using the MS model in rodents suggest that even the absence of the mother alone is highly deleterious for the proper development of the brain and the nociceptive system. Non-pharmacological strategies like KMC used to reduce parental separation and to treat early pain hence seem promising. However, the identification of the mechanisms underlying the effects of ELS, being early pain, MS or other type of adversities, could lead to new strategies to prevent or treat the development of pain related hypersensibilities or chronic disease.

# L) Complementary papers and reviews

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# Characterization of the fast GABAergic inhibitory action of etifoxine during spinal nociceptive processing in male rats

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ABSTRACT

Etifoxine (EFX) is a non-benzodiazepine anxiolytic which potentiate $GABA_A$ receptor ($GABA_AR$) function directly or indirectly via the production of 3α-reduced neurosteroids. The later effect is now recognized to account for the long-term reduction of pain symptoms in various neuropathic and inflammatory pain models. In the present study, we characterized the acute antinociceptive properties of EFX during spinal pain processing in naive and monoarthritic rats using in vivo electrophysiology. The topical application of EFX on lumbar spinal cord segment, at concentrations higher than 30 μM, reduced the excitability of wide dynamic range neurons receiving non-nociceptive and nociceptive inputs. Windup discharge resulting from the repetitive stimulation of the peripheral receptive field, and recognized as a short-term plastic process seen in central nociceptive sensitization, was significantly inhibited by EFX at these concentrations. In good agreement, mechanical nociceptive thresholds were also significantly increased following an acute intrathecal injection of EFX. The acute modulatory properties of EFX on spinal pain processing were never seen in the simultaneous presence of bicuculline. This result further confirmed EFX antinociception to result from the potentiation of spinal $GABA_A$ receptor function.



## 1. Introduction

Several mechanisms of spinal disinhibition associated with pathological pain states have recently been characterized and potentiation of GABAergic inhibitory controls in the spinal cord is thought to be an efficient strategy to limit or prevent pain symptoms (Zeilhofer et al., 2012b). Indeed, intrathecal injections of positive allosteric modulators of $GABA_A$ receptors ($GABA_ARs$) function, such as benzodiazepines or 3α-reduced neurosteroids, reduced pain responses in various animal models and in human pain states (Goodchild and Serrao, 1987; Serrao et al., 1992). Results from these studies have, however, been difficult to interpret because of sedative, anxiolytic, and rewarding properties of these compounds. To overcome this difficulty, a growing number of studies are now attempting to use subtype-selective benzodiazepines (Zeilhofer et al., 2012a). In the case of neurosteroids, we recently adopted an alternative strategy aimed at stimulating their endogenous production with translocator protein (TSPO) agonists (Rupprecht et al., 2010). This strategy already appeared to be efficient in some models of pain while using olesoxime (TRO19622), a cholesterol derivative with neuroprotective properties (Bordet et al., 2008), for example. In our laboratory, we demonstrated that 3α-reduced neurosteroids produced after TSPO stimulation with the benzoxazine etifoxine (EFX) were responsible for the long-lasting analgesic effects, seen in several animal models of neuropathic and inflammatory pain (Aouad et al., 2009, 2014a, 2014b). Analgesic mechanisms included amplification of $GABA_AR$-mediated transmission, protection from prostaglandin E2-induced glycinergic disinhibition, reduction of pro-inflammatory processes and maintenance of proper chloride gradients (Aouad et al., 2014b).

Apart from these long-term effects mediated by TSPO, little is known on the acute modulation of $GABA_AR$ function by EFX in the spinal cord and its impact in spinal pain processing. Etifoxine (EFX) is commercially-available as a non-benzodiazepine anxiolytic in several countries (Micallef et al., 2001; Nguyen et al., 2006; Servant et al., 1998) and exerts positive allosteric modulation of β2/β3-containing $GABA_ARs$ (Hamon et al., 2003; Schlichter et al., 2000; Verleye et al., 1999, 2001). These subunits are likely to constitute most of the $GABA_ARs$ since they are widely expressed in all laminas of the spinal cord in rodents (Bohlhalter et al., 1996; Paul et al., 2012) and human (Waldvogel et al., 2010). So far, the precise

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location of EFX binding site on β subunit is not known. It is, however, apparently not overlapping with specific sites for benzodiazepine and neurosteroids since the potentiating action of EFX is not altered after binding of the silent benzodiazepine site antagonist flumazenil or of the neurosteroid allopregnanolone (Verleye et al., 1999, 2001). Using freshly dissociated spinal cord neurons, potentiation of $GABA_AR$ currents was observed with low micromolar concentrations of EFX (Schlichter et al., 2000).

To characterize the acute action of EFX on spinal nociceptive processing, we recorded deep dorsal horn neurons (i.e. wide dynamic range neurons), integrating peripheral noxious and non-noxious informations, in anesthetized adult rats. Action potential (AP) discharges resulting from the activation of non-noxious and noxious sensory neurons were analyzed in protocols of acute nociceptive stimulation of the receptive field (RF) and during short-term potentiation of action potential discharge (windup) induced by repetitive stimulation, as previously published (Juif and Poisbeau, 2013). Complementary experiments on a model of knee monoarthritic have also been performed.

## 2. Material and methods

Male Sprague Dawley rats (250–350 g; Janvier, Le Genest St Isle, France) were used for this study. They were housed by group of 4 under standard conditions (room temperature [22 °C], 12–12 h light–dark cycle) with *ad libitum* access to food and water. All experiments were conducted in conformity with the recommendations of the European Union directive on animal experimentation (2010/63/EU adopted on September 22, 2010) and were evaluated by the regional ethic committee in charge of animal experimentation (CREMEAS, authorization AL 01/01/02/11). This study was conducted under the responsibility of authorized personnel (license 67-116 from the French Department of Agriculture to PP).

### 2.1. In vivo electrophysiology

Single unit extracellular recordings were made from dorsal horn neurons in the lumbar enlargement of the spinal cord of the rat following the procedure previously described elsewhere (Juif and Poisbeau, 2013). Briefly, a laminectomy was performed in anesthetized rats (isoflurane; Vaporizer Isotec 3 datex-Ohmeda) to expose the L4–L5 segments of the spinal cord. Before recordings, the cord was firmly attached by vertebral clamps and meninges were delicately removed, and the spinal cord surface was covered with a thin layer of mineral oil. Single-unit extracellular recordings were made with a stainless steel electrode (FK#02; FHC, UK) connected to a differential amplifier (DAM80, WPI). An electrode was lowered into the dorsal horn to record neurons located in the deep dorsal horn of the spinal cord. Data were acquired and analyzed by a CED 1401 analog-to-digital interface coupled to a computer with Spike 2 software (Cambridge Electronic Design, Cambridge, UK). All neurons included in the present study were wide dynamic range (WDR) neurons (Le Bars and Cadden, 2009) responding to both innocuous and noxious stimuli after electrical stimulation of the peripheral hind paw RF and located in the medial part of the deep layers of the dorsal horn (752 ± 27 μm; n = 12). Note that 25% of them were found to project to supraspinal structures (Juif and Poisbeau, 2013). After stimulation of the RF, the recorded neuron emitted APs. Two protocols of stimulation were done: (i) 60 stimulations at a frequency of 0.2 Hz (*i.e.* 5 min of recording); stimulus intensity of 1.5 times C-fiber threshold; pulse duration of 1 ms and (ii) wind up (30 stimulations, frequency: 1 Hz, intensity: 3xC-fiber threshold, pulse duration: 1 ms). Wind up efficiency was assessed through the slope and was calculated as a ratio (number of action potentials emitted by the neuron after last stimulation and divided by the number of APs triggered by the first stimulation).

Post-stimulus histograms were built by counting the number of APs corresponding to the activation of fast-conducting Aβ (delay to stimulus artifact <20 ms), slow-conducting Aδ (delay of 20–90 ms) and very slow-conducting C fibers (delay of 90–300 ms), as it is described in the literature for rats weighting about 250 g or more and used for electrophysiology experiments (Urch and Dickenson, 2003). With such animals, APs observed 300–800 ms after the stimulus artifact were considered as being part of the postdischarge. For this experimental approach, it is assumed that most of the non-nociceptive and nociceptive informations are mostly transmitted via Aβ fibers and C fibers, respectively in naïve animals. AP changes were compared before and immediately after the topical EFX application on the spinal cord.

### 2.2. Behavioral testing

All animals were habituated to the room and to the tests at least one week before starting the experiments. Mechanical nociceptive thresholds were measured using a calibrated forceps (Bioseb, Vitrolles, France) as previously (Aouad et al., 2009). Briefly, the habituated rat was loosely restrained with a towel masking the eyes in order to limit stress by environmental stimulations. The tips of the forceps are placed at each side of the paw and a gradually increasing force was applied. The pressure, in gram, producing withdrawal of the paw or in some cases the vocalization of the animal, corresponds to the nociceptive threshold value. This manipulation was performed three times for each hindpaw and the values were averaged.

### 2.3. Drugs and treatments

EFX (Biocodex, Gentilly, France) was prepared in saline (NaCl 0.9% in distilled water) containing 1% tween 80 (v/v; Sigma, St Louis, USA) and was injected intrathecally 20 min before behavioral testing (dose: 0.6 μg in 20 μl). EFX was applied at the surface of the spinal cord during in vivo electrophysiological recording at 3 different concentrations: 5, 30 and 60 μM. Bicuculline (Sigma–Aldrich, France) was diluted in saline and administered on the spinal cord at a final steady-state concentration of 10 μM. Analysis was performed 15 min after EFX application. After this period, EFX was washed out from the saline solution covering the exposed spinal cord segment.

At the end of the study, EFX was also tested using in vivo electrophysiology of WDR neurons on monoarthritic rats, one week after a unilateral knee injection of 50 μl CFA (complete Freund's adjuvant; Sigma St Louis, MO, USA). The control animals received an equivalent volume of mineral oil, the vehicle of CFA, as previously published (Aouad et al., 2014b).

### 2.4. Statistics

All data are expressed as mean ± standard error of the mean (SEM). Repeated measure one-way ANOVA followed by Bonferroni comparisons was used to analyze the effects on AP firing while recording from spinal neurons in vivo. Student's t test helped compare the electrical thresholds between two groups (unpaired) or before/after drug application (paired). When parametric tests were inappropriate (low N number of observations or data not normally distributed, A Kruskal–Wallis test or Wilcoxon matched pair test was used. In this later case, Dunn's multiple comparisons posthoc test was used to compare the experimental value to the control. Differences were considered to be statistically significant for $p < 0.05$.

## 3. Results

### 3.1. Etifoxine increases mechanical nociceptive threshold and reduces the excitability of WDR neurons after peripheral nociceptive stimulation

In freely-moving animals, mean mechanical thresholds were of 583.3 ± 19.7 g (n = 7) and they remained unchanged after injection of the vehicle of EFX (Fig. 1B; 542.4 ± 14.3 g; n = 5). In sharp contrast, EFX injection resulted in a significant increase of mechanical threshold, which reached a mean value of 768.1 ± 22.2 g (n = 5; Wilcoxon, $p < 0.05$). Compared to control, this corresponded to an increase by about 42% thus confirming the acute anti-nociceptive properties of EFX when spinally administered.

To further analyze EFX action on spinal nociceptive processing, we recorded WDR neurons and first characterized its possible effect of the electrical activation threshold required to observe an A and C-mediated action potential (AP) discharge in WDR neurons (representative traces in Fig. 1A). Compared to control (i.e. before application), we failed to see any changes in the thresholds while using concentration of 5 μM (Fig. 1C). When compared to control (basal in Fig. 1C), this increase was particularly robust after application of EFX at 30 μM (Aβ: from 4.3 ± 0.3 V to 6.3 ± 0.5 V, n = 8; one-way Anova, $F_{4,40} = 17.83$, Bonferroni $p < 0.01$; C: from 21.7 ± 0.4 V to 26.4 ± 0.3 V, n = 12; Bonferroni $p < 0.001$) and at 60 μM (Aβ: 7.4 ± 0.3 V, Bonferroni $p < 0.001$, Aδ: from 15.0 ± 1.2 V to 23.2 ± 0.6 V, n = 5; Kruskal Wallis, $p < 0.01$, Dunn's comparisons $p < 0.05$ and C: 32.1 ± 0.6 V; Bonferroni $p < 0.001$). This increase was fully abolished when EFX (60 μM) was co-administered with bicuculline (10 μM) confirming that this change was mediated by $GABA_A$ receptors.

EFX effects on spinal nociceptive processing were next characterized by quantifying changes in the number of APs emitted by WDR with respect to the respective contribution of Aβ, Aδ and C sensory neurons. Using a stimulation intensity of 1.5 times the threshold for C fibers (frequency: 0.2 Hz; pulse duration: 1 ms), we only observed a significant decrease in the number of APs after

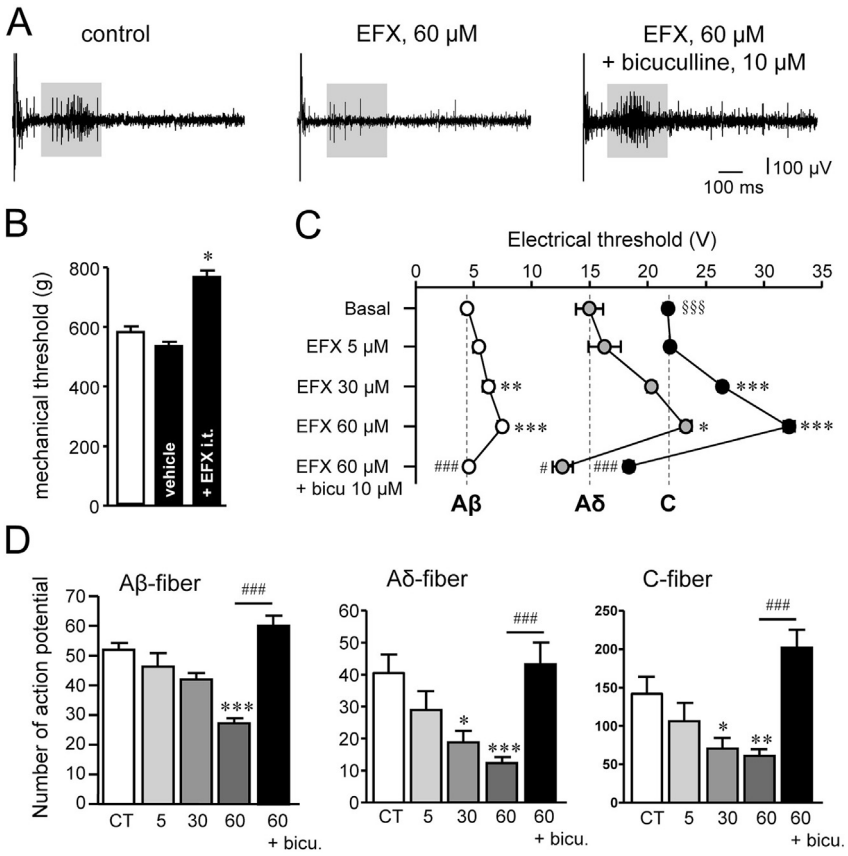


**Fig. 1.** A. Typical traces representing a single electrical stimulation (intensity: 3 times C-fiber threshold) under basal conditions (left) and following etifoxine (EFX, 60 µM; middle) or EFX (60 µM) + bicuculline (10 µM; right). B. Mechanical threshold (in gram) before (white bar, n = 7) and following (black bar) intrathecal injection of EFX (0.6 µg/20 µl, n = 5) or its vehicle (n = 5). C. Electrical threshold of Aβ- (white, n = 12), Aδ- (grey, n = 4) and C-type (black, n = 12) fiber measured before and following EFX application on the spinal cord. D. Histograms representing the dose response effects of EFX on Aβ (left, n = 12), Aδ (middle, n = 12) and C (right, n = 12) fiber-related AP discharge. Compared to control (CT, white bars), EFX effects are indicated at 5, 30 and 60 µM. Black bar on the right represent the application of EFX at 60 µM in the presence of bicuculline (bicu.).Statistics: Wilcoxon matched pairs test (panel B) and ANOVA followed by Bonferroni comparisons (control vs. treatment): (*)$p < 0.05$, (**)$p < 0.01$, (***)$p < 0.001$; In panel C: (§§§) $p < 0.001$ while comparing Aβ vs C-fiber threshold; (###) $p < 0.001$: EFX vs EFX + bicuculline and (*): control vs treatment).

application of EFX at 30 µM and 60 µM (Fig. 1D). At the maximal concentration tested of 60 µM, EFX significantly reduced the number of Aβ-mediated APs (from 51.9 ± 2.8 to 27.2 ± 1.7, n = 12; one-way Anova, $F_{4,40} = 14.5$; $p < 0.001$; Bonferroni $p < 0.001$), Aδ- (from 40.6 ± 5.9 to 12.4 ± 1.9, n = 12; one-way Anova, $F_{4,40} = 8.0$; $p < 0.001$; Bonferroni $p < 0.001$) and C-mediated fibers (from 142.2 ± 21.9 to 61.2 ± 8.5, n = 12; one-way Anova, $F_{4,40} = 8.4$; $p < 0.01$; Bonferroni $p < 0.01$). Note that APs, mediated by Aδ- and C-, but not Aβ fibers were already significantly reduced with EFX at 30 µM. We also observed that reduction in the number of AP by EFX was not seen when bicuculline was co-applied at 10 µM (number of APs emitted by C-type fibers: control: 142.2 ± 21.9 EFX + bicuculline: 202.1 ± 23.1, n = 6; Bonferroni $p > 0.05$).

### 3.2. Intrathecal EFX limits short-term potentiation of action potential discharge through a $GABA_A$R-mediated action

Short-term potentiation of AP discharge (windup) has been triggered here using a train of repetitive stimulation of the peripheral RF at constant intensity (3 times the C-fiber threshold, pulse duration: 1ms) and frequency (1 Hz). With the selection procedure used to record WDR neurons (i.e. displaying A and C discharges), we found that all neurons exhibited windup with our stimulation procedure (see representatives traces in Fig. 2A). Windup remained unaffected by a concentration of 5 µM EFX (not shown) whereas it was strongly limited at 30 µM and above (n = 8). This effect can be quantified with the windup ratio (see methods, Fig. 2B). It was significantly reduced in the presence of EFX at 30 µM (ratio = 11.0 ± 1.6, n = 8; one-way Anova, $F_{4,40}$: 48.91; $p < 0.001$; Bonferroni $p < 0.001$) and at 60 µM (ratio = 11.3 ± 1.5, n = 12; one-way Anova, $F_{4,40}$: 48.91; $p < 0.001$; Bonferroni $p < 0.001$), compared to the control windup ratio (17.4 ± 3.1, n = 12). When EFX was co-administered with bicuculline, the windup ratio was similar to the control (15.2 ± 1.7; Bonferroni $p > 0.05$). EFX-induced reduction in the number of APs during windup could also be seen as a reduction in the windup slope before reaching the plateau phase as illustrated in Fig. 2C–D. Note that the reduction in windup ratio and slope was absent when EFX (60 µM) was co-applied with the $GABA_A$R antagonist bicuculline (10 µM). All effects were transient (seen after 15 min) and rapidly washed out (30 min) after removal of EFX.

### 3.3. EFX action in the monoarthritic pain model

To go one step further, EFX action was characterized in a rat model of persistent inflammatory pain. Knee monoarthritis was induced by an intra-articular injection of complete Freund's adjuvant giving rise to a local inflammation and the development of mechanical and thermal hot hyperalgesia (Aouad et al., 2014b). While recording from WDR neurons in anesthetized CFA-injected rats, we first confirmed this nociceptive hypersensitivity since electrical threshold to observe C-related action potentials was very low (15.1 ± 1.8 V, n = 10) compared to the control groups (oil

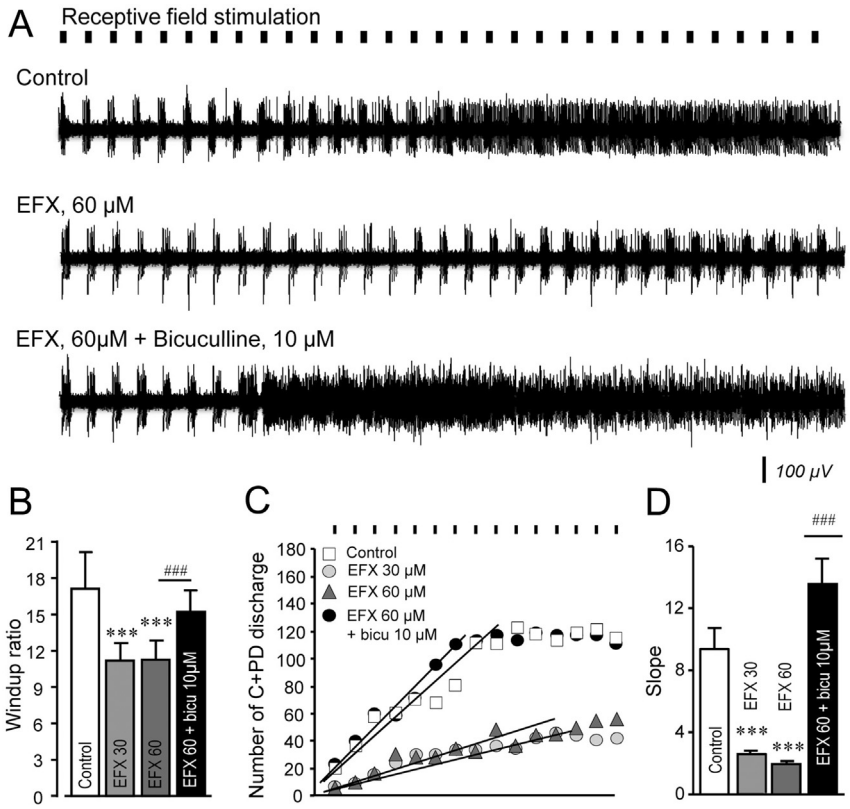


**Fig. 2.** A: Representative traces illustrating single unit wide dynamic range neuron displaying windup (30 stimulations, 3 times the C-fiber threshold, frequency: 1 Hz) before (CT, top trace) and after (middle trace) EFX (60 μM). The bottom trace represents the responses when EFX (60 μM) is co-applied with the $GABA_AR$ antagonist bicuculline (10 μM). In order to clarify the figure, all the artifacts of stimulation have been removed. B: Histogram showing the windup ratio for the different experimental conditions: control conditions (white bars, n = 12); after EFX application at 30 μM (light grey, n = 8) and at 60 μM (dark grey, n = 12). Black bar shows the windup ratio after application of EFX at 60 μM in the simultaneous presence of the $GABA_A$ receptor antagonist (at 10 μM). C, D: Evolution of the initial slope for a representative WDR neuron (graph in C) and mean slope values for all recorded neurons (graph in D, same N number than in panel B). Statistical code: (*** control vs. EFX treatment; ###, EFX 60 μM vs. EFX 60 μM + Bicuculline 10 μM) significativity at $p < 0.001$ with Bonferroni comparisons after repeated measures one-way ANOVA.

injected: 21.7 ± 0.5 V, n = 8; Student's t = 3.443; df = 10.31; $p < 0.01$). Hyperexcitability of the neighboring neurons and of the recording WDR cell after electrical stimulation did not allowed us to accurately measure threshold for A fibers and forced us to focus our attention on C fiber threshold. After application of EFX at 60 μM, we did observe a significant increase in C fiber threshold which reached 20.2 ± 2.3 V (n = 10, Student's: t = 4.594, df = 9, $p < 0.01$). As illustrated in Fig. 3, EFX effect on C fiber threshold (panel A) was not accompanied by a change in windup ratio (panel B–C, n = 7, Students, t = 1.427, df = 6, $p > 0.05$).

## 4. Discussion

In this study, we show that the fast non-genomic antinoceptive effect of EFX is mediated by a $GABA_AR$-mediated inhibition in the dorsal horn of the spinal cord. This inhibition is associated with an increase in the mean mechanical nociceptive threshold in freely-moving naïve animals, an overall increase in the electrical threshold to observe A- and C-mediated discharges in WDR neurons and a limitation of windup during repetitive RF stimulation. In monoarthritic rats, EFX antinociception was seen as an increase in C fiber threshold since windup appeared unchanged.

This fast inhibitory action of EFX on spinal nociceptive processing, seen about 15 min after the in vivo application, is consistent with a potentiation of $GABA_AR$ function (Hamon et al., 2003; Schlichter et al., 2000; Verleye et al., 1999, 2001). This potentiation may occur at different places in the dorsal horn of the spinal cord because EFX-sensitive $GABA_ARs$, containing β2-3 subunits, are theoretically expressed by primary afferents of sensory neurons and by most, if not all, dorsal horn neurons (Bohlhalter et al., 1996; Waldvogel et al., 2010). Here, we observed a rather non-specific inhibition of the AP discharge resulting from the activation of C- and A-fibers. Without excluding other possibilities, this likely suggests an elevated $GABA_AR$ inhibitory control onto the recorded WDR neurons limiting their general excitability after a noxious stimulation of the peripheral RF. Another alternative deals with a non-specific increase in the presynaptic inhibition of primary afferents. In any case, an increased efficacy of GABAergic interneurons is involved and perfectly explains the observed EFX effects. Indeed, intrathecal injections of $GABA_AR$ agonists and of allosteric-positive $GABA_AR$ modulators have previously proven their efficacy to reduce pain thresholds in human and animal models (Clavier et al., 1992; Eaton et al., 1999; Edwards et al., 1990; Goodchild and Serrao, 1987; Knabl et al., 2008; Serrao et al., 1992). On the other hand, GABAergic transmission is essential to prevent the appearance of pain symptoms as for example observed after intrathecal injections of bicuculline in vivo (Charlet et al., 2008; Ishikawa et al., 2000; Sivilotti and Woolf, 1994). Our results on monoarthritic rats revealed that EFX antinociception is mediated by an increase in the activation threshold of C fibers and apparently no longer by an inhibition of windup. This interesting result may suggest that presynaptic inhibition of C-type primary afferent fibers by EFX, presumably by potentiating $GABA_AR$ function, is preserved in inflammatory pain states whereas windup inhibition

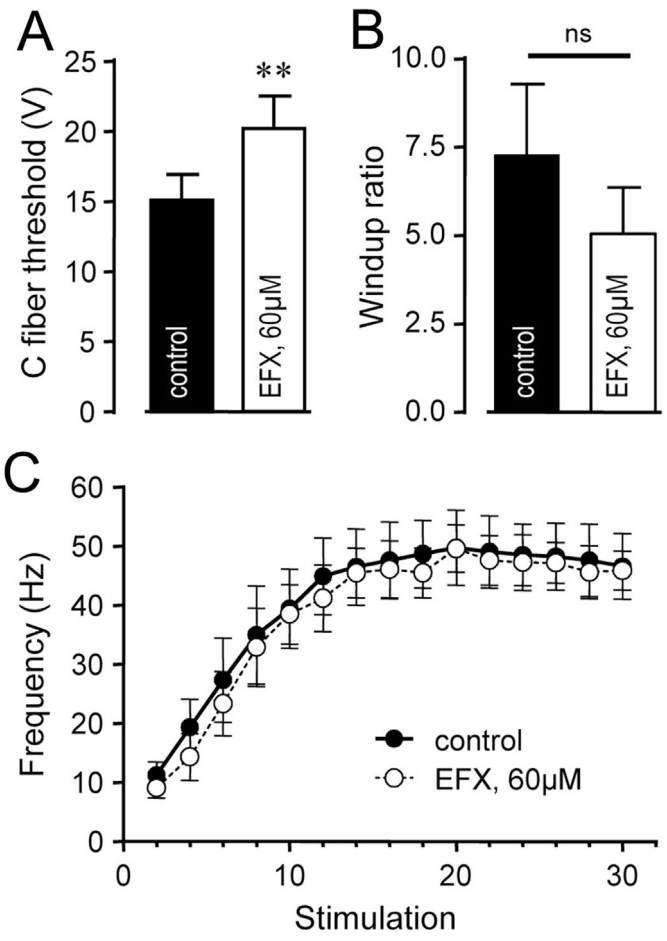


**Fig. 3.** Etifoxine effects in monoarthritic rats. A: Histogram illustrating C fiber threshold before and 15 min after local spinal application of EFX (60 μM) B,C: Windup ratio (B) and development (C) before and 15 min after EFX application at 60 μM. Statistical code: (**), $p < 0.01$ Student's t-test, control vs. EFX).

exhibited by WDR neurons is no longer possible. This result can be fully explained by a preserved presynaptic shunting effect of $GABA_A$Rs on the primary afferent C-fibers combined to spinal disinhibition of dorsal horn layer neurons (like WDR neurons) processing nociceptive messages. The alteration of chloride gradients in spinal cord neurons of inflamed rats, reported by several groups around the world (Aouad et al., 2014b; Morales-Aza et al., 2004; Zhang et al., 2008), could fully explain the effects of EFX seen here. In summary, these results suggest that EFX fast action in pathological pain states may be weaker than in physiological pain conditions.

Etifoxine is an interesting $GABA_A$R modulator because it has clear anxiolytic properties (Nguyen et al., 2006; Servant et al., 1998; Ugale et al., 2007) and limited adverse side effects (Micallef et al., 2001), compared to the classical benzodiazepines (e.g. amnesia, sedation, functional tolerance …). There are also growing evidences suggesting that EFX promotes nerve regeneration (Girard et al., 2008) and several molecular mechanisms have been recently proposed to explain this property (Zhou et al., 2013, 2014). In our laboratory, we did observe that EFX prevents the apparition of inflammatory and neuropathic pain symptoms in several pain models (Aouad et al., 2009, 2014a, 2014b). So far, we concentrated on the long-term therapeutic effects and confirmed that they were mostly mediated by the local production of the endogenous neurosteroid analgesics such as allopregnanolone (Poisbeau et al., 2014). Interestingly, the potent analgesia resulting from EFX treatment involves several synergistic mechanisms. In the spinal cord, this include a limitation of pro-inflammatory processes, a proper maintenance of chloride gradients, a protection from glycinergic disinhibition and an amplification of $GABA_A$R inhibitory function (Aouad et al., 2014b). In pathological pain states, alterations of these processes have all been demonstrated to contribute for a large part to the expression of pain symptoms (Zeilhofer et al., 2012b).

In the present study, we have characterized the fast non-genomic effects of EFX when administered in the spinal cord of rats during nociceptive processing. In this work, dedicated mostly to the electrophysiological analysis of EFX action during spinal nociceptive processing, we did not characterize potential motor or sedative side effects of the compound. Sedative effects are clearly limited in human studies when compared to similar doses of lorazepam (Micallef et al., 2001). In animal studies, they are only observed after intraperitoneal injection of concentration higher than 70 mg/kg (Poisbeau & Kamoun, unpublished data). Up to now, we have no evidence for such an effect after intrathecal injection. In addition to the *princeps* studies describing EFX potentiating effects on $GABA_A$ receptors (Hamon et al., 2003; Schlichter et al., 2000; Verleye et al., 1999, 2001), we provide here novel evidence of this mechanism, but during processing of nociceptive messages in vivo. Without having any other binding site described so far for EFX at the membrane level, our results strongly suggest that EFX reduces the spinal nociceptive processing by potentiating $GABA_A$R function at presynaptic and postsynaptic sites. In naïve rats, the inhibitory effect of EFX on the excitability of WDR neurons is not restricted to nociceptive messages mediated by Aδ and C fibers, but also affects the processing of non-nociceptive Aβ sensory messages. In monoarthritic rats, we confirmed EFX antinociception on C fiber activation threshold. In summary, the fast (present study) and long-lasting effects of EFX (Aouad et al., 2014b), mediated at least by a modulation of spinal $GABA_A$R, are of significant interest to limit spinal pain processing and the expressed pain symptoms, in physiological and pathological pain states.

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FAITES LE POINT

# Conséquences des perturbations périnatales sur les réponses douloureuses

## *Consequences of perinatal disturbances on pain responses*

Pierrick Poisbeau

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**MOTS CLÉS**
Douleur chez l'enfant ;
Interaction mère—enfant ;
Soins intensifs ;
Prématurité ;
Développement du système nociceptif ;
Épigénétique

**Résumé** Pendant de nombreuses années, le monde médical n'a pas reconnu la capacité du jeune enfant à ressentir la douleur. Parmi les arguments avancés, la question de l'immaturité du système nociceptif et de la myélinisation des circuits nociceptifs ont été des arguments majeurs utilisés pour expliquer l'absence de souvenirs douloureux dans les premières années de vie. Les travaux d'Anand et al., publiés à la fin des années 1980, ont permis de corriger ce préjudice. Il est maintenant clairement établi que l'organisation neuroanatomique du système nociceptif est fonctionnelle au début du troisième trimestre de la vie fœtale permettant d'observer une intégration corticale des messages nociceptifs périphériques. L'immaturité du système nociceptif, évoquée en fin de grossesse et au début de la vie extra-utérine, touche plus spécifiquement les filtres inhibiteurs au sein du système nerveux central dont ceux de la moelle épinière. De nombreux progrès doivent encore être réalisés dans la prise en charge de la douleur chez l'enfant. Plus de 20 ans après la publication des travaux d'Anand, il était constaté que seulement 20 à 30 % des interventions douloureuses était effectuées à l'aide d'une analgésie préemptive chez les enfants nouveau-nés prématurés, par exemple. Dans cette revue, le développement du système nociceptif du nouveau-né et son expression douloureuse seront tout d'abord présentés. Évaluer la douleur chez le nouveau-né reste effectivement un challenge puisqu'il lui est impossible de l'exprimer sous forme d'une plainte intelligible. Des approches indirectes, basées sur l'observation de « signes » de douleur sont donc nécessaires et ont été développées. Il s'agit par exemple de l'évaluation des réponses autonomes à la douleur : fréquence cardiaque, fréquence respiratoire, pression artérielle ou mesure de la saturation en oxygène du sang. Ces données peuvent être associées utilement à la mesure d'indicateurs comportementaux basés sur l'observation générale des mouvements du corps, de l'état d'agitation de l'enfant, de la durée des pleurs ou encore de l'analyse de l'expression faciale (froncement

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des sourcils, ouvertures des lèvres, contraction des paupières...). Au-delà de la question de la reconnaissance de la douleur chez l'enfant, les conséquences à long terme de la prise en charge des nouveau-nés dans les unités de soins intensifs sont un sujet d'inquiétude. Ces perturbations périnatales douloureuses et non-douloureuses (c.-à-d. excès de stimulations sensorielles, déficit d'interaction mère—enfant...) semblent laisser une empreinte à long terme au sein même du système nociceptif. L'enfant devenu adulte montre ainsi une réponse accentuée ou inappropriée vis-à-vis des stimulations douloureuses, un risque plus élevé de développer des douleurs chroniques et plus généralement, fait preuve d'une mauvaise adaptation vis-à-vis des stress environnementaux. Les altérations à long terme de la réponse douloureuse, induites par les procédures douloureuses néonatales ou par la mauvaise qualité des interactions mère—enfant, seront présentées à la fin de cette revue. Les données récentes de la recherche fondamentales sur des modèles animaux et sur les mécanismes associés seront également évoquées.


**KEYWORDS**
Child pain;
Mother—child interactions;
Intensive care;
Development of the nociceptive system;
Epigenetic

**Summary** For several years, the medical world did not recognize the capacity of the young child to feel pain. Among the advanced arguments, the question of the immaturity of the nociceptive system and the myelination of nociceptive circuits were major arguments used to explain the absence of painful memories in the first years of life. The works of Anand et al., published in the end of the 1980s, allowed to correct this dramatic mistake. It is now clearly established that the neuroanatomical organization of the nociceptive system is functional at the beginning of the third trimester of fetal life and allows observing a cortical integration of peripheral nociceptive messages. The so-called immaturity of the nociceptive system described at the end of pregnancy and during the first weeks of extra-uterine life touches, more specifically, the inhibitory filters within the central nervous system, including those present in the spinal cord. Numerous progresses must be again realized in the child pain care. Indeed, more than 20 years after the publication of Anand's works, it was noticed that only 20—30% of the painful interventions were made by means of a preemptive analgesia on premature newborn children. In the first part of this review, the development of the nociceptive system of the newborn child and his painful expression will be described. To evaluate pain in newborn child stays actually a challenge. Indirect approaches, based on the observation of ''signs'' of pain are thus necessary and have been developed. This includes evaluation of the autonomous responses to pain stimulus: changes in heart rate, respiratory frequency, blood pressure or blood oxygen saturation. These data can be usefully associated with the measure of behavioral indicators such as abnormal body movements, cry duration or facial expression scores. Beyond the question of recognizing child pain, the long-term consequences of intensive neonatal care are also critical. Painful and non-painful perinatal disturbances (i.e. excess of sensory stimulations, deficit of mother-child interactions, etc.) seem to leave a long-term imprint within the nociceptive system. Adults with intensive neonatal care history often express a higher response to pain stimulus, inappropriate stress adaptation and a higher risk of developing chronic neuropathologies including chronic pain. Long-term consequences of neonatal pain, led(inferred) by the neonatal painful procedures or by the bad quality of the interactions mother—child, will be presented to the end of this magazine(review). The recent data of basic researches on animal models and on associated mechanisms will be also evoked.


## Reconnaître la douleur de l'enfant : une affaire d'adulte

Pendant de nombreuses années, le monde médical n'a pas reconnu la capacité du jeune enfant à ressentir la douleur. Parmi les arguments avancés, la question de l'immaturité du système nociceptif et de la myélinisation des circuits nociceptifs ont été des arguments majeurs utilisés pour expliquer l'absence de souvenirs douloureux dans les premières années de vie. Les travaux d'Anand et al., publiés à la fin des années 1980 [1], ont permis de corriger ce préjudice. En conclusion de ce travail, il est ainsi clairement établi que l'organisation neuroanatomique est fonctionnelle au début du troisième trimestre de la vie fœtale pour véhiculer l'influx nociceptif de la périphérie aux structures centrales. L'immaturité du système nociceptif touche plus particulièrement les filtres inhibiteurs au sein du système nerveux central dont la moelle épinière.

De nombreux progrès doivent encore être réalisés dans la prise en charge de la douleur chez l'enfant. Plus de 20 ans après la publication des travaux d'Anand, il était constaté que seulement 20 à 30 % des interventions douloureuses était effectuées à l'aide d'une analgésie préemptive chez les enfants nouveau-nés prématurés, par exemple [2—4]. Le développement du système nociceptif du nouveau-né et son expression douloureuse seront tout d'abord présentés dans la première partie de cette revue. Évaluer la douleur chez le nouveau-né reste effectivement un challenge puisqu'il lui est impossible de l'exprimer sous forme d'une plainte intelligible [3]. Des approches indirectes, basées sur l'observation de « signes » de douleur sont donc nécessaires et ont été développées. Il s'agit par exemple de l'évaluation des réponses autonomes à la douleur : fréquence cardiaque, fréquence respiratoire, pression artérielle ou mesure de la saturation en oxygène du sang. Ces données peuvent être associées utilement à la mesure d'indicateurs comportementaux basés sur l'observation générale des mouvements du corps, de l'état d'agitation de l'enfant, de la durée des pleurs ou encore de l'analyse de l'expression faciale (froncement des sourcils, ouvertures des lèvres, contraction des paupières...).

*Plusieurs échelles d'évaluation existent pour associer les paramètres physiologiques et comportementaux et tenter d'établir « un score » de douleur du nouveau-né pour des douleurs aiguës, prolongées ou postopératoires.*

Il existe également une échelle spécifiquement adaptée aux enfants prématurés, le PIPP [5].

Au-delà de la question de la reconnaissance de la douleur chez l'enfant, les conséquences à long terme de la prise en charge des nouveau-nés dans les unités de soins intensifs (USI) sont un sujet d'inquiétude. Ces perturbations périnatales douloureuses et non-douloureuses (c.-à-d. excès de stimulations sensorielles, déficit d'interaction mère—enfant...) semblent laisser une empreinte à long terme au sein même du système nociceptif. L'enfant devenu adulte montre ainsi une réponse accentuée ou inappropriée vis-à-vis des stimulations douloureuses, un risque plus élevé de développer des douleurs chroniques et plus généralement, une mauvaise adaptation vis-à-vis des stress environnementaux. Ces altérations à long terme de la réponse douloureuse, induites par les procédures douloureuses néonatales ou par la mauvaise qualité des interactions mère—enfant, seront présentées à la fin de cette revue. Les données récentes de la recherche fondamentales sur des modèles animaux et sur les mécanismes associés seront également évoquées.

## Le système nociceptif est fonctionnel bien avant la naissance

Plusieurs substrats anatomiques sont nécessaires pour permettre la sensation douloureuse et son expression. Ces circuits se développent en grande partie avant la naissance mais continuent leur maturation pendant la période postnatale [6,7]. L'élaboration d'une sensation douloureuse consciente nécessite des neurones sensoriels périphériques activables et couvrant les différents territoires périphériques, une articulation de ces derniers avec les neurones de deuxième ordre au sein de la moelle épinière et, des voies ascendantes fonctionnelles capables d'atteindre les régions corticales afin de donner lieu à une intégration consciente des messages nociceptifs afférents. En plus des réflexes sensorimoteurs médullaires mis en place précocement, une réponse douloureuse comportementale adaptée peut être élaborée à tous les étages du névraxe (y compris par les structures corticales) afin d'éviter ou de supporter les stimulations potentiellement nocives agressant notre organisme (c.-à-d. *fight or flight*).

Le développement cérébral débute dès les premières semaines de gestation avec la neurulation et la formation des différentes vésicules encéphaliques. Ces étapes sont rendues possibles par l'importante prolifération des cellules nerveuses qui atteint son apogée vers la 20e semaine de gestation et qui permet le début des processus de myélinisation (Fig. 1). À ce stade se constitue le milliard de neurones qui, après migration et différentiation, formera le futur système nerveux du nouveau-né. L'innervation cutanée par les neurones sensoriels débute dès le 2e trimestre de gestation et certains arcs réflexes médullaires, non nociceptifs, sont d'ores et déjà établis. Les connections avec la moelle épinière vont continuer à se renforcer tout au long du 3e trimestre de gestation. Tout particulièrement, les fibres de type C, amyéliniques et de petits diamètres, sont les dernières fibres sensorielles à établir des connections fonctionnelles avec les neurones médullaires. Le 3e trimestre de grossesse constitue donc une période critique pour le développement du système nociceptif, puisque les connexions fonctionnelles avec les structures supraspinales vont pouvoir être observées. Les potentiels évoqués corticaux sont ainsi observés dès la 30e semaine de gestation après stimulation des champs récepteurs périphériques. Cette observation est bien corrélée avec la myélinisation des fibres ascendantes, observée au moins jusqu'au thalamus à la 30e semaine de gestation. Les contrôles descendant se mettent également en place lors du 3e trimestre et vont permettre une modulation plus fine des informations et des réponses nociceptives. Comme indiqué précédemment pour les potentiels évoqués corticaux mais également par des approches de spectroscopie proche infrarouge (*near infrared spectroscopy* [NIRS]), le cortex semble capable d'intégrer les informations sensorielles (y compris nociceptives) dès le milieu du 3e trimestre de gestation [8,9]. Durant cette fenêtre temporelle les processus de dendritogenèse et de synaptogenèse sont à l'œuvre dans le cortex, menant à une augmentation du volume de la substance grise. Les analyses d'imagerie par ultrason chez les prématurés suggèrent également la présence d'un cortex cingulaire antérieur (entre 28 et 31 semaines) et insulaire (après 31 semaines), deux régions corticales clés dans l'élaboration des réponses émotionnelles à la douleur [10]. Enfin, le traitement des stimulations non-nociceptives ou nociceptives semble faire appel à des zones corticales distinctes entre la 35e et 37e semaine de gestation [11].

Finalement, les différents constituants du système nociceptif semblent mis en place et fonctionnels dès le milieu du 3e trimestre de gestation. Il reste encore immature chez

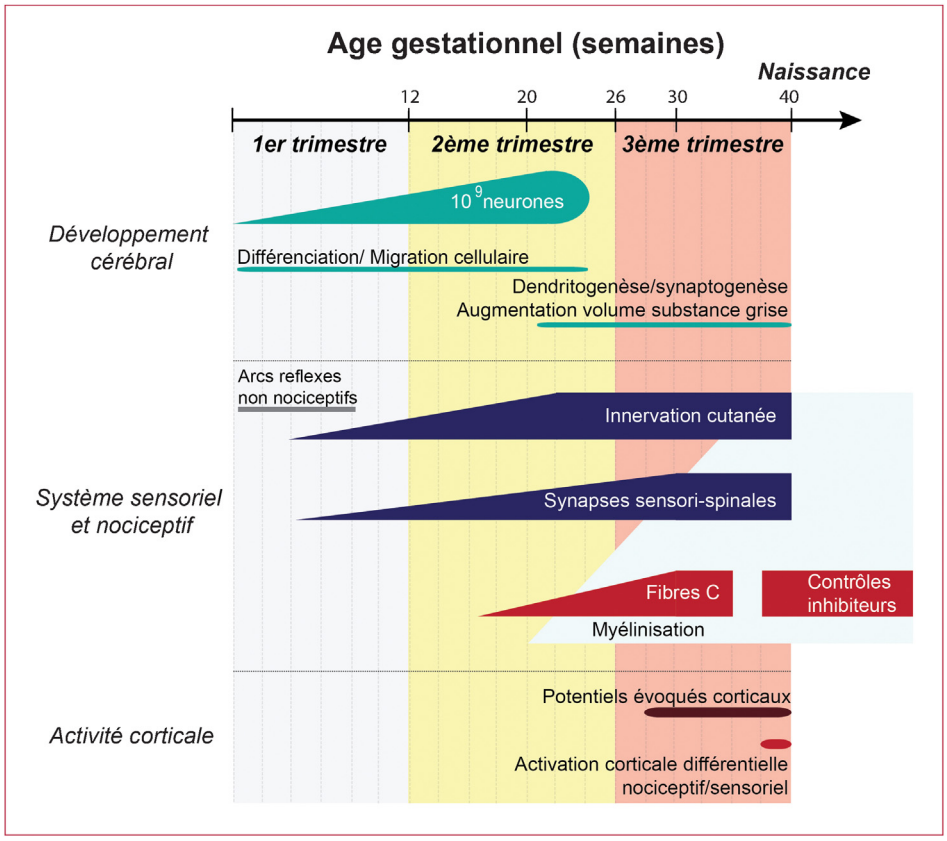


**Figure 1.** Schéma simplifié de quelques processus développementaux qui contribuent à la formation du système nociceptif au sein du système nerveux du fœtus, pendant la gestation. Voir les détails dans le texte.

le nouveau-né et sera finement remodelé dans les jours qui suivent la naissance. Ceci comprend, entres autres, la consolidation des filtres inhibiteurs tellement importants dans les contrôles segmentaires de la moelle épinière.

## Le nouveau-né est hyper-réactif lors de stimulations douloureuses

En se basant sur les données obtenues en recherche fondamentale, il semble en effet que les réseaux médullaires sont particulièrement hyperexcitables immédiatement après la naissance chez les rongeurs. Plusieurs mécanismes peuvent expliquer ce phénomène. Le premier est anatomo-fonctionnel et concerne la mise en place des fibres C. Avant cette mise en place, la fonction nociceptive semble assurée par les fibres Aβ (en charge de la sensibilité non nociceptive chez l'adulte) qui sont capables d'induire une forte activité spinale suite à des stimulations douloureuses [12]. Chez le rat nouveau-né, les fibres afférentes primaires Aβ innervent largement les couches superficielles de la moelle épinière [13], d'où elles vont se rétracter progressivement pour laisser la place aux fibres C, selon un processus dépendant de l'activité [14,15]. Si ce phénomène existe également chez le nouveau-né humain, ceci pourrait en partie expliquer l'induction de réflexes spinaux par les stimulation nociceptives avec des mouvements peu organisés, de forte amplitude et qui concernent chacun des quatre membres [16]. Toujours en se basant sur des données récentes en recherche fondamentale, l'homéostasie des ions chlorures (fortes concentrations intracellulaires) dans les neurones médullaires semble favoriser les processus excitateurs [17,18]. Ces deux éléments militent en faveur d'un déséquilibre des mécanismes excitateurs dans la corne dorsale de la moelle épinière.

Les mécanismes identifiés pourraient parfaitement expliquer pourquoi les nouveau-nés sont en réalité hyper-réactifs vis-à-vis des stimulations douloureuses. Ceci a par exemple été confirmé par des études cliniques qui montrent que plus le nouveau-né est jeune en âge, plus sa réponse douloureuse est importante [19]. Dans cet exemple, le score d'expression faciale a été utilisé pour mesurer la réaction des nouveau-nés vis-à-vis d'une prise de sang de routine et combiné à la mesure de la fréquence cardiaque et respiratoire. De même, lorsqu'une pression calibrée est exercée sur l'abdomen de nouveau-nés par l'intermédiaire des filaments de Von Frey, un réflexe de flexion bilatéral des membres inférieurs est systématiquement observé chez les plus jeunes d'entres eux, et encore accentué chez les prématurés [20]. Les seuils de déclenchement de ces réflexes sont également plus bas aux plus jeunes âges.

*Au-delà de la reconnaissance de la douleur chez l'enfant, il apparaît donc aujourd'hui que les nouveau-nés sont plus sensibles aux stimulations douloureuses et cela pose une question fondamentale quant à leur prise en charge par les unités de soin intensif.*

Ce passage est obligatoire et potentiellement problématique pour les enfants nés prématurés qui sont les plus sensibles aux stimulations douloureuses [9,20]. La prise en charge thérapeutique de la douleur dans les USI, rendue nécessaire face à ce constat, est particulièrement délicate. Elle ne fait pas l'objet de cette revue mais les stratégies actuellement utilisées sont résumée en détail dans une revue récente [21].

## Conséquences à long terme des procédures douloureuses néonatales

Du fait de l'immaturité du système nociceptif du nouveau-né, il n'est donc pas surprenant qu'il soit particulièrement vulnérable aux hyperstimulations périnatales. L'hypothèse actuelle est que ces hyperstimulations sensorielles affectent son développement normal et laissent une empreinte suffisante pour altérer durablement son fonctionnement à l'âge adulte. Cette hypothèse a été bien étayée en ce qui concerne les conséquences à long terme des stimulations douloureuses néonatales.

Les études de Taddio et al. ont montré très tôt l'importance de prendre en charge les procédures douloureuses néonatales. Ainsi, la circoncision des nouveau-nés peu après la naissance augmente significativement les scores de douleurs de ces mêmes enfants, âgés de 4 à 5 mois, lors de vaccination de routine [22,23]. L'utilisation d'anesthésiques locaux lors de telles interventions semble également contribuer à diminuer la réponse douloureuse vis-à-vis de la vaccination. Cette premier série de travaux est riche d'enseignement car elle montre qu'une seule procédure douloureuse, la circoncision, suffit par son intensité à construire une réponse douloureuse exacerbée, et quantifiable quelques mois après l'intervention. L'enfant placé en unité de soin intensif subit, pour sa part, des stimulations douloureuses beaucoup plus nombreuses. En effet, des piqûres répétées du talon chez l'enfant induisent une hypersensibilité sur le territoire concerné [24]. Cette hypersensibilité n'est pas observée si les piqûres sont effectuées après avoir appliqué un anesthésique local sur la peau. De la même façon, les besoins de l'enfant en anesthésiques et en analgésique (c.-à-d. morphine) dans la période postopératoire sont augmentés dès la deuxième chirurgie sur un même territoire [25]. Opérés initialement dans les trois premiers mois de vie, les enfants présentent des scores de douleur plus élevés à la suite de cette deuxième chirurgie. Les procédures douloureuses ont des conséquences d'autant plus dommageables qu'elles sont effectuées sur des enfants nés prématurés. Les études cliniques récentes, basées sur les techniques d'imagerie cérébrales, suggèrent que ces procédures pourraient entraîner une diminution du volume de la substance blanche et une perturbation de la maturation de la substance grise sous corticale chez des enfants prématurés [26]. Une perturbation de l'activité oscillatoire corticale a pu ainsi être observée chez des enfants grands prématurés, à l'âge où ils entrent à l'école [27].

Plusieurs études cliniques suggèrent également que les nouveau-nés hospitalisés en soins intensifs, y compris les prématurés, présentent des réponses atypiques à la douleur au moment de l'adolescence ou arrivés à l'âge adulte. Pour se faire une idée et selon les études publiées, les prématurés peuvent subir une douzaine de procédures douloureuses par jour, deux tiers d'entres-elles étant réalisées sans analgésie préemptive [2,4]. Sur une moyenne de 14 jours d'hospitalisation en soins intensifs, les procédures douloureuses varient entre 50 (prématurés) et 200 (très grands prématurés). Pour ces enfants devenus adolescents ou adultes, une hypersensibilité vis-à-vis des stimulations nociceptives thermiques peut être parfois observée [28]. Dans une autre étude, les seuils nociceptifs sont indifférents entre les enfants nés à terme ou prématurés, cependant, un déficit de leur contrôles inhibiteur diffus nociceptifs (CIDN) est notables [29]. Les adultes ayant eu une histoire de prématurité semblent avoir un plus grand risque de développer des douleurs chroniques [30] et présentent une tolérance plus faible à la douleur [31]. Une étude démontre également qu'ils sont susceptibles de développer très tôt des migraines nécessitant un traitement médicamenteux plus important [32].

Les données fondamentales obtenues à l'aide de modèles de douleur chez le rongeur permettent progressivement d'identifier les mécanismes responsables de ces hypersensibilités et pourraient à terme, faciliter l'identification de cibles thérapeutiques potentielles pour pallier à ces déficits. Les hypothèses de travail dans ce domaine font l'objet d'une revue récente [33]. Dans la plupart des cas, les procédures douloureuses néonatales induisent des hypersensibilités chez l'animal arrivé à l'âge adulte. Ces hypersensibilités sont parfois associées à une hyper-innervation périphérique par les fibres de type C, à une désinhibition glycinergique dans la moelle épinière, à une activation microgliale notable dans la moelle épinière ou encore à des modifications supraspinales. Le modèle du rongeur apporte un avantage non négligeable dans ces études puisque les processus neurodéveloppementaux interviennent selon la même séquence temporelle que chez l'homme. Ainsi, le système nerveux du rongeur à la naissance est en réalité au même stade de développement que celui du fœtus lors du 3$^{e}$ trimestre de gestation [34,35]. Cette particularité permet alors d'étudier des mécanismes pathophysiologiques que l'on peut associer à la prématurité chez l'homme. Un intérêt grandissant est porté aujourd'hui autour de la qualité des interactions entre la mère et son enfant.

## Séparation maternelle et hypersensibilité douloureuse

Dans certaines conditions, les nouveau-nés sont séparés de leur mère dès la naissance. C'est le cas des prématurés mis en couveuse et pris en charge dans les unités de soins intensifs. Dans d'autres situations, l'état de la mère après l'accouchement ne lui permet pas d'être en contact immédiatement avec son bébé. Une étude américaine montre par exemple que la mère est hospitalisée avec une prévalence de 0,9 pour 1000 naissances [36]. La séparation maternelle s'ajoute donc aux perturbations sensorielles que subissent les nouveau-nés en soins intensifs. Ce facteur pourrait également suffire à rendre le nouveau-né devenu adulte hyper-réactif à la douleur.

Dès la naissance, les stimulations sensorielles de la mère (odeur, toucher, son...) sont cruciales pour le

développement de l'enfant, en particulier pour la mise en place des processus cognitifs et sociaux. Plusieurs études cliniques ont déjà démontré que les stimulations tactiles chez les prématurés permettent d'améliorer leur croissance et de diminuer leur réponse au stress et à la douleur [37,38]. Dans cette optique, la séparation maternelle serait tout à fait dommageable pour le développement des systèmes biologiques de l'enfant, comme l'ont suggéré très tôt plusieurs études chez des orphelins et enfants hospitalisés (voir revue [39]). Ces derniers ont un risque élevé de développer des troubles comportementaux et une détresse émotionnelle à l'âge adulte, dont est responsable l'isolation maternelle précoce selon les auteurs. Par ailleurs, les procédures liées à l'intégration en soin intensifs constituent un événement traumatisant pour les parents, et peut conduire à une dépression sévère de la mère [40—42]. Cette situation pourra également altérer durablement les liens mère—enfant, au-delà de la période périnatale.

Chez l'homme, peu de preuves directes des effets négatifs de la séparation maternelle sur les réponses à la douleur ont pu être démontrées mais plusieurs évidences suggèrent une corrélation importante entre la séparation précoce et la présence d'une hypersensibilité nociceptive. Tout d'abord, les études effectuées sur le « peau-à-peau » soulignent les effets bénéfiques des interactions sensorielles avec la mère sur les réponses nociceptives de l'enfant. Cette stratégie consiste à porter l'enfant au plus près de la mère, lui permettant de suivre les mouvements rythmiques de sa respiration et de bénéficier de sa chaleur et de son odeur. Elle permet de diminuer les réponses du nouveau-né face à des stimulations de type piqûre de routine tout en diminuant les réponses autonomes induits par la procédure [43]. Si le peau-à-peau améliore la prise de poids des nouveau-nés, l'effet bénéfique à long terme est encore peu étudié. Il semblerait qu'il atténue les réponses au stress chez l'enfant de 10 ans tout en améliorant ses fonctions

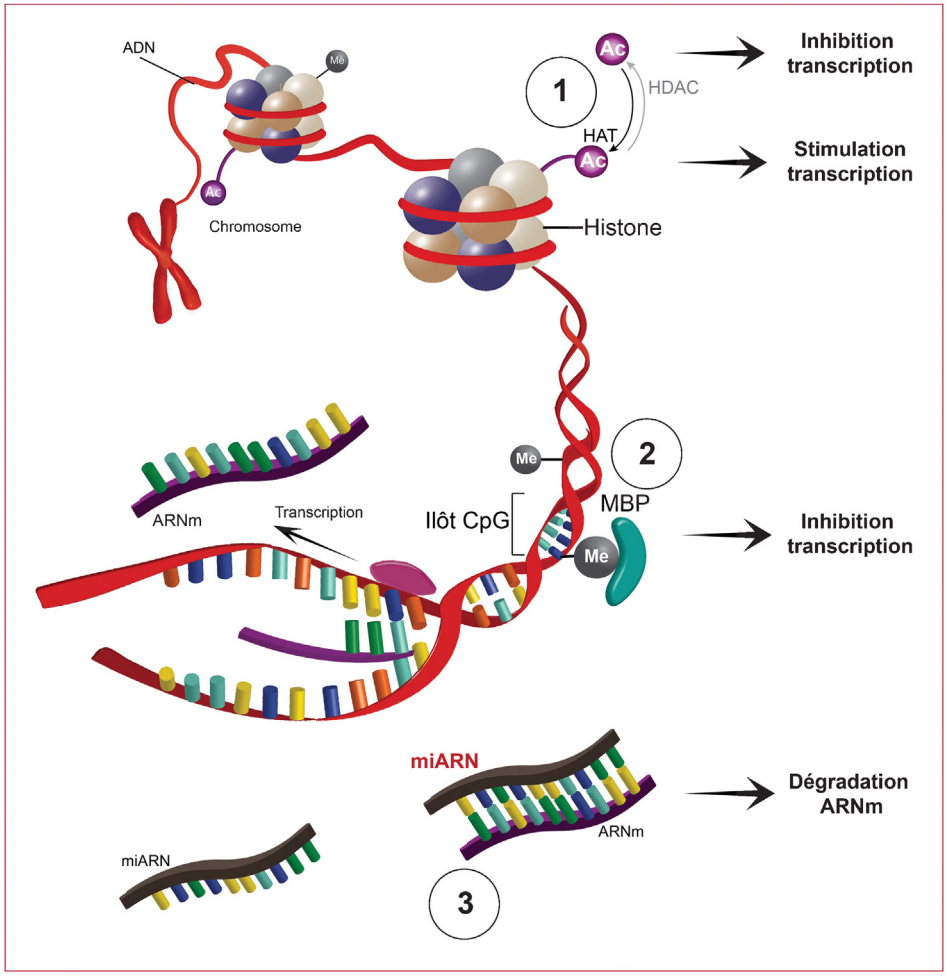


**Figure 2.** Mécanismes épigénétiques susceptibles de moduler l'expression de gènes affectant durablement la nociception. (1) Modification des histones : l'ajout d'un groupement acétyl au niveau des histones, catalysé par les histones acétylases (HAT), affaibli l'interaction histone/ADN, favorise un déroulement de la chromatine et permet son accès à des facteurs de transcription. La désacétylation, catalysée par les histones désacétylases (HDAC), provoque l'effet opposé et diminue l'efficacité de la transcription. (2) Modification de l'ADN : La méthylation de l'ADN, sur les cytosines présentes au niveau des îlots CpG, induit une compaction des nucléosomes et diminue l'accès aux facteurs de transcription. Certaines protéines sont capables de lier spécifiquement l'ADN méthylé, comme les MethylCpG *binding protein* (MBP), et répriment la transcription des gènes cibles. Elles sont également capables de recruter les enzymes de modifications des histones de type HDAC. (3) Modifications post-transcriptionnelles par les miRNA. De petits ARN d'environ 22 nucléotides, capables de lier des ARNm complémentaires et formant un ARN double brin qui sera dégradé par la cellule, inhibant ainsi la traduction de l'ARNm ciblé.

cognitives et exécutives ainsi que l'architecture du sommeil [44]. Le peau-à-peau pourrait tout à fait exercer un effet positif sur les dysfonctionnements du système nociceptif induits par les procédures de soins intensifs, même si des études complémentaires sont nécessaires. Les données chez l'animal semblent en faveur de cette hypothèse, puisque les variations naturelles d'intensité du comportement maternel chez le rat peuvent moduler les réponses nociceptives à long terme. Effectivement, les petits dont les mères ont un comportement maternel important ont une moins grande sensibilité vis-à-vis des stimulations thermiques nociceptives [45]. Ces études démontrent l'importance des contacts mère—enfant qui semblent :

- programmer précocement les réponses nociceptives de l'adulte ;
- suggérer que leur perturbation peut être à l'origine de réponses pathologiques à la douleur.

*D'avantages de données sur les effets de la séparation maternelle ont pu être obtenues chez le rongeur dans un modèle de séparation répétée de 3H par jour entre le 2e et le 12e jour postnatal.*

Ces études permettront à terme de définir par quels mécanismes l'altération des interactions mère—enfant peut moduler les réponses douloureuses à long terme. Ses conséquences ont d'ores et déjà été mises en évidence concernant la sensibilité viscérale, puisqu'une hypersensibilité nociceptive viscérale est observée chez des rats et souris adultes ayant subi le protocole de séparation maternelle [46—49]. Le système opioïdergique semble tout particulièrement perturbé par la séparation maternelle, dans la mesure où l'efficacité de la morphine est diminuée chez le rat mâle adulte suite à la séparation [50]. De plus, il semblerait que la séparation maternelle rende les individus adultes plus sensibles aux processus inflammatoires comme l'indiquent les hypersensibilités observées suite à des injections intraplantaires de formol [51] ou de prostaglandines E2 [52].

## Conclusions et perspectives

Les données issues de la recherche fondamentale, bien qu'encore incomplètes, sont capitales puisqu'elles soulignent la possibilité que les traitements analgésiques actuels n'aient pas la même efficacité chez des enfants ayant subi ce type de perturbation périnatale. Par ailleurs, plusieurs systèmes de contrôles endogènes de la douleur utilisant les opioïdes, mais éventuellement d'autres neuromodulateurs, pourraient être altérés par la séparation maternelle néonatale. Ces modifications importantes, si elles sont confirmées, plaident pour la nécessité de préserver au mieux les liens mère—enfants chez les prématurés et nouveau-nés pris en charge dans les unités de soins intensifs.

D'un point de vue mécanistique, le comportement maternel pourrait participer à la répression fonctionnelle de l'axe hypothalamo-hypophyso-surrénalien (HHS). Une hypothèse séduisante serait que la séparation maternelle lève précocement cette répression, menant à une hyperactivité de l'axe chez l'adulte [53]. Une autre piste majeure mettrait en jeu la modulation épigénétique de l'expression de certains gènes liés à la nociception. Ceci a justement déjà été évoqué dans le contexte des réponses au stress et de la dérégulation de l'axe HHS [54]. L'expression des gènes est contrôlée par plusieurs facteurs épigénétiques agissant sur les histones et la compaction de l'ADN, son état de méthylation ou encore par la répression fonctionnelle de la traduction des ARN messagers par les micro-ARN (Fig. 2). Ces processus épigénétiques constituent une piste prometteuse pour expliquer les modifications à long terme observées à la suite des perturbations périnatales mais pourraient aussi être à l'œuvre dans les phénomènes de chronicisation de la douleur. Des travaux dans ce sens devraient rapidement voir le jour et confirmer/infirmer ces hypothèses de travail. Ils pourront également permettre de déterminer si des thérapies ciblant ces processus épigénétiques peuvent être mise au point pour protéger le nouveau-né contre la sensibilisation durable de son système nociceptif ou contre la chronicisation des douleurs neuropathiques/inflammatoires.

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Les auteurs déclarent ne pas avoir de conflits d'intérêts en relation avec cet article.

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# REVIEW

# INSIGHTS INTO THE MECHANISMS AND THE EMERGENCE OF SEX-DIFFERENCES IN PAIN

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**Abstract—Recent studies describe sex and gender as critical factors conditioning the experience of pain and the strategies to respond to it. It is now clear that men and women have different physiological and behavioral responses to pain. Some pathological pain states are also highly sex-specific. This clinical observation has been often verified with animal studies which helped to decipher the mechanisms underlying the observed female hyper-reactivity and hyper-sensitivity to pain states. The role of gonadal hormones in the modulation of pain responses has been a straightforward hypothesis but, if pertinent in many cases, cannot fully account for this complex sensation, which includes an important cognitive component. Clinical and fundamental data are reviewed here with a special emphasis on possible developmental processes giving rise to sex-differences in pain processing.**

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**Key words: pain, nociception, sex hormones, gender, sex, development.**

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## INTRODUCTION

Women and men do not experience pain equally. Women perceive painful stimuli as more intense than men and are overrepresented in the majority of clinical pain conditions (Mogil, 2012). Interestingly, the same is true in animals, females having a lower pain threshold than males, supporting the implication of biological differences (Craft et al., 2004).

A great interest in the problematic of sex-differences in pain responses and pain processing has emerged in recent years. The number of dedicated clinical and animal studies has widely increased and supports the idea that pain seems to be processed differentially in men and women. Animal studies have been a huge help in deciphering potential mechanisms linked to the differences observed, raising the question of inherent anatomical differences between the two populations, and of the role of gonadal hormones in the modulation of pain responses.

Pain is a complex phenomenon relying on intricate excitatory and inhibitory psychophysiological mechanisms. Chronic pain frequently results in an

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*Abbreviations:* ANS, autonomic nervous system; BP, blood pressure; CFA, Complete Freund's adjuvant; DRG, dorsal root ganglion; fMRI, functional magnetic resonance imaging; FSH, Follicle-stimulating hormone; GABA, gamma-Aminobutyric acid; KOR, kappa-opioid receptor; LH, Luteinizing hormone; LPS, Lipopolysaccharide; MK801, dizocilpine; MOR, mu-opioid receptor; NICU, neonatal intensive care unit; NMDA, N-methyl-D-aspartate; PAG, periaqueductal gray matter; PBn, parabrachial nucleus; PET, positron emission tomography; RVM, rostro-ventral medulla; SIA, stress induced analgesia; TLR4, Toll Like Receptor 4.

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excessive recruitment of excitatory mechanisms often referred to as peripheral and central sensitization. Excitation is often further amplified by a reduced efficacy of inhibitory controls exerted by local interneuronal networks or by supraspinal axonal projections (e.g. Conditioned Pain Modulation – CPM) (Yarnitsky et al., 2014; Todd, 2015). Recent studies have highlighted that patients exhibiting an increased sensitization by temporal summation versus a deficit of CPM are expected to respond positively to different classes of drugs (Yarnitsky et al., 2012; Olesen et al., 2013) .

Beside the physiological responses after recruitment of somatic and autonomic systems, the role of cognitive and emotional processes is of utmost importance. In classical views, the term sex refers to a person's biological status as defined by sex chromosomes, gonads, internal reproductive organs and external genitalia. Gender refers to the attitudes, feelings, and behaviors that a given culture associate with a person's biological sex (American Psychological Association, 2012). In this review, we will use the term sex-specificities as a generic term, also covering any gender-specific differences in pain processing and expression. We will put some emphasis on the developmental origins of sex-differences in pain and provide some clinical and experimental observations supporting the differential pain responses during the development from childhood to adulthood in both animal models and human. The implication of the environment linked to early life events which could differentially imprint and alter pain processing in a long-term manner in a sex-specific manner will also be discussed.

## PERCEPTION AND REACTIVITY TO PAIN

### Experimental pain

Numerous clinical studies have highlighted differences in the perception of pain between men and women. Stimulating different tissues like skin, muscles or even visceral sites using electrical, thermal or mechanical stimuli can be used to assess the pain response. These experiments allow measuring pain threshold (when the subject describes the first sensation of pain), pain tolerance (how long the subject can support pain before it becomes unbearable) or the efficacy of endogenous descending controls of pain using dynamic models of experimental pain. Evidence from experimental pain studies report that women display higher pain responses for both electrical and thermal stimuli (Fillingim et al., 2009), but the results seem, however, highly dependent on the modality of pain stimulation (Riley et al., 1998; Racine et al., 2012).

For example, women exhibit lower threshold and tolerance of pressure pain when cutaneous territories are stimulated with pressure algometers or Von Frey filaments (Racine et al., 2012). Suprathreshold mechanical stimulation induces greater reported pain sensitivity (i.e. hyperalgesia) in women compared to men, and an associated greater autonomic response as measured by pupil dilation (Ellermeier and Westphal, 1995). However, using a dynamic model of experimental pain (temporal summation of pain using a train of 10 mechanical stimulation of the finger by a sharp probe), Sarlani and Greenspan describe a higher pain rating by women of the 5th and 10th stimulation (Sarlani and Greenspan, 2002), and a greater unpleasantness together with painful after sensations at the end of the train of stimulation (Sarlani et al., 2004). However, this difference in pain ratings could not be reproduced in another independent study stimulating the tibialis anterior (Nie et al., 2005).

Most studies tested pain responses to thermal stimulation after immersion in cold or hot water. Although few studies failed to describe any sex-differences, a large amount of data conclude that women display lower pain thresholds and pain tolerance compared to men, as seen in a recent meta-analysis (Racine et al., 2012).

On the contrary, studies assessing ischemic pain sensitivity were inconclusive regarding sex-specificities in thresholds or tolerance to ischemic pain (Bragdon et al., 2002; Racine et al., 2012).

### Brain imaging

Results from imaging studies are sometimes inconclusive, but clearly indicate that nociceptive information could be processed differently by the pain matrix in men and women. As studied by functional magnetic resonance imaging (fMRI) and positron emission tomography (PET), several brain regions activated by a painful stimulus have comparable activities in men and women, among which the premotor cortex, primary motor (M1) and somatosensory (S1) cortices and the cerebellum (Paulson et al., 1998). A sex-specific activation of few brain regions is also reported using nociceptive stimuli adjusted for individual pain perception. For example, Derbyshire and colleagues used a calibrated thermal stimulus to induce the same pain response in both men and women, but a different pattern for cortical activation (Derbyshire et al., 2002). In men, the thermal stimulus induced activation of the parietal cortex bilaterally, the contralateral secondary somatosensory cortex, the prefrontal cortex and the insula, while the ipsilateral perigenual and ventral cingulate cortex were preferentially activated in women. Using the same stimulation paradigm, another research team reported a sex-specific activation of the prefrontal cortex contralateral to the stimulation in male and ipsilateral to the stimulation in females (Paulson et al., 1998). This observation, if confirmed, suggests a possible sex-specific lateralization in pain processing and particularly with regard to the emotional dimension of pain. The same study also demonstrated a greater activation of the contralateral thalamus and anterior insula in women, who described the stimulus as more painful than men. Differential brain activation has also been detected using electrical stimulation of the finger, as indicated by a greater activation of the contralateral medial prefrontal cortex in women and a greater activation of the ipsilateral posterior insular cortex in men (Straube et al., 2009). On the contrary, Moulton and colleagues, using fMRI and BOLD signal, demonstrated a greater deactivation of the primary

sensory and of prefrontal cortices for the same painful stimulus in females than in males (Moulton et al., 2006).

Using a painful rectal distension (internal pressure of 44 mmHg) in healthy subjects, a male-specific activation of the left thalamus and ventral striatum was observed in men together with a greater activation of the insula (Berman et al., 2006). In the same study, women only showed a greater deactivation of the midcingulate cortex. In another visceral pain model induced by electrical esophageal stimulation, Hobson and colleagues failed to detect any sex-differences using magneto-encephalography and evoked potentials (Hobson et al., 2005).

The endogenous pain modulation systems could also play an important role in brain activity differences between men and women. Using PET combined with a mu-opioid receptor (MOR) selective radiotracer, Zubieta and colleagues found that men showed a larger activation of MOR than women in the thalamus, ventral basal ganglia and amygdala while women had a reduced activity in the nucleus accumbens during matched levels of pain intensity (Zubieta et al., 2002). It is important to mention here that the testing was strictly done during the luteal phase in healthy women. Indeed, sex hormones (estrogens, progesterone) seem to be a key factor in determining the efficacy of women endogenous inhibitory controls (Tousignant-Laflamme and Marchand, 2009a). Even testosterone levels in the serum of women are positively correlated with the activation of structures such as the rostral ventromedial medulla, implicated in descending inhibitory pathways (Vincent et al., 2013).

Altogether, these results suggest that the difference in pain perception and occurrence between men and women are related to both excitatory and inhibitory mechanisms. Imaging studies also support the idea that areas involved in the sensory-discriminative response to pain are unlikely to imply sex-specificities, contrary to other brain areas of the pain matrix coding for the emotional and self-focused response to pain (Fig. 1).

## Pain and the autonomic nervous system (ANS)

Pain is not only associated with the activation of specific brain areas (the so-called "pain matrix") but also involves a cascade of autonomic responses when nociceptive information progresses from the spinal cord to cortical areas. These autonomic responses can also be used as indirect markers of pain reactivity. Indeed, pain leads to an increase of arousal following activation of the sympathetic autonomic system. This results in an increase in cardiovascular activity (blood pressure (BP) and heart rate) and respiration rate, the dilatation of the pupils and corticosterone release (Möltner et al., 1990; Leone et al., 2006; Cortelli et al., 2013). One could hypothesize that the changes in autonomic variables would parallel the intensity of the painful stimulus. Surprisingly, Tousignant-Laflamme and colleagues measured heart rates in response to a 2-min immersion of the hand in cold water in healthy volunteers and described a correlation between heart rate variability and the intensity of the painful stimulus only in men (Tousignant-Laflamme et al., 2005). This difference has been further explored in a

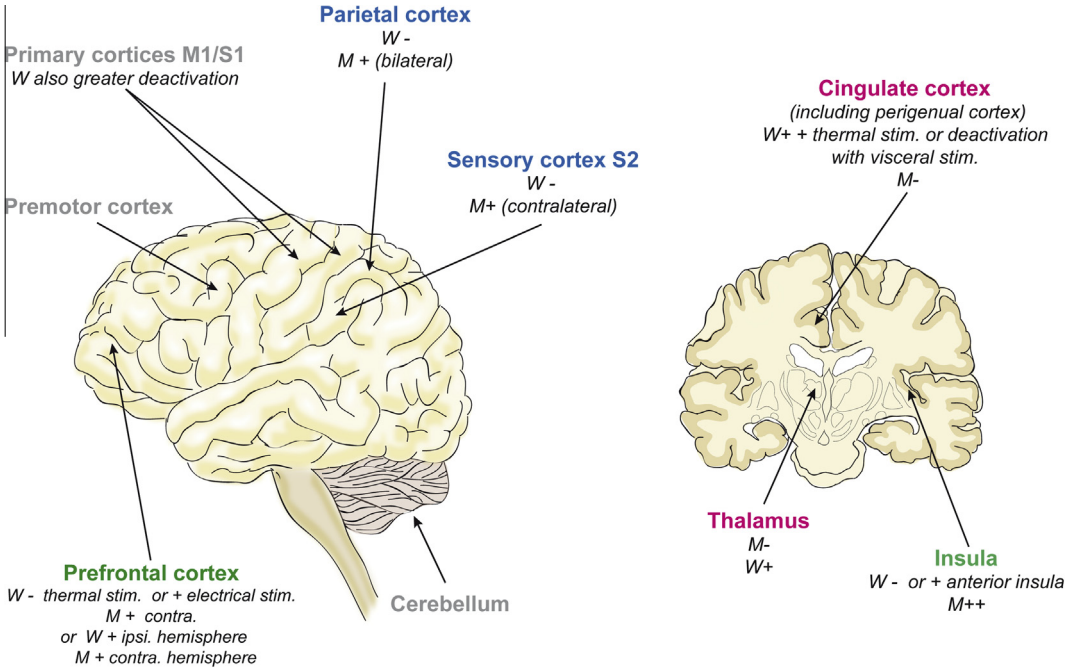


**Fig. 1.** Summary of brain imaging studies in adult showing differential activation in the pain matrix in men and women. Regions activated by a painful stimulus have comparable activity in men and women (in gray), an increased reactivity in men (in blue), or an increased reactivity in women (in pink). In green are indicated the areas where the results are more conflicting and vary between different studies. contra. = contralateral to the stimulation, ipsi. = ipsilateral to the stimulation, M = Men, W = Women. + indicate activation of related structures, – indicate no activation of related structures.

population of chronic pain patients suffering from low back pain (Tousignant-Laflamme and Marchand, 2006), confirming a differential regulation of the autonomic response to pain in males and females. Moreover, when measuring salivary cortisol levels, it appears that women display lower cortisol reactivity than men in response to a thermal stimulus (Zimmer et al., 2003; Dixon et al., 2004). It has been proposed that the lack of change in heart rate variability in females is concealed by a differential autonomic response to pain across the menstrual cycle. A correlation between heart rate and pain intensity was indeed described only during the menstrual phase (Tousignant-Laflamme and Marchand, 2009b). However, heart rate variability and BP do not consistently vary in response to pain during the menstrual cycle (Tousignant-Laflamme and Marchand, 2009b). The ANS response to pain may also be dependent on its relation with the recruitment of conditioned pain modulation (CPM). While measuring the resting BP and BP reactivity to the immersion of the arm in cold water (conditioning stimulus), the author found that resting BP was positively correlated to heat pain tolerance while BP reactivity to the conditioning stimuli was correlated to the analgesic amplitude of the CPM (Chalaye et al., 2013). The same research group also found that this relation was present in chronic pain conditions particularly present in women, fibromyalgia and irritable bowel syndrome (Chalaye et al., 2012, 2014). Interestingly, in a neuropathic pain condition, trigeminal neuralgia, the authors found that the patients had more sympathetic activity during the cold pressor test as compared to healthy controls (Léonard et al., 2015) and that the autonomic activity was negatively correlated to the average number of daily pain paroxysms, suggesting that the hyper-reactivity of the ANS could be a protective reaction in this chronic pain condition.

## Chronic pain

Chronic pain is a major issue in human health, as it concerns as much as 30% of adult individuals in the US and Canada (Moulin et al., 2002; Johannes et al., 2010), and around 20% in Europe (Breivik et al., 2006), Australia (Henderson et al., 2013), Japan and Thailand (Sakakibara et al., 2013). Epidemiological studies reveal that about 50% of the chronic pain pathologies have a higher prevalence in women whereas only 20% prevail in men (Berkley, 1997; Mogil, 2012) (Table 1).

This is particularly clear for migraine (Stewart et al., 1992), as 17.6% of females and 5.7% of males reports one or more migraine episodes per year. Other examples are fibromyalgia (Wolfe et al., 1995) and irritable bowel syndrome (Heitkemper et al., 2003) where only around 10% of patients are men. Moreover, in most of these pathologies, it seems that women have stronger and longer lasting pain symptoms. For musculoskeletal pain, Kindler and colleagues also demonstrated that among patients suffering from shoulder pain, women have a lower mechanical and thermal pain threshold than men (Kindler et al., 2011). Musculoskeletal pain in general, with no regard on pain site, is also more reported by women, as well as widespread pain (Leveille et al., 2005). In a population of patients suffering from fibromyalgia, women have significantly more ‘‘tender points” and fatigue symptoms (Wolfe et al., 1995). In the case of irritable bowel syndrome, women also describe longer pain episodes than men (Heitkemper et al., 2003).

If there is a consensus regarding prevalence of chronic pain states, a detailed and cautious analysis has to be done to identify any possible bias. For example, women are more likely to seek medical care and report pain than men. This psychosocial determinant of pain will be discussed later in the review. Note also that this review will not focus on clinical pain conditions associated with female-specific or male-specific organs (gynecological disorders, breast cancer, prostate cancer, etc.).

## Animal studies

To study the sex-specific differences in pain responses, various animal models mimicking the human situation have been used. Unfortunately, these numerous studies are still insufficient to fully elucidate the underlying mechanisms involved. Indeed, results can be highly variable depending on the testing procedures, acute/persistent pain models and animal species (in particular strains when mice were used) (DeLeo and Rutkowski, 2000; Mogil et al., 2000; Leo et al., 2008).

Animal models used to highlight sex-differences in pain are summarized in recent reviews (Kuba and Quinones-Jenab, 2005; Leo et al., 2008). Overall, animal studies emphasize observations made in humans, suggesting that females are more sensitive to acute, inflammatory and neuropathic pain. For example, using the formalin test which induces a typical biphasic response, Gaumond et al. (2002) were able to reveal a stronger nociceptive score in female rats in both the acute and late phases of the inflammatory nociceptive sensitization response. The response to complete Freund’s adjuvant is also higher in proestrus females than in male rats (Bradshaw et al., 2000). There are several reports indicating the crucial role of sex hormones in determining nociceptive threshold and, sometimes, showing that female rats are less sensitive to noxious stimuli (Tall and Crisp, 2004). It should be emphasized again here that the estrus cycle is very short in rodents (i.e. difficulty to identify the different phases of the estrus cycle) and this might contribute to the apparent contradictory results of the literature. Experimental data also parallelled the clinical studies on chronic pain, indicating a higher neuropathic pain condition, revealed by a stronger mechanical allodynia in female Sprague–Dawley rats following spinal nerve transection (DeLeo and Rutkowski, 2000), which also lasts longer in females in a mouse model of chronic constriction injury (Vacca et al., 2014), but not in paclitaxel chemotherapy inducing generalized neuropathy (Hwang et al., 2012). Beside rat models, the use of mice allowed the study of genetic differences in nociceptive behaviors. Indeed, a quantitative trait locus on mouse chromosome 4 has been identified to be linked to the baseline thermal sensitivity measured by a hot plate test in male mice but not females (Mogil et al., 1997b). Another quantitative trait locus, Fsia1 on chromosome 8, has been identified to be linked to stress-induced anal-

**Table 1.** Summary table of some pathological pain states associated with women prevalence and higher chronic pain symptom intensities in adult. In the table, observations made on children are also indicated. Note that chronic pain states with men prevalence are not listed in this table but some examples can be found in the text. Abbreviations: B = boys, CRPS = complex regional pain syndrome, G = girls, M = men, OA = osteoarthritis, RA = rheumatoid arthritis, W = women

| | | Adults | | | Children | |
|---|---|---|---|---|---|---|
| | | Prevalence W/M | Symptom intensity | References | Observations | References |
| Migraine/headache | | 14–18% W<br>6% M<br>Ratio 3 | Longer duration and intensity in W/More migraine with aura in W | Stewart et al. (1992), Weitzel et al. (2001), Stovner et al. (2007) | Earlier onset in B. Higher prevalence in G after puberty (9% in G vs 7% in B) | Stewart et al. (1991), Hasvold and Johnsen (1993), Brun Sundblad et al. (2007), Stovner et al. (2007) |
| Musculo-skeletal pain | Any site | 35–59% W<br>23–49% M<br>Ratio ~1.25 | Lower thermal & mechanical thresholds in W | Cunningham and Kelsey (1984), Rollman and Lautenbacher (2001), Leveille et al. (2005), Kindler et al. (2011) | Higher prevalence in G. (same for neck pain and pain in upper limb) | Zapata et al. (2006), Brun Sundblad et al. (2007), El-Metwally et al. (2007), Wirth et al. (2013) |
| | Fibromyalgia | 2–4% W<br>< 0.5% M | More tender points in W | Wolfe et al. (1995) | More tenderness in G | Buskila et al. (1993), Buskila and Ablin (2012) |
| | Back pain | 18–40% W<br>11–32% M<br>Ratio 1.2–2.1 | | Thomas et al. (1999), Carmona et al. (2001), Bingefors and Isacson (2004), Schneider et al. (2006) | Higher prevalence in G | Wirth et al. (2013) |
| Arthritis | Rheumatoid arthritis | Ratio ~2<br>age 55 + | W daily pain is 72% higher than M (RA and OA independently) | Cunningham and Kelsey (1984), Verbrugge et al. (1991), Affleck et al. (1999), Carmona et al. (2001), Schuna (2002) | Juvenile arthritis: Greater prevalence in G, around 19.4% in G and 11.2% in B | Oberle et al. (2014), Thierry et al. (2014) |
| | Osteoarthritis | 14–24% W<br>6–18% M<br>Ratio ~1.5 | Higher pain ratings for F aged 50–74<br>Higher pain ratings for M aged 75 + | Cunningham and Kelsey (1984), Verbrugge et al. (1991), Affleck et al. (1999), Carmona et al. (2001), Christmas et al. (2002), Jinks et al. (2008) | | |
| Irritable bowel syndrome | | Ratio 2–5:1 | Longer pain episodes and more non-pain related symptoms in W | Heitkemper et al. (2003), Adeyemo et al. (2010), Mulak and Taché (2010), Tang et al. (2012) | Slightly higher in G (G: 21.06 vs B: 20.88). Increased risk associated with early life trauma for G | Zhou et al. (2010), Bradford et al. (2012), Devanarayana et al. (2015) |
| Neuropathic pain | | 6–8% W<br>3–6% M<br>Ratio ~1–2<br>CRPS ratio 3–4:1 | | Sandroni et al. (2003), Bouhassira et al. (2005), Torrance et al. (2006), de Mos et al. (2007) | Neuropathic pain occurs mostly in later childhood and adolescence<br>Complex regional pain syndrome: around 85% are G | Wilder et al. (1992), Low et al. (2007), Tan et al. (2008), Walco et al. (2010) |

**Table 2.** Examples of sex-specific differences in rodent models of inflammatory sensitization and of neuropathic pain

| | Pain model | Symptomatic sex-difference | Strain | Behavioral measure | References |
|---|---|---|---|---|---|
| Inflammatory sensitization | Formalin | Females display higher pain scores in both phases of formalin response | Sprague–Dawley rats<br>Wistar rats | Licking, flexing of the paw | Gaumond et al. (2002)<br>Aloisi et al. (1994) |
| | CFA (hindpaw) | Higher responses in females, but only in proestrus | Sprague–Dawley rats | Plantar (thermal) | Bradshaw et al. (2000) |
| | | Lower mechanical threshold in females | Lewis rats | Analgesia meter (mechanical pressure) | Cook and Nickerson (2005) |
| | Carrageenan | Less thermal pain symptoms in females after 2H | Fischer 344 FBNF1 hybrid rats | Plantar (thermal) and Von Frey (mechanical) | Tall and Crisp (2004) |
| | Spinal LPS | Allodynia in males at 1-6H post injection, lasting 24H<br>No effect in females | CD-1 mice | Von Frey (mechanical) | Sorge et al. (2011) |
| | Capsaicin | Stronger hyperalgesia in females with same dose<br>Requires a 3× higher dose in M to get the same amount of hyperalgesia | Fischer 344 rats | Hot water tail withdrawal | Barrett et al. (2003) |
| Neuropathic | L5 nerve transection | Stronger mechanical allodynia in Sprague Dawley-rats<br>No differences in Holtzmann rats | Sprague–Dawley rats | Von Frey test (mechanical) | DeLeo and Rutkowski (2000) |
| | Chronic constriction injury | Longer thermal hyperalgesia (14 days in male and 35 days in females) | Fischer 344 FBNF1 hybrid rats | Plantar (thermal) And Von Frey (mechanical) | Tall et al. (2001) |

F = females, M = males.

gesia efficiency but only in female mice (Mogil et al., 1997a) (Table 2).

These animal models have the advantage of allowing a huge range of hormonal, neurochemical and behavioral manipulations that can be very useful to unravel the mechanisms underlying the sex-specific pain responses observed in human studies, as it will be detailed later in this review.

## ENDOGENOUS AND EXOGENOUS CONTROLS OF PAIN

### Endogenous control of pain

The descending controls of pain are apparently also submitted to strong sex-specific variations, as supported by human studies on diffuse noxious inhibitory controls of pain (DNIC, also referred to as CPM for conditioned pain modulation) and animal studies using stress induced analgesia (SIA) paradigms.

In humans, efficacy to recruit DNIC gives an interesting measure of endogenous pain controls (Le Bars et al., 1979a, 1979b). It can be assessed using two noxious stimuli applied on different sensory territories (Yarnitsky et al., 2010). Most of the time, a cold pressor test by immersion on the hand/arm in noxious cold water is used (Conditioning Stimulus) to inhibit pain responses resulting from a mechanical or thermal stimulus applied on the other arm (Test Stimulus). The analgesic effect of DNIC is classically attributed to the activation of brainstem descending inhibitory controls rapidly decreasing the activity of spinal nociceptive neurons. Overall, DNIC seems more efficient in men than in women (Arendt-Nielsen et al., 2008; Granot et al., 2008; Goodin et al., 2009) even if some results have blurred this main conclusion (France and Suchowiecki, 1999; Baad-Hansen et al., 2005; Pud et al., 2005). The inconsistency of these results should partly be explained by technical biases, and the need to consider women's hormonal status. In line with this explanation, DNIC varies highly with the estrus cycle and the level of the corresponding sex hormones. DNIC are for example more efficient in the ovulatory phase (Tousignant-Laflamme and Marchand, 2009a). With this in mind, a literature review on the subject suggested that

DNIC is less efficient in women than in men (Popescu et al., 2010).

In both human and rodents, acute stress is known to recruit endogenous pain controls, leading preferentially to analgesia. In rodents, SIA is often induced by a cold forced-swim test, recruiting different descending controls based on the duration of the swim test and temperature of the water. Both opioidergic and non-opioidergic SIA are weaker in female rodents than in male (Romero and Bodnar, 1986). Interestingly, non-opioidergic SIA in male mice can be blocked by the N-methyl-D-aspartate (NMDA) antagonist MK801, but not in female, suggesting a differential neurochemical recruitment of descending pathways in rodents (Mogil et al., 1993; Sternberg et al., 2004). This NMDA sensitivity seems to rely on sex hormones, since ovariectomizing the mouse restores the effect of MK801, which is suppressed by a pretreatment by progesterone (Sternberg et al., 2004) or estrogens (Mogil et al., 1993). The differential effects of opiates could also be related to the genotype, as it has been showed that MC1R alleles modulated the response to a kappa-opioid receptor (KOR)-mediated analgesics in human and that a blockade of MC1R in mice restores the blockage of SIA by NMDA antagonists (Mogil et al., 2003).

### Differential effects of opioidergic analgesics

Both pharmacological and non-pharmacological analgesic interventions aimed at alleviating pain revealed important sex-specificities, in rodent studies as well as in clinical human studies. Most particularly, studies on opioidergic analgesia reveal an important contribution of sex and gender. In humans, opioid-induced analgesia using mu- and kappa-type opioid receptor agonists (MOR and KOR, respectively) seemed to be achieved with lower doses compared to men and, for a given dose, produced a greater analgesia in women (Craft, 2003). Surprisingly, the opposite effect seems to emerge from some animal studies in rodents (see review (Kest et al., 2000)), demonstrating in their majority greater morphine analgesia in males (Islam et al., 1993; Cicero et al., 1996). Beside the acute effect of morphine, repeated exposure is known to induce functional tolerance and possibly hyperalgesia in both human and rodents. Animal studies demonstrate that the onset of morphine-induced hyperalgesia is delayed in male mice compared to females and lasts longer in female mice when using low doses of morphine (Holtman and Wala, 2005; Juni et al., 2008; Waxman et al., 2010). During the formalin test, the inhibitory interphase seems to be contributed by opioids in female rats, but not in male (Gaumond et al., 2007). Moreover, the underlying mechanisms of hyperalgesia seem to differ in males and females. A systemic or central injection of an NMDA receptor antagonist can reverse hyperalgesia in males only, while in females a melanocortin-1 receptor antagonist reverse hyperalgesia (Juni et al., 2010; Waxman et al., 2010; Arout et al., 2015). The increased morphine-induced hyperalgesia in females has been linked to progesterone, which produces the same hyperalgesic response when injected subcutaneously to males (Waxman et al., 2010).

These observations seem to be, at least partly, related to the density of opioid receptors in the corresponding brain regions. First, binding studies show an overall greater KOR binding in women brain (Zubieta et al., 1999). As previously mentioned, the administration of radiolabeled MOR agonist [11C]Carfentanil measuring the brain activity by PET scan, Zubieta and colleagues found a higher MOR activation in men's brain regions related to anti-nociception (e.g. anterior thalamus, basal ganglia and amygdala) in response to muscle pain. In women who were scanned during the follicular phase of the menstrual cycle, significant activation of MOR was detected only in the ventral pallidum for the same muscle stimulation intensity. In sharp contrast, a decrease in the activation of MOR was seen in the nucleus accumbens of women compared to the saline control (Zubieta et al., 2002).

### Differential effects of non-opioidergic analgesics

Sex-differences in non-opioidergic analgesia are less clear. Some authors studied the response to Nonsteroidal anti-inflammatory drug (NSAID) in men and women, and showed that men seem to be more responsive to ibuprofen in a paradigm of electrically induced-pain (Butcher and Carmody, 2012), but not in response to dental pain by molar extraction (Averbuch and Katzper, 2000). Using ketorolac, Compton and colleagues found no sex-differences in the response to cold pressor test (Compton et al., 2003). There are several animal studies supporting that other analgesic targets, such as gamma-Aminobutyric acid (GABA) (Tonsfeldt et al., 2016) and cholinergics (Chiari et al., 1999), are acting differently between male and females, stressing out the need for more clinical studies on sex and gender differences in responses to different analgesic classes.

## SEX-DIFFERENCES DURING THE DEVELOPMENT

Altogether laboratory and clinical studies reveal clear sex differences in pain processing in adults and this raises the question of whether similar differences could also occur in children. Studying sex-specificity during development may also help to better understand when and how the organizational and activational events occur to install these differences in pain processing in the adult population.

Assessing pain in children could be an uneasy task, especially in younger children, and yet could be a useful tool to understand the setting-up of sex-differences in pain responses. In neonates in particular, no direct evaluation of pain severity is possible. Yet physiological and behavioral measures are used, by recording cry duration, body movement, facial expressions, cardiovascular and respiratory response, or the hormonal response to a potentially painful stimulus. Auto-evaluation scales can be used by children (starting around 5 yo) to score their pain sensation, in addition to evaluation scales based on behavioral observations

(McGrath and Gillespie, 2001). In clinical studies, experimental pain is often assessed using the cold pressor test, allowing measuring both pain threshold and pain tolerance/endurance. As for other variables, such as pain in everyday life or strategies to cope with pain, self-report questionnaires and parent-report questionnaires are often used.

### Pain reactivity in children

Experimental pain studies in young children gave contrasting results. Blanckenburg and colleagues failed to reveal any sex-differences using mechanical sensory testing with Von Frey filaments, calibrated sharp metal electrodes, pressure algometer and vibration detection among participants aged from 7 to 14 years (Blankenburg et al., 2011). They demonstrated, however, that thermal sensitivity tends to be higher in girls as measured with warm detection test, cold detection tests and thermal sensory limen (alternating warm and cold stimuli) even if it was not statistically significant for the last two tests. With the cold pressor task, other independent groups demonstrated that girls have a lower pain threshold (Schmitz et al., 2013; Boerner et al., 2014) and less tolerance to heat stimuli (Boerner et al., 2014). The unpleasantness score to venipuncture pain, a standard procedure in children, also seems to be higher in girls than boys in the group of children older than 8 years (Goodenough et al., 1999). Using the neonatal facial coding system, another independent study failed to describe differences between boys and girls in preterm babies in response to puncture (Valeri et al., 2014). As for adults, pain induces activation of cortical areas in the newborn brain. Compared to preterm neonate girls, it has been described that pain-related increases in oxygenated hemoglobin [$HbO_2$] in somatosensory cortices are more pronounced in boys (Bartocci et al., 2006). Inhibitory controls of pain are submitted to major changes during the early postnatal development, since DNIC efficacy is significantly lower in children (8–11 yo) compared to adolescents (12–17 yo) in both boys and girls (Tsao et al., 2013). Moreover, early pain experienced after birth in the neonatal intensive care unit (NICU), especially for premature babies in the first months of their life, reduces DNIC efficacy in those children when compared to their counterparts less submitted to painful stimuli during their NICU stays (Goffaux et al., 2008). Only a few data are available regarding possible sex-differences in children during central pain processing, but Tsao and colleagues failed to demonstrate any sex differences in the efficacy of DNIC in children and adolescent (Tsao et al., 2013).

Similarly, sex specificity in the autonomic response to pain has been poorly reported. Girls, aged from 8 to 18 years, seem to have a higher pre-trial heart rate when facing an experimental pain (Lu et al., 2005). Interestingly, some studies have supported a direct relationship between pain and resting BP, possibly indicating a sex-specificity in children. In adults, resting BP seem to be correlated with a decreased pain sensitivity, which has also been demonstrated in young girls from 8 to 18 years but not in boys, since girls with high resting BP have lower thermal intensity ratings (Haas et al., 2011). Even in babies, it has been shown that boys born preterm display a higher increase in heart rate in response to venipuncture (Valeri et al., 2014). Moreover, long-term abnormal heart rate variability is reported in adolescent and young adult if they had past-experience of long NICU stay or surgery at birth, although no sex difference is reported (Morin et al., 2014).

### A matter of time or sexual maturity

Sex differences in experimental pain highly depend on the age at the moment of the measurements. A recent meta-analysis, using a sample without any age concern, globally conceal the sex-differences that seem to exist (Boerner et al., 2014). A global higher sensitivity to pain in girls, comparable with what is found in adults, starts to emerge in children above 12 years old. These results seems to suggest that sex-differences in pain are regulated by activational rather than structural effects since no differences between boys and girls can be found before 12–14 years old, when reaching puberty (Roth-Isigkeit et al., 2004). However, as we previously stated, there are some structural differences in pain modulation pathways between sexes. We can probably conclude that the interactions of activational and structural differences are necessary to produce the differences. Pain sensitivity seems to develop according to different pattern in boys and girls. Indeed, pain threshold tends to decrease in girls whereas it stays mostly the same in boys. This observation could be due to a hormonal transition which supports the hypothesis that the differences recorded in adults could be partially due to a differential action of sex hormones on nociceptive processing. This important hypothesis will be discussed in the next paragraph of the review.

### Chronic pain in children

The literature also reports that chronic pain seems to be experienced more often by girls than boys (Perquin et al., 2000; King et al., 2011), and with greater intensity (Perquin et al., 2000), while other studies show no differences in the intensity of chronic pain in children (Lynch et al., 2007). This is in agreement with what was demonstrated in the adult population, suggesting a similar mechanism of sex-differences in pain in both adult and children.

Epidemiological studies suggest that pathological pain such as migraine with or without aura develops at younger age in boys, but that girls are more likely to report new onset of migraine (Stewart et al., 1991). In general, headaches are almost reported twice as much by girls aged of 9, 12 and 15 years-old (Brun Sundblad et al., 2007). In a study conducted in adolescents (with a mean age of 14.2 years) authors failed to demonstrate any sex-specificity in low back pain as reported by a self-questionnaire (Zapata et al., 2006). This result contrast with another study demonstrating a higher prevalence of low back pain and neck pain in girls aged of 14 years-old (Wirth et al., 2013). Apart from low back pain, Zapata and colleagues also demonstrated that pain in the upper limb seemed to be reported more often by girls (11% vs 7% in boys). Furthermore, the medical exam revealed that musculoskeletal pain syndrome is detected in girls

more often, especially benign joint hypermobility syndrome (27% vs 9%) and myofascial syndrome (7% vs 2%). Again, this observation is in apparent contradiction with another work which reports no sex-differences for musculoskeletal pain (Brun Sundblad et al., 2007). Sundblad and colleagues also reported that abdominal pain, occurring once a week or more, is reported more often in girls than in boys aged 12 (8% vs 4% in girls) and 15 years (11% vs 5% in girls), respectively (Brun Sundblad et al., 2007).

As for adults, several neuropathic pain syndromes can be experienced by children even if they are pretty rare. This includes (not exhaustive) complex regional pain syndromes, phantom limb pain, spinal cord injury or trauma and postoperative neuropathic pain (Walco et al., 2010). Neuropathic pain mostly occurs in later childhood and adolescence. In the case of complex regional pain syndromes, a huge sex-specificity is observed, since around 85–90% of patients are girls (Wilder et al., 1992; Low et al., 2007; Tan et al., 2008).

Arthritis also concerns mostly adults, with a strong prevalence in population above 55 years and in women (Cunningham and Kelsey, 1984; Verbrugge et al., 1991; Affleck et al., 1999; Schuna, 2002). However, a juvenile form of arthritis exists and parallels the observations made with rheumatoid and osteoarthritis in adults, indicating a higher prevalence in girls (≈20% in girls and 11% in boys) (Oberle et al., 2014; Thierry et al., 2014).

In conclusion, studies about experimental and chronic pain in children highlight developmentally regulated sex-differences in pain responses that are similar to the ones found in adults. Overall, post-pubescent girls appear to be more sensitive to pain and more likely to develop chronic pain states. The emerging of sex-specificity matches with the time of puberty, when the hormonal state of both girls and boys is subjected to strong adjustment. The mechanism behind these specificities could then rely, at least partly, on a direct effect of sex-hormones on the ascending nociceptive pathways, and/or to a more cognitive explanation, resulting in a different emotional response to pain.

### Strategies to cope with pain

Questions regarding pain coping and the cognitive responses to pain in children should be assessed shrewdly, as a developmental bias probably contributes. Indeed, the ability to understand pain in its multidimensional aspect and to cope with it increases with age and experience (Piira et al., 2002). An interesting fact here is that it has long been hypothesized that girls are able to report on the emotional nature of pain at earlier age than boys (Gaffney and Dunne, 1987). This was supported by a study by Goodenough et al. (1999), where children were asked to rate pain and unpleasantness in response to venipuncture, as an indicator of the affective dimension of pain. The authors demonstrated that girls older than 7 years perceive pain as more unpleasant. Two hypotheses were given, the first one being that girls may be able to discriminate the multidimensional aspects of pain at younger age, and the second one being that they are more willing to report pain than boys at the same age. This last working hypothesis is of great interest, as the reluctance to express pain in boys should partly explain the differences reveals by clinical studies (Fowler-Kerry and Lander, 1991), even in the adult population.

As for adults, children develop different strategies to cope with pain; girls are apparently more likely to seek social support to friends and family (Lynch et al., 2007) and more likely to respond with catastrophizing (Schmitz et al., 2013), whereas boys preferentially engage in alternative behavior and other activities (Lynch et al., 2007).

## MECHANISMS

### Structural and functional hypothesis

When studying the role of sex and gender on pain we need to take into account structural and functional differences that either be solely responsible or interacts to change pain perception. Are pain pathways different from males and females? This is an important question, which has not been fully assessed. If central circuits are apparently structurally similar between males and females, the structural plasticity of peripheral nociceptive nerve tissues is expected to be strongly influenced by the biological (e.g. hormonal) environment. For example, animal models of nociceptive hypersensitivity are often associated with a stronger innervation of the wounded skin by nociceptive afferent fibers (Chakrabarty et al., 2011). If different between men and women, such processes could easily support sex-specificities. Mowlavi and colleagues who measured the density of nerve fibers in cadaver skins have made one interesting observation. They describe that the average nerve fiber density in the skin of women is twice the average of men's (Mowlavi et al., 2005). In rats, Hendrich and colleagues compared the electrophysiological properties of nociceptive primary afferents *in vivo* and of dorsal root ganglion (DRG) neurons that innervate the gastrocnemius muscle in primary cultures (Hendrich et al., 2012). They found clear differences between male and female rats but their results did not tend toward the hypersensitivity in females. Compared to male, IB4 positive DRG neurons (i.e. non-peptidergic C-type sensory neurons) in females had a more hyperpolarized resting membrane which was not linked to a change of electrical excitability *in vitro* but to an increased mechanical threshold *in vivo*. This hypothesis, although insufficiently explored, should reveal interesting differences supporting sex-specificity in peripheral nociceptive processing. These studies suggest that many factors and/or physical dimorphisms may also contribute in other structures of the nociceptive pathways.

Within central structures, anatomical or functional differences may partially explain sex-differences in the response to pain. Among the "pain matrix", the periaqueductal gray matter (PAG) is of great interest since it is implicated in one of the major descending pathway controlling pain response, projecting first to the rostro-ventral medulla (RVM) and then to the spinal cord. Loyd et al. (2008) studied the anatomical and functional differences of the PAG-RVM pathway among male

and female rats. They found that males have a higher MOR expression in the PAG than females. The density of MOR in the PAG is positively correlated with morphine-induced analgesia, but in males only (Loyd et al., 2008). They also conducted an anatomical study, investigating the projections from the PAG to RVM in rats. The qualitative distribution of PAG RVM projections was similar in males and females. However, females had more PAG-RVM output neurons across the rostrocaudal axis of the PAG, in particular within the lateral and ventrolateral regions which are important to consider here since they contain a high distribution of MOR. In response to peripheral inflammation, PAG neurons are similarly activated in male and female rats, as revealed by c-fos staining. A sex-difference appears only when considering PAG neurons projecting to the RVM which displayed a stronger activation in males but no associated reduced behavior in response to inflammation. Retrograde labeling studies indicated that efferent and afferent projections of the PAG also differ in males and females. Amygdala, ventromedial hypothalamus and periventricular nucleus have more projections to the PAG in females, while projections to male PAG were more numerous from the medial preoptic area, parabrachial nucleus (PBn) and locus coeruleus. (Loyd and Murphy, 2009)

Tonsfeldt et al. (2016) studied sex-differences in GABAergic signaling within the PAG. This structure is placed under a tonic GABAergic inhibition mediated by $GABA_A$ receptors and when inhibition is reduced analgesia is produced by recruitment of the PAG-RVM pathway. Interestingly, $GABA_A$ inhibition is modulated by peripheral Complete Freund's adjuvant (CFA) inflammation, resulting in an increase of presynaptic GABA release in females but not in males, as well as a decrease in high affinity tonic $GABA_A$ currents (Tonsfeldt et al., 2016).

The spinoparabrachial pathway has also been studied in the context of visceral pain. Murphy and colleagues used retrograde labeling from the PBn to the spinal cord and studied c-fos expression in response to colorectal distension in rats. No clear structural differences in the distribution of projection neurons from the spinal cord to PBn could be observed but greater c-fos expression following colorectal distension was detected in spinoparabrachial neurons in male rats (70% of spino-PBn neurons in males vs. 40% in females). The use of morphine as an analgesic was able to decrease c-fos expression in males (70%–40%) but not in females, which has been linked to a decreased MOR expression in PBn of female rats (Murphy et al., 2009).

### Hormonal hypothesis

Sex hormones have an obvious role in the control of the reproductive system, but also influence many other physiological systems, including the nervous system. Receptors for these hormones are found in a variety of brain areas, among which are areas dedicated to the transmission and the control of pain responses. It is then logical to hypothesize that sex hormones modulate nociceptive transmission and pain responses in humans and animals. As mentioned above, sex-differences in pain are developmentally regulated further supporting the important role of sex-hormones in the modulation of pain. Sex-differences appear only around puberty, when the hormonal condition changes in both boys and girls.

Another observation supporting the role of sex hormones shaping pain has been made while studying the variations of pain responses in females during the ovarian cycle [see review (Kuba and Quinones-Jenab, 2005)]. In women, the follicular phase is characterized by an increasing level of Follicle-stimulating hormone (FSH) and estrogens, and then ovulation occurs triggered by a peak in Luteinizing hormone (LH) levels, followed by the luteal phase when estrogens, LH and FSH remain low whereas progesterone increases. Studies of pain responses during the menstrual cycle, although highly discussed, suggest a possible decrease in pain sensitivity during the late follicular phase (Riley et al., 1999). Sex hormone levels can also affect the descending controls of pain. The efficacy of DNIC is, for example, dependent on the phase of menstrual cycle in women, particularly more efficient during ovulation, a phase characterized by high levels of estradiol and low levels of progesterone (Tousignant-Laflamme and Marchand, 2009b; Rezaii et al., 2012). Other regions linked to the descending controls of pain, such as the PAG, are also differentially activated throughout the menstrual cycle. Stress-induced hyperalgesia (SIH) is then observed in female rats in late diestrus only (Devall and Lovick, 2010). These observations should be directly related to a modulation of the sensory-discriminative pathways or of the descending controls of pain by sex-hormones, since the affective modulation of pain is not affected by the phase of the menstrual cycle (Rhudy and Bartley, 2010).

Animal studies also helped decipher the mechanisms inducing sex-specificity in pain, as described in the review by Kuba and Quinones-Jenab (2005). It should be noted, however, that the estrus cycle in rodents lasts only a few days [4 or 5 days in the rat (Nequin et al., 1979) and 5–10 days in mice (vom Saal and Bronson, 1980)] and is characterized by high progesterone and estrogen levels in proestrus, with a secondary increase in progesterone during diestrus. Using rats, Gaumond and colleagues described that a similar response to the formalin test can be obtained with castrated males and ovariectomized females, strongly suggesting a role of gonadal hormones in nociceptive sensitization processes (Gaumond et al., 2002). Using the same model and using gonadectomized and intact male rats expressing no difference in the basal response to formaline, Aloisi and colleagues found that intact rats displayed habituation (decrease of pain behavior) to repeated formalin injections which was absent in gonadectomized rats (Aloisi et al., 2003; Ceccarelli et al., 2003). They hence suggested that male hormones have the ability to inhibit nociceptive response to a repeated painful stimulation. Indeed, injections of testosterone decreases licking behavior in response to formalin in female rats only, suppressing the female hyperresponsiveness to formalin (Aloisi et al., 2004). Testosterone seems to also play an important role in women, since endogenous pain inhibition is positively correlated to the level of testosterone in healthy women in a low estradiol state (controlled by oral contraceptives) as mea-

sured by fMRI brain activity in the RVM and pain perception (Vincent et al., 2013). The effect of progesterone and testosterone on pain responses is efficiently mediated through their active metabolites, chemically reduced in 3α5α compounds (i.e. allopregnanolone and 3α-pregnane-diol, respectively). These 3α5α compounds, when injected systemically (intravenous) or in the central nervous system (intrathecal, intra-cerebro-ventricular) have been showed to attenuate nociceptive responses in rodents (Frye and Duncan, 1994; Poisbeau et al., 2014). Moreover, the analgesic effect of testosterone is absent in mice deficient for 5α-reductase, one of the enzymes responsible for the local production of 3α-pregnane-diol (Frye et al., 2004). Sex steroids-derived 3α5α metabolites are known to be the most potent positive modulators of $GABA_A$ receptor function and it is likely that they exert their analgesic actions through this receptor (Poisbeau et al., 2014). Apart from this GABAergic effect, other rapid action might be mediated by other neurotransmitter systems such as cholinergic, serotonergic and catecholaminergic systems (Rupprecht et al., 1996; McEwen and Alves, 1999). A longer lasting action involving genomic changes might also be involved as demonstrated by Macguire & Mody. It is well illustrated by the expression changes of $GABA_A$receptor containing α4/δ subunits which are strongly regulated by estrus cycle (Maguire and Mody, 2009). During pregnancy, high expression of α4/δ subunit-containing $GABA_A$ receptors is detected in the hippocampus and strongly controls neuronal excitability. This could also be mediated by estrogen receptors alpha and beta which have specific anti- and pro-nociceptive actions based on the already published work (Coulombe et al., 2011) .

More recently, a differential implication of Toll Like Receptor 4 (TLR4) receptors has been proposed in the establishment of mechanical allodynia induced by LPS, CFA or spared nerve injury in CD-1 mice. Indeed, mRNA coding for TLR4 receptors are upregulated in both sexes after LPS injections, but TLR4 seem implicated in the observed allodynia only in males and dependent on testosterone availability, since the sensitivity to LPS disappears in castrated mice. In LPS, CFA and neuropathic models, mechanical allodynia was reversed by pharmacological blockade of TLR4 receptors only in males, suggesting another signaling pathway underlying allodynia in females (Sorge et al., 2011). This has been linked to a differential activation of microglia in males but not in females who preferentially recruit adaptive immune cells (Sorge et al., 2015).

## Socio-cognitive aspect of pain and gender expectation

Pain is a multidimensional sensation, and has a strong cognitive, emotional and psychosocial component that one should take into account when studying the sex-specificity of pain. Clinical studies demonstrate that men and women are using different strategies to cope with pain (Fillingim et al., 2009). Men are more likely to use behavioral distraction, whereas women preferentially seek social support, or use emotion-focused techniques as positive-self management (Unruh et al., 1999; Thompson et al., 2008).

Other factors can influence pain behaviors, such as anxiety or catastrophizing. It is indeed suggested that women respond to pain with a higher anxiety (Ramírez-Maestre and Esteve, 2014). In a recent study measuring pain perception while recording spinal (nociceptive reflex) and brain (somatosensory evoked potentials), authors found that women's higher sensitivity and brain activation to pain disappeared when controlling for trait anxiety (Goffaux et al., 2011). Catastrophizing corresponds to the tendency to exaggerate the magnitude of pain, to focus more on the threatening aspect of pain and to underestimate one's own capacity to endure pain. It can be assessed using the Pain Catastrophizing Scale (Sullivan et al., 1995), measuring helplessness, rumination and magnification. Catastrophization alters the feeling of a painful stimulus, possibly increasing its reported intensity and negatively affects the development and occurrence of chronic pain pathologies. A greater tendency to engage in pain catastrophization could explain some of the sex-differences observed in chronic pain and/or in experimental pain, as women seem more likely to engage in this strategy when facing pain (Jensen et al., 1994; Keefe et al., 2000; Edwards et al., 2004). In contrast, some publications failed to highlight sex-differences in catastrophizing (Rhudy et al., 2010; Ramírez-Maestre and Esteve, 2014). Empathy is also an important phenomenon in pain perception. Observing someone else in pain, especially a loved-one, triggers comparable brain activity as if it was our own personal experience (Lamm et al., 2011). Women are known to be more empathetic than men (Eisenberg et al., 2014). Interestingly, in a study demonstrating that it was possible to trigger CPM by observing a video of ourselves or of our spouse during the conditioning stimulus (cold pressor test), the effect was correlated with pain catastrophizing in both men and women and was correlated with empathy, but only in women (Gougeon et al., 2015). These results suggest that cognitive and emotional factors such as anxiety, catastrophizing and empathy are playing an important role in sex and gender differences in pain.

Social pressure could affect how men and women react in response to a painful stimulus. Indeed, a gender expectation seems to lead to the belief that men are supposed to express less pain. This is likely to start in childhood, when boys seem to be reluctant to express their pain (Fowler-Kerry and Lander, 1991). In children, the way the mother interacts with her children can also have an impact on their reported pain. For example, Chambers and colleagues exposed children aged from 8 to 12 years to a cold pressor pain in front of their mothers, and asked the mother to act either in a pain promoting manner or in a pain reducing manner. They demonstrated that girls are more likely to be sensitive to the social maternal influence in pain, as they report more pain when the mother presents a pain promoting behavior although maternal behavior has no effect on boys reported pain (Chambers et al., 2002). This could be part of a ''boy/girl education on pain'' that will determine the future pain response. In the adult population, Robinson

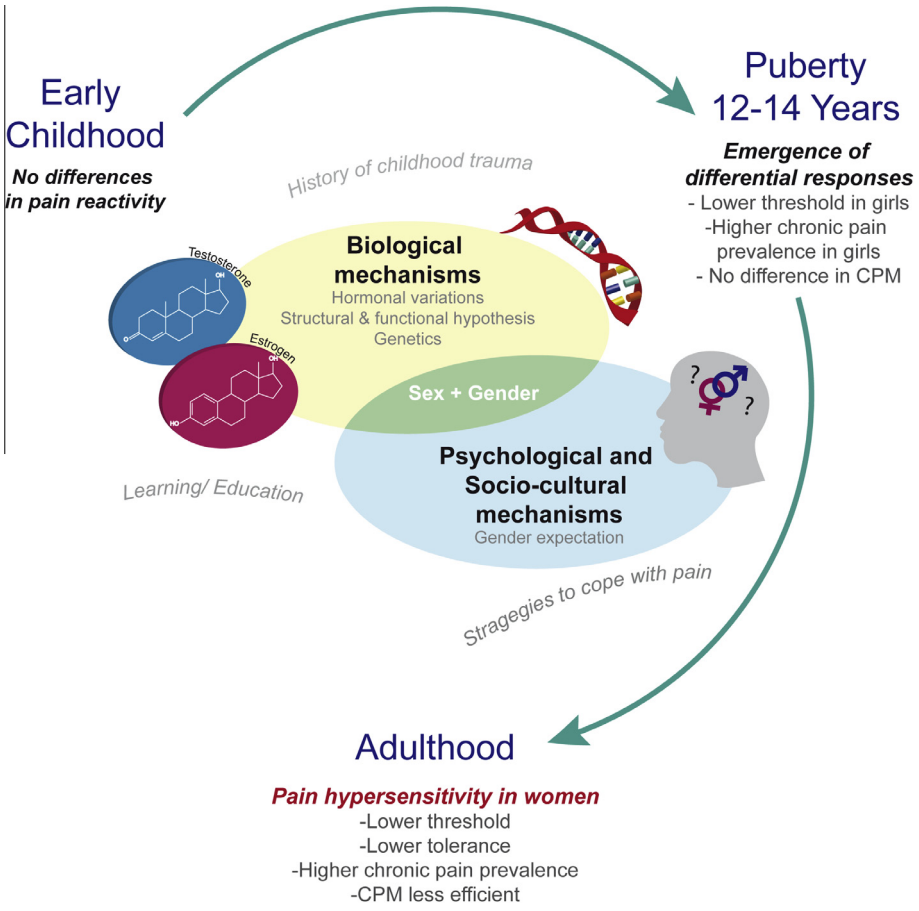


**Fig. 2.** Schematic representation of sex and gender mechanisms possibly underlying the emergence of sex-specific differences of pain processing all along the human lifespan. Biological, psychological and sociocultural mechanisms have been pointed out as critical determinants of female hypersensitivity to pain at adulthood. Beside pure biological explanations in the modulation of pain processing, by sex hormones for example, the development of pain circuits can also be imprinted in a sex-dependant manner by early childhood history and by social expectations from childhood to adulthood.

and colleagues demonstrated that people globally expect men to express pain less than women; a social expectation that could lead men to minimize and endure pain (Robinson et al., 2001).

### History of physical pain and psychological distress

Early childhood adversity can include violence, physical and sexual abuse, parental neglect, repeated pain or chronic stress, and is known to impact hugely on anxiety and stress behavior in adults, but it also induces long-term alterations in the response to pain (reviewed in (Davis et al., 2005)). For example, history of childhood trauma is suggested to be connected to the development of irritable bowel syndrome (Talley et al., 1995; Bradford et al., 2012), a pathology affecting mostly women. Evidences from clinical and preclinical studies suggest than men and women are differentially vulnerable to early life trauma.

Fillingim and Edwards demonstrated that a history of childhood abuse decreases pain sensitivity in adults, measured by a lower unpleasantness rating in responses to a pain stimulus, but only in women (Fillingim and Edwards, 2005). Early life trauma (physical punishment, emotional abuse and sexual abuse) is also reported more often by women suffering from irritable bowel syndrome than men (Bradford et al., 2012). This suggests that women could have an increased vulnerability to early negative events in terms of long-term impact on the pain response.

Traumatic events in the perinatal period could also induce long-term alteration of pain behaviors. For example, preterm children are integrated in NICU and subjected to a huge number of painful procedures (Simons et al., 2003; Carbajal et al., 2008); which not only has an impact on the later-reported pain responses but also on maternal stress and maternal way to deal with its child pain all along its childhood. In adolescence, children born preterm indeed describe an altered sensitivity to thermal and mechanical stimuli (Buskila et al., 2003; Hermann et al., 2006; Walker et al., 2009), have less efficient CPM (Goffaux et al., 2008) and are more prone to develop chronic pain (Maneyapanda and Venkatasubramanian, 2005). Among this population, girls appear to have more tender points and lower tender threshold (Buskila et al., 2003), but are not more affected by NICU integration in terms of migraine development (Maneyapanda and Venkatasubramanian, 2005). Moreover, it has been demonstrated that mother of

preterm children act preferentially in a pain promoting manner (Hohmeister et al., 2009), which could shape their latter responses to pain.

In fundamental studies on rodents, pain and/or stressful events in the neonatal period also alter the nociceptive responses in a long-term manner. Repeated painful procedures decrease or increase pain sensitivity in adults, depending on the procedure [see review (Schwaller and Fitzgerald, 2014)], as well as neonatal stress like long and repeated maternal separation which increases visceral sensitivity in adult (Coutinho et al., 2002) and causes chronic pelvic pain associated with increased inflammatory markers in the urogenital system in adult male and female mice (Pierce et al., 2014; Fuentes et al., 2015). Sex-differences in the long-term effects of neonatal stress/pain have not been consistently assessed since the studies were conducted mostly in males, but Bhutta et al. (2001) describe that male rats exposed to neonatal inflammation (formalin injection on P1–P7) display a longer thermal latency than females. However, LaPrairie and Murphy describe an opposite effect with a single neonatal injection of carrageenan on the hindpaw, with females being more sensitive to the long-term effects (LaPrairie and Murphy, 2007). At adulthood, they display a greater hypoalgesia and a greater CFA-induced hyperalgesia. In a visceral hyperalgesia model consisting of a paired odor-shock paradigm in rat neonates from P8 to P12, Chaloner and colleagues describe that in adulthood, female rats express more nociceptive behavior (visceromotor response in response to colorectal distension) than males, indicating a higher susceptibility to early life events, possibly explaining the higher prevalence of IBS in women (Chaloner and Greenwood-Van Meerveld, 2013).

Non-painful stress in the neonatal period affects the nociceptive function in a sex-specific manner, as a long and repeated separation (3H over the first 2 weeks) decreases morphine sensitivity preferentially in male rats (Kalinichev et al., 2001). The same protocol in the neonatal period induces a stronger emotional response to neuropathic pain in adult female mice only. Indeed, they display a depressive-like behavior, measured by an increased immobility time in a forced swim stress (Nishinaka et al., 2015). No sex-differences have been highlighted in a model of visceral hyperalgesia induced by neonatal limited bedding in rats (Guo et al., 2015), but female rats subjected to repeated 2-h maternal and littermates separation between P1 and P14 did express higher visceral hyperalgesia to colorectal distension, which was reversed by ovariectomy in adulthood but reinstalled with 1 week of estradiol treatment (Rosztóczy et al., 2003) .

Altogether, these observations could rely on a hormonal basis, since the hormonal levels are subjected to strong variations just before and after birth. In boys there is a peak of testosterone prenatally which is centrally aromatized into estradiol. This peak of testosterone leads to the masculinization of the brain, hence possibly underlying some of the sex-differences observed in pain behavior (Weisz and Ward, 1980). Estradiol levels in the brain are significantly higher in boys 2 h after birth, and remain higher 2 days after birth but only in the hypothalamus (Amateau et al., 2004). Neonatal hormonal levels indeed seem to play a key role in the later nociceptive function, as a single neonatal testosterone exposure in female mice suppresses the adult sex-differences in non-opioidergic SIA (Sternberg et al., 2004), with female SIA being transferred into NMDA-sensitive SIA and displaying a greater SIA than vehicle treated females (Sternberg et al., 1995) (Fig. 2).

## CONCLUSION

There are several basic and clinical reports supporting sex and gender differences in pain. Several mechanisms are related to these differences that seems to affect both excitatory (hyperalgesia, allodynia, central sensitization) and inhibitory (Conditioned Pain Modulation) mechanisms. The influence of physiological factors such as sex hormones and psychological factors such as anxiety seems to play important roles in women and men differences in the perception of pain, but also on the frequency of pain symptoms.

Women are overrepresented in clinical pain. They also have a lower pain threshold in most of experimental pain modalities and more brain activity in cortical regions that are related to the affective pain component (Paulson et al., 1998). In fact, when controlling for state anxiety, brain activity related to painful stimuli is no longer different between women and men, suggesting that psychological factors are playing a very important role (Goffaux et al., 2011). However, sex hormones have been demonstrated to be major contributors in sex-specificities of pain. Through the control of local inhibitory neuronal networks they are likely to influence the integration of pain messages as well as the efficacy of endogenous pain controls in both animal and human studies (Gaumond et al., 2005; Tousignant-Laflamme and Marchand, 2009a).

Sex difference in autonomic response to pain and the role of the autonomic system in pain modulation, raises the question of possible sex specificity in pain autonomic reactivity. However, it could also be just the opposite; differences in pain responses between men and women could trigger different ANS activity. They could be two independent phenomena activated independently and in parallel with the same external stimuli, in this case a nociceptive stimulus or an emotion. Regardless of the mechanisms, it is important to remember that ANS measurement could lead to different conclusions on the pain experience of men and women and could then not be sufficient to conclude on the efficacy of an analgesic in a non-communicating subject.

Finally, developmental factors, both psychological and physiological changes during the development from intrauterine to adulthood, could influence sex and gender differences in pain.

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Meggane MELCHIOR

# Caractérisation du contrôle descendant inhibiteur ocytocinergique et de sa modulation par un stress de séparation maternelle


## Résumé

L'ocytocine est un petit peptide synthétisé par des neurones de l'hypothalamus. Il est connu pour ses rôles dans la reproduction et les interactions sociales, en particulier dans les interactions mère-enfants, mais possède également un effet analgésique endogène. Au cours de cette thèse, j'ai cherché à comprendre plus en détail les circuits qui sous-tendent son effet analgésique. Dans un second temps j'ai cherché à déterminer si une séparation maternelle précoce, qui affecte les interactions mère-enfants, perturbe les réponses à la douleur et l'analgésie ocytocinergique chez la descendance. Ces travaux ont permis d'identifier un groupe de neurones ocytocinergiques dans l'hypothalamus, capables de diminuer la douleur par une double action. D'une part ils inhibent directement la transmission de l'information nociceptive dans la moelle épinière, et d'autre part contrôlent l'activité de neurones à ocytocine libérant la molécule dans la circulation sanguine. Notre étude sur la séparation maternelle démontre qu'elle induit une hypersensibilité à la douleur à l'âge adulte et un dysfonctionnement de l'analgésie endogène ocytocinergique


**Mots-clés** : douleur, ocytocine, séparation maternelle, stress post-natal, hypothalamus, contrôles descendants de la douleur


## Summary

Oxytocin is a small peptide synthesized in hypothalamic neurons. She is well known for its roles in reproduction and social interactions, especially in mother-infant interactions, but also displays analgesic effects. During this thesis, I tried to get a better understanding of the circuits underlying OT analgesia. Then, I tried to determine if neonatal maternal separation, affecting mother-infant interactions, alters adult pain responses and oxytocin analgesia. This work allowed to identify a subgroup of oxytocinergic neurons in the hypothalamus, able to decrease pain through a dual action. They directly inhibit nociceptive transmission in the spinal cord and control the activity of another population of oxytocinergic neurons releasing the peptide in the bloodstream. Our work on maternal separation shows that it induces nociceptive hypersensitivity at adulthood, and a dysfunction in oxytocin analgesia.


**Key-words**: pain, oxytocin, maternal separation, early life stress, hypothalamus, descending controls of pain