Université de Strasbourg

UNIVERSITÉ DE STRASBOURG

École Doctorale des Sciences de la Vie et de la Santé STRASBOURG

ÉCOLE DOCTORALE des Sciences de la Vie et de la Santé

Institut des Neurosciences Cellulaires et Intégratives, CNRS, UPR 3212

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soutenue le : **09 novembre 2021**

pour obtenir le grade de : **Docteur de l'université de Strasbourg**

Discipline/ Spécialité : Neurosciences

Contribution de la voie amygdalo-cingulaire dans la dépression et sa comorbidité avec la douleur chronique

Contribution of the amygdalo-cingulate pathway in depression and its comorbidity with chronic pain

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Mr Jorge MENDOZA	Directeur de Recherche, CNRS UPR3212

“There is some good in this world […], and it’s worth fighting for”

The Two Towers
J.R.R. Tolkien

Léa BECKER

Contribution de la voie amygdalo-cingulaire dans la dépression et sa comorbidité avec la douleur chronique

Résumé

Le trouble dépressif majeur (TDM) est une maladie chronique et invalidante, souvent aggravée par la présence de comorbidités telles que la douleur chroniqueIl est urgent de mieux comprendre les mécanismes sous-tendant le TDM. Des travaux récents suggèrent un rôle crucial du cortex cingulaire antérieur (CCA) et de l'amygdale basolatérale (BLA) dans ce trouble. Grâce à l'utilisation d'approches optogénétiques et à une évaluation comportementale détaillée nous avons mis en évidence le rôle crucial de la voie BLA-CCA dans la mise en place de comportements de type dépressif chez la souris. En effet, alors que l'activation de cette voie induit des comportements de type dépressif chez des animaux naïfs, son inhibition empêche leur développement suite à une douleur chronique. Au niveau moléculaire, nous avons montré que la dépression induite par stimulation de la voie BLA-CCA reposait sur des altérations transcriptomiques similaires à celles observées chez le patient dépressif. Ainsi, ce projet amène une caractérisation nouvelle et détaillée du rôle de la voie BLA-CCA dans la régulation émotionnelle, tant au niveau comportemental que moléculaire.

Mots-Clés : Depression, Douleur chronique, Cortex cingulaire antérieur, Amygdale basolatéral, Transcriptomique

Résumé en anglais

Major depressive disorder (MDD) is a chronic and debilitating disease, often worsened by the presence of comorbidities such as chronic pain. There is an urgent need to uncover the mechanisms underlying MDD. Recent work suggests a crucial role of the anterior cingulate cortex (ACC) and basolateral amygdala (BLA) in this disorder. Using optogenetics and a battery of behavioral testings, we highlighted the crucial role of the BLA-ACC pathway in the establishment of depressive-type behaviors. Indeed, the activation of this pathway induces depressive-like behaviors in naïve animals while its inhibition alleviates the depression induced by chronic neuropathic pain. At the molecular level, we have shown that depression induced by stimulation of the BLA-CCA pathway recapitulate similar transcriptomic alterations to those observed in depressed patients. Thus this project brings an extensive behavioral and molecular characterization of the role of the BLA-ACC pathway in emotional processing.

Key-words: Depression, Chronic-pain, Anterior cingulate cortex, Basolateral amygdala, Transcriptomic

Publications and communications

Publications resulting from thesis work

Becker LJ, Fillinger C, Waegaert R, Waltisperger E, Hener P, Journée SH, Ayazgok B, Humo M, Karatas M, Santin M, Barrot M Veinante P, Hugel S, Harsan LA, Turecki G, Lutz PE, Yalcin I (2021) A bottom-up pathway linking the amygdala to the cingulate cortex contributes to depression and its comorbidity with chronic pain. In prep

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Hideo Hagihara, Hirotaka Shoji, **International Brain pH Project Consortium**, Tsuyoshi Miyakawa Systematic analysis of brain lactate and pH levels in 65 animal models related to neuropsychiatric conditions. bioRxiv 2021.02.02.428362; doi: https://doi.org/10.1101/2021.02.02.428362. preprint

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Becker LJ, Fillinger C, Waegaert R, Hener P, Waltisperger E, Ayazgok B, Journée SH, Barrot M, Veinante P, Hugel S, Lutz PE, Yalcin I. Role of the amygdalo-cingulate pathway in depression, chronic pain and their comorbidity. EBBS 2021 Meeting, Lausanne, Switzerland.

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LJ Becker, C Fillinger, E Waltisperger, S Hugel, L Harsan, P Veinante, M Barrot, I YalcinRole of amygdalo-cingulate pathway in major depressive disorder. NeuroFrance Meeting 2019. Marseille, France.

Thesis Funding

My thesis was funded by a doctoral contract from the University of Strasbourg (Life & Health Doctoral School competition, University of Strasbourg) from 01/10/2017 to 30/09/2020. I then obtain a grant from the Fondation pour la Recherche Médicale (FRM) covering a fourth year of thesis (from 01/10/2020 to 30/09/21). Finally, I was recruited as an assistant engineer in biology on a private team contract from 01/10/2021 to 31/12/2021.

Award and Prizes

2021:

- Post-Doctoral mobility grant, the French National Research Agency (ANR) through the Programme d'Investissement d'Avenir EURIDOL graduate school of pain under the contract ANR-17- EURE-0022

2020:

- Regional finalist of the popularization challenge "My thesis in 180 seconds"

2019:

- Travel Grant of the Neuropole Federation for the NeuroFrance meeting 2019
- Financial support of the RIKEN CBS for the 2019 Summer School
- Biotechne Travel Grant for the 2019 SfN meeting

Outline

Acknowledgments

First, I would like to sincerely thank **Dr. Jorge Mendoza**, **Dr. Esther Berroccoso**, **Dr. Naguib Mechawar** and **Dr. Rohini Kuner** for agreeing to take a bit of their precious time to evaluate my work, I am very grateful to them.

Ces quatre dernières années rassemblent une grande partie de ce que je pense être les meilleurs moment de ma vie. Cela vient en grande partie des personnes que j'ai rencontré lors de cette aventure et bien sûr de celles qui ont été présentes bien avant et m'ont soutenus tout du long. Je m'excuse par avance pour ceux et celles que je vais oublier et pour ceux et celles qui n'auront pas les remerciements qu'ils/elles méritent, il me faudrait probablement un second manuscrit dédié uniquement à ces remerciements pour pouvoir exprimer ma gratitude comme il se doit.

Je vais commencer par la personne qui a eu l'impact le plus positif sur ce travail mais également sur tous les aspects annexes à un travail de thèse. **Ipek**, je n'aurais pas pu rêver meilleure encadrante. D'un point de vue scientifique j'ai pu apprendre énormément de choses grâce à toi, que cela soit à la paillasse ou sur les autres « devoirs » d'un chercheur. Tu as su me laisser la liberté nécessaire pour que je puisse m'épanouir dans ce monde scientifique tout en restant présente pour me soutenir et entretenir ma motivation à chaque instant. Tu m'as également laissé la possibilité d'explorer d'autres aspects de la science, notamment en me laissant m'investir dans des activités associatives, sans jamais me reprocher que cela puisse empiéter sur mon travail de thèse. J'ai pu voir avec les années que cela n'est pas si fréquent dans notre communauté, je tiens donc à te remercier pour ça. Enfin, et c'est probablement le plus important, merci d'être la personne que tu es. Tu es la preuve qu'il est possible d'être brillant tout en restant humain, que faire preuve de compassion, de patience et de bienveillance est le meilleur choix possible. Tu m'as fait énormément grandir en tant que personne et donné envie d'être une meilleure version de moi-même. Tu as été un merveilleux mentor, tu es devenue une amie et tu resteras un modèle pour moi.

Sylvain, même si tu vis dans le labo d'en face tu as également eu une influence positive sur la réalisation de ce travail de thèse et sur la scientifique je suis en train de devenir. Grâce à nos discussions autour de boissons plus ou moins alcoolisées tu as su me transmettre une autre vision de la science et du monde scientifique mais aussi me rappeler qu'il existe une vie après la thèse. Je pense que cela a grandement contribué au fait que je sorte de cette thèse sans avoir jeté mes convictions et ma motivation à faire bouger certaines choses à la poubelle. Merci à toi.

Pierre (Veinante), tu as été (une des) raisons qui m'a poussé à choisir un master en neurosciences, tu as ensuite entretenu mon intérêt avec tes cours de neuroanatomie et c'est en grande partie grâce à toi que je n'ai pas abandonné lors des années de master. A la fin de la première année je ne pensais pas être taillée pour réussir dans cette voie. Lors de l'entretien individuel que tu organises avec les étudiants tu m'as dit que le chemin pour arriver à être chercheur était long et compliqué mais que tu pensais que je pouvais y arriver. Cela m'a redonné la motivation et la confiance dont j'avais besoin à ce moment-là, alors un grand merci à toi. Je suis bien contente de m'être accroché, ça en valait la peine. Merci également de ne pas déjà m'avoir assommé quand je venais te harceler pour Doctoneuro, les cagnottes de thèse ou pour un briquet… (j'en ai un dans mon bureau maintenant !)

Pierre-Eric je tiens à te remercier pour m'avoir fait découvrir le monde de la génomique et de la bio-informatique, pour être honnête je n'aurais jamais pensé que cela pouvait autant m'intéresser, mais

c'est finalement le cas ! Un grand merci également pour l'aide que tu m'as apportée lors de la rédaction de l'article, j'ai beaucoup apprécié nos séances de travail.

Merci également à **Michel**, qui m'a accepté dans l'équipe et m'a fourni une très bonne formation scientifique.

Mélanie, **Jenny**, **Marie-José** et **Dominique**, nous faisons partie d'une (très) grande équipe et chacune d'entre vous a contribué à ce travail et plus largement à mon parcours scientifique par vos suggestions, discussions et astuces pour choisir un post-doc aux États-Unis. Je vous en suis extrêmement reconnaissante.

Bien que les résultats ne soient pas présentés dans ce manuscrit j'ai eu la chance de participer à une expérience d'IRM chez la souris et pour cela je remercie le **Dr. Laura Harsan** qui m'a appris un peu de son univers ainsi qu'à **Laetitia** et **Marie-des-Neiges** qui ont géré toute une partie de l'expérimentation et de l'analyse.

Toutes les expériences transcriptomiques n'auraient pas été possibles sans l'aide de la formidable équipe de la plateforme GenomEast qui a réalisé le séquençage, l'analyse d'expression différentielle et le Fluidigm, je remercie donc chaleureusement **Christelle**, **Damien** et **Violaine** pour leur aide précieuse.

Pour réaliser une thèse il faut bien plus qu'un doctorant et son superviseur, « it takes a village ». Un énorme merci donc à **Dom**, **Sophie**, **Julie**, **Cédric**, **Christophe**, **Momo** et plus généralement tout le staff du Chronobiotron et du bâtiment qui se démène tout les jours pour que nous puissions faire notre travail.

Tout ce travail n'aurait pas été possible sans l'aide des incroyables ingénieurs que sont **Betty**, **Pierre** (Hener) et (G)**Robin**. Un grand grand merci à vous trois. Nicolas Cage serait fier de toi Grobin !

I'll switch back to English for this one. As I already said, the Barrot/Yalcin team is huge (and cosmopolitan), so to the past members: **Florian**, **Muris**, **Fanny**, **Alessandro**, **Beyza**, **Heba**, to my beloved little interns: **Léa**, **Claire** and **Charlotte** and to the current members: **Camille**, **Marion** and **Marion** (we seriously need to stop with the similar names at the 3rd floor it is really confusing), I'm really grateful that our path crossed, you made this journey way better and less stressful!

A l'équipe d'en face, je tiens à remercier **Ben**, **Charlotte** et **Taddy**, je suis contente d'avoir passé un peu de temps à vos côtés, on a vécu les mêmes galères, on s'est encouragé mutuellement mais surtout on a beaucoup ri et c'est ça que je vais retenir.

Aux bureaux de **Doctoneuro** dont j'ai fait partie ces 5 (6 ?) dernières années, un grand merci à tous, j'ai été heureuse de faire partie de cette aventure qui m'a permis de rencontrer des personnes fantastiques. A ce titre je tiens à adresser un merci tout particulier à **Clémence** (meilleure trésorière) **Guillaume** et **Baptiste** (meilleurs secrétaires) vous êtes tout simplement géniaux. Un grand merci à **Robin** également (un des meilleurs VP pédagogie, et qui a visiblement « *souvent le souffle chaud sur ta nuque* », ne demande pas d'explication j'ai lancé une idée stupide et j'en récolte maintenant les fruits) ça a été très agréable de voir quelqu'un avec un vrai esprit associatif cette dernière année, tu as mis en place des choses fantastiques malgré la situation, keep going ! Et faites en sorte que cette association perdure, je veux pouvoir faire une entrée spectaculaire au gala des 10 ans ! Et bien évidemment un grand merci aux anciens, **Max**, **Lucas** et **TB** plus particulièrement, la plus part de mes meilleurs souvenirs depuis mon entrée en master sont avec vous et je ne vous en remercierai jamais assez.

J'ai encore du mal à croire que les mots qui vont suivre seront à jamais écrit à la vue et au su de tous… mais merci **Valod** (Vanod ? Valessandro ? Mohammed ? Jean-Michel ?) d'avoir été la personne la plus exaspérante que j'ai rencontré. Je ne sais pas si j'irais jusqu'à dire que je suis heureuse que tu fasses partie de ma vie mais disons que je ne le regrette pas. (Fais encadrer ces quelques lignes sérieux).

Ce qui m'amène à toi **Ludo**. J'ai vaguement aperçu au travers du sus-mentionné Valod ce que je t'ai fait enduré pendant des années… Donc tout d'abord je suis navrée (un peu, pas vraiment en fait), ceci dit tu t'es toujours bien défendu et tu m'a donné le bon (mauvais ?) exemple. Merci pour ton aide et tes conseils, mais surtout, merci pour tous ces soirs et week-end passé à déplacer/démonter des bureaux ça m'a beaucoup manqué quand tu es parti. Je ne sais pas si c'est une bonne chose mais je t'ai toujours un peu vu comme un modèle. Je ne sais pas ce qu'il en est pour la partie scientifique, mais pour la partie casse couille je pense que je suis sur la bonne voie ! Et prépare toi, j'arrive bientôt au pays du hot-dog !

Il y a des gens qui sont entrés dans ma vie lors de cette thèse et qui ont heureusement décidé de ne plus en sortir. **Baptiste**, **Elaine**, **Xavier** on en a chié ensemble, on s'est soutenu mutuellement, on s'est plaint de nos manips de von Frey mais on s'en est tous sorti (plus ou moins dans un bon état). Vous avoir à mes côté a définitivement rendu ces années meilleures et j'ai hâte de voir ce que la suite nous réserve. Vous êtes top ne changez rien !

Sarah, si j'avais su que donner de mon temps pour aider à la rentrée des Erasmus me conduirait à passer mon temps avec toi dans le même bureau, eh bien je n'aurais absolument rien changé. Déjà parce que sans toi j'aurais manqué une bonne partie de la fine gastronomie de Strasbourg et les meilleures astuces pour créer des posters géants (et gênants). Soit sûre que ton héritage concernant ce dernier point sera perpétué outre-atlantique ! Mais plus important encore tu m'as apporté un réel soutient notamment avec des blagues de qualité et de très mignonnes vidéos de chiens (et autres animaux mignons). Tu as également été un des piliers de ma santé mentale en temps de confinements et tu m'a très clairement évité un burn-out associatif. Merci pour tout, tu déchires, ait confiance en toi tu as les capacités d'accomplir tout ce que tu entreprendras.

Victor et **Clémentine**, nous n'avons au final pas passé tellement de temps ensemble, mais je n'en retiens que de bons souvenirs. Clémentine, sans toi je n'aurais pas pu arriver où j'en suis, tu m'as tout appris en ce qui concerne les chirurgies, l'opto ou le comportement et surtout tu es la seule personne avec un humour aussi fin et raffiné que le mien ! Victor, champion, ton retour au labo a été un vent de fraicheur pour moi. La fin de ce doctorat a parfois été compliquée car j'avais beaucoup d'incertitudes et de doutes que je n'arrivais pas à partager avec la plupart des autres étudiants. Nos discussions m'ont vraiment permis de surmonter une bonne partie de ces peurs et de retrouver la motivation qui commençait à me faire défaut. Et puis soyons honnête ta seule présence illumine mes journées, sinon pourquoi est-ce qu'il y aurait une photo de toi au-dessus de mon bureau ? Je vous souhaite à toi et Clémentine tout le bonheur possible pour les années à venir, et j'ai hâte de rencontrer le petit Eliott (Igor) Fillinger-Mathis !

Il y des gens qui sont comme des anges gardiens, qui nous retiennent de faire des choses débiles et qui nous poussent à prendre des décisions sages et raisonnées. Et puis il y a toi, **Damien**, tu es un peu mon Sheitan personnel. Même si je me suis souvent réveillée en regrettant de t'avoir suivi dans tes « nan mais juste une bière et après on rentre » ces soirées improvisées m'ont permis de souffler un bon coup. Tu as été une sorte de grand frère (un peu maléfique), merci d'avoir été là quand j'en avais le plus besoin.

Merci à **mes parents**, vous vous êtes démenés tout au long de votre vie pour que je puisse accomplir tout ce que j'avais envie d'accomplir et pour ça je ne vous remercierai jamais assez.

Mélissa, **Lise**, **Gaëtan**, **Gautier**, **Sophie**, **Cynthia**, je pourrais écrire des pages et des pages pour vous dire à quel point je suis heureuse de vous compter parmi mes amis. Mais les chances que vous lisiez ces mots étant proches de 0 je vais épargner les lecteurs de ce manuscrit et dirai simplement que je vous aime de tout mon cœur. Merci de ne jamais m'avoir lâché alors que je n'ai pas toujours pu vous accorder le temps qui vous méritez ces six dernières années.

Noëmi (j'ai bien écrit ton nom t'as vu !), malgré que nous nous soyons rencontré tardivement tu as rapidement pris une place importante dans ma vie. Tu es vraiment une personne formidable ne l'oublie jamais. Merci pour les pauses clopes, les sessions de tététravail sur ton balcon, les (trop nombreux) verres au Wawa et les randos (un peu moins pour les déménagements). J'espère sincèrement que notre amitié résistera à la distance. Et souviens toi que tu es une excellente jeune scientifique, tu vas tout déchirer !

Lou, je ne sais même pas par où commencer, j'ai tellement de choses à te dire. On a démarré cette aventure au début du master, ça me parait tellement loin, c'est un peu comme si tu avais toujours fait partie de ma vie. On a fait énormément de chemin depuis ces premiers oraux d'IDSN (**PTSD noises**) et je suis ravie d'avoir passé ces 6 dernières années à tes côtés. Tu es sans aucun doute la personne la plus merveilleuse que j'ai pu rencontrer lors de ma thèse et peut-être même au cours de ma vie. Tu as toujours été là pour me soutenir, tu as toujours su trouver les mots pour me réconforter, je ne t'en remercierais jamais assez. Mais probablement le plus impressionnant, tu as toujours ris à mes blagues, même (surtout) les plus nulles. Merci pour tout, merci d'être telle que tu es tout simplement.

En parlant de blagues nulles, « c'est l'histoire d'un mec, il rentre dans un bar, puis dans une table, puis dans une chaise… Merde, j'ai oublié de dire qu'il était aveugle. ». **Quentin**, j'ai bien du passer 4/5 heures à trouver un moyen de formuler ce que j'avais à te dire de façon intelligible et concise. Ce fut un échec je vais donc partir pour de l'improvisation. De garçon morose et doté de réactions émotionnelles limitées tu es passé à un homme un peu moins morose avec un panel émotionnel légèrement plus varié et un corps de grimpeur (je ne sais pas où je vais avec ça mais je devais bien le caler quelque part alors voilà). Plus sérieusement tu es l'une des personnes qui compte le plus à mes yeux, chaque moment passé avec toi, que ce soit au labo ou en dehors, m'est extrêmement précieux. Dépendamment des jours et de mon humeur du moment tu as été le Boyle de mon Peralta ou le Sam de mon Frodo. Je ne sais pas s'il existe des âmes sœurs en amour mais tu es la preuve qu'elles existent bel et bien en amitié.

« Et là normalement il me faut une citation latine, mais pfff… j'en ai marre ! »
François Rollin – Roi Loth D'Orcanie
Kaamelott Livre V, Tome 1

List of abbreviations

ACC : Anterior Cingulate Cortex

ADs : Antidepressant Drugs

AMPA : α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Aspa : Aspartoacylase

BA : Basolateral amygdala

BLA : Anterior part of the basolateral nucleus of the amygdala

BLP : Posterior part of the basolateral nucleus of the amygdala

BNST : Bed nucleus of the stria terminalis

CeA : Central amygdala

CEN : Central executive network

CPED : Chronic pain-induced emotional disorder

CPID : Chronic pain-induced depression

CRS : Chronic restrained stress

CS : Conditioned stimulus

CSDS : Chronic social defeat stress

CVS : Chronic variable stress

DEGs : Differentially expressed genes

dlPFC : Dorsolateral prefrontal cortex

DMN : Default mode network

DRN : Dorsal raphe nucleus

DSM : Diagnostic and statistical manual of mental disorders

FC : Fold-change

FST : Forced Swim Test

FS-PV : Fast-spiking parvalbumine neurons

GABA : Gamma Aminobutyric Acid

$GABA_A$: GABA receptor A

Gad65 : glutamic acid decarboxylase 65

Glu : Glutamate

GO : Gene ontology

HPA : Hypothalamic-pituitary-adrenal

HPC : Hippocampus

ICD : International classification of diseases

IL : Infralimbic cortex

INS : Insula

LA : Lateral amygdala

LC : Locus coeruleus

LD : Light/Dark box test

Lingo-1/3 : Leucine rich repeat and Immunoglobin-like domain-containing protein 1/3

LTP : Long-term potentiation

Mal : Myelin and lymphocyte protein

MAO : Monoamine oxidase inhibitors

MAPK : Mitogen-activated protein kinase

Mbp : Myelin basic protein

MDD : Major depressive disorder

MKP-1 : Mitogen-activated protein kinase phosphatase-1

Mog : Myelin oligodendrocyte glycoprotein

mPFC : Medial prefrontal cortex

M2 : Secondary motor cortex

NAc: Nucleus accumbens

NSF : Novelty Suppressed Feeding Test

MRI : Magnetic resonance imaging

NMDA : N-Methyl-D-aspartic acid

OL : Oligodendrocytes

Olig2 : Pligodendrocyte transcription factor 2

OPC : Oligodendrocytes progenitors cells

PAG : Periaqueductal grey area

PCC : Posterior cingulate cortex

PET : Positron emission tomography

PF : Parafascicular thalamic nucleus

PFC : Prefrontal cortex

Plp1 : Proteolipid protein 1

PNs: Projection neurons

PO : Posterior thalamic nucleus

POU3F1 : POU Class 3 Homeobox 1

Ppp1r1b+ : Protein phosphatase 1 regulatory inhibitor subunit 1B

PrL : Prelimbic cortex

PTSD : Post-traumatic stress disorder

PV : Parvalbumin

RRHO2 : Rank-rank hypergeometry overlap

Rspo : R-spondin 2

rTMS : Repetitive Transcranial Magnetic Stimulation

RT-qPCR : Real-time quantitative polymerase chain reaction

sACC : Subgenual anterior cingulate cortex

Sema4A : Semaphorin 4A

SI : Social isolation

SN : Salient network

SNI : Spared nerve injury

SNRI : Selective norepinephrin reuptake inhibitor

SSRI : Selective serotonin reuptake inhibitor

SST : Somatostatin

ST : Splash Test

S1 : Primary somatosensory cortices

S2 : Secondary somatosensory cortices

TCA : Tricyclic Antidepressant

UCMS : Unpredictable chronic mild stress

US : Unconditioned stimulus

vGlut2 : Vesicular glutamate transporter 2

vHPC : Ventral hippocampus

VIP : Vasointestinal peptide

vlPAG : Ventrolateral periaqueductal grey area

WGCNA : Weighted gene co-expression network analysis

WHO : World health organization

5-HT : Serotonin

Foreword

I. Background of my PhD project

Emotional disorders are among the most common diseases worldwide, with major depressive disorder (MDD) being the leading cause of mental disability nowadays. In addition, MDD is often found to be comorbid with other debilitating conditions which worsen the burden of the patients. Notably, MDD is frequently observed in patients suffering from chronic pain, leading to poorer prognosis for both conditions. Yet, the pathophysiology underlying this comorbidity remains unclear.

Fortunately, the research efforts put during the past decades to decipher the underlying mechanisms of emotional disorders and chronic pain brought new insights for both pathologies and their comorbidity. It has been proposed that there are some shared neuroanatomical structures processing pain as well as affective information. Thus, molecular alterations induced in these regions by long-term chronic pain could lead to altered processing of affective information which may result in mood disorders. The anterior cingulate cortex (ACC) is one of the brain areas that displays the strongest morphological and functional alterations in chronic pain and depressed patients. These alterations can be recapitulated in animal models of both pathologies. However, none of these diseases can be resumed to the dysfunction of a single brain structure. Therefore, the recent works in the field are now rather focusing on the underlying brain circuits. Among the numerous inputs and outputs of the ACC its connection with the basolateral amygdala, particularly its anterior part (BLA) appears to be of great interest in the scope of depression, chronic pain and their comorbidity. Indeed, the BLA is a major center for emotional regulation and several evidences document its role in pain as well. Yet, despite the large body of evidences underlining the importance of the ACC and the BLA in the regulation of emotions and pain, the role of the connection between these two structures is surprisingly still largely unknown.

II. Goals and Study Conduct

The main goal of my PhD project was to characterize the role of the BLA-ACC pathway in emotional processing in mice in an integrated manner. This project is constructed around three aims: 1) determining the implication of the BLA-ACC pathway in the sensory and affective component of chronic pain, 2) assessing the impact of manipulating the activity of the BLA-ACC pathway on emotional processing in naïve conditions and 3) deciphering the underlying molecular mechanisms of depression by comparing transcriptomic data from mice and human.

To fulfill the first aim we used our well established and behaviorally characterized neuropathic pain model, cuff model. In this model a peripheral nerve injury induces an immediate mechanical hypersensitivity and delayed anxiodepressive-like behaviors. First, we combined c-fos protein immunohistochemistry and retrograde tract tracing approaches to demonstrate that the BLA-ACC pathway is indeed hyperactive when the negative affective consequences of chronic pain emerge. We found an overall increase c-os immunoreactivity in the BLA that was preferentially found in neurons projecting toward the ACC. Therefore, we hypothesized that this hyperactivity might drive the chronic pain-induced emotional deficits. To test this, we used an optogenetic approach to selectively inhibit the BLA-ACC pathway and demonstrated that acute inhibition does not improved the mechanical hypersensitivity caused by nerve injury in the von Frey test (either at early or late stages of neuropathic pain). Interestingly, while the acute inhibition has no impact on anxiety-like behaviors (Light-Dark test, LD), it efficiently reversed the depressive-like behaviors (Splash test, ST and Forced Swim Test, FST). Altogether these results suggest that chronic pain-induced depression trigger a hyperactivity of the BLA-ACC pathway which is necessary for the emergence of such behaviors.

Building on the previous results we wondered if enhancing the activity of the BLA-ACC pathway could selectively drives depressive-like behaviors outside the context of pain (aim 2). Therefore, we used the opposite strategy and activated the BLA-ACC pathway in naïve mice. First, we observed that acute activation did not modify anxiodepressive-like behaviors (ST and Novelty Suppressed Feeding test, NSF). Nonetheless, considering that mood disorders usually take time to develop, this result was not surprising and we then sought to determine the effect of repeated activation of this pathway. We set up a paradigm in which animals were

optogenetically stimulated three times a week during three weeks. To assess the anxiodepressive-like behaviors a battery of behavioral tests (LD, NSF, Nest test, ST and FST) was performed at different time points (weeks 1, 2 and 3). In mirror to the conclusion of the first aim, the overall results of this experiment showed that BLA-ACC repeated activation induces depressive-like behaviors with no effect on anxiety. However, the development of depression-like phenotype was not immediate. Indeed, while no changes were observed at week 1, a tendency toward increased depressive-like behaviors appeared at week 2 (Nest test) that became significant at week 3 (Nest test and ST). Noteworthy, these behavioral deficits disappeared when the optogenetic stimulations were interrupted. To go further and decipher the cellular actors of the observed behavioral effect, we used the RNAscope technology to assess the neurochemistry nature of the activated neurons within the ACC at the week 3 time point and looked at the colocalization between *c-Fos*, *Gad65* and *Vglut2* markers. The large majority of *c-Fos+* cells colocalized with the *vglut2* marker, indicating a strong engagement of glutamatergic neurons within the ACC. However, while the raw number of *c-Fos+*/*vglut2+* was strongly increased in stimulated animals compared to controls, its relative proportion was not modified. On the other hand, the number of *c-Fos+* cells colocalizing with the *gad65* marker was much smaller but with a significantly higher relative proportion in stimulated animals. Altogether these results underline a selective role of the BLA-ACC pathway in depressive-like behaviors that can only be achieved through repeated activation and mostly involve glutamatergic neurons of the ACC with a possible recruitment of local interneuron circuits.

The third aim of this project was dedicated to deciphering the molecular mechanisms underlying the optogenetically-induced depressive-like behaviors observed in aim 2. Therefore, we performed RNA-sequencing on the ACC at the week 3 time point when depressive consequences are maximal. The differential expression analysis highlighted strong differences between stimulated and unstimulated animals. Furthermore, Gene Ontology and Gene Set Enrichment Analysis (GO, GSEA) unraveled major dysregulation in neurogenesis, intracellular transport or myelination processes. However, even if our optogenetic paradigm triggers strong depressive-like phenotype it remains artificial and could lack translational value. Therefore, we conducted the rest of our transcriptomic analysis by comparing our mice results with human data obtained recently by Turecki's lab in the same brain region (ACC).

Using a threshold-free method (Rank-Rank Hypergeometry Overlap, RRHO), which allow for comparison across the whole genome, we showed that human and mice MDD share a robust pattern of gene dysregulation, with gene expression altered in the same direction for both species. Then, we conducted a weighted gene co-expression network analysis (WGCNA) on both gene-set to compare the modular organization of gene expression between mice and human. In both species we observed strong correlation between several gene networks and the condition (stimulation for mice and MDD diagnosis for human) and part of them were conserved across species. We proceed to GO analysis of these conserved networks and found enrichment in gene related to myelination. This was of great interest because it goes along with the conclusion previously obtained by Turecki's group for the human data used in this study. In addition, studies from other researchers also showed a strong association between MDD and myelin or oligodendrocyte deficits. Therefore we choose to focus on the genes involved in oligodendroglial function that were the most central within gene networks and differentially expressed in both species. We thus started by confirming our RNAsequencing results through a microfluidic qPCR (Fluidigm) approach. Overall, results were concordant with RNA-sequencing, with a down-regulation of myelin components *(mbp*, *plp1*, *mog*, *mal*) and synthesis enzyme (*aspa*) and an up-regulation of myelin and oligodendrocyte inhibitors (*lingo1*, *sema4A*). Accordingly, we hypothesized that the behavioral deficits induced by repeated activation of the BLA-ACC rely on a deficit in myelination and/or oligodendrocyte integrity within the ACC, possibly through the up-regulation of *lingo1* and/or *sema4A*.

At the time of writing this thesis, experiments aiming at determining the causal link between molecular alterations and behavioral outcomes were ongoing. Through virally mediated strategy, we plan to knock-down *lingo1* and *sema4A* in the ACC using our optogenetic model of depression and investigate the impact on depressive-like behaviors.

With the completion of this experiment we hope to draw a comprehensive and integrated characterization of the role of the BLA-ACC pathway in depression with and without chronic pain comorbidity.

III. Thesis Content

This manuscript is based on the format of scientific articles and is organized as follow:

The general introduction is divided in four parts. The first one reviews the evolution of the concept of emotions and gives an overview of mood disorders associated with a dysfunction in emotional processing. The second part focus on major depressive disorder, its symptomatology, epidemiology and physiopathology, with a special focus on the recent discoveries regarding the involvement of oligodendrocytes and myelination. It ends up with a overiew of the brain circuits underlying emotion regulation. The third section is centered on chronic pain and its comorbidity with depression. After a short definition of chronic pain it includes a review (I'm the second author) published in the European Journal of Neuroscience, untitled "How to study anxiety and depression in rodent models of chronic pain?". It is then followed by a description of the brain networks involved in pain and a commentary, published in Neuroscience and Biobehavioral Reviews (I'm the first author), which focuses on the circuits underlying chronic pain and anxiodepressive disorders comorbidity. The fourth part of this introduction deals with the ACC circuitry in emotional disorders and chronic pain with a special focus on the basolateral amygdala. In this part the organization, connectivity, physiological role and involvement in pain and emotional disorders are described for both structures. Finally, the current knowledge on the interaction between the ACC and the BLA in physiological and pathological conditions is described.

All the results and material and methods used during my thesis is organized as a scientific paper that we are currently working on. It includes all of the three aforementioned aims. This thesis manuscript is finalized with a general discussion and perspectives.

I. Emotions: from physiology to pathology

Nowadays, it would be impossible to deny the direct link between the brain and the emotions. However, the first attempts to describe the organization of the human mind associated the brain only with the intellect while emotions were thought to arise from the heart (Democritus circa 460 BC). Of course, this vision did not last long and observations of disturbed emotional responses in brain-damaged subjects make it impossible to dissociate the brain and the emotions. Yet, emotions were for long left aside because they were seen as a brake for human intellect (*The Passions of the Soul*, René Descartes, 1649) or without evolutionary purpose (*The Expression of the Emotions in Man and Animals*, Charles Darwin 1872). This could partially explain the long lack of investigation of the neurobiological basis of emotions compared to other cerebral functions (like memory, attention or decision making). Nevertheless, this vision was questioned during the XVIII and XIX centuries, as it appeared that emotions were entangled with every other cognitive and executive processes (Brosch et al., 2013; Burghardt, 2019). The brain is, by essence, a very integrated system and as pointed by Damasio, emotions play a significant part in this system and should not be put aside (A. R. Damasio, 1998). Moreover, dysfunction in emotional processing is a feature of many psychiatric disorders such as anxiety or depression. While those conditions affect more and more people worldwide, their pathophysiology remain unclear. Therefore, it is now crucial to gain a better understanding of the mechanisms underlying emotions and their dysregulation.

1. Definition of emotion, where we are?

The field of emotion has remained little explored until the late seventies and has known an exponential grow over the past three decades. Several attempts have been made to define different emotional terms as discussed by many specialists of the field (Gu et al., 2019; J. LeDoux, 2012; J. E. LeDoux, 1995; Russell, 2003). Yet a consensus is still missing since several notions seem to overlap. The first attempt to define emotions goes back to 1884 when William James asked, “What is an emotion?”. Even though the foundations of his work were later oversimplified (Ellsworth, 1994) his idea that, at least in humans, emotions are the conscious perception of bodily changes is still extensively reused by his successors (Critchley & Garfinkel,

2017; A. Damasio & Carvalho, 2013; Keltner, 2019; J. LeDoux, 2012). Indeed, Ledoux suggested that emotions are “a subjective experience that you have in a particular situation”[1]. In the same article, Phelps adds up to this vision and postulates that emotions need a subjective experience and a physiological response. However, while this definition may be suitable when considering human research, it is delicate or even impossible to transpose it in animals since they cannot verbalize how and what they feel. As outlined by Ledoux, this does not mean that animals do not exhibit any kind of emotions but only that our methodological approaches do not allow us to assess them if we strictly stick to this definition. Interestingly, Damasio brought a slightly different view (A. Damasio & Carvalho, 2013). He introduced the notion of “action programmes” that aim at detecting changes and maintaining the homeostasis through modifications of the physiology and the cognition of the individual. Those action programmes are divided into “drives” and “emotions”. The “drives” aim at satisfying basic instincts like hunger or thirst and are induced by internal stimuli (blood osmolality or sugar). In contrast, emotions follow the perception or the recall of exteroceptive stimuli and trigger physiological changes (facial muscle contraction, changes in heart rate, sweating, shift of attention etc.) (A. Damasio & Carvalho, 2013). In turn, the nervous system reacts to these physiological alterations and the conscious perception gives rise to what Damasio calls the “feelings” (**Figure 1**). Thereby, the perception of a predator induces the emotion of fear by increasing the heart rate or enhancing the attention directed to the predator but also the feeling of fear through the conscious perception of these phenomena. According to that view, the observation of the behavior or physiological parameters of animals can give us access to their emotions, but their feelings will remain inaccessible.

In the end, the definition of emotions seems to depend highly on the considered aspect of emotion (physiological manifestations, behavioral outcomes). Nonetheless, each definition shares some common ground. As proposed by Coppin and Sander in the latest edition of Emotion Measurement (2021), we could define emotions as fast processes, focused on

[1] What We Talk About When We Talk About Emotions. Cell. 2016 Dec 1;167(6):1443-1445. doi: 10.1016/j.cell.2016.11.029. PMID: 27912051.

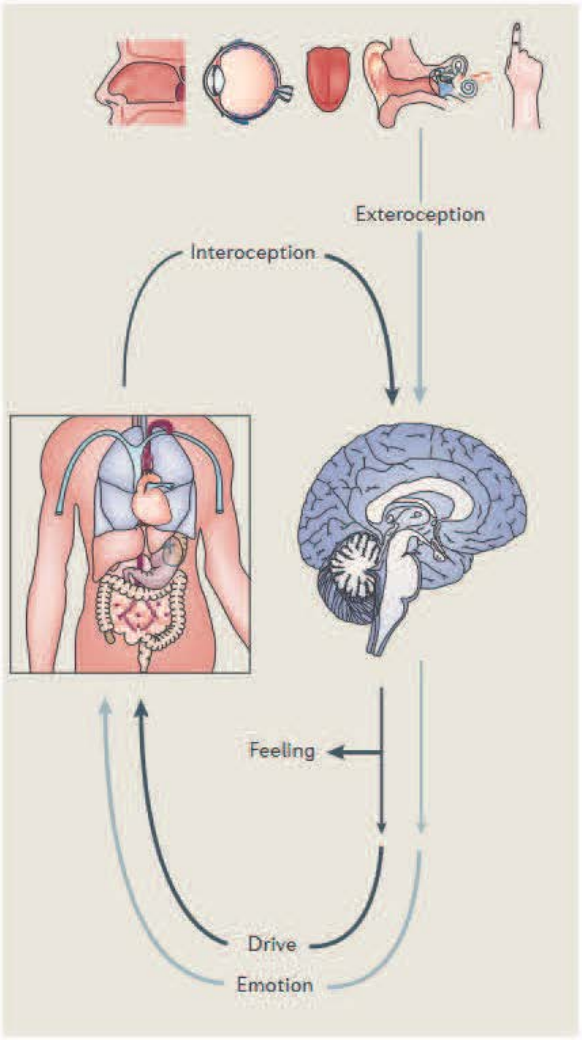

Figure 1 Drives, emotions and feelings

Schematic representation of the processing of external and internal stimuli that give rise to drive, emotions and feelings.

Adapted from Damasio and Carvalho 2013

relevant event, resulting in the modification of several component (bodily function, facial expression, behavior).

2. Emotional disorders

Despite the lack of consensus around the definition of an emotion, the research in the field is powered by the fact that dysfunction in emotional processes leads to severe and debilitating disorders. Indeed, emotions have a purpose of adaptation to maximize the chances of survival (R. Nesse, 1998; R. M. Nesse & Ellsworth, 2009). However, when emotions, especially the negative ones, become excessive they may trigger several disorders that severely impair the quality of life. For instance, at low level, fear will restrain us from engaging in potentially harmful situation and sadness acts as a way to communicate with conspecific and seek help. However, when fear and/or sadness are experienced too frequently or with an excessive intensity, they will end up restricting us in our daily activity and leading to the development of emotional disorders. These disorders can be regrouped mainly as anxiety disorders, post-traumatic stress disorders (PTSD) and depressive disorders. Despite being described separately in the two main classification of psychiatric disorders (DSM and ICD), those disorders share common features, and it was therefore proposed to create a separate cluster to regroup them (Goldberg et al., 2009). First, similarities in etiology are frequent, like early-life adversity or increased risks to develop an emotional disorder if a close relative is affected. The symptomatology also appears similar, with high prevalence of sleep disturbance, energy loss, irritability or worrying and a high prevalence of neuroticism among these patients. Likewise, some neurophysiological changes seem to be consistent between each pathology. Patients suffering from emotional disorder for instance show an activation of brain structures such as the prefrontal cortex (PFC), amygdala, locus coeruleus (LC), hypothalamus, dorsal raphe nucleus (DRN) or insula. Moreover, patients often display alteration in serotonin and norepinephrine neuromodulation and tend to respond to the same kind of treatments (antidepressant drugs, cognitive behavioral therapy). Finally, it exists a high rate of comorbidity between all emotional disorders (Charlson et al., 2019) that can suggest shared underlying mechanisms. Nowadays, it is estimated that 21% of the world population is affected by at least one disorder related to emotional dysfunction during their lifespan (Charlson et al., 2019). Unfortunately, our understanding of the mechanisms underlying these

emotional disorders remains limited leaving a great number of patients with few suitable therapeutic options. In the meantime, the prevalence of these mental disorders is expected to grow further as a consequence of the increasing number of risks factors like sanitary crisis (Bah et al., 2020; Peretti-Watel et al., 2020) climate change (Khafaie et al., 2019; Majeed & Lee, 2017), war and conflict (Charlson et al., 2019). It is thus urgent to implement our knowledge of the underlying neurobiology of these disorders. In this work, we choose to focus on major depressive disorder, which constitutes the most frequent emotional disorder worldwide.

II. Major depressive disorder

1. Definition and epidemiology

Major depressive disorder or MDD is a chronic and debilitating disease. According to the fifth edition of the DSM, it is characterized by the presence, for two weeks minimum, of at least five of the following symptoms, one of them being either depressed mood or loss of interest or pleasure: appetite, sleep or concentration disorder, hyper- or hypoactivity, energy loss, feeling of worthlessness or excessive/inappropriate guilt and suicidal ideation. The world health organization (WHO) estimates that 20% of the world population encounters at least one episode of MDD during their lifespan, placing MDD as a leading cause of disability worldwide and a major contributor to the global disease burden (WHO 2017). Beside the dramatic consequences of MDD on the patient's life-quality, it also affects patients work-life balance, education and their relationship with family, friend or other close-relative thus contributing to socio-economic burden as well. In certain cases, MDD can even lead to suicide, which is responsible for 80.000 deaths per year and is the second cause of mortality in adolescent and young adults (15-29 years-old). Despite these alarming observations, the treatments remain poor in part because of the gaps in the understanding of the underlying mechanisms of MDD. Luckily, new therapeutic strategies and new tools for fundamental research emerged during the last decades and brought new insights on MDD neurobiology.

2. The pathophysiology of MDD

The research on the pathogenesis of MDD did not cease to increase during the past half century. Several hypotheses on the mechanisms underlying depression have been proposed over the decades, but the exact neurobiology underlying this disorder remains unclear. Among

the factors involved in that incomplete understanding of MDD we can cite the difficulties to access the brain abnormalities in patients, the idiopathic nature of the disease (most of the time no direct causes are identified) or the impossibility to entirely model the disease in animals (Krishnan & Nestler, 2008). Nonetheless, recent observations and discoveries led to new views of the pathophysiology of MDD and unlocked new therapeutic strategies.

i. *From monoamine to glutamate*

The first hypothesis advanced to explain the development of MDD was a dysfunction of the monoaminergic system, particularly of serotonin and noradrenaline (Hirschfeld, 2000; Krishnan & Nestler, 2008; Nemeroff, 2020; Perez-Caballero et al., 2019). Indeed, patients diagnosed with MDD present a general decrease in the functioning of the monoaminergic system (Krishnan & Nestler, 2008) as well as a decrease in monoamine metabolites in blood, cerebrospinal fluid and urine (Nemeroff, 2020). In addition, early serendipitous observations reported an antidepressant effect of antihistaminic (that will later lead to the synthesis of chlorpromazine), antitubercular (iproniazide) and antihypertensive (reserpine) agents, all playing on the monoaminergic system (Hirschfeld, 2000; Pereira & Hiroaki-Sato, 2018). Accordingly, the first antidepressant drugs (ADs) were designed to enhance the monoaminergic system. They are divided in three categories, the tricyclic antidepressants (TCA), the monoamine oxidase inhibitors (MAO) and the selective serotonin/norepinephrin reuptake inhibitors (SSRIs/SNRIs) and are still the most largely prescribed ADs (Hirschfeld, 2000; Krishnan & Nestler, 2008; Pereira & Hiroaki-Sato, 2018; Perez-Caballero et al., 2019). However, numerous side effects subsist in all families of ADs (nausea, anxiety, insomnia, and headache), that sometimes lead to cessation of treatment and a significant number of patients (around 50%) never fully recovers despite the medication (Pereira & Hiroaki-Sato, 2018). Moreover, while ADs induce an almost immediate increase in monoamine availability, the antidepressant effect is only achieved after at least 4 to 5 weeks of treatment and depletion of monoamine is not sufficient to induce mood changes in healthy subjects (Krishnan & Nestler, 2008). This suggests that monoaminergic dysfunction is not the primary cause of MDD.

With the advent of neuroimaging (MRI, PET scan), it became possible to investigate the anatomical and functional alterations of brain structures in MDD patients. Volumetric analysis

report decrease volume in the PFC, the insula and the hippocampus (HPC) (Cattarinussi et al., 2021; Demenescu et al., 2017; Malykhin & Coupland, 2015; Travis et al., 2015; Wise et al., 2017) and increased amygdala size (Sandu et al., 2017). The decrease observed in PFC and HPC being potentially linked to decrease in density and/or size of excitatory neurons, astrocytes and oligodendrocytes (Hercher et al., 2009). The activity of several brain regions also appears altered in depression, with decrease glucose metabolism in dorsal PFC, posterior cingulate cortex, HPC, ventromedial striatum or amygdala and increase in the hypothalamic-pituitary-adrenal (HPA) axis and the ACC (Drevets, 2003; Drevets, Price, et al., 2008; Drevets, Savitz, et al., 2008; Holsboer, 1999; Videbech, 2000). However, these finding are not consistent across all studies, a possible explanation coming from the heterogeneity of the patients included (recurrence of the depressive episode, severity, age of onset etc.) (Gray et al., 2020). Nonetheless, the discovery of abnormal activity led to the development of new therapeutic strategies aiming at normalizing these changes. Therefore, deep brain stimulation of the ACC, the ventral capsule/ventral striatum the medial forebrain bundle or the lateral habenula seem to exert promising antidepressant effect, especially in treatment resistant patients (Drobisz & Damborská, 2019; Kisely et al., 2018; Mayberg et al., 2005), as well as repetitive transcranial magnetic stimulation (rTMS) at the level of the PFC (L. Thompson, 2020).

From the observations of impaired brain activity, and particularly in the PFC, a new hypothesis involving a disturbance of the excitation/inhibition balance also emerged (Choudary et al., 2005; Duman et al., 2019; Lener, Niciu, et al., 2017). Indeed, decrease in glutamate level in the PFC (Yildiz-Yesiloglu & Ankerst, 2006), the dorsolateral PFC (Hasler et al., 2007) and the ACC (Auer et al., 2000) are observed in MDD patients. Decreased GABA levels in the cerebrospinal fluid and cortical regions (Fee et al., 2017; Godfrey et al., 2018) are also reported. These diminutions in glutamate and GABA concentrations seem to predict the reduction in functional connectivity of the subgenual ACC in depressed patients (Horn et al., 2010). Therefore, drugs targeting the glutamatergic and GABAergic neurotransmission system became of great interest for the treatment of MDD. For instance, allopregnanolone analogs ($GABA_A$ receptor positive allosteric modulator) display rapid antidepressant effect (Lener 2017). Likewise, injection of ketamine (NMDA receptor antagonist) at a subanesthetic dose has a rapid antidepressant action lasting up to one week (Fond et al., 2014; Lener, Kadriu, et

al., 2017; Newport et al., 2015) and Esketamine (stereoisomer of ketamine) was approved by the Food and Drug Administration for MDD treatment. Further investigations are needed to decipher the underlying mechanisms of these new treatments and to optimize them, but those recent advances bring a promising evolution for the therapeutic strategies of MDD.

ii. *Oligodendrocyte and myelin*

As stated above, the structural changes observed in MDD patients at least partially result from a reduction in the density and integrity of glial cells and particularly of oligodendrocytes (OL). In patients diagnosed with MDD, MRI investigations uncovered a decrease in white matter volume (e.g. corpus callosum, cingulate bundle, uncinated fasciculus) (Bae et al., 2006; Bhatia et al., 2018; Hyett et al., 2018), myelin integrity impairment (Tham et al., 2011) and reduction in the number and morphology of OL and their progenitors (B. Zhou et al., 2021). In addition, patients presenting more severe white matter impairment display poorer response to ADs (Peng et al., 2013; Serafini et al., 2015). At the molecular level, transcriptomic analysis conducted in the ACC of patients diagnosed with severe depression highlighted a down-regulation of genes encoding myelin constituents, protein controlling myelin synthesis and regulators of OL differentiation. This alteration was associated with changes in methylation level, notably at the *lingo3* and *Pou3f1* loci (Lutz et al., 2017). Single-nucleus transcriptomic analysis, conducted by the same group, also reports major alteration in oligodendrocyte gene expression. Indeed, the transcriptomic changes observed in the dorsolateral PFC of MDD patients rely at 47% on alterations in deep layer excitatory neurons and oligodendrocyte progenitor cells (OPC) and most of the dysregulated genes found in OPC are known to be associated with MDD (Nagy et al., 2020). The same conclusions are found in preclinical studies. Therefore, different paradigm of chronic or early-life stress (chronic unpredictable stress, chronic social stress, social isolation, neonatal separation) result in decrease white matter volume (Wang et al., 2014), impaired OL and OPC integrity (Y. Yang et al., 2017), and decrease in myelin-related genes in the PFC (J. Liu et al., 2012; Makinodan et al., 2012) and the amygdala (Cathomas et al., 2019). Moreover, genetic ablation of OPC in the PFC induces depressive-like behaviors in mice, which is spontaneously reversed with OPC proliferation (Birey et al., 2015). Interestingly, myelin and OL/OPC impairment induced by chronic stress are normalized with ADs (SSRI and SNRI) treatment (Czéh et al., 2007; Elsayed et al., 2012; Kodama et al., 2004;

Wang et al., 2014) and pro-myelinating agents like clemastine show an antidepressant-like effect in mice subjected to social isolation (J. Liu et al., 2016). Because of the expression of the NMDA receptor by OL, they are also thought to play a role in the antidepressant effect of ketamine (Duman et al., 2019). Overall, impaired oligodendroglial function appears strongly associated with the development of MDD. Further studies are needed to understand the entire role of OL and OPC in the pathogenesis of depression and to determine the potential benefit of acting on myelination.

3. Brain circuitry underlying emotional regulation

Imaging and lesion studies allowed deciphering of the implication of various brain structures in neurological pathologies such as mood disorders. However, focusing on isolated structures is a limitation to the understanding of the physiopathology of these brain diseases. Instead, studying the networks and circuits linking all these structures is expected to give more relevant and precise information. Thanks to the advent and development of functional imaging studies (fMRI), opto- and chemogenetics, it is now easier to dissect the contribution of pathways and networks implicated in emotions and mood.

Functional imaging studies in human unraveled disruption in several brain circuits in anxiety and mood disorders (**Figure 2**). Among the networks altered in depression, the most extensively studied is the default mode network (DMN), composed of the dorsolateral prefrontal cortex (dlPFC), the anterior and posterior cingulate cortex (ACC and PCC), the amygdala, and the HPC. As the DMN is responsible for resting introspection and rumination, it is therefore not surprising that a hyperactivity of this network is found amongst depressed patient in which higher level of rumination is reported (Hamilton et al., 2015; H.-X. Zhou et al., 2020). Beside the DMN, the salient network (SN) and the central executive network (CEN) are disrupted in MDD. The former is composed of the ACC, anterior insula, amygdala, hypothalamus and ventral striatum and allows the detection and integration of emotionally

relevant stimuli (Seeley et al., 2007). The CEN is recruited when subjects are engaged in a demanding attentional task and comprise the dlPFC and the posterior parietal cortex (Seeley et al., 2007). Even though the alterations in the SN and CEN are less consistent compared to the DMN, a general decrease in activity of the SN and the CEN exists in MDD patients (Brakowski et al., 2017; Hamilton et al., 2015). The connectivity between those three networks

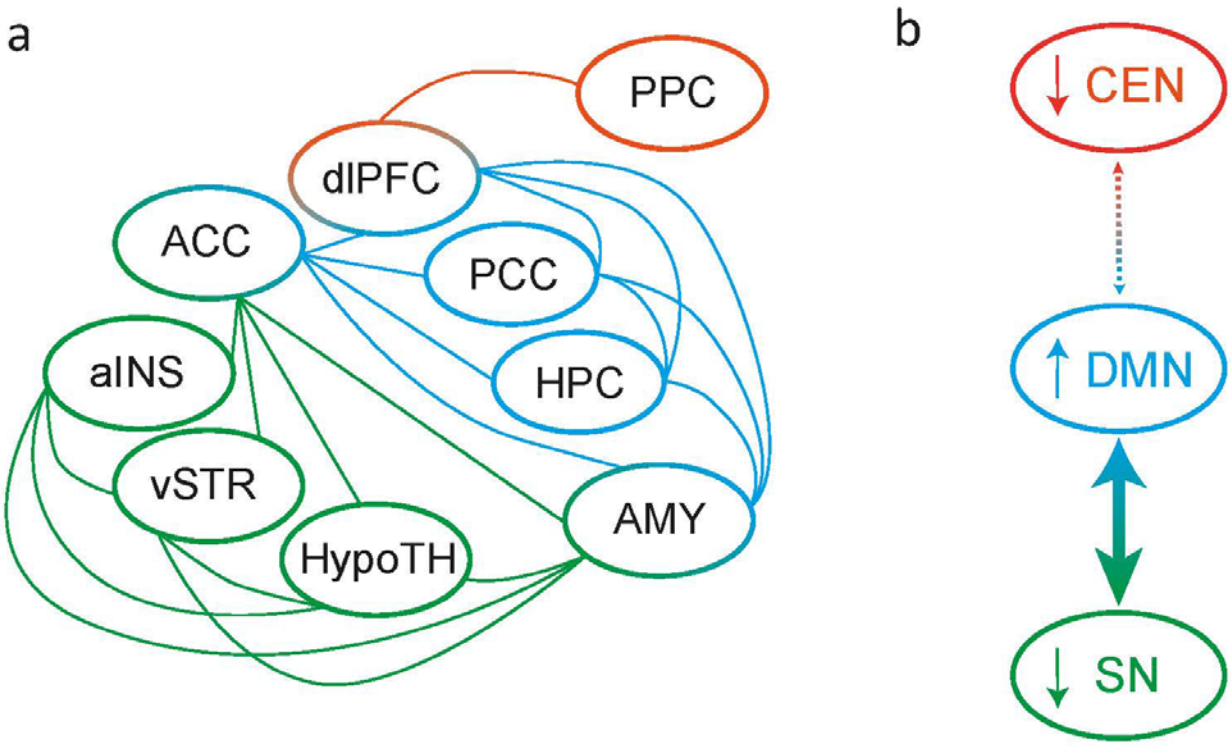

Figure 2: Brain networks alteration in depressed patients

(a) Schematic representation of the brain areas forming the DMN (blue), the CEN (red) and the SN (green).

(b) Depressed patients present a general increase activity of the DMN and decrease activity of the CEN and SN (single headed arrows). In addition, a decrease connectivity between the CEN and the DMN and an increase connectivity between the DMN and the SN is observed in these patients (double headed arrows)

ACC: anterior cingulate cortex, aINS: anterior insula, AMY: amygdala, CEN: central executive network, dlPFC: dorso-lateral prefrontal cortex, DMN: default mode network, HPC: hippocampus, HypoTH: hypothalamus, PCC: posterior cingulate cortex, PPC: posterior parietal cortex, SN: salience network, vSTR: ventral striatum.

is also altered in MDD patients with notably an increased connectivity between the DMN and the SN (Brakowski et al., 2017) and a decreased connectivity between the DMN and CEN (Mulders et al., 2015). Comparably, studies performed on anxiety disorders highlighted connectivity disruption among DMN, CEN and SN (Coutinho et al., 2016; Northoff, 2020) and particularly at the level of the PFC, insula and the cingulate cortex (Picó-Pérez et al., 2017; X. Yang et al., 2019).

In rodents, the same regions are altered, with atrophy of the PFC and the hippocampus principal neurons or increased complexity of the dendritic tree of amygdalar and nucleus accumbens (NAc) neurons (Duman et al., 2019; McEwen et al., 2016; McEwen & Morrison, 2013; Ménard et al., 2016; Popoli et al., 2011; Vyas et al., 2002; Warren et al., 2014). Besides anatomical alterations, rodent models of mood disorder also exhibit functional changes. For instance, in a model of unpredictable chronic mild stress (UCMS) the LC exhibits a decrease of its spontaneous activity as well as an increase of the number of tyrosine hydroxylase (TH) cells (Bravo et al., 2014). In the same model, the 5-HT release undergo pre-sysnaptic plasticity at the level of synapse between the DRN and the lateral habenula (LHb) (Zhang et al., 2018). Likewise, chronic stress triggers a modulation of the synaptic plasticity between the neurons of the basolateral amygdala (BA) and the PFC (Lowery-Gionta et al., 2018). However, those observations remain correlative while the real strength of these animal models lies in the possibility to reach a causal relationship between pathway activity and emotional regulation. To this end, chemo- and optogenetic approaches are very useful and already brought primordial information (**Figure 3**).

Circuits encompassing afferents and efferents to the PFC are the most extensively studied in animal studies, particularly when it comes to its relationship with the BA. Projection from the BA to the prelimbic (PrL) and infralimbic (IL) cortices are involved in fear memory but at different fashion. Indeed, the manipulation of BA inputs onto the PrL interferes with fear memory formation while BA→IL is more implicated in fear extinction (Senn et al., 2014). Photostimulation or photoinhibition of the BLA-PrL pathway respectively increases or decreases freezing in mice, a common feature of fear expression (Burgos-Robles et al., 2017). The PFC also sends projections back to the BA, and this connection is involved as well in emotional processing. Therefore, ACC to BA connections modulate fear in a selective manner.

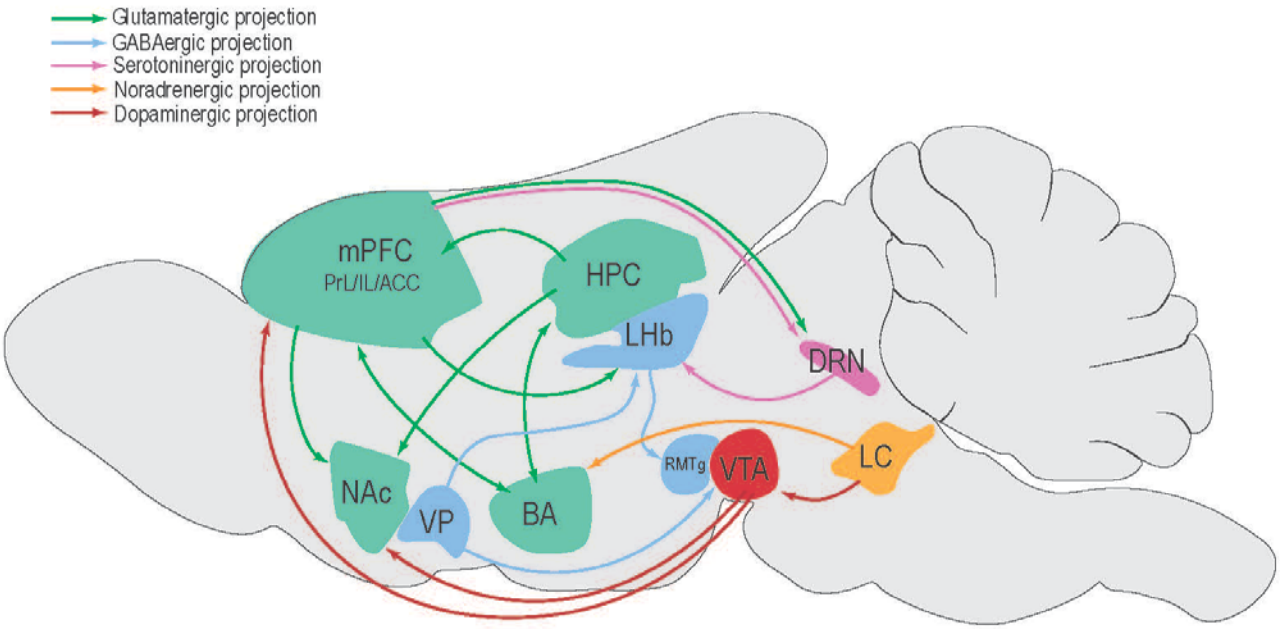

Figure 3: Circuitries implicated in emotional regulation

Schematic representation of the pathways involved in the regulation of negative emotions and their neurochemistry in rodents.

ACC: anterior cingulate cortex, BA: basolateral amygdala (anterior and posterior part); DRN: dorsal raphe nucleus; HPC: hippocampus; IL: infralimbic cortex; LC: locus coeruleus; mPFC: medial prefrontal cortex; NAc: nucleus accumbens; PrL: prelimbic cortex; RMTg: rostromedial tegmentum nucleus; VP: ventral pallidium; VTA: ventral tegmental area.

Adapted from Biselli et al., 2019

Indeed, inactivation of this projection inhibits novelty-induced but not trained fear (Ortiz et al., 2019). In addition, low frequency stimulation of the dorsomedial PFC projection to the BA increases glutamate release in the latter and alleviates anxiety-like behavior induced by chronic restrain stress (W.-Z. Liu et al., 2020). The afferent from the LC to the BA also participate to anxiety modulation, since activation of this projection in naïve animals promotes anxiety-like behaviors through a release of noradrenaline (McCall et al., 2017). The hippocampus and particularly its ventral part (vHPC) is another central hub in the circuits regulating emotions and mood. Similarly to the ACC, the inputs from the vHPC to the BA modulate fear behaviors but only in the case of generalized fear, not in a context of trained fear (Ortiz et al., 2019). The BA in turn sends projection to the vHPC, and those projections mediate social and anxiety behaviors. Indeed, inhibiting this pathway increases social behaviors and promotes anxiolytic effect (Felix-Ortiz et al., 2013; Felix-Ortiz & Tye, 2014). Anxiety-like behaviors also require the connection between the vHPC and IL/PrL. Disruption of the activity between these structures impair the expression of anxiety-like behaviors in both the elevated-plus maze and open field tests (Padilla-Coreano et al., 2016). Finally, through its connection with the NAc, the vHPC mediates susceptibility and resilience to stress. Indeed, inhibition of the vHPC-NAc fosters resilience in a model of chronic social defeat stress (CSDS) while its activation increases the susceptibility to develop anxiodepressive-like behaviors (Bagot et al., 2015).

As evidenced by the studies mentioned above, the neurobiology of mood disorders was principally deciphered in the context of chronic stress, which is the main determinant for the development of depression. However, MDD also results from various other causes (Fava & Kendler, 2000; Nemeroff, 2020) and a complete understanding of the underlying mechanisms of this pathology will only be achieved if other etiologies are accounted for.

III. Chronic pain as a major predisposition for MDD

Beside chronic stress, chronic pain is the most important factor for the development of anxiodepressive disorders (Attal et al., 2011). Indeed, 50% of patients suffering from chronic pain also develop mood and anxiety disorders (Bair et al., 2003). This comorbidity worsens the burden of the patient and complicates treatment strategies (Arnow et al., 2006; Kirsh, 2010).

1. Chronic pain, definition and clinical implication

Pain is defined as "*an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage*" (IASP 2020). When pain is acute, it has a protective and adaptive function since it allows us to avoid potentially harmful situation (Morrison et al., 2013). However, a pain that lasts or recurs for more than three months, well beyond the regular healing process, is considered as chronic and become pathological (Merskey H & Bogduk N, 1994). All causes combined, chronic pain affects around 30% of the population (Bouhassira et al., 2008) and severely impairs the patients in their daily life activities. In addition, the burden of chronic pain is worsened by the high prevalence of mood and anxiety disorders among these patients that affect half of them on average (Attal et al., 2011; Bair et al., 2003; Radat et al., 2013). Among the different type of chronic pain, neuropathic pain arises from a lesion or a disease affecting the somatosensory system (Jensen et al., 2011; Treede et al., 2008). It affects 7-8% of the population and involves positive (hyperalgesia and hypersensitivity) and negative (hypoesthesia and hypoalgesia) symptom (Bouhassira et al., 2008). Around 30% of the patients experiencing neuropathic pain develop mood and/or anxiety disorders. Noteworthy, the reverse association exists as well with depression acting as a risk factor for neck and back pain (Carroll et al., 2004). Although the comorbid relation between chronic pain and mood disorders is clinically well-established, the two conditions are often treated distinctly, which can represent an issue for the relief of both pathologies. Instead of considering this comorbidity as the sum of both disorders, it should be approached as a separate pathology with its own specificities in physiological and behavioral aspects. To this end, animal models turn out to be valuable as they offer the possibility for precise investigation at the molecular, cellular, circuits and behavioral levels. In recent decades, the development of preclinical models allowed the exploration of the anxiodepressive consequences of chronic pain. In parallel, various tests were developed in rodents to provide tools for fundamental and translational research on the topic.

2. How to study anxiety and depression in rodent models of chronic pain?

The following section is a review of the models and behavioral tests used in basic research to evaluate pain-, anxiety- and depressive-like behaviors in rodents. We compiled the literature published during the past two decades and discussed the important parameters that should

be taken into consideration when conducting studies on the comorbidity between chronic pain and anxiety/mood disorders. A particular emphasis was made on the time-dependant nature of the comorbidity, the limitation of the tests and models. We then further proposed solutions to homogenize the research in the field.

Received: 29 October 2019 | Revised: 6 January 2020 | Accepted: 14 January 2020

DOI: 10.1111/ejn.14686

SPECIAL ISSUE REVIEW

EJN European Journal of Neuroscience FENS WILEY

How to study anxiety and depression in rodent models of chronic pain?

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Funding information
Centre National de la Recherche Scientifique, Grant/Award Number: UPR3212; University of Strasbourg; Agence Nationale de la Recherche, Grant/Award Number: ANR-17-EURE-0022; Brain & Behavior Research Foundation, Grant/Award Number: 24736

Abstract

Mood disorders such as depression and anxiety are frequently observed in patients suffering from chronic pain. Over time, different tests and models have been developed in rodents to study the anxiodepressive-like consequences of chronic pain. This review describes these preclinical tools (models and tests) used for studying behavioural aspects of the comorbid relationship between chronic pain and anxiety and/or major depressive disorder. Three major types of chronic pain strongly associated with anxiodepressive-like comorbidity as well as their animal models are presented: neuropathic pain, inflammatory pain and fibromyalgia. After a description of chronic pain animal models and of the tests that allow determining nociceptive responses, this review presents and discusses the various behavioural tests that have been used to assess anxiety and depressive-like behaviours in these models of chronic pain. Finally, this review highlights the progress that remains to be made to homogenize the results in the field of pain-induced mood disorders and summarizes the recent advances achieved through these tests and models.

KEYWORDS

anxiety, depression, models, pain, tests

1 | INTRODUCTION

Pain is a multidimensional and subjective experience which is considered as a debilitating disease when it becomes chronic. Chronic pain does indeed affect various aspects of the patient's quality of life, including mood, sleep and cognitive processes (Haanpaa et al., 2011; Maletic & Raison, 2009; Radat, Margot-Duclot, & Attal, 2013). In chronic pain patients, mood disorders such as major depressive disorders and anxiety are frequently observed (Bair, Robinson, Katon, & Kroenke, 2003; Gustorff et al., 2008; Maletic & Raison, 2009; Radat et al., 2013).

Major depressive disorders are highly disabling psychiatric disorder which affects around 16% of the population at

Abbreviations: ACC, anterior cingulate cortex; BT, burrowing test; CCI, chronic constriction injury; CeA, central nucleus of the amygdala; CFA, complete Freund's adjuvant; CION, infraorbital nerve constriction; DM3T, dimethyl-3-transferase; EPM, elevated plus maze; EZM, elevated zero maze; FST, forced swim test; HB, hole-board test; HDAC, histone deacetylase; HIV, human immunodeficiency virus; IASP, International Association for the Study of Pain; ICS, intermittent cold stress; IDO1, indoleamine 2,3-dioxygenase 1; IL, interleukin; LC, locus coeruleus; LDB, light/dark box test; MAPK, mitogen-activated protein kinases; MB, marble burying test; MDD, major depressive disorder; NAc, nucleus accumbens; NSF, novelty-suppressed feeding; OF, open field; PAG, periaqueductal grey; PFC, prefrontal cortex; PSNL, partial sciatic nerve ligation; RCS, repeated cold stress; SCI, spinal cord injury; SI, social interaction test; SNI, sciatic nerve injury; SNL, spinal nerve ligation; SPT, sucrose preference test; ST, splash test; TIC, trigeminal inflammatory compression; TNF, tumour necrosis factor; TNT, tibial nerve transection; TST, tail suspension test.

Edited by John Foxe.

The peer review history for this article is available at https://publons.com/publon/10.1111/ejn.14686.

some point over their lifespan (Bromet et al., 2011) and is among the main contributors to the disease burden worldwide (Kessler et al., 2003; Olesen, Gustavsson, Svensson, Wittchen, & Jonsson, 2012). Due to its multifactorial nature and heterogeneous symptomatology, the precise aetiology of this debilitating disorder remains poorly understood (Menard, Hodes, & Russo, 2016). However, besides chronic stress and psychosocial trauma (Liu & Alloy, 2010), chronic pain can also be cited among the first determinants of mood disorders (Attal, Lanteri-Minet, Laurent, Fermanian, & Bouhassira, 2011; Breivik, Collett, Ventafridda, Cohen, & Gallacher, 2006; McWilliams, Goodwin, & Cox, 2004), as shown by the mean prevalence rate around 50% for major depressive disorder reported in these patients (Bair et al., 2003). Besides, anxiety is also among mood disorders frequently diagnosed in chronic pain patients. Indeed, this psychopathology, defined by excessive fear and worry, affects up to 60% of chronic pain patients. This prevalence is however highly dependent on the considered type of pain, with the lowest rate (between 1% and 27%) for neuropathic pain and the highest (between 18% and 60%) for fibromyalgia (Hooten, 2016).

Different models have been developed in rodents to study the various types of clinically observed chronic pain conditions (Fischer, Adeyemo, O'Leary, & Bottaro, 2017; Jaggi, Jain, & Singh, 2011; Kumar, Kaur, & Singh, 2018; Mogil, 2009; Muley, Krustev, & McDougall, 2016; Sluka & Clauw, 2016), as well as their anxiodepressive consequences (Leite-Almeida, Pinto-Ribeiro, & Almeida, 2015; Liu & Chen, 2014). While several groups successfully achieved to model this comorbidity in animals (Goncalves et al., 2008; Matsuzawa-Yanagida et al., 2008; Narita, Kaneko, et al., 2006; Narita, Kuzumaki, et al., 2006; Suzuki et al., 2007), many initial studies and some of the recent ones failed to show any association between chronic neuropathic or inflammatory pain and anxiety- and depression-related behaviours (Hasnie, Wallace, Hefner, Holmes, & Rice, 2007; Kontinen, Kauppila, Paananen, Pertovaara, & Kalso, 1999; Pitzer, Porta, Treede, & Tappe-Theodor, 2019; Urban, Scherrer, Goulding, Tecott, & Basbaum, 2011). However, some of the negative studies (but not all) were done at early pain stages, that is during the first week or the first 3 weeks following inflammatory and neuropathy induction, respectively. While it may not be the only aspect that allows explaining the presence or not of anxiodepressive-like consequences in rodent models of chronic pain, the time factor appears as critical (Humo, Lu, & Yalcin, 2019; Yalcin & Barrot, 2014; Yalcin et al., 2011). Others factors, such as the species, the strains of animals, the chosen models of chronic pain, and the time of the day–night cycle when the animals are tested, could also be involved.

In this review, based on the literature up to December 2019, we summarized the preclinical tools (models and tests) used over the past two decades and pertaining to behavioural aspects of the comorbid relationship between chronic pain and anxiety and/or major depressive disorder (MDD). After a brief description of the types of chronic pain and of their animal models, as well as the tests that allow determining nociceptive responses, we will present and discuss the various behavioural tests that have been used to assess anxiety and depressive-like behaviours in these models of chronic pain.

2 | ANIMAL MODELS OF PAIN THAT HAVE BEEN USED IN RESEARCH ON ANXIETY AND/OR DEPRESSION

Pain is defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." Acting as an alarm signal, acute pain triggers reactions to preserve the integrity of the organism (Morrison, Perini, & Dunham, 2013; Scholz & Woolf, 2002). When pain persists beyond several months, it is considered as chronic. Contrary to acute pain, chronic pain is regarded as an illness per se, and mood disorders are among its comorbidities (Attal et al., 2011; Haanpaa et al., 2011). According to the epidemiological studies, the prevalence rate of major depressive disorder in patients with chronic pain (Bair et al., 2003; Maletic & Raison, 2009) varies from around 30% for patients suffering from neuropathic pain (Gustorff et al., 2008; Radat et al., 2013), to around 80% in fibromyalgia patients (Fietta, Fietta, & Manganelli, 2007). Up to recently, the mechanism(s) underlying this comorbidity remained unclear (Doan, Manders, & Wang, 2015; Fasick, Spengler, Samankan, Nader, & Ignatowski, 2015; Yalcin & Barrot, 2014; Zis et al., 2017). However, main advances have been achieved in the past decade, thanks to the development of animal models that allow studying anxiodepressive consequences of chronic pain (Leite-Almeida et al., 2015; Yalcin & Barrot, 2014). We will focus here on three major types of chronic pain strongly associated with anxiodepressive-like comorbidity: neuropathic pain, inflammatory pain and fibromyalgia.

2.1 | Neuropathic pain and its animal models

By definition, neuropathic pain arises as a direct consequence of a lesion or disease affecting the somatosensory system (Treede et al., 2008). It is a syndrome usually chronic, with various possible aetiologies. For most patients it has a peripheral origin, arising as a consequence of peripheral nerve injury (nerve section or compression), or as a consequence of a metabolic disease such as diabetes. Nerve injuries and diabetic peripheral neuropathy account for almost two-thirds of

the patients. However, neuropathic pain can also result from infectious diseases, as in post-herpetic neuralgia, from exposure to neurotoxic compounds, such as those used for cancer chemotherapy, or be of central origin, as observed after spinal cord injury or local post-stroke ischaemia (Attal et al., 2008; Colloca et al., 2017; Scholz et al., 2019; Zilliox, 2017).

A large number of animal models of neuropathic pain has been developed (Colleoni & Sacerdote, 2010; Jaggi et al., 2011; Kumar et al., 2018; Sorkin & Yaksh, 2009), but their exhaustive presentation is beyond the scope of this review. Here, we will simply focus on the few models in which most of the comorbidity studies on anxiodepressive aspects were performed. Almost 90% of the published studies used trauma models based on chronic nerve compression (see Figure 1). This nerve compression has been achieved either by ligation, as for the partial sciatic nerve ligation (PSNL) which is a tight ligation of one-third to half of the sciatic nerve (Seltzer, Dubner, & Shir, 1990), the spinal nerve ligation (SNL) which is a tight ligation of L5 and L6 spinal nerves (Kim & Chung, 1992) or of L5 spinal nerve only (LaBuda & Little, 2005), the chronic constriction injury (CCI) consisting in four loose ligatures applied around the sciatic nerve (Bennett & Xie, 1988), or by the implantation of a polyethylene cuff around the main branch of the sciatic nerve (Mosconi & Kruger, 1996). Another frequently used trauma model is the spared nerve injury (SNI), which relies on the axotomy of two of the three branches of the sciatic nerve (Decosterd & Woolf, 2000). The tibial nerve transection (TNT) is a variant of this model in which the axotomy concerns the tibial nerve only (Andrews et al., 2012). The anxiodepressive comorbidity has also been studied using trigeminal neuralgia models, such as the infraorbital nerve constriction (CION) which corresponds to CCI of the infraorbital nerve (Vos, Strassman, & Maciewicz, 1994), or in animals with trigeminal inflammatory compression (TIC) induced by a chromic gut suture alongside the infraorbital nerve (Ma, Zhang, Lyons, & Westlund, 2012).

Besides nerve lesion, diseases affecting the somatosensory system constitute another main aetiology of neuropathic pain (Jolivalt et al., 2016). In this regard, anxiodepressive-like comorbidities have also been studied in a streptozotocin model, which is a common model of diabetic polyneuropathy (Lenzen, 2008), and in models of human immunodeficiency virus (HIV) 1 protein gp120 (Wallace, Blackbeard, Segerdahl, et al., 2007) or varicella zoster virus (Hasnie, Breuer, et al., 2007) as infection models. Exposure to neurotoxic drugs, such as the HIV antiretroviral stavudine (Joseph, Chen, Khasar, & Levine, 2004) or the chemotherapy drug oxaliplatin (Cavaletti et al., 2001) or paclitaxel (Cavaletti, Tredici, Braga, & Tazzari, 1995), has also been used to study the comorbidity between neuropathic pain and anxiety/depressive-like behaviours.

Concerning central neuropathies, studies of anxiety/depressive-like behaviours have been done using a model of spinal cord injury (SCI) induced by dropping a weight over the exposed spinal cord (Behrmann, Bresnahan, Beattie, & Shah, 1992), or by using a photochemical reaction to form a thrombosis and occlusion in small vessels supplying the spinal cord (Verdu et al., 2003).

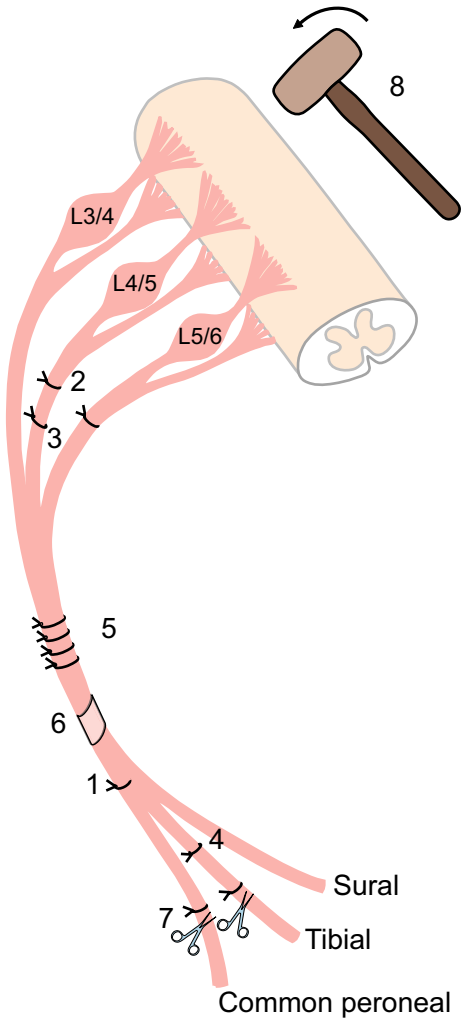


FIGURE 1 Experimental traumatic models of neuropathic pain. (1) Partial sciatic nerve ligation (PSNL; Seltzer et al., 1990); (2) sciatic nerve ligation (SNL; Kim & Chung, 1992); (3) L5 spinal nerve ligation (variant of the SNL model; LaBuda & Little, 2005); (4) tibial nerve transection (TNT; Lee, Park, Won, Park & Sohn, 2000); (5) chronic constriction injury (CCI; Bennett & Xie, 1988); (6) sciatic nerve cuffing (Cuff; Benbouzid et al., 2008; Mosconi & Kruger, 1996); (7) spared nerve injury (SNI; Decosterd & Woolf, 2000); (8) spinal cord injury (SCI; Behrmann et al., 1992). The level of insertion of the nerve roots and therefore the associated dorsal root ganglia may vary according to the animals' strain, hence the double level numbering on the dorsal root ganglia.

2.2 | Inflammatory pain and its animal models

When inflammation becomes chronic, it loses its role as natural physiological response to tissue injury or infection,

and it becomes a maladaptive and physiopathological condition. The chemical mediators that are responsible for tissue inflammation affect nociceptive nerve endings to lower neuronal excitation thresholds and sensitize afferent firing rate, leading to the development of allodynia and hyperalgesia, respectively (Kidd & Urban, 2001; Lipnik-Stangelj, 2013). One of the main organ systems that is particularly susceptible to the development of inflammatory pain is joints.

Arthritis, which literally means joint inflammation, refers to a group of rheumatic diseases and other conditions that can cause pain, stiffness and swelling in the joints. It includes forms ranging from those related to wear and tear of cartilage (such as osteoarthritis; Kuyinu, Narayanan, Nair, & Laurencin, 2016), to those associated with inflammation resulting from an overactive immune system (such as rheumatoid arthritis; Di Paola & Cuzzocrea, 2008). Recent surveys report that rheumatoid arthritis is one of the most common chronic inflammatory pain conditions in developed countries, affecting approximately 15 million people worldwide (Fiest et al., 2017). Rheumatoid arthritis is a chronic debilitating autoimmune disorder characterized by synovitis that leads to cartilage and bone erosion by invading fibrovascular tissue (Scott, Wolfe, & Huizinga, 2010). The pathogenesis of rheumatoid arthritis is complex and involves genetic predispositions as well as environmental factors (Imboden, 2009). Psychiatric disorders are highly associated with rheumatoid arthritis (Nerurkar, Siebert, McInnes, & Cavanagh, 2019). Depression is diagnosed in up to 66% and anxiety in up to 70% of individuals with rheumatoid arthritis (Fiest et al., 2017).

Animal models have contributed to improve our understanding of the pathophysiological mechanisms responsible for the generation of chronic inflammatory pain and its associated comorbidities (Fischer et al., 2017; Kuyinu et al., 2016; Muley et al., 2016). The most widely used model for studying the comorbidity between inflammatory pain and anxiodepressive-like disorders has been the complete Freund's adjuvant (CFA)-induced inflammation of the paw (Fehrenbacher, Vasko, & Duarte, 2012). This model is based on a unilateral intraplantar injection of CFA, which can produce a long-lasting (>3 weeks) decrease in mechanical and thermal thresholds compared to the contralateral non-inflamed paw (Cook & Moore, 2006). Carrageenan can also be injected into the paw to model arthritis, but this model is more acute than chronic because the associated mechanical and thermal hypersensitivity usually lasts no longer than 72 hr (Mert, Ocal, Cinar, Yalcin, & Gunay, 2014). Monoarthritis models have also been used, by injecting an inflammatory agent such as CFA, uric acid or a kaolin–carrageenan mix directly into the tibiotarsal (Butler, Godefroy, Besson, & Weil-Fugazza, 1992), knee (Lopez-Munoz & Salazar, 1993; Radhakrishnan, Moore, & Sluka, 2003) or temporomandibular (Harper, Kerins, McIntosh, Spears, & Bellinger, 2001) joint. Obesity is also a main risk factor for the development of arthritis (Georgiev & Angelov, 2019), and a model of arthritis based on dietary obesity (Silberberg & Silberberg, 1950) has also been used to test anxiodepressive-like aspects (Griffin et al., 2010).

2.3 | Fibromyalgia and its animal models

Fibromyalgia is a condition characterized by chronic widespread musculoskeletal pain, which includes widespread tenderness to pressure stimuli and morning stiffness (Clauw, 2014; Hauser et al., 2015; Sluka & Clauw, 2016). The pathogenesis of fibromyalgia is complex and controversial, but some recent advances in the field showed the possible involvement of lipid mediators (Hsu et al., 2019), autoimmunity, neuroinflammation and small fibre neuropathy (Ryabkova, Churilov, & Shoenfeld, 2019). There is however no evidence of any single event causing this condition; instead, it is considered to be triggered or aggravated by multiple physical and/or emotional stressors, such as infections, or emotional and physical trauma (Schmidt-Wilcke & Clauw, 2011; Sluka & Clauw, 2016). Fibromyalgia is more common in women than in men, and its worldwide prevalence is 2%–3% (Cabo-Meseguer, Cerda-Olmedo, & Trillo-Mata, 2017). Fibromyalgia is also associated with a number of other symptoms, including pronounced fatigue, sleep disturbances and psychological disturbances (depression and/or anxiety) (Hauser et al., 2015). Overall depression and anxiety are among the most common comorbidities of fibromyalgia, with prevalence rates ranging from 20% to 80% and 13 to 64% respectively (Maletic & Raison, 2009).

Some studies tried to address the anxiodepressive consequences in animal models of fibromyalgia. However, as there is no well-defined aetiology of fibromyalgia, the validity of animal models remains imperfect and simply based on symptoms and on the response to treatment (mostly antidepressant drugs). The most frequently used fibromyalgia model addressing depression-like behaviours is based on biogenic amine depletion by systemic reserpine administration (Nagakura, Oe, Aoki, & Matsuoka, 2009). However, the chronic widespread musculoskeletal pain model induced by repeated intramuscular acid injections in rodents has been suggested to have better face validity to human's conditions, but few studies have dealt with the anxiodepressive consequences in this model yet (Liu, Shao, Yen, & Shaw, 2014). Anxiodepressive consequences have also been described in stress-induced fibromyalgia models, such as the cold stress models, that is intermittent (ICS; Nishiyori & Ueda, 2008) or repeated cold stress (RCS; Nasu, Kubo, Queme, & Mizumura, 2019), the unpredictable sound stress model (Khasar et al., 2008), or the subchronic swim stress inducing chronic widespread-like pain (Nazeri et al., 2018). Although

existing models mimic some symptoms of fibromyalgia, it is still critical to develop new models which can reflect the different aspects of this syndrome.

3 | NOCICEPTIVE TESTS FREQUENTLY USED TO EVALUATE CHRONIC PAIN

An important notion in the field of pain is the distinction between pain and nociception. If nociception corresponds to "neural process of encoding noxious stimuli" (i.e., stimuli presenting a risk for the integrity of the body; Basbaum & Jessell, 2000), pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Box 1; Apkarian & Reckziegel, 2019; Baliki & Apkarian, 2015; Basbaum, Bautista, Scherrer, & Julius, 2009; Loeser & Melzack, 1999). These two facets of the same phenomenon distinguish the sensation (nociception) from its interpretation (pain). In patients, pain is evaluated verbally, which is not possible in rodents. Thus, rodent “pain tests” are actually nociceptive tests, based on reflex responses, and the preclinical measurement of pain itself is still a challenge for this field of research (Barrot, 2012; Deuis, Dvorakova, & Vetter, 2017). For a long time, fundamental research on pain and its preclinical treatments has been based on nociceptive tests performed on naive animals, that is "not painful" (Le Bars, Gozariu, & Cadden, 2001). But in the past decades, the combination of nociceptive tests and pain models has improved the relevance of studies in the field of pain research (Mogil, 2009).

The main nociceptive tests used in animal studies addressing the comorbidity between pain and anxiodepressive-like disorders are based on thermal or mechanical stimuli (Barrot, 2012; Leite-Almeida et al., 2015). Some of them, like the tail-flick test, the hot- or cold-plate tests and the radiant heat paw-withdrawal test, rely on the latency for avoidance behaviour: a withdrawal reflex of the paw or the tail (Barrot, 2012; Deuis et al., 2017). Here, the stimulus may be considered as fixed. The tail withdrawal test, the first developed (D'Amour & Smith, 1941), is based on the latency of the withdrawal reflex after applying a heat beam at the end of the tail or after having immersed it in a bath at fixed temperature. Although the observed response results from a spinal reflex, it remains under the influence of supraspinal controls as well as mechanisms of thermoregulation (Barrot, 2012). This test, sensitive to opiates, has been used extensively for analgesic research. Nevertheless, this test is much less used in models of chronic pain such as inflammatory pain or trauma models of neuropathic pain as these models rather focus on one of the paws of the animal. The hot-plate test (O'Callaghan & Holtzman, 1975; Woolfe & MacDonald, 1944) is another classic test developed in the 1940s, most often with a plate temperature set at 52 or 55°C for rodents. These temperatures, which are 10–15°C higher than the nociceptor response thresholds, are necessary for the increase in skin temperature to activate the nociceptors and thus to observe a supraspinal behavioural response within less than 10 s. The measurement is most often the latency of withdrawal and licking of the paw, but in the mouse the jump of the animal is sometimes taken into consideration (Deuis et al., 2017). In the late 1980s, Hargreaves et al. have described a test to differentiate the nociceptive response of the two hind paws in rodents: the Hargreaves test or Plantar® test (Hargreaves, Dubner, Brown, Flores, & Joris, 1988; Muley et al., 2016). The animal is placed on a glass floor and the point source of heat is brought under the paw to be tested, the system automatically detects the withdrawal of the paw. This test is useful in unilateral pain models, like most models of inflammatory and neuropathic pain. Similarly, the cold nociceptive response can be tested using a cold-plate test (Bennett & Xie, 1988; Choi, Yoon, Na, Kim, & Chung, 1994), but this measure can be more difficult to establish as the response latency is sometimes unreliable, and the number of responses over a given period of time is generally preferred (Deuis et al., 2017).

Finally, some of the thermal nociceptive tests are based on the observation and quantification of nociceptive behaviours, as is the case with the acetone test. To assess cold allodynia, a drop of acetone can be applied on the hind paws (Choi et al., 1994). Its evaporation produces a cold stimulus, which is usually not detected as nociceptive by naive animals but results in cold allodynia in pain models (Barrot, 2012; Deuis et al., 2017).

Other nociceptive tests, such as the von Frey filaments or the Randall–Selitto analgesimeter, can rely on the mechanical stimulus threshold necessary to elicit an avoidance behaviour (Barrot, 2012; Deuis et al., 2017; Muley et al., 2016). In this case, the stimulus is variable with increasing value. An advantage of these tests is to measure allodynia, that is the response to a normally non-nociceptive stimulus, or hyperalgesia, an exaggerated response to a nociceptive stimulus. While all mechanical tests are feasible in rats, the von Frey test mostly remains preferred in mice. These filaments of various diameters are generally applied to the plantar surface, until they bend exerting a calibrated pressure. The threshold filament inducing a response gives the value of the mechanical sensitivity threshold. This value is nevertheless influenced by the speed and the duration of application of the filaments, and the standardization of the procedure is therefore particularly important (Barrot, 2012). In recent years, automated versions of this test have been developed (Deuis et al., 2017). Manual or motorized, based on pressure gauges, they have the advantage of offering a continuous scale and no longer logarithmic values. With the Randall–Selitto analgesimeter (Kayser, Basbaum, & Guilbaud, 1990; Randall & Selitto, 1957), the plantar surface of the rat's paw

Box 1 Definition and assessment of sensory symptoms or signs in pain.

Terms	Definitions
Pain	An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (IASP)
Allodynia	Pain due to a stimulus that does not normally provoke pain (IASP)
Analgesia	Absence of pain in response to stimulation which would normally be painful (IASP)
Arthritis	An informal way of referring to joint pain or joint disease (COFER)
Dysesthesia	An unpleasant abnormal sensation, whether spontaneous or evoked (IASP)
Fibromyalgia	Syndrome characterized by chronic widespread pain at multiple tender points, joint stiffness and systemic symptoms (e.g., mood disorders, fatigue, cognitive dysfunction and insomnia) without a well-defined underlying organic disease (ICD)
Hyperalgesia	Increased pain from a stimulus that normally provokes pain (IASP)
Hyperpathia	A painful syndrome characterized by an abnormally painful reaction to a stimulus, especially a repetitive stimulus, as well as an increased threshold (IASP)
Hypoalgesia	Diminished pain in response to a normally painful stimulus (IASP)
Inflammatory pain	Inflammatory nociceptive pain is associated with tissue damage and the resulting inflammatory process (COFER)
Monoarthritis	Inflammation of one joint (arthritis) at a time (ICD)
Neuropathic pain	Pain caused by a lesion or disease of the somatosensory nervous system (IASP)
Nociception	The neural process of encoding noxious stimuli (IASP)
Nociceptive pain	Pain that arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors (IASP)
Nociceptive stimulus	An actually or potentially tissue-damaging event transduced and encoded by nociceptors (IASP)
Nociceptor	A high-threshold sensory receptor of the peripheral somatosensory nervous system that is capable of transducing and encoding noxious stimuli (IASP)
Osteoarthritis	Group of distinct, but overlapping diseases, which may have different aetiologies, but similar biological, morphological and clinical outcomes affecting the articular cartilage, subchondral bone, ligaments, joint capsule, synovial membrane and periarticular muscles (ICD)
Pain threshold	The minimum intensity of a stimulus that is perceived as painful (IASP)
Paraesthesia	An abnormal sensation, whether spontaneous or evoked (IASP)
Rheumatoid arthritis	Persistent and/or erosive disease that is defined as the confirmed presence of synovitis in at least one joint, absence of an alternative diagnosis that better explains the synovitis, and achievement of a total score of 6 or greater (of a possible 10) from the individual scores in four domains: number and site of involved joints, serologic abnormality, elevated acute-phase response and symptom duration (ICD)
Sensitization	Increased responsiveness of nociceptive neurons to their normal input, and/or recruitment of a response to normally subthreshold inputs (IASP)

Abbreviations: COFER, Collège français des enseignants en rhumatologie; IASP, International Association for the Study of Pain; ICD, International Classification of Diseases.

is placed on a fixed element and a mobile element exerts an increasing pressure on the other face. According to the protocols, the parameter measured will be either the threshold expressed in grams of appearance of a withdrawal reflex or that of a vocalization. This test gives highly stable and reproducible values, but requires a strong behavioural expertise (Barrot, 2012; Deuis et al., 2017). Indeed, the rat is restrained in a vertical non-natural position to maintain its paw on the apparatus. A similar system has been developed to assess mechanonociception in models of arthritis where calibrated forceps are oriented along the joint line and an increasing compression force is applied (Amorim, David-Pereira, Pertovaara, Almeida, & Pinto-Ribeiro, 2014; Ji, Fu, Ruppert, & Neugebauer, 2007).

The various tests that we described above have been used to validate the presence of nociceptive hypersensitivity in animal models of pain used to study the anxiodepressive comorbidity. Most of these studies were based on measuring the evoked nociceptive response instead of spontaneous or ongoing pain as the latter cannot be easily measured in rodents.

In the past decade, however, an effort from some research groups has focused on the search for objective and quantitative measures of pain, or at least for indirect parameters that may reflect the ongoing pain in the animal (Deuis et al., 2017; Mogil, 2009). One of the strategies to obtain such parameters has been based on the evaluation of the affective dimension of the pain experience, which comprises its unpleasantness and salient fear negative-stimuli-related escape and avoidance behaviours (Fuchs & McNabb, 2012; Navratilova, Xie, King, & Porreca, 2013; Price, 2000). One of the most frequently used methods to evaluate such avoidance consists in giving the choice to the animal between environments associated or not with the painful experience. This can for example be done in response to mechanical stimulation (LaBuda & Fuchs, 2000; Llorca-Torralba, Mico, & Berrocoso, 2018), by using plates with temperature gradient or allowing the choice between two surfaces with different temperatures (Moqrich et al., 2005). Another indirect measure of ongoing pain has been done by using a modified version of the conditioned place preference test, which is based on an animal's preference for a context paired with a pain-relieving treatment (Barthas et al., 2015; King et al., 2009; Sellmeijer et al., 2018), such as intrathecal or systemic administration of non-rewarding analgesic drugs (Navratilova et al., 2013; Sufka, 1994).

Another strategy is to consider the emotional component of pain through facial or vocal expression. The "grimace scale" of pain can actually be recognized and evaluated in rats (Sotocinal et al., 2011) and mice (Langford et al., 2010) exposed to acute or short-term pain. Unfortunately, as observed in chronic pain patients, this facial signature may not necessarily be present in chronic pain models (Langford et al., 2010). The evaluation of pain through ultrasonic vocalizations has also been explored by some teams (Calvino, Besson, Boehrer, & Depaulis, 1996; Han, Bird, Li, Jones, & Neugebauer, 2005; Kurejova et al., 2010), but the reliability of this parameter still remains low, limited to certain pain models and not necessarily relevant in a context of chronic pain (Jourdan, Ardid, & Eschalier, 2002; Wallace, Norbury, & Rice, 2005).

4 | EVALUATING ANXIETY-LIKE AND DEPRESSION-LIKE BEHAVIOURS IN ANIMAL MODELS OF CHRONIC PAIN

4.1 | Anxiety-like behaviours

4.1.1 | Elevated plus maze

One of the most widely used test to assess anxiety-like behaviours in rodents is the elevated plus maze (EPM). It has been pharmacologically validated in rats (Pellow, Chopin, File, & Briley, 1985) and in mice (Lister, 1987) using chlordiazepoxide, diazepam, phenobarbitone and yohimbine. The EPM apparatus consists in a cross composed of two open and two closed arms, joined by a common central platform (Narita, Kaneko, et al., 2006). This apparatus is set 40–80 cm above the floor and the closed arms are enclosed by 15–40 cm walls depending on the considered species (Goncalves et al., 2008; Hasnie, Wallace, et al., 2007; Narita, Kaneko, et al., 2006; Roeska, Doods, Arndt, Treede, & Ceci, 2008). Animals are placed in the middle of the apparatus and let free to explore the maze. The number of entries as well as time spent in both closed and open arms is assessed over 5–15 min depending on the protocols (Ji, Yakhnitsa, Kiritoshi, Presto, & Neugebauer, 2018; Narita, Kaneko, et al., 2006). Arm entry and exit are often considered when all four paws are into or out of a given arm. This paradigm induces a conflict between the innate exploratory behaviour of the rodent and the fear generated by the open and heighten environment. Thus, a decrease in the amount of time spent in open arms is thought to reflect anxiety-like behaviour. To strengthen the aversion created by the open arms, it is possible to create a contrast in light setting between open and close arms. Thus, light intensity in closed arms may sometimes be below 10 lux while it can go up to 100 lux for the open arms. However, as very few studies indicate the light intensity used in their paradigm it is difficult to conclude about the optimal light setting. Besides, analysis of the number of arm entries can be used as an indicator of the animal locomotion, a decrease in the number of arm entries suggesting a deficit in locomotion. This internal control is then very useful to make sure that the effect seen in the EPM arises from anxiety-like behaviour and not from motor impairment (Benbouzid et al., 2008).

Concerning pain-induced anxiety, the majority of published studies using the EPM test succeeded in highlighting the presence of anxiety-like behaviours in both mouse and rat pain models (Figure 2). Indeed, a decreased time spent in the open arms of the EPM has been observed after 5–14 post-induction days in fibromyalgia models (Green, Alvarez, Gear, Mendoza, & Levine, 2011; Liu et al., 2014; Nazeri et al., 2018; Wu et al., 2017) (see also Table 3) and within a few hours to one day (Fernandez-Guasti, Reyes, Martinez-Mota, & Lopez-Munoz, 2005; Ji et al., 2007; do Nascimento & Leite-Panissi, 2014) in inflammatory models (Table 2). The anxiety-like behaviours observed in the EPM for rodents with inflammatory pain have also been shown to last up to 3–4 weeks post-pain induction (Amorim et al., 2014; Borges, Neto, Mico, & Berrocoso, 2014; Narita, Kaneko, et al., 2006; Narita, Kuzumaki, et al., 2006; Parent et al., 2012; Wang, Zhong, et al., 2015). In traumatic neuropathic pain models, a decrease in the time spent in the open arms has mainly been observed after 3–4 weeks post-surgery (Table 1; Figure 3; Benbouzid et al., 2008; Caspani, Reitz, Ceci, Kremer, & Treede, 2014; Chen, Wei, Sagalajev, Koivisto, & Pertovaara, 2019; Ferreira-Chamorro, Redondo, Riego, Leanez, & Pol, 2018; Ji et al., 2017; Jiang et al., 2014; Leite-Almeida et

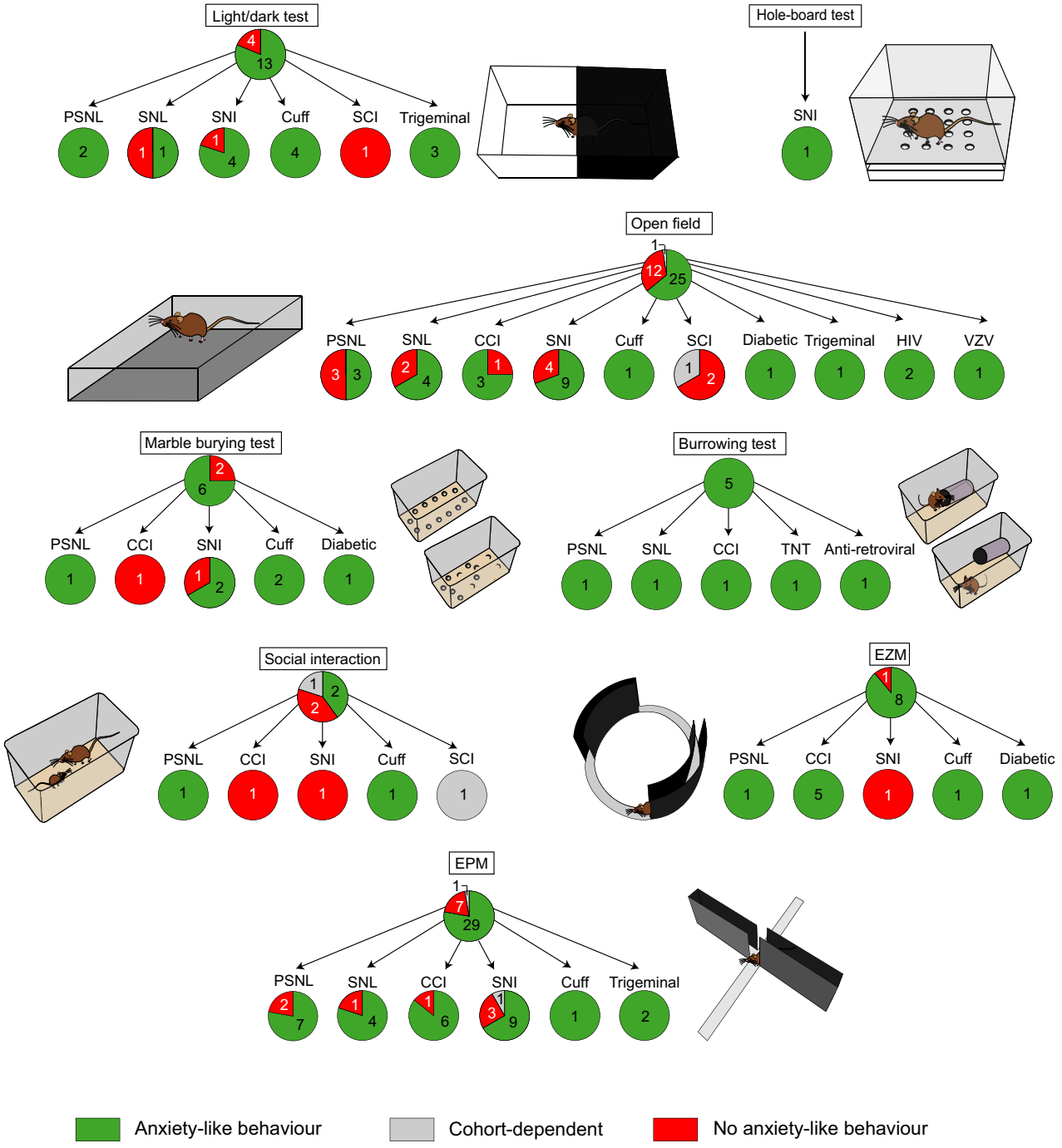


FIGURE 2 Anxiety-like behaviours in animal models of neuropathic pain. "*n*" displayed in the figure corresponds to the number of publications relative to the test (see Table 1 for more details and references). EPM, elevated plus maze; EZM, elevated zero maze.

al., 2012; Li et al., 2014; Matsuzawa-Yanagida et al., 2008; Narita, Kaneko, et al., 2006; Narita, Kuzumaki, et al., 2006; Roeska, Ceci, Treede, & Doods, 2009; Sawada et al., 2014; Wang, Li, Cai, & Pan, 2017). This phenotype is usually still present at 8 weeks post-surgery (Descalzi et al., 2017; Lyons et al., 2015; Sang et al., 2018; Suzuki et al., 2007) or even at 19–24 post-operative weeks in the SNI model (Seminowicz et al., 2009), but it can also start to recover at this time point in PSNL (Gonzalez-Sepulveda, Pozo, Marcos, & Valverde, 2016). Some studies, however, failed to show the presence of anxiety-like behaviours, in some cases due to the time point chosen for the test. Indeed, anxiety-like behaviours related to peripheral neuropathic pain in this test tend to mostly develop around 3–4 weeks following induction of the chronic pain, so testing too early may sometimes explain the lack of anxiety-like behaviours in some studies (Gregoire, Michaud, Chapuy, Eschalier, & Ardid, 2012; Hasnie, Wallace, et al., 2007; Kontinen et al., 1999; Pitzer et al., 2019; Roeska et

TABLE 1 Summary of studies on the affective consequences of neuropathic pain

Pain model	Species	Behavioural test	Results	References
PSNL	Mouse	EPM; LD	ALB at PO day 28	Narita, Kaneko, et al. (2006) and Narita, Kuzumaki, et al. (2006)
PSNL	Rat	OF	No ALB at PO day 14	Hasnie, Breuer, et al. (2007)
PSNL	Mouse	EPM; OF; TST	No ALB at PO days 7, 14 and 28; no DLB at PO days 8, 15 and 29	Hasnie, Wallace, et al. (2007)
PSNL	Rat	OF	ALB at PO day 14	Wallace, Segerdahl, et al. (2007)
PSNL	Mouse	EPM; LD	ALB at PO day 27	Matsuzawa-Yanagida et al. (2008)
PSNL	Rat	EPM	No ALB at PO week 4	Roeska et al. (2008)
PSNL	Mouse	OF	No ALB at PO day 10	Kodama et al. (2011)
PSNL	Rat	BT	Burrowing behaviour deficits at PO day 14	Andrews et al. (2012)
PSNL	Mouse	SPT	DLB at PO day 16	Bura et al. (2013)
PSNL	Mouse	EPM	ALB at PO day 28	Sawada et al. (2014)
PSNL	Mouse	FST; TST	DLB at PO week 4	Gai et al. (2014)
PSNL	Rat	EPM; OF; FST; SPT	ALB at PO day 28; no DLB at PO day 28	Wang, Zhong, et al. (2015)
PSNL	Mouse	FST	DLB at PO week 4	Bruning et al. (2015)
PSNL	Mouse	EPM; TST; MB	ALB at PO days 5–47; DLB at post-surgery days 20–62	Gonzalez-Sepulveda et al. (2016)
PSNL	Mouse	EPM; FST; SPT	ALB at PO weeks 1 and 3; DLB at PO week 3	La Porta et al. (2016)
PSNL	Mouse	EPM; OF; FST	ALB and DLB at PO day 30	Wang et al. (2017)
PSNL	Mouse	EPM; SPT	ALB at PO day 15; DLB at PO day 10	Martinez-Navarro et al. (2019)
PSNL	Mouse	SI; FST; NSF	ALB at PO week 6; DLB at PO week 8	Hisaoka-Nakashima et al. (2019)
PSNL	Mouse	FST; ST	DLB at PO day 28	Birmann et al. (2019)
SNL	Rat	EPM; OF; LD	No ALB at PO day 14	Kontinen et al. (1999)
SNL	Mouse	EPM; OF; LD; FST	ALB at PO days 30 and 56; DLB at PO days 15, 30 and 56	Suzuki et al. (2007)
SNL	Rat	OF	ALB at PO day 14	Hasnie, Breuer, et al. (2007)
SNL	Rat	FST	DLB at PO day 29	Hu et al. (2010)
SNL	Rat	BT	Burrowing behaviour deficits at PO day 14	Andrews et al. (2012)
SNL	Rat	EPM; OF	ALB at PO day 10	Jiang et al. (2014)
SNL	Rat	OF; FST	No ALB at PO day 20 DLB at PO day 23	Chung et al. (2017)
SNL	Rat	EPM; SPT	ALB and DLB at PO week 4	Ji et al. (2017)
SNL	Mouse	OF; FST; SPT	ALB at PO days 7 and 14; DLB at PO days 7, 14 and 21	Zhu et al. (2017)
SNL	Rat	EPM; FST	ALB and DLB at PO week 4	Ji et al. (2018)
SNL	Mouse	FST; SPT	DLB at PO day 14	Wu et al. (2018)
SNL	Rat	FST; TST; SPT	DLB at PO day 15	Zong et al. (2018)
CCI	Rat	FST; HST	DLB at PO days 3 and 7	Zeng et al. (2008)
CCI	Rat	EPM	ALB at PO week 4	Roeska et al. (2008)
CCI	Rat	EPM	ALB at PO day 36	Roeska et al. (2009)
CCI	Rat	FST	DLB at PO days 21–28	Hu et al. (2009)
CCI	Mouse	EPM; OF; MB	No ALB at PO day 3 to week 7	Urban et al. (2011)
CCI	Rat	FST	DLB at PO days 14–21	Fukuhara et al. (2012)
CCI	Rat	EPM; OF; SI; SPT	No ALB and no DLB at PO days 14–21	Gregoire et al. (2012)
CCI	Rat	EZM; FST	ALB and DLB at PO day 28	Alba-Delgado et al. (2013)
CCI	Rat	EPM; FST	ALB at PO day 25; DLB at PO day 32	Caspani et al. (2014)

(Continues)

TABLE 1 (Continued)

Pain model	Species	Behavioural test	Results	References
CCI	Rat	EPM; FST	ALB and DLB at PO days 7 and 21	Li et al. (2014)
CCI	Mouse	FST; TST	DLB at PO weeks 2, 4 and 6	Zhao, Wang, et al. (2014)
CCI	Mouse	FST	DLB at PO weeks 2–5	Zhao, Yu, et al. (2014)
CCI	Mouse	SPT	DLB at PO weeks 4–10	Dellarole et al. (2014)
CCI	Rat	OF; EZM	ALB at PO week 4	Alba-Delgado et al. (2016)
CCI	Rat	BT	Burrowing behaviour at PO days 3–14	Muralidharan et al. (2016)
CCI	Mouse	OF	ALB at PO week 2	Missig et al. (2017)
CCI	Rat	FST	DLB at PO days 7, 14, 21 and 28	Garg et al. (2017)
CCI	Rat	FST; SPT	DLB at PO day 28	Li et al. (2017)
CCI	Mouse	FST; TST	DLB at PO days 7–34	Jiang et al. (2018)
CCI	Rat	EZM; FST	ALB and DLB at PO weeks 4 and 6	Alba-Delgado et al. (2018)
CCI	Mouse	EPM; TST	ALB and DLB at PO day 28	Ferreira-Chamorro et al. (2018)
CCI	Rat	EZM	ALB at PO week 4	Llorca-Torralba et al. (2018)
CCI	Rat	FST; NSF	DLB at PO day 29	Jiang et al. (2019)
CCI	Mouse	FST; SPT	DLB at PO days 35–42	Wang et al. (2019)
CCI	Rat	FST	DLB at PO week 2	Li et al. (2019)
SNI	Rat	EPM; OF; FST	No ALB at PO week 7; DLB at PO week 7	Goncalves et al. (2008)
SNI	Rat	EPM; OF; FST	ALB and DLB at PO day 28	Leite-Almeida et al. (2009)
SNI	Rat	EPM	ALB at PO weeks 19 and 24	Seminowicz et al. (2009)
SNI	Mouse	OF; FST	No ALB at PO day 6; DLB at PO day 7	Norman et al. (2010)
SNI	Rat	FST; SPT	DLB at PO days 14 and 56	Wang et al. (2011)
SNI	Mouse	OF; EZM; SI; MB; FST; SPT	No ALB and DLB at PO day 3 to week 7	Urban et al. (2011)
SNI	Rat	EPM	ALB cohort-dependent at PO day 28	Leite-Almeida et al. (2012)
SNI	Mouse	LD; NSF	ALB at PO day 12	Mutso et al. (2012)
SNI	Rat	FST; SPT	DLB at PO day 14	Goffer et al. (2013)
SNI	Mouse	FST	DLB at PO day 15	Stratinaki et al. (2013)
SNI	Rat	FST	DLB at PO day 14	Le et al. (2014)
SNI	Rat	OF	ALB at PO day 21	Avila-Martin et al. (2015)
SNI	Mouse	TST; MB	DLB at PO day 30	Guida et al. (2015)
SNI	Rat	OF	ALB at PO 21	Galan-Arriero et al. (2015)
SNI	Mice	FST	DLB at PO day 7	Zhou et al. (2015)
SNI	Rat	EPM; OF	No ALB at PO weeks 2, 5, 9, 14 and 19	Hubbard et al. (2015)
SNI	Mouse	EPM; OF; FST; SPT	ALB at PO month 2 DLB at PO week 9	Descalzi et al. (2017)
SNI	Mouse	EPM; OF	ALB at PO day 28	Zhang et al. (2017)
SNI	Mouse	FST	DLB at PO day 7	Laumet et al. (2017)
SNI	Rat	FST; SPT	DLB cohort-dependent at PO days 14 and 21	Xie et al. (2017)
SNI	Mouse	TST; MB	DLB at PO year 1	D'Aniello et al. (2017)
SNI	Rat	FST; SPT	DLB at PO day 14	Xu et al. (2017)
SNI	Rat	EPM; OF; SPT	ALB at PO days 20–40 DLB at PO day 45	Gong et al. (2018)
SNI	Rat	EPM; OF	ALB at PO weeks 4 and 8	Sang et al. (2018)
SNI	Rat	EPM; OF; LD; SPT	ALB at PO week 3 No DLB at PO week 3	Chen et al. (2018)

(Continues)

TABLE 1 (Continued)

Pain model	Species	Behavioural test	Results	References
SNI	Mouse	EPM; LD; HB	ALB at PO weeks 4–6	Sieberg et al. (2018)
SNI	Rat	SPT	DLB at PO week 11	Thompson et al. (2018)
SNI	Rat	FST; SPT	DLB at PO days 14 and 18	Pan et al. (2018)
SNI	Rat	FST; SPT	DLB at PO days 42 and 56	Fu et al. (2018)
SNI	Mouse	EPM; LD; FST	No ALB and DLB at PO days 3–97	Pitzer et al. (2019)
SNI	Rat	LD	ALB at PO day 14	Chen et al. (2019)
SNI	Rat	FST; TST; SPT	DLB at PO day 25	Zhang et al. (2019)
SNI	Mouse	SPT	Anhedonia cohort-dependent at PO days 5, 12, 19	Yang, Fang, et al. (2019)
SNI	Rat	FST	DLB at PO day 14	Yang, Liu, et al. (2019)
SNI	Rat	FST; SPT	DLB at PO day 42	Fang, Xu, et al. (2019)
SNI	Rat	SPT	DLB cohort-dependent at PO days 12 and 19	Fang, Zhan, et al. (2019)
SNI	Mouse	OF; EPM; TST; SPT	ALB and DLB at PO week 6	Zhou et al. (2019)
Cuff	Mouse	EPM; SI; MB; TST	ALB at PO days 30 and 41 No DLB at PO days 23 and 35	Benbouzid et al. (2008)
Cuff	Mouse	LD; MB; FST; NSF; ST	ALB at PO weeks 4–9; DLB at PO weeks 6–9	Yalcin et al. (2011)
Cuff	Mouse	OF; EZM; FST	ALB at PO day 35; DLB at PO day 40	Dimitrov et al. (2014)
Cuff	Mouse	FST; NSF; ST	DLB at PO weeks 6–8	Barthas et al. (2015)
Cuff	Mouse	LD; FST; NSF; ST	ALB at PO week 8; DLB at PO weeks 8–17	Barthas et al. (2017)
Cuff	Mouse	LD; FST; NSF; ST	ALB at PO week 8; DLB at PO weeks 7 and 8	Sellmeijer et al. (2018)
Cuff	Mouse	LD; FST	ALB at PO week 8; DLB at PO week 8	Liu et al. (2019)
TNT	Rat	BT	Burrowing behaviour deficits at PO days 56–77	Andrews et al. (2012)
SCI	Rat	OF	No ALB at PO day 42	Galan-Arriero et al. (2014)
SCI	Mouse	TST; SPT	DLB at PO week 10	Wu et al. (2014)
SCI	Rat	OF; SI; FST; SPT	ALB and DLB cohort-dependent at PO days 10 and 21	Maldonado-Bouchard et al. (2016)
SCI	Mouse	OF; LD; FST	No ALB at PO weeks 1, 4 and 8; DLB at PO week 8	Boadas-Vaello et al. (2018)
Oxaliplatin	Mouse	TST; NSF	ALB and DLB at PI day 7	Hache et al. (2015)
Oxaliplatin	Mouse	FST; NSF	DLB at PI day 28	Poupon et al. (2018)
Paclitaxel	Mouse	FST; SPT; NSF; nest test	ALB at PI weeks 3–9; DLB at PI weeks 2 and 3	Toma et al. (2017)
Streptozotocin	Rat	OF; EZM	ALB at PI week 4	Alba-Delgado et al. (2016)
Streptozotocin	Rat	FST	DLB at PI weeks 2 and 4	Redivo et al. (2016)
Streptozotocin	Mouse	FST; TST; MB	DLB at PI day 46	Aguilar-Avila et al. (2019)
CION	Rat	EPM; LD; FST; SPT	ALB at PO day 15; no DLB at PO day 14–46	Gambeta et al. (2018)
TIC	Mouse	EPM; OF; LD	ALB at PO week 8	Lyons et al. (2015)
TIC	Mouse	LD	ALB at PO week 8	Lyons et al. (2018)
Antiretroviral	Rat	BT	Burrowing behaviour at PI day 21	Huang et al. (2013)
gp120	Rat	OF	ALB at PI 14	Wallace, Blackbeard, Pheby, et al. (2007) and Wallace, Blackbeard, Segerdahl, et al. (2007)
VZV	Rat	OF	ALB at PI day 14	Hasnie, Breuer, et al. (2007)

Abbreviations: ALB, anxiety-like behaviour; BT, burrowing test; CCI, chronic constriction injury; CION, infraorbital nerve constriction; DLB, depression-like behaviour; EPM, elevated plus maze; EZM, elevated zero maze; FST, forced swimming test; gp120, immunodeficiency virus type 1 envelope glycoprotein 120; HB, hole-board test; HST, horizontal suspension test; LD, light–dark test, MB, marble burying test; NSF, novelty-suppressed feeding; OF, open field; PI, post-induction; PO, post-operative; PSNL, partial sciatic nerve ligation; SCI, spinal cord injury; SI, social interaction; SNI, spared nerve injury; SNL, sciatic nerve ligation; SPT, sucrose or saccharin preference test; ST, splash test; TIC, trigeminal inflammatory compression; TNT, tibial nerve transection; TST, tail suspension test; VZV, varicella zoster virus.

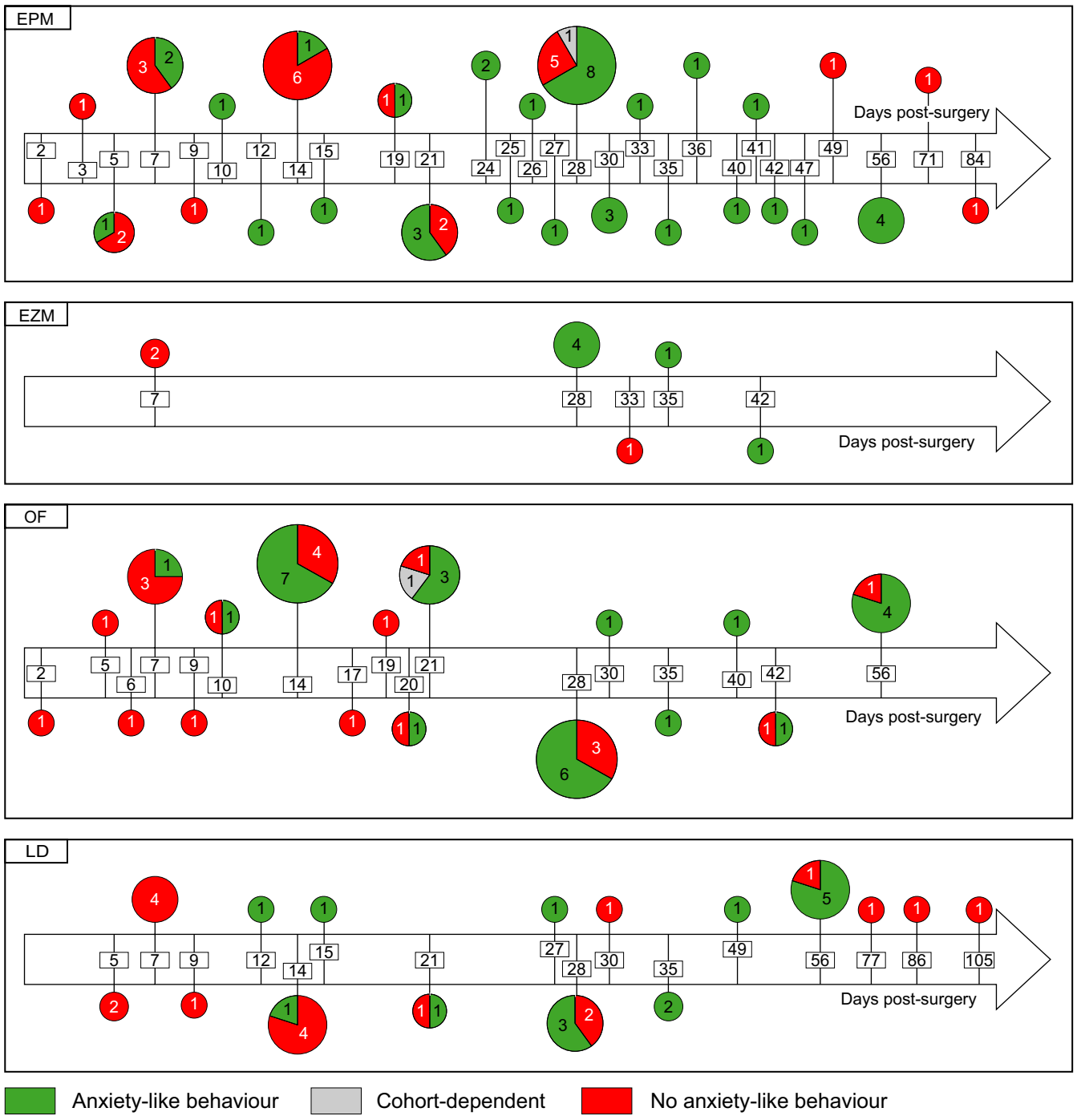


FIGURE 3 The impact of time on the anxiety-like behaviour in animal models of neuropathic pain. "*n*" displayed in the figure corresponds to the number of publications relative to the test (see Table 1 for more details and references). EPM, elevated plus maze; EZM, elevated zero maze; LD, light–dark test; OF, open field.

al., 2008). Conversely, anxiety-related behaviours generally disappear after a certain delay, thus testing this component of chronic pain at a very late time points can be misleading (Goncalves et al., 2008; Pitzer et al., 2019). Moreover, it has been shown that the side of the nerve lesion might also impact the affective consequences of chronic pain. For instance, Leite-Almeida et al. (2012) showed that when SNI is performed on the left nerve, male Wistar Han rats displayed more pronounced anxiety-like profile than when SNI was performed on the right nerve. Finally, the repetition of the EPM procedure on the same animals can also effect the interpretation of results (Hubbard et al., 2015). Indeed, the EPM has been depicted as sensitive to one-trial tolerance phenomenon, namely a decrease of time spent in open arms with the repetition of the test and independent of experimental groups (Tucker & McCabe, 2017).

TABLE 2 Summary of studies on the affective consequences of inflammatory pain

Pain model	Species	Behavioural test	Results	References
CFA	Mouse	EPM; LD	ALB at PI day 28	Narita, Kaneko, et al. (2006) and Narita, Kuzumaki, et al. (2006)
CFA	Mouse	OF; EZM; MB; SI; FST; SPT	No ALB and DLB at PI days 7–35	Urban et al. (2011)
CFA	Rat	OF; FST; TST; SPT	ALB at PI days 7 and 14; DLB at PI day 14	Kim et al. (2012)
CFA	Rat	EPM; OF; LD; SPT	ALB at PI day 28	Parent et al. (2012)
CFA	Rat	BT	Burrowing behaviour at PI day 10	Andrews et al. (2012)
CFA	Mouse	EPM; OF	ALB at PI days 3 and 7	Chen et al. (2013)
CFA	Mouse	FST; TST	DLB at PI days 7–21	Maciel et al. (2013)
CFA	Rat	EPM; LD	ALB at PI days 1, 3 and 10	do Nascimento and Leite-Panissi (2014)
CFA	Rat	EPM; FST	ALB and DLB at PI day 28	Borges et al. (2014)
CFA	Rat	OF; SI; SPT	ALB and DLB at PI weeks 2–3	Gregoire et al. (2014)
CFA	Rat	FST	DLB at PI day 7	Le et al. (2014)
CFA	Mouse	EPM	ALB at PI day 21	Wang, Tian, et al. (2015)
CFA	Rat	FST	DLB at PI day 7	Hamann et al. (2016)
CFA	Rat	FST; SPT	DLB at PI days 7–14	Zhang et al. (2016)
CFA	Mouse	EPM; OF	ALB at day 21	Guo et al. (2016)
CFA	Mouse	EZM; SPT	ALB at PI day 1; DLB at PI day 2	Refsgaard et al. (2016)
CFA	Mouse	EPM; OF	ALB at PI day 21	Sun et al. (2016)
CFA	Rat	BT	Burrowing behaviour at PI days 2–10	Muralidharan et al. (2016)
CFA	Mouse	EPM; OF	ALB at PI week 2	Tian et al. (2017)
CFA	Mouse	LD; TST	ALB and DLB at PI day 14	Omorogbe et al. (2018)
CFA	Mouse	EPM; OF	ALB at PI week 1	Yue et al. (2018)
CFA	Mouse	EPM; OF; LD; FST	No ALB and DLB at PI days 1–22	Pitzer et al. (2019)
CFA	Mouse	OF; EPM; TST; SPT	ALB and DLB at PO week 3	Zhou et al. (2019)
K/C	Rat	EPM	ALB at PI hours 5–6	Ji et al. (2007)
K/C	Rat	EPM; OF; FST; SPT	ALB and DLB at PI week 4	Amorim et al. (2014)
Uric acid	Rat	EPM; MB	ALB at PI hour 3	Fernandez-Guasti et al. (2005)
Osteoarthritis	Mouse	EZM; TST	ALB and DLB at PI week 41	Griffin et al. (2010)

Abbreviations: ALB, anxiety-like behaviour; BT, burrowing test; CFA, complete Freund's adjuvant; DLB, depression-like behaviour; EPM, elevated plus maze; EZM, elevated zero maze; FST, forced swimming test; HB, hole-board test; K/C, kaolin/carrageenan; LD, light–dark test; MB, marble burying test; NSF, novelty-suppressed feeding; OF, open field; PI, post-induction; SI, social interaction; SPT, sucrose or saccharin preference test; ST, splash test; TST, tail suspension test.

4.1.2 | Elevated zero maze

To avoid the one-trial tolerance phenomenon, the elevated zero maze (EZM) was developed and validated for anxiety assessment using diazepam and chlordiazepoxide treatments (Shepherd, Grewal, Fletcher, Bill, & Dourish, 1994). It consists of an elevated (40–70 cm above from floor) annular platform (5–10 cm wide; 46–120 cm diameter) with two opposite closed quadrants and two open quadrants (Alba-Delgado, Cebada-Aleu, Mico, & Berrocoso, 2016; Urban et al., 2011). Animals are placed in one of the enclosed quadrants and let free to explore the apparatus. As for the EPM, the number of entries and time spent in open quadrants are usually recorded during 5 min (Alba-Delgado et al., 2016; Llorca-Torralba et al., 2018; Martinez-Navarro et al., 2019). Other parameters can also be considered like head dips or "stretch attend" postures if a finest assessment of anxiety-related behaviours is searched for (Alba-Delgado et al., 2016; Alba-Delgado, Llorca-Torralba, Mico, & Berrocoso, 2018). As for the EPM, the EZM produces a conflict between exploratory behaviour and fear induced by a bright (the light intensity is set between 40 and 100 lux) and open environment (Kulkarni, Singh, & Bishnoi, 2007). Thereby, a decrease in the time spent in open quadrants suggests the development of anxiety-like behaviours.

Using this test, studies showed that neuropathic pain conditions, either caused by traumatic event (Alba-Delgado et al.,

TABLE 3 Summary of studies on the affective consequences of fibromyalgia

Pain model	Species	Behavioural test	Results	References
USS	Rat	EPM	ALB at PI day 14	Green et al. (2011)
BAD	Rat	FST	DLB at PI day 5	Nagakura et al. (2009)
BAD	Rat	FST	DLB at PI day 2	Arora and Chopra (2013)
BAD	Mouse	FST; TST	DLB at PI day 3	Klein et al. (2014)
BAD	Mouse	FST	DLB at PI day 4	de Souza et al. (2014)
BAD	Rat	NSF	DLB at PI days 4–5	Blasco-Serra et al. (2015)
BAD	Rat	OF; EZM	ALB at PI day 5	Wu et al. (2017)
BAD	Rat	FST	DLB at PI day 3	Siemian et al. (2019)
Acid-induced hyperalgesia	Rat	OF; EPM; FST; SPT	ALB at PI day 13; DLB at PI days 19–20	Liu et al. (2014)
ICS	Mouse	HB	DLB at PI day 3	Montserrat-de la Paz et al. (2015)
RCS	Rat	FST	DLB at PI days 10–14	Nasu et al. (2019)
SSS	Rat	EPM	ALB at PI week 1	Nazeri et al. (2018)

Abbreviations: ALB, anxiety-like behaviour; BAD, biogenic amine depletion; DLB, depression-like behaviour; EPM, elevated plus maze; FST, forced swimming test; HB, hole-board test; ICS, intermittent cold stress; NSF, novelty-suppressed feeding; OF, open field; PI, post-induction day; RCS, repeated cold stress; SPT, sucrose preference test; SSS, subchronic swim stress; TST, tail suspension test; USS, unpredictable sound stress.

2016, 2013, 2018; Dimitrov, Tsuda, Cameron, & Usdin, 2014; Llorca-Torralba et al., 2018) or by streptozotocin-induced diabetes (Alba-Delgado et al., 2016), can decrease the time spent in open quadrants at 4–6 weeks post-surgery, indicating the development of chronic pain-induced anxiety-like behaviours (Table 1, Figure 3). Interestingly, Martinez-Navarro and collaborators selected Swiss albino male mice based on their high or low anxiety trait at the beginning of the experiment, and demonstrated that animals expressing higher anxiety-like behaviours prior neuropathic pain induction developed anxiety-like behaviours at earlier time points (2 weeks; Martinez-Navarro et al., 2019). In CFA-induced inflammatory pain, anxiety-like behaviours were observed using the EZM one day after induction (Refsgaard, Hoffmann-Petersen, Sahlholt, Pickering, & Andreasen, 2016), while these behaviours were observed at week 41 in an osteoarthritis model (Griffin et al., 2010; Table 2). However, Urban and collaborators could not observe anxiety-like behaviours using the EZM in the SNI, CCI and CFA models (Urban et al., 2011). For the CFA model, the late time point used in this study, 33 days post-induction (against 1–10 days for other studies), could explain the lack of effect in EZM. Indeed, conversely to Urban and collaborators, other studies assessing anxiety-like behaviours (with either the EPM or the EZM) in CFA-induced inflammation never tested it after four post-induction weeks (Chen et al., 2013; do Nascimento & Leite-Panissi, 2014; Refsgaard et al., 2016). Regarding the results obtained in the SNI and CCI models, the authors suggest that the absence of effect could arise from the protocol used in their study (Urban et al., 2011).

4.1.3 | Open field

Anxiety-related behaviours can also be assessed with the open field test (OF). The OF consists in a square arena (40 × 40 × 30 cm: mice, 100 × 100 × 60 cm: rats), lighted with dim to bright light (4–580 lux; Wallace, Blackbeard, Segerdahl, et al., 2007; Zhu, Xu, Wang, Mao, & Lin, 2017). Animals are placed in the centre of the OF (Hasnie, Breuer, et al., 2007) or facing one wall of the arena (Zhu et al., 2017) and let free to explore the test. The time spent as well as the number of entries in the centre of the arena (area located 10 cm [mice] or 40 cm [rats] to the walls) are measured during 5–15 min. Again, this paradigm creates a conflict between the innate exploratory behaviour and the fear generated by an open and bright area. Administration of anxiolytic drugs such as diazepam or chlordiazepoxide was shown to elicit an increase in time spent and entries in the centre area (Choleris, Thomas, Kavaliers, & Prato, 2001).

When conducted in an inflammatory pain model, a decrease in time spent in the centre of the OF is observed from one to 28 post-induction days (Amorim et al., 2014; Chen et al., 2013; Gregoire, Wattiez, Etienne, Marchand, & Ardid, 2014; Guo et al., 2016; Kim et al., 2012; Parent et al., 2012;

Sun et al., 2016; Tian et al., 2017; Yue et al., 2018; Table 2). Liu and collaborators tested anxiety-like behaviours in the acid-induced hyperalgesia model of fibromyalgia and observed an effect 13 days after induction (Liu et al., 2014; Table 3). Regarding models of neuropathic pain, OF results are quite heterogeneous (Table 1, Figure 2). Indeed, over 39 published studies, 25 showed decreased time spent in the centre of the OF at time points mainly between 2 and 8 weeks after pain induction; while the other 14 studies failed to find any effect in this test (Figure 3). A main explanation for these discrepancies could again arise from the time point used to assess anxiety-related behaviours. Indeed, some studies (Kodama, Ono, & Tanabe, 2011; Kontinen et al., 1999; Norman et al., 2010) only tested animals at early or very late stage of chronic pain (Goncalves et al., 2008) although it is known that the temporality of the development of affective consequences of chronic pain is a critical parameter (Yalcin et al., 2011). The choice of the neuropathic model has also an impact on results obtained in OF. For instance, in spinal cord injury models either no differences or cohort-dependent effect was observed (Boadas-Vaello et al., 2018; Galan-Arriero et al., 2014; Maldonado-Bouchard et al., 2016). Also, in the PSNL model (Figure 1), near 50% of the studies did not report change in OF (Hasnie, Breuer, et al., 2007; Hasnie, Wallace, et al., 2007; Kodama et al., 2011). The reason why huge variability is frequently observed with this test can also be due to the protocol parameters, such as light setting (from 4 to more than 100 lux), test duration (4–60 min), area size and definition of the central zone. Indeed, over the 18 studies for which light parameters are provided in methods, 10 out of the 14 studies conducted with a light intensity set at 60 lux or less succeeded in demonstrating pain-induced anxiety-like behaviours (Avila-Martin et al., 2015; Galan-Arriero et al., 2015; Gong et al., 2018; Hasnie, Breuer, et al., 2007; Missig et al., 2017; Suzuki et al., 2007; Wallace, Blackbeard, Pheby, et al., 2007; Wallace, Blackbeard, Segerdahl, et al., 2007; Wallace, Segerdahl, et al., 2007; Zhang, Jiang, & Gao, 2017), while the four studies using an intensity equal or higher than 100 lux failed (Chen, Wei, Pertovaara, Wang, & Carlson, 2018; Kodama et al., 2011; Kontinen et al., 1999; Urban et al., 2011). These data suggest that too high light intensity might induce anxiety strong enough in controls to mask the difference between experimental groups and that milder light setting should perhaps be preferred for better discrimination between groups. Finally, it has been shown that even when the test conditions are strictly controlled, a strong variability between different laboratories can still be present (Robinson, Spruijt, & Riedel, 2018). Regarding these results, it seems that the OF should not be the only test used to assess anxiety in pain condition, and indeed, several groups rather employ a battery of behavioural tests to depict rodent emotional state (Descalzi et al., 2017; Leite-Almeida et al., 2009; Suzuki et al., 2007; Wang, Zhong, et al., 2015) and often use the OF as a marker of locomotor activity rather than an anxiety test (Goncalves et al., 2008; Lyons et al., 2015; Pan et al., 2018; Wu et al., 2016).

4.1.4 | Light/dark box test

Another test aiming at assessing anxiety-like behaviour is the light/dark box test (LDB) developed by Crawley and Goodwin (1980) who also showed the sensitivity of this test to benzodiazepines. Several other groups reproduced the results obtained by Crawley and Goodwin with various benzodiazepines, as well as with drugs acting on the serotonergic neurotransmission system (for an extensive review, see Bourin & Hascoet, 2003). The LDB is formed by two communicating chambers, one dimly lit with black walls and one brightly lit (100–500 lux) with white (Narita, Kaneko, et al., 2006) or transparent (Gambeta et al., 2018) walls. Animals are placed in the dark compartment and let free to explore the apparatus for 5–10 min. Entries and time spent in the light box are recorded, and latency to enter in the light box and rearing can also be considered among parameters (Lyons et al., 2015). As the bright compartment represents an aversive environment for rodents, a decrease in the time spent/or number of entries in this zone is defined as an anxiety-like behaviour.

As for the previously described tests, when LDB is performed in neuropathic pain models anxiety-like behaviours can be observed 4 (Chen et al., 2013; Guimaraes et al., 2019; Matsuzawa-Yanagida et al., 2008; Narita, Kaneko, et al., 2006; Narita, Kuzumaki, et al., 2006; Sieberg et al., 2018; Yalcin et al., 2011) to 8 weeks post-surgery (Barthas et al., 2017; Lyons et al., 2015, 2018; Sellmeijer et al., 2018; Suzuki et al., 2007; Yalcin et al., 2011), except for the SCI model in which no decrease in time spent in light box was detected (Boadas-Vaello et al., 2018; Table 1, Figures 2 and 3). Three studies also demonstrated a decrease in the time spent in the light box already detectable at 2 weeks after neuropathic pain induction (Chen et al., 2019; Gambeta et al., 2018; Mutso et al., 2012). Again difficulties to show anxiety-like behaviours can generally be explained by the testing time point being too early (Kontinen et al., 1999; Pitzer et al., 2019) or too late (Pitzer et al., 2019). When done in inflammatory condition, anxiety-like behaviour was observed in the LDB from 1 to 28 days after induction (Narita, Kaneko, et al., 2006; Narita, Kuzumaki, et al., 2006; do Nascimento & Leite-Panissi, 2014; Omorogbe et al., 2018; Parent et al., 2012; Table 2).

4.1.5 | Social interaction

The social interaction test (SI) was first developed to enable a measure of anxiety based on ethological behaviours and to replace tests including electric shock or food deprivation used

so far (File & Seth, 2003). This test was shown to be sensitive to anxiogenic (yohimbine, benzodiazepine receptor antagonists, picrotoxin) and anxiolytic drugs (lorazepam, diazepam, buspirone), as well as to stress-related hormones like the corticotropin-releasing factor and the adrenocorticotropic hormone (File & Seth, 2003). Originally, animals were isolated for several days prior to testing, but recent studies only used an isolation of 5–120 min (Gregoire et al., 2012; Hisaoka-Nakashima et al., 2019). After this isolation period, a juvenile congener is introduced in the cage and the amount of time spent in interaction (sniffing, following, grooming, licking) is recorded during 5 min (Hisaoka-Nakashima et al., 2019). For a more global recording of rodent social spontaneous behaviours, Benbouzid and collaborators did a video monitoring in home cage during 6 hr (Benbouzid et al., 2008). In this case the SI was performed with littermates instead of a juvenile congener.

The number of studies using the SI as a measure of anxiety-like behaviours in pain conditions is quite limited. Yet, when conducted in neuropathic pain models, a decrease in SI has been observed at 4 (Benbouzid et al., 2008) and 6 (Hisaoka-Nakashima et al., 2019) weeks post-surgery (Table 1, Figure 4). The studies conducted by Maldonado-Bouchard (Maldonado-Bouchard et al., 2016) or Gregoire (Gregoire et al., 2012) only tested animals before 3 weeks post-surgery, which might explain the absence of decrease in SI (Table 1, Figure 4). Indeed, the work conducted by Benbouzid and collaborators even report an increase in time dedicated to social contact at two post-operative weeks (Benbouzid et al., 2008). Regarding the sole study conducted in an inflammatory model (Gregoire et al., 2014), a decrease in SI was observed 2–3 weeks after CFA injection.

4.1.6 | Marble burying test

Originally used to assess anxiety-like behaviours because of its sensitivity to anxiolytic drugs such as diazepam or buspirone (Njung'e & Handley, 1991), the marble burying test (MB) is now thought to also be a potential test for the detection of compulsive behaviours (Angoa-Perez, Kane, Briggs, Francescutti, & Kuhn, 2013; Thomas et al., 2009). MB is performed in cages (same dimension as the home cages) containing 3–5 cm of fine sawdust. Twelve to 25 glass marbles (1 cm diameter) are evenly spaced on top of the sawdust. Animals are placed individually into the cages and left undisturbed for 15–30 min. After this period, animals are removed, and buried marbles are counted. Marbles are considered buried if two-thirds or more of their surface is covered by sawdust. The number of buried marbles is considered as a measure of animal anxiety and/or compulsive behaviour (Jimenez-Gomez, Osentoski, & Woods, 2011).

When used in neuropathic pain models, the MB highlights compulsive/anxiety-like behaviours at time points between 28 and 46 days post-surgery (Aguilar-Avila et al., 2019;

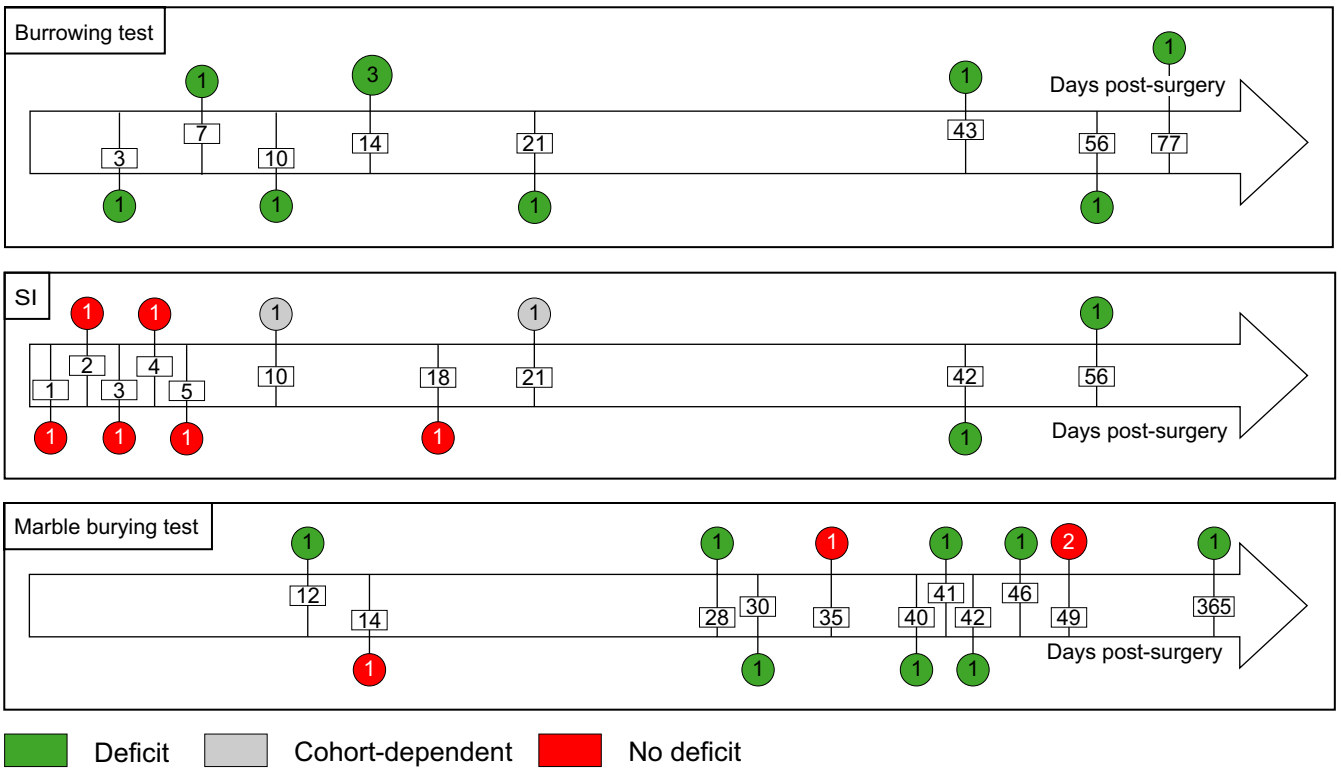


FIGURE 4 The impact of time on well-being in animal models of neuropathic pain. "*n*" displayed in the figure corresponds to the number of publications relative to the test (see Table 1 for more details and references). SI, social interaction.

Benbouzid et al., 2008; Guida et al., 2015; Yalcin et al., 2011; Table 1; Figure 4). According to D'Aniello and collaborators, the changes seen in burying behaviours, at least in the SNI model, can last up to 1 year (D'Aniello et al., 2017; Table 1, Figure 4). In an inflammatory pain model induced by intraplantar CFA injection, the increase in buried marbles occurred within 5 weeks post-CFA injection (Urban et al., 2011).

4.1.7 | Hole-board test

While the hole-board test (HB) can also give an insight into anxiety-like behaviours, it is less commonly used in the study of pain and mood disorder comorbidity. The HB device consists of a square plate (around 40 × 40 cm dimension) surrounded by walls and containing 9 (Sieberg et al., 2018) or 16 holes (Montserrat-de la Paz, Garcia-Gimenez, Angel-Martin, & Fernandez-Arche, 2015), around 3 cm diameter each, equally spaced on 3–4 rows. Animals are placed in the periphery (Sieberg et al., 2018) or in the centre (Montserrat-de la Paz et al., 2015) of the arena and let free to explore the apparatus for 5–15 min (Montserrat-de la Paz et al., 2015; Sieberg et al., 2018). The number of nose-pokes, recorded by infrared photocells in the hole, is used as a measure of anxiety-like behaviour as diazepam or chlordiazepoxide administration increases nose-poke numbers while anxiogenic compounds (FG7142, beta-CCM; inverse agonists of the benzodiazepine site of GABAa receptors) decrease it (Takeda, Tsuji, & Matsumiya, 1998). A decrease in the number of nose-pokes then reflects anxiogenic condition. Besides, the time spent in the centre of the HB is also recorded as a measure of anxiety (like in the OF), and the total distance travelled is an indicator of locomotor activity and thus serves as an internal control for motor function (Sieberg et al., 2018).

In a neuropathic pain model, a decrease in nose-poke activity has been observed at 4–6 post-operative weeks (Sieberg et al., 2018). In a fibromyalgia model, this effect is already present at three post-induction days (Montserrat-de la Paz et al., 2015).

4.1.8 | Burrowing test

This test was first introduced by Deacon et al. in 2001, and relies on the natural burrowing behaviour of rodents (Deacon, 2012; Deacon, Raley, Perry, & Rawlins, 2001). The burrowing test (BT) was proposed as a measurement of laboratory animals well-being as burrowing deficit are among the first behaviours to be detectable when rodents undergo stressful condition (Jirkof, 2014). The testing takes place in new cages similar to animal's home cages. Hollow plastic tubes (32 cm long, 10 cm diameter for rats) sealed at one end and opened at the other end, are filled with gravel and disposed 6 cm above the ground to avoid loss of gravel (Andrews et al., 2012; Huang et al., 2013). Test sessions are usually 2 hr long and latency to start burrowing and the amount of gravel displaced are recorded. In the BT, an increase in latency to burrow and a decreased amount of material displaced suggests the development of anxiety-like behaviours. Interestingly BT can be repeated on the same animals over time and thus gives the opportunity to conduct longitudinal studies. Note that several other protocols exist for burrowing assessment in rodent, and notably some using food pellet instead of gravel (for a review, see Jirkof, 2014).

In a model of inflammatory pain, burrowing behaviour deficits were seen at 10 post-induction days (Andrews et al., 2012). Regarding neuropathic pain models, a decrease in burrowing has been observed at 10 post-operative days in SNL and PSNL (Andrews et al., 2012) and between 21 (Huang et al., 2013) and 77 days (Andrews et al., 2012) in TNT (Table 1, Figure 4). Notably, a standardization of the procedure and a comparison across laboratories have been recently done (Muralidharan et al., 2016; Wodarski et al., 2016), making this test particularly interesting and reinforcing its validity for studying pain-related deficits.

4.2 | Depression-like behaviours

4.2.1 | Novelty-suppressed feeding test

The novelty-suppressed feeding (NSF) test can be used to assess both anxiety- and depressive-like behaviours, as demonstrated by its sensitivity to both anxiolytic (lorazepam, buspirone) and antidepressant drugs (imipramine, fluoxetine, amitriptyline; Dulawa & Hen, 2005). The NSF consists in a 40x40x30 cm plastic box with the floor covered with 2 cm of sawdust. Animals are usually food-restricted for twenty-four hours prior to the test. At the time of testing, a single pellet of food is placed on a paper in the centre of the box. An animal is then placed facing a corner of the box and the latency to first contact and onset of eating the pellet is recorded within a 5 min period. This test induces a conflict between the drive to eat the pellet and the fear of venturing in the centre of the box. The increase in the latency to eat suggests anxiodepressive-like behaviours.

In each neuropathic pain model used so far, an increase in the latency to feed has been observed, mainly between 2 and 9 weeks post-surgery (Barthas et al., 2017, 2015; Hisaoka-Nakashima et al., 2019; Jiang et al., 2019, 2018; Mutso et al., 2012; Poupon et al., 2018; Sellmeijer et al., 2018; Yalcin et al., 2011; Table 1; Figures 5 and 6). In a model of chemotherapy-induced neuropathy, Hache and collaborators showed an earlier onset of anxiodepressive-like behaviours compared to traumatic models (Hache et al., 2015) (Table 1, Figures 5 and 6). In the biogenic amine depletion model of fibromyalgia, an increase in the latency to feed was seen 4–5 post-induction days (Blasco-Serra et al., 2015).

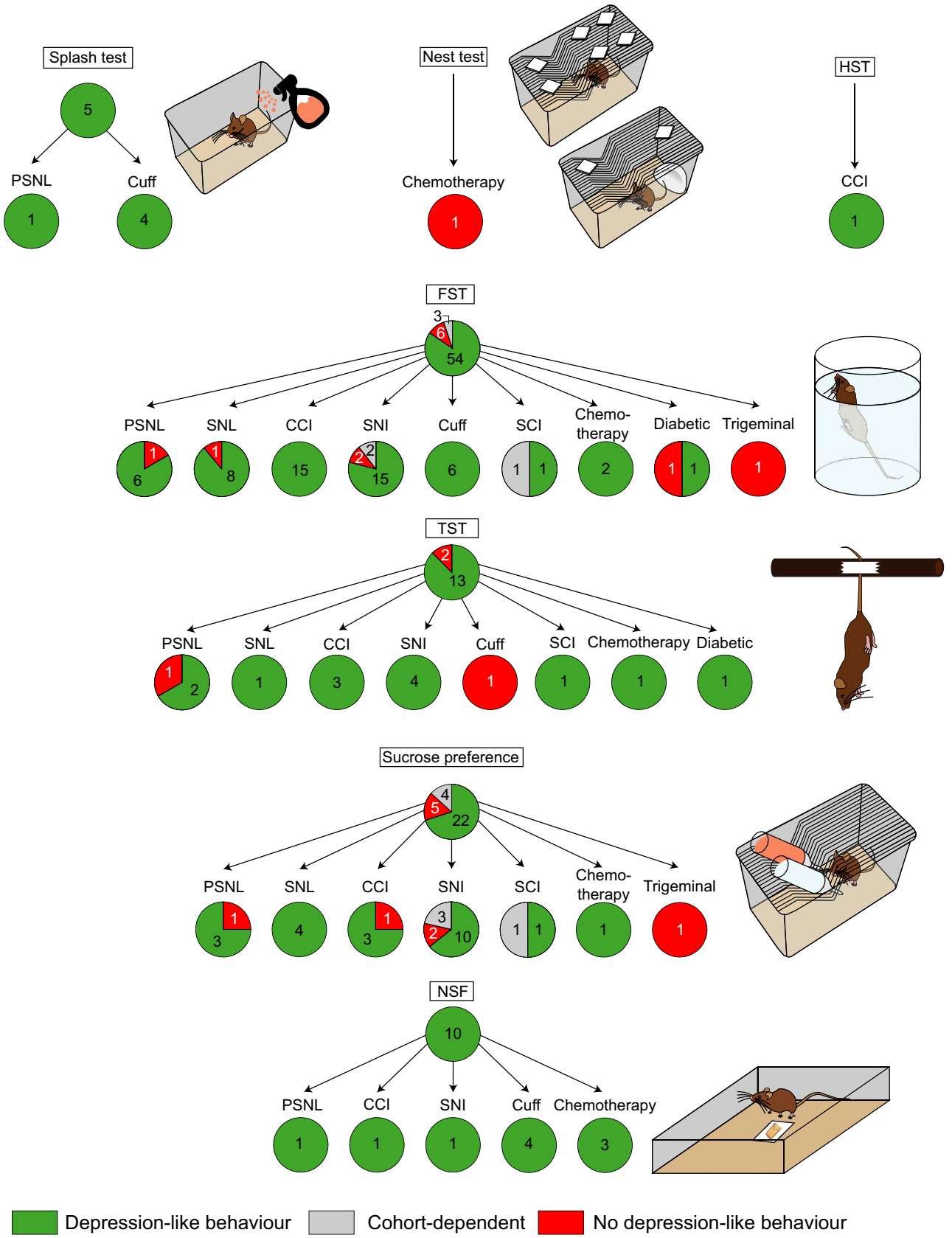
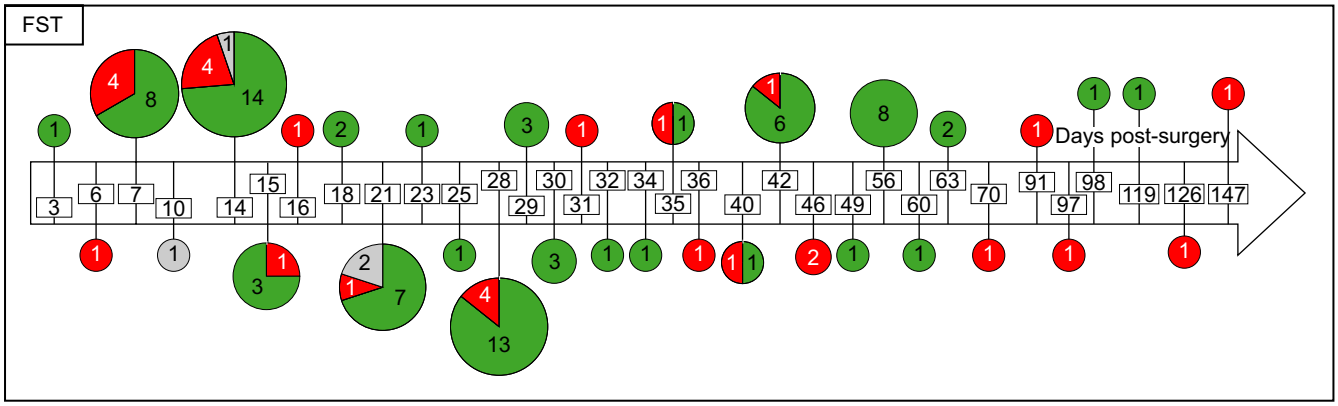
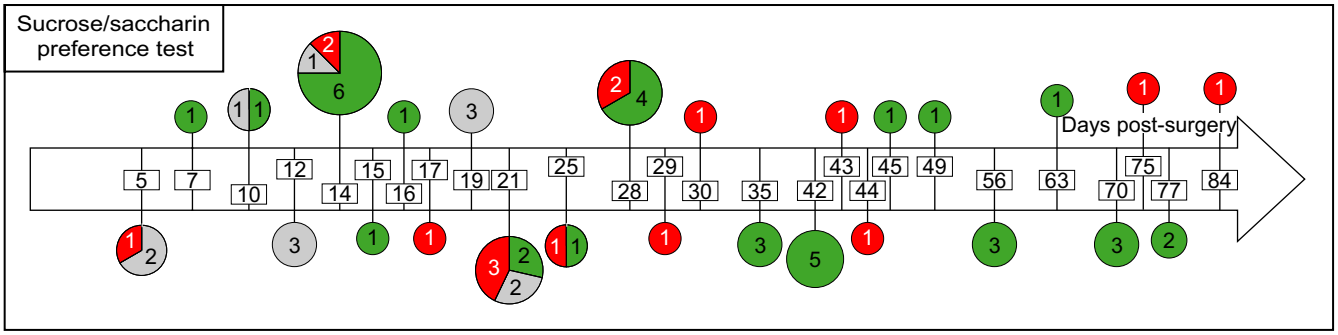
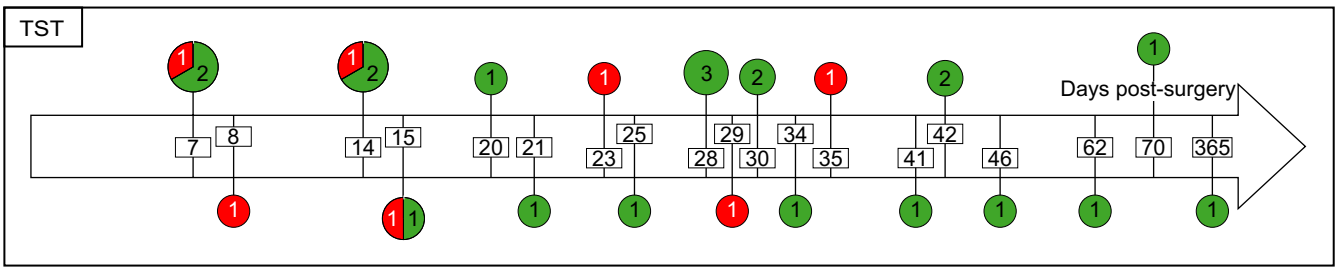
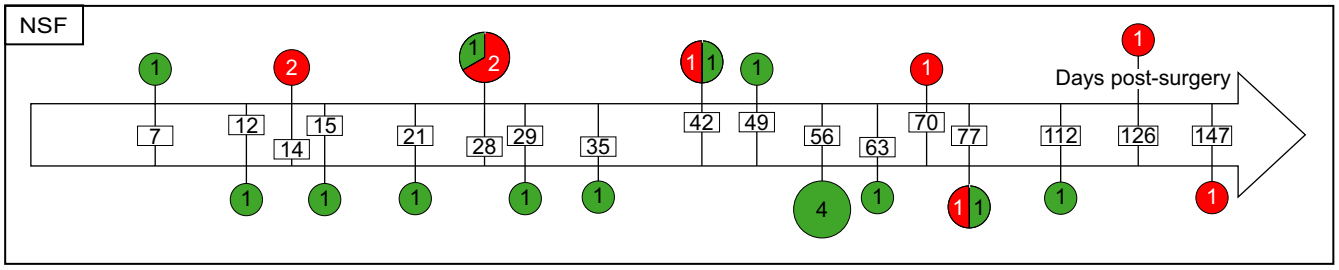


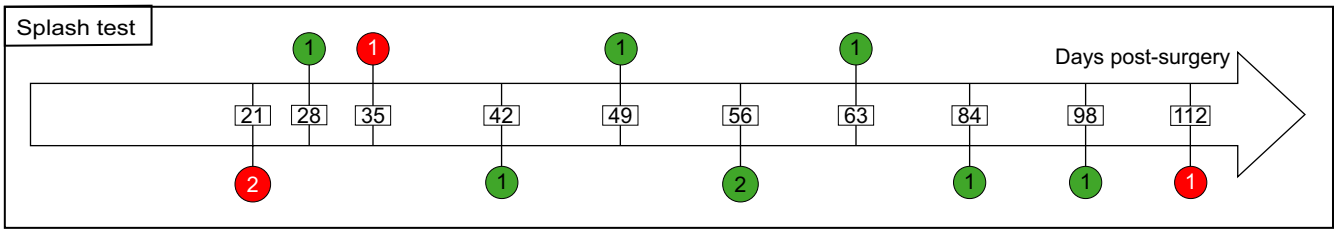
FIGURE 5 Depressive-like behaviours in animal models of neuropathic pain. "*n*" displayed in the figure corresponds to the number of publications relative to the test (see Table 1 for more details and references). FST, forced swimming test; HST, horizontal suspension test; NSF, novelty-suppressed feeding; TST, tail suspension test.











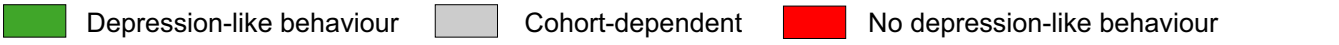


FIGURE 6 The impact of time on depressive-like behaviour in animal models of neuropathic pain. "n" displayed in the figure corresponds to the number of publications relative to the test (see Table 1 for more details and references). FST, forced swimming test; NSF, novelty-suppressed feeding; TST, tail suspension test.

4.2.2 | Forced swim test

A more common test to assess depressive-like behaviours in rodents is the forced swim test (FST), initially developed by Porsolt et al. in rats (Porsolt, Pichon, & Jalfre, 1977) and mice (Porsolt, Bertin, & Jalfre, 1977) in order to rapidly screen antidepressant drugs. Indeed, it was shown that immobility in the FST was decreased by tricyclic antidepressants, such as imipramine or amitriptyline (Porsolt, Le Pichon, et al., 1977). From this initial validation and use as a simple drug-screening test, the FST has since been more widely used to also study depressive-like behaviours. In rats, a pretest phase is required. Animals are gently lowered in an inescapable cylinder (height 50 cm, diameter 20 cm) containing 30 cm of water (23–25°C) for 15 min. The following day the rat is placed once again in the same apparatus and the duration of immobility phase is scored during 5 min (Alba-Delgado et al., 2013). For mice, there is no need to expose them to the pretest, so animals are directly lowered into an inescapable cylinder (height 20–46 cm, diameter 10–25 cm) containing 14–20 cm of water (21–25°C; Descalzi et al., 2017; Dimitrov et al., 2014; Gai et al., 2014; Yang, Fang, et al., 2019). Here, the test duration is 6 min, and as little immobility is generally observed during the first 2 min, the duration of immobility is quantified over the last 4 min of the 6 min test (Yalcin et al., 2011). The animals are considered immobile when they float in the water, in an upright position, and made only small movements to keep their head above water. In this paradigm, the impossibility to escape an aversive situation creates a despair state in the animals, reflected by immobility time; thus, an increase in immobility time is considered as a depressive-like behaviour. Besides immobility time, differentiating active behaviours between climbing, defined as forepaw vigorous upward movements in and out of the water, and swimming can also be recorded (Alba-Delgado et al., 2013; Hu, Doods, Treede, & Ceci, 2009). This distinction will then allow a finest assessment of despair behaviour as it has been shown that climbing behaviour was increased by norepinephrine-targeting antidepressant drugs, while swimming was modified by serotonin-targeted antidepressant drugs (Detke & Lucki, 1996).

For most of the studies using FST to assess neuropathic pain-induced depression, an increased immobility time was observed at 4 weeks (Bruning et al., 2015; Chung et al., 2017; Gai et al., 2014; Hu, Yang, Hu, Wang, & Li, 2010; Poupon et al., 2018; Wang et al., 2017; Wang, Zhong, et al., 2015) and 8 weeks (Boadas-Vaello et al., 2018; Descalzi et al., 2017; Goncalves et al., 2008; Hisaoka-Nakashima et al., 2019; Suzuki et al., 2007) post-surgery (Table 1, Figures 5 and 6). Interestingly, in the SNI and CCI models a shift to an earlier development of depressive-like behaviours can be observed. Indeed, over the 15 studies reporting an increase in immobility time in CCI, eight showed it at or even before 2 weeks post-surgery (Fukuhara et al., 2012; Garg, Deshmukh, & Prasoon, 2017; Li et al., 2014; Li, Chen, Li, & Jiang, 2019; see also Table 1 and Figures 5 and 6). For the SNI, among the 17 studies presenting depressive-like behaviour, 11 observed the development of deficits in active behaviour in this test before 2 weeks post-surgery (Laumet et al., 2017; Pan et al., 2018; Stratinaki et al., 2013; Xu et al., 2017; Yang, Liu, et al., 2019; Zhou et al., 2015; see also Table 1 and Figures 5 and 6). This timing could however be related to the severity of the model(s), and the fact that pain related to paw might by itself also affect motor (swimming) capacity, thus interfering with FST. In the CCI model, fluctuation in the time-dependency of depressive-like behaviours could also be explained by the inter-individual variability in pain induction that may be present in this model. Similarly, in a chemotherapy-induced neuropathy an early onset of depressive-like behaviour has been reported (Redivo et al., 2016; Toma et al., 2017). Regarding inflammatory pain models, increased immobility time in FST has been detected between 7 and 35 post-induction days in CFA injected animals (Borges et al., 2014; Hamann et al., 2016; Kim et al., 2012; Le, Lee, Su, Zou, & Wang, 2014; Maciel, Silva, Morrone, Calixto, & Campos, 2013; Urban et al., 2011; Zhang et al., 2016) and at four post-induction weeks in kaolin/carrageenan injected animals (Amorim et al., 2014). Finally, when conducted in an acid injection-induced model of fibromyalgia, a decrease in immobility time was observed after 19–20 post-operative days (Liu et al., 2014), after 10–14 days in an intermittent cold stress model (Nasu et al., 2019), and within few days in biogenic amine depletion models (Arora & Chopra, 2013; Klein et al., 2014; Nagakura et al., 2009; Siemian et al., 2019; de Souza et al., 2014).

4.2.3 | Tail suspension test

A variant of the FST, also developed for antidepressant drug screening (Steru, Chermat, Thierry, & Simon, 1985) and based on behavioural despair, is the tail suspension test (TST) in mice. It is sensitive to desipramine and amitriptyline (Steru et al., 1985). In TST, mice are suspended by the tail from a bar, 50 cm above the floor using an adhesive tape (1–2 cm from the proximal tail tip; D'Aniello et al., 2017; Jiang et al., 2018). The test duration is 6 min and the immobility is measured during the whole test. Mice are considered as immobile when they hung down motionless (Gai et al., 2014). An increase immobility time is thought to represent depressive-like behaviour.

As for the FST, when the TST is used in CCI or SNI an early development of depressive-like behaviour (around 1–2 weeks) is observed (Jiang et al., 2018; Yang, Fang,

et al., 2019; Zhao, Wang, et al., 2014; Zhao, Yu, et al., 2014). Similar results were found when using a chemotherapy-induced neuropathic pain model (Hache et al., 2015). For other neuropathic pain models, increased in immobility time was observed between 4 weeks and 2 months (Aguilar-Avila et al., 2019; Ferreira-Chamorro et al., 2018; Gai et al., 2014; Guida et al., 2015; Wu et al., 2014; Zhang et al., 2019; Table 1; Figures 5 and 6). For inflammatory pain models, depressive-like behaviours have been highlighted by the TST 7–14 days after CFA injection (Kim et al., 2012; Maciel et al., 2013; Omorogbe et al., 2018) and at 41 weeks in an osteoarthritis model (Griffin et al., 2010). Finally, in a fibromyalgia model, an increase in immobility time was observed within three post-induction days (Klein et al., 2014).

4.2.4 | Sucrose preference test

The sucrose or saccharine preference test (SPT) was first described by Katz (1982) and pharmacologically validated five years later by Willner and collaborators using the tricyclic antidepressant desipramine (Willner, Towell, Sampson, Sophokleous, & Muscat, 1987). The purpose of this test is to assess in animals the decrease ability to feel pleasure, also called anhedonia, often reported in patients suffering from depression. Animals are individually housed for the duration of the test and are free to choose between water and a sweet solution. An index of sweet solution preference is then calculated as the ratio of the sweet solution intake over total liquid intake. A decrease in this index suggests depressive-like behaviour (Dellarole et al., 2014; Xie et al., 2017). Today, a multitude of protocols are used to assess anhedonia in rodents. The sweet solution is mainly obtained using sucrose at a concentration varying from 0.5% (Gambeta et al., 2018) to 20% (Liu et al., 2014), but concentrations of 1% or 2% are the most used (Bura, Guegan, Zamanillo, Vela, & Maldonado, 2013; La Porta, Lara-Mayorga, Negrete, & Maldonado, 2016; Martinez-Navarro et al., 2019; Wang, Zhong, et al., 2015; Zhu et al., 2017). To avoid a potential bias due to sucrose caloric value, it can be replaced by saccharine; the concentrations used are then comprised arround 0.25%–0.3% (Refsgaard et al., 2016; Wu et al., 2014). Usually, a period of habituation is required prior testing. During this period, from 2 hr (Zong, Liao, Ren, & Wang, 2018) to 10 days (Wang et al., 2011), the bottles of water and sweet solution are often interchanged to prevent any side preference. Before the test session, a water deprivation is sometimes done with a duration varying between 2 (Bura et al., 2013) and 24 hr (Yang, Fang, et al., 2019). Finally, when considering the test duration, it varies from 15 min (Ji et al., 2017; Liu et al., 2014) to 48 hr (Urban et al., 2011; Wu et al., 2014), but test sessions of 24 hr were the most commonly used (Gong et al., 2018; La Porta et al., 2016; Li et al., 2017; Martinez-Navarro et al., 2019; Pan et al., 2018; Wu et al., 2018; Xie et al., 2017).

Despite all these protocol variations, the majority of reported studies could asses a presence of anhedonia from one week (Fang, Zhan, et al., 2019; Goffer et al., 2013; Martinez-Navarro et al., 2019; Wu et al., 2018; Xu et al., 2017; Zhu et al., 2017) to 10–11 weeks (Fang, Xu, Lin, & Liu, 2019; Fu et al., 2018; Thompson et al., 2018; Wu et al., 2014) after neuropathic pain induction (see also Figures 5 and 6 and Table 1). However, this time window depends on the considered model. Regarding the CCI model, anhedonia tends to arise at four post-surgery weeks (Dellarole et al., 2014; Li et al., 2017; Wang et al., 2019). Thus, the lack of such behavioural deficit in the Gregoire and collaborator study could result from the fact that the test was performed at an earlier time point (Gregoire et al., 2012). Furthermore, two studies conducted in the SNI model showed the presence of individual differences (in pro-inflammatory cytokines or in gut microbiota) between animals that are resilient or sensitive to chronic pain-induced depression (Xie et al., 2017; Yang, Fang, et al., 2019). These inter-individual differences might explain that effect in the SPT is sometimes cohort-dependent. In inflammatory pain models, a decrease in sucrose consumption was observed at 2 days post-induction (Refsgaard et al., 2016) and lasted at least for 4 weeks (Amorim et al., 2014). Finally, in a fibromyalgia model, Liu and collaborators observed anhedonia-like behaviour at 19–20 post-induction days (Liu et al., 2014).

4.2.5 | Nesting test

Nesting is an innate behaviour in rodents, which allows them to shelter from environment, maintain a certain heat and reproduce (Jirkof, 2014). As for the burrowing test described before, a deficit in nesting is an early sign of decreased well-being that can be rescued with chronic fluoxetine treatment (Farooq et al., 2018). In the study of Toma and collaborators, mice were individually housed with all the previous nesting material removed from the cage and placed in a dark room for an acclimation period of 30 min. Then, a compressed cotton nestlet was weighted and cut into six pieces placed on the top of the wire cage lid, evenly spaced. After 120 min the nestlet pieces remaining on the cage lid were weighted and a score given to the nest constructed. A score of 0 was given if no nest was formed, a score of 1 if the mice build a partial nest and a score of 2 if the nest was completely constructed. A poor nest score and a high amount of remaining nestlet on the cage lid are thought to indicate depressive-like behaviours. With this nesting protocol, no nesting deficit was found in a paclitaxel-induced neuropathic pain model (Toma et al., 2017).

If one wants to assess the nesting behaviour more precisely, other protocols have been used in mood

disorder-related studies (see Jirkof (2014) for a review); the most common protocol being the one described by Deacon in 2003, with a score from 0 (no nest formed) to 4 (established nest). This protocol also takes into account the shape of the nest (flat or dome shaped), the position in the cage (in the centre or at a corner) and is performed overnight (Deacon, 2006a, 2012). When using the nest test to assess depressive-like behaviour it is essential to make sure that the chosen protocol fits with the strain and sex of the animals used in the study. Indeed, differences in nest quality have been reported between males and females and between mice strains (Gaskill et al., 2012) or even between sub-strains (Sluyter, Marican, & Crusio, 1999).

4.2.6 | Splash test

The splash test (ST) is based on grooming, which is an important aspect of rodent behaviour and is often altered in animal models of depression (Santarelli et al., 2003; Yalcin et al., 2011). Animals are placed in a new cage with 1 cm of sawdust and a solution of 10% sucrose is sprayed on their back. The time spent for grooming as well as the grooming location (head or body) is measured for 5 min. A grooming deficit in this test is thought to be related to the loss of interest in performing self-oriented minor tasks, thus indicating the development of depressive-like behaviours. Moreover, the administration of antidepressant drugs, such as imipramine, desipramine, fluoxetine or maprotiline, rescued the grooming deficits in the unpredictable chronic mild stress mouse model of depression (Yalcin, Belzung, & Surget, 2008). When studied in a neuropathic pain model, more particularly in the cuff model, grooming deficits were reported between 6 and 9 post-operative weeks (Barthas et al., 2015; Sellmeijer et al., 2018; Yalcin et al., 2011; Table 1; Figures 5 and 6). Interestingly, Sellmeijer and collaborators studied the long term effect of neuropathic pain on affective behaviours and found that grooming deficits were still present at 14 post-operative weeks but no more at 16 weeks. In this study the extinction of depressive-like behaviours seemed to follow the recovery of ongoing pain.

5 | DISCUSSION

For this review, we found and analysed 144 articles related to the study of anxiodepressive-like disorders induced by chronic pain. The first articles on the topic were published shortly before the 2000s, but there has been a sharp increase in the number of scientific publications in the recent years, pointing out the growing interest in the comorbidity between pain and anxiety and depression. While most published studies demonstrated the possibility to model anxiodepressive-like consequences of chronic pain in animals (and potentially highlighted the essential role of time in the development of these symptoms), contradictory results can still be observed. Besides the time factor, these differences between studies may be due to the chosen animal models and tests, as well as to the protocols used to perform the tests. Rather than relying on a single parameter in animal models, it may thus be important to prefer strategies that would include behavioural profiling based on performing several tests evaluating anxiety and depression (Sellmeijer et al., 2018; Yalcin et al., 2011). In this respect, choosing the appropriate tests and control condition is one of the critical steps to assess the affective consequences of chronic pain. For example, most of the tests measuring anxiety and depression in rodent depend on motor activity of animals (exploration of a novel environment, swimming), which might be altered by pain models and lead to bias in interpreting the results. It is thus critical to take into consideration the limit of each test. For instance FST is one of the most common paradigms used to detect depressive-like behaviours, despite the fact that besides antidepressants most psychostimulants could also decrease the immobility time (Bogdanova, Kanekar, D'Anci, & Renshaw, 2013). Similarly when using the NSF, nesting, burrowing and marble burying tests, it should be kept in mind that these tests can in fact reflect various behavioural alterations at the same time, such as anxiety- or depressive-like behaviours for NSF (Dulawa & Hen, 2005), as well as obsessive compulsive- and autism-like behaviours for marble burying (Angoa-Perez et al., 2013; Deacon, 2006b). Furthermore, the experimenter should be careful with the order of tests when using several tests on the same animals. Indeed, some tests are anxiogenic per se, like the FST or TST, and should preferably be performed at the end of the experiments to avoid risks of interactions with other tests. In addition, tests based on the fear generated by novelty can often only be done once (EPM, NSF, EZM). On the other hand, tests based on ethologically relevant rodent behaviours, such as grooming, nesting or burrowing assessment, are potentially less anxiogenic. These tests, together with social interaction and the sucrose preference test, have the advantage to be repeatable and thus useful for a longitudinal follow-up of the animals (Kaidanovich-Beilin, Lipina, Vukobradovic, Roder, & Woodgett, 2011; Thomas et al., 2009). For chronic pain studies, such longitudinal testing strategy allows evaluating emotional state before pain induction and at various time points afterwards, thus using the animal as its own control. However, the development of new devices, such as PhenoWorld, can reinforce the possibility of testing animals in their habitual environment in automatic fashion, with limited interaction with the experimenter and on long term periods (Castelhano-Carlos, Costa, Russig, & Sousa, 2014). In addition, apparatuses combining multiple tests (EPM, LDB and OF) in one testing paradigm (Ramos, 2008) can also be a good alternative.

In this issue, we aimed at describing anxiodepressive tests that are already used for studying the comorbidity of chronic

pain and mood disorders, but it is worth to mention that other paradigms such as intracranial self-stimulation (ICSS), sexual behaviours, electroencephalography or circadian rhythm analysis (Castagne, Moser, & Porsolt, 2009) can also be used to address motivation, anhedonia and sleep pattern which are frequently altered in chronic pain.

The diversity of tests has already enabled notable breakthrough in the understanding of mood disorders and chronic pain comorbidity in animal models. However, in order to homogenize and increase the reproducibility of results, it is critical that the description of the parameters used for the testing is detailed in articles' methods. Indeed, important information such as handling and housing conditions or habituation to the testing room are often missing and thus prevent to conclude on the best practices to adopt. For example, as already mentioned previously, light setting in tests like OF, EPM or EZM are rarely specified although the use of a too bright light can induces anxiety-like behaviours even in control animals. Indeed, creating more relevant procedures and standardization of some of the most variable procedures (such as OF and SPT) among laboratories would be of great interest for the field. NIMH proposed a Research Domain Criteria system (RDoC) defining the good practices when evaluating psychopathologies in animal models that might be of great use for achieving this goal (Anderzhanova, Kirmeier, & Wotjak, 2017). Moreover, an effort is still needed in developing new methods to measure pain that are not based on nociceptive reflex response. Indeed, no study so far could show a correlation between the degree of nociceptive hypersensitivity and the anxiodepressive-like consequences. Thus, animals with or without anxiety- and/or depression-like phenotype can show similar mechanical or thermal withdrawal thresholds in animal models of pain (Gui et al., 2016; Xie et al., 2017; Yang, Fang, et al., 2019). Conversely, a recent study showed that rats that do not develop mechanical allodynia after SNI can still develop anxiodepressive-like behaviours similarly to painful rats (Guimaraes et al., 2019). There is thus no correlation in rodent models of chronic pain between the nociceptive response and the development of anxiodepressive disorders, which is similar to clinical reports (Bagnato et al., 2015; Dickens, Jayson, & Creed, 2002; Jensen et al., 2010; Keltner et al., 2012).

The above results may apparently question the causality relation between pain and emotional/cognitive disturbances. However, a causal link between pain and anxiodepressive consequences should not necessarily imply direct correlation between the intensity of nociceptive and anxiodepressive symptoms. Some observations suggest a temporal dissociation and partly independent mechanisms between these aspects of chronic pain (Gui et al., 2016; Guimaraes et al., 2019; Zhou et al., 2015). As example, in a model of sciatic nerve compression, mechanical hypersensitivity is no longer present 2–3 months after the surgery, while anxiodepressive-like behaviours can persist after the recovery of hypersensitivity (Dimitrov et al., 2014; Sellmeijer et al., 2018). Interestingly, one of these studies showed that ongoing pain can also persist beyond the recovery of nociceptive hypersensitivity (Sellmeijer et al., 2018), suggesting that reflex responses might not always be the best marker of pain.

Another limitation in the field relates to the fact that most published data used trauma models of neuropathic pain. While the cause of neuropathic pain is peripheral in a large set of patients, recent studies on anxiodepressive consequences of pain showed the potential involvement of distinct mechanisms with different neuropathic pain aetiologies, that is diabetic versus trauma (Alba-Delgado et al., 2016). For mechanistic aspects, it will thus be necessary to study these comorbidities in the context of the considered aetiologies, and in particular distinguish neuropathy, inflammation and fibromyalgia; and within neuropathic pain also consider metabolic diseases or neurotoxicity beyond the mostly used lesion models. Indeed, only three preclinical articles so far focused on the anxiodepressive-like aspects in chemotherapy-induced murine models of neuropathic pain (Hache et al., 2015; Poupon et al., 2018; Toma et al., 2017). Similarly, models mimicking neuropathic pain following viral infection (HIV or varicella zoster virus) have been poorly studied in regard to anxiodepressive-like symptoms. The study of central neuropathic pain also requires improvements. Indeed, preclinical studies using spinal cord injury either failed in showing anxiodepressive phenotype or reported a lack of reproducibility, whereas the comorbidity is clinically well established (Attal et al., 2011; Haythornthwaite & Benrud-Larson, 2000; Lim et al., 2017). Studies testing similar mechanistic hypotheses in models relying on different aetiologies would likely be important in the field in order to identify shared mechanistic features (which may illustrate core or converging mechanisms of anxiety or of depression) and distinct mechanistic features (which may be relevant to individualized medicine).

Despite their imperfections, animal models have proved to be useful in dissecting the mechanisms underlying the comorbidity between chronic pain and mood disorders. Thus, the involvement of several brain structures, such as the prefrontal cortex (PFC) including the anterior cingulate cortex, the hippocampus, the amygdala, the nucleus accumbens (NAc), the lateral habenula, the ventral tegmental area or the locus coeruleus (LC) has been shown (Doan et al., 2015; Humo et al., 2019; Yalcin, Barthas, & Barrot, 2014). Not only morphological, structural and functional modifications have been observed in these brain structures, but the implication of several neurotransmitter systems was also identified. For example, there is an increase in glutamatergic transmission in PFC, NAc, LC and amygdala (Goffer et al., 2013; Gonzalez-Sepulveda et al., 2016; Llorca-Torralba et al., 2018; Martinez-Navarro et al., 2019) leading to hyperactivities in these brain structures. Besides the glutamatergic system, the implication

of the endocannabinoid system (Hasnie, Breuer, et al., 2007; Jiang et al., 2019; Wallace, Segerdahl, et al., 2007), and of serotoninergic, noradrenergic and opioidergic transmissions have also been identified in structures such as the LC, the amygdala, the striatum or the anterior cingulate cortex (ACC; Alba-Delgado et al., 2018; Ji et al., 2017; Narita, Kuzumaki, et al., 2006; Sang et al., 2018; Thompson et al., 2018).

Beyond these anatomical and neurotransmitter-related information, animal studies on neuropathic pain also reported an implication of inflammatory mechanisms such as microglia activation (Ferreira-Chamorro et al., 2018; Galan-Arriero et al., 2014, 2015; Sawada et al., 2014; Wu et al., 2014; Xu et al., 2017) and/or pro-inflammatory cytokine production (Dellarole et al., 2014; Gonzalez-Sepulveda et al., 2016; Norman et al., 2010) in mood disorders accompanying chronic pain. At intracellular level, changes in the mitogen-activated protein kinases (MAPK) and indoleamine 2,3-dioxygenase 1 (IDO1) were also reported (Barthas et al., 2017; Sawada et al., 2014; Zhou et al., 2015). Looking at the mechanisms down to epigenetic level, the animal models and tests also allowed showing the recruitment of histone deacetylases (HDACs; Descalzi et al., 2017) and of dimethyl-3-transferase (DM3T) (Wang et al., 2019). From anatomy to neural transmission and cellular, molecular and epigenetic changes, the animal models and tests thus provided major contributions to our understanding of the mechanisms linking pain and mood (for review; Humo et al., 2019; Leite-Almeida et al., 2015; Yalcin & Barrot, 2014; Yalcin et al., 2014).

In conclusion, despite their drawbacks that are highlighted in this review, preclinical models so far allowed exploring the anxiodepressive-like consequences of chronic pain. However, translational studies combining animal models and human condition, as well as side-by-side comparison of different animal models, should help us further improving the existing tests and guide us in developing new approaches to model this comorbidity in animals. Moreover, not all the patients suffering from chronic pain develop mood disorders. To better understand the mechanisms underlying the comorbidity between pain and mood disorders, it would thus also be critical to develop studies on such resiliency/susceptibility in animal models. In parallel, an effort is still needed in developing other measurements than the behavioural testing, such as the neuroimaging or biochemical biomarkers, in order to better characterize the anxiodepressive consequences of chronic pain.

ACKNOWLEDGEMENTS

This work was supported by the "Centre National de la Recherche Scientifique" (contract UPR3212), the University of Strasbourg, the "Agence Nationale de la Recherche" (Euridol ANR-17-EURE-0022), and a NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation (24736).

CONFLICT OF INTERESTS

The authors declared no potential conflict of interest with respect to the research, authorship and/or publication of this article. The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

M.K. and L.B. did the bibliographic research; M.K. wrote parts 1, 2, 3 and 5; L.B. wrote parts 4 and 5; M.K. realized all the figures and L.B. has completed Figures 2 and 5; I.Y. and M.B. have supervised and revised the article.

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How to cite this article: Kremer M, Becker LJ, Barrot M, Yalcin I. How to study anxiety and depression in rodent models of chronic pain?. *Eur J Neurosci*. 2021;53:236–270. https://doi.org/10.1111/ejn.14686

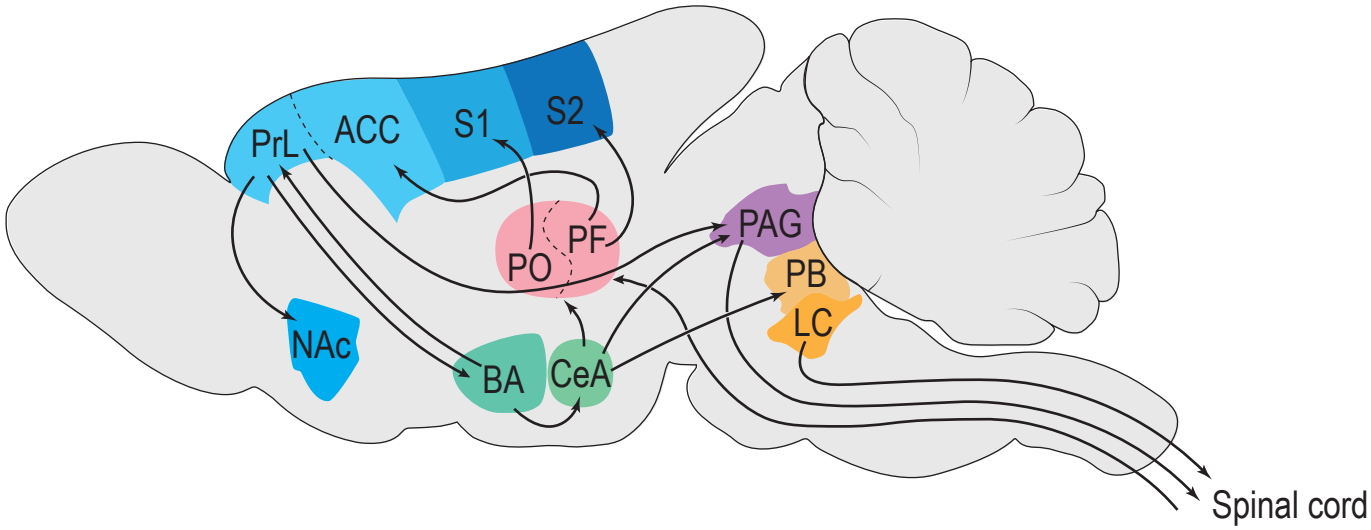

Figure 4: Circuitry underlying pain regulation

Schematic representation of the pathways involved in the regulation of pain.

ACC: anterior cingulate cortex, BA: basolateral amygdala (anterior and posterior part); CeA: central amygdala; LC: locus coeruleus; NAc: nucleus accumbens; PAG: periacqueducal grey; PB: parabrachial nucleus; PF: parafascicular nucleus; PO: posterior thalamic nucleus; PrL: prelimbic cortex; S1/S2: primary and secondary somatosensory cortices.

3. Brain networks underlying chronic pain and its comorbidity with emotional disorders

i. *Neuronal networks in pain*

Magnetic Resonance Imaging (MRI) studies conducted in human and rodents affected by chronic pain highlighted functional alterations in similar brain region for both species. Following nociceptive stimulation, the most consistently activated structures are primary and secondary somatosensory cortices (S1, S2), insula, ACC, periaqueductal grey area (PAG) and thalamus (Apkarian et al., 2005; Da Silva & Seminowicz, 2019; Ossipov et al., 2010; Tan & Kuner, 2021; Tanasescu et al., 2016). In the recent years, efforts have been made to uncover the circuits linking all these structures and underlying pain behaviors (**Figure 4**).

In a general manner, nociceptive information arrives from the periphery to the thalamus and are then transferred to cortical and subcortical regions to be integrated (Brooks and Tracey 2005). Interestingly, the recruited thalamo-cortical pathways are dependant of the aetiology of pain. On one hand, when allodynia is induced by tissue injury, it engages the projection from posterior thalamic nucleus glutamatergic neuron (PO^{Glu}) to S1 cortex. On the other hand, when allodynia is triggered by depressive states (chronic restrain stress (CRS) or chronic unpredictable stress) it recruits the circuit between parafascicular thalamic nucleus glutamatergic neurons (PF^{Glu}) and ACC GABAergic interneuron (Zhu et al., 2021). Likewise, hypersensitivity induced by chronic stress involves the PF^{Glu} neurons. In a model of chronic restrained stress, (CRS) mice show an enhanced inhibition of the circuit involving central amygdala (CeA) GABAergic neurons, PF^{Glu} and S2. Chemogenetic and optogenetic manipulation of this circuit alleviates the pain symptoms induced by chronic stress (Zhu et al., 2019). Additionally in CRS and in a model of peripheral neuropathy (SNI), the CeA displays a hyperexcitability resulting in a disinhibition of the ventrolateral PAG (vlPAG) associated with mechanical hypersensitivity. While activation of the glutamatergic neurons in the vlPAG cells only reversed nociceptive behaviors in CRS mice, inactivation of the GABAergic ones was efficient in both CRS and SNI mice (Yin et al., 2020). Whereas ascending pathways convey nociceptive information from the periphery to supraspinal centres in order to integrate them, descending pathway possess a crucial role in the modulation of pain-related behaviors (Ossipov et al., 2014). Those pathways notably involve brainstem regions

like the LC or the PAG. In a model of chronic constriction injury, the LC display an increased bursting activity, along with an increase of the α2 adrenoreceptor expression (Alba-Delgado et al., 2012). Interestingly, this effect is stronger a month following the induction of the neuropathic pain than after one or two weeks. Therefore, the activity changes in the LC seems to coincide more with the onset of chronic pain-induced anxiodepressive-like behaviors (Alba-Delgado et al., 2021). Nonetheless, the LC participates to nociception control through its connections with the spinal cord. Indeed, activation of the LC neurons projecting to the spinal cord drives a strong anti-nociceptive effect (Hirschberg et al., 2017). The PAG is a second hub for the top-down modulation of pain and exerts pro- and anti-nociceptive influence through a balance between the activity of GABAergic and glutamatergic neurons (Samineni et al., 2017). The activity of the PAG is driven by afferents coming from the prelimbic cortex since the photostimulation of this connection alleviates mechanical hypersensitivity and thermal hyperalgesia. Interestingly, the PrL to PAG connection is necessary for the antinociceptive action of the BA to PrL input. Indeed, while inhibition of projection from the BA to the PrL reduces pain-related behaviors, lesion of the PrL to PAG pathway prevents this effect (J. Huang et al., 2019).

As for emotional regulation, pain and nociception strongly rely on dysregulation of the communication between the prefrontal cortex and subcortical structures, notably with the amygdalar complex. The group of Neugebauer proposed a mechanism to explain the dysfunction in amygdala and PFC in a model of arthritis pain (**Figure 5**). According to their electrophysiological observations, following chronic pain, the feed forward inhibition of the PFC normally induced by the BA is lost, resulting in an increased activity of the BA and an overdrive of the CeA (Kiritoshi & Neugebauer, 2018; J. M. Thompson & Neugebauer, 2019). The CeA is then involved in nociception modulation through its connection with the parabrachial nucleus (Raver et al., 2020) or the PAG (Yin et al., 2020). The PFC also drives pain behavior through its projection to the NAc. Indeed, the inhibition of these projections enhances the sensory and affective component of both acute and chronic pain (H. Zhou et al., 2018). In addition, ACC projection to the NAc is involved in empathy related to pain. While the presence of a cagemate experiencing inflammatory pain was sufficient to trigger hyperalgesia

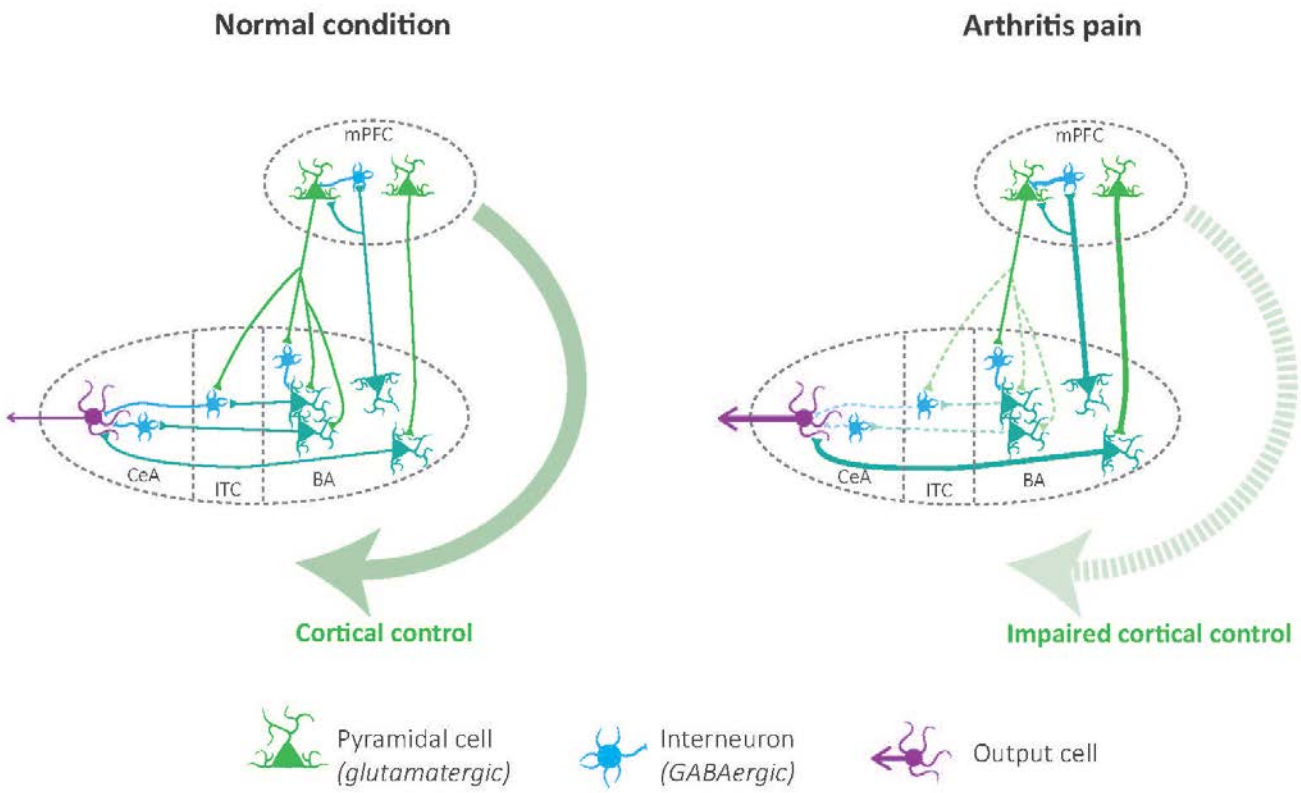

Figure 5: Possible alteration of cortico-amygdala pathway in painful condition

Schematic representation of possible alterations in synaptic connectivity between the BLA and the mPFC leading to impaired cortical control in a model of arthritic pain
BA: basolateral amygdala; CeA: central amygdala; ITC: intercaled cells; mPFC: medial prefrontal cortex

Adapted from Thompson and Neugebauer 2019 and Kiritoshi and Neugebauer 2018

in a bystander mouse, photoinhibition and photostimulation of the ACC-NAc pathway respectively blocks and enhances the social transfer of pain (Smith et al., 2021).

Because of the similarities of brain structures and circuits involved in pain and emotional regulation, it is reasonable to think that the comorbidity observed between chronic pain and anxiodepressive disorders could arise from a dysfunction in those common circuits. Therefore, it is important to dissect these pathways in models of chronic pain induced emotional disorders (CPED) to reach a better understanding of this comorbidity.

ii. *Comorbidity of chronic pain and anxiodepressive disorders: Deciphering underlying brain circuits*

In 2020, Bravo and collaborators published a review compiling the latest advances in preclinical studies focusing on the relation between pain and several psychiatric disorders (Bravo et al., 2020). They nicely reviewed the bidirectional relation between stress and pain, summarized the functional involvement of different brain structures (PFC, amygdala, NAc, LC and HPC) in pain, emotion and cognition and described the results obtained so far on the effect of several medication for pain and depression. Finally, they extended their literature review by including findings on the comorbidity of pain with other psychiatric disorders (schizophrenia, autism spectrum disorders). Based on this review, we wrote a commentary describing few studies focusing on the neuronal circuits of the comorbidity of chronic pain and emotional disorders and highlighted the need for a more extensive study of the brain networks involved in this comorbidity.

Neuroscience and Biobehavioral Reviews 115 (2020) 131–133

Contents lists available at ScienceDirect

Neuroscience and Biobehavioral Reviews

journal homepage: www.elsevier.com/locate/neubiorev

Comorbidity of chronic pain and anxiodepressive disorders: Deciphering underlying brain circuits

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ARTICLE INFO

Keywords:
Chronic pain
Anxiety
Depression
Brain circuits
Comorbidity
Optogenetic
Chemogenetic

Chronic pain and anxiodepressive disorders are well-known for being intimately related and for influencing each other, but the mechanisms underlying this clinically significant comorbidity remain largely unknown. In this issue, Bravo et al. (2020) address this challenging topic by focusing on brain structures implicated in their bidirectional relationship. By reviewing lesion, pharmacologic, optogenetic or chemogenetic studies, they nicely recapitulate the role of individual brain structures (such as the prefrontal cortex - PFC, the amygdala, the nucleus accumbens - NAc, the locus coeruleus - LC, and the hippocampus) in the anxiodepressive disorders-pain dyad.

Even though studies focusing on individual brain regions improve our understanding of pathophysiology, it is also essential to reach circuitry level of understanding. Indeed, most central nervous system disorders are linked to alterations in brain circuits rather than to changes occurring in single structures. While this issue was difficult to explore until recently, the rapid development of optogenetic and chemogenetic tools now allows the targeting of anatomically defined neuronal pathways (Fig. 1A). Regarding chronic pain-induced emotional dysregulation (CPED), a few preclinical studies have started to unravel critical brain circuits (Fig. 1B), which link together some of the brain regions highlighted by Bravo et al.

Lee and collaborators (2015) first showed that optogenetic activation of PFC neurons terminals within the NAc blocks both nociceptive and negative affective behaviors in neuropathic animals (Fig. 1A). To further identify the neurochemical identity of this pathway, they combined optogenetic and pharmacological manipulations, and infused an AMPA receptor antagonist into the NAc, which blocked the effects of the optogenetic modulation.

The amygdala is another major hub for CPED. Llorca-Torralba and colleagues (2019) demonstrated that chemogenetic silencing of LC noradrenergic neurons projecting to the basolateral amygdala (Fig. 1A) relieved pain-induced anxiety-like behaviors in neuropathic rats, without affecting mechanical hypersensitivity.

To decipher potentially divergent contributions from subpopulations of neurons located in the same brain structure but sending projections to distinct target sites, Hirschberg and colleagues highlighted another approach (2017). The authors used retrograde viruses injected in the PFC or spinal cord (SC) to target ascending and descending projections from noradrenergic neurons of the LC, respectively (Fig. 1A). They showed that chemogenetic activation of the LC-SC pathway had an anti-nociceptive effect, while the activation of LC-PFC projections enhanced aversive and anxiety-like behaviors. This study along with that of Llorca-Torralba et al. (2019) point out that refining the analysis of specific neuronal subpopulations based on their efferences can reveal distinct and even functionally opposite effects in the modulation of the sensory and affective components of pain.

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https://doi.org/10.1016/j.neubiorev.2020.05.013
Received 23 April 2020; Accepted 26 May 2020
Available online 30 May 2020
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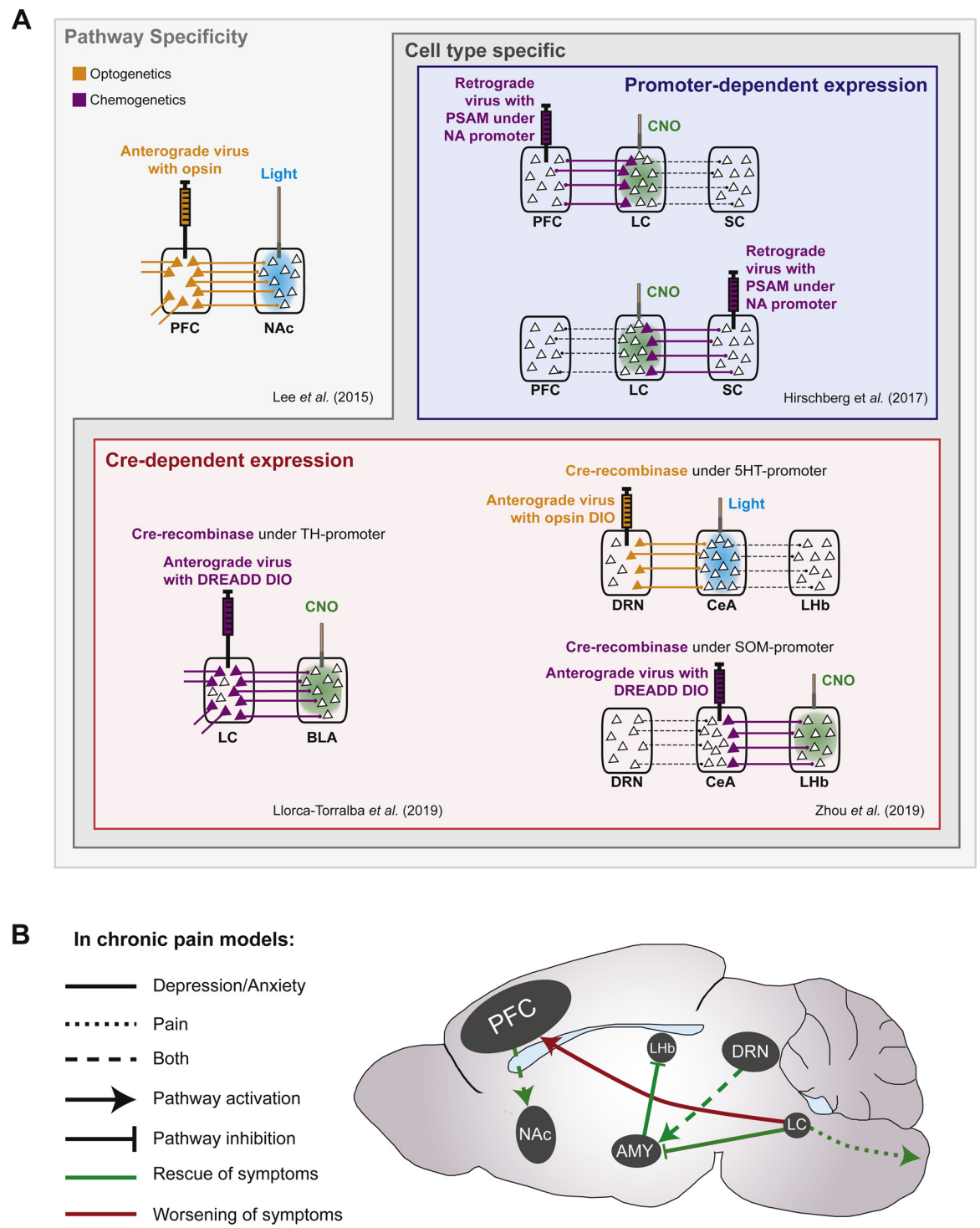

Fig. 1. Tools and approaches used to dissect neuronal circuitry underlying chronic pain-induced emotional dysregulation. A. Optogenetic and chemogenetic technics used to dissect brain circuits. *Upper left panel:* mPFC-NAc specificity reached by injection of anterogradely transported opsin in the PFC and by an optic fiber implantation in the NAc (Lee et al., 2015). *Upper right panel*: Cell-type specificity by promoter-dependent expression: injection in the PFC or SC of a retrograde virus expressing DREADD under the synthetic promoter PRS targeting noradrenergic (NA) neurons. Cell bodies of NA neurons expressing DREADD and projecting either the PFC or SC are stimulated by CNO injection in the LC through a cannula (Hirschberg et al., 2017). *Lower panel*: Cell-type specificity by Cre-dependent expression. *Left*: Injection of an anterogradely transported DREADD in the LC. DREADD will be expressed in neurons expressing the Cre recombinase under the TH promoter. Axon terminals expressing DREADD in the BLA are stimulated by CNO injection through a cannula (Lorca-Torralba et al., 2019). *Right:* Injection of an anterogradely transported opsin or DREADD in the DRN or the CeA. The opsins/DREADD will only be expressed in neurons expressing Cre-recombinase under the Pet-1 (5 H T neurons, injected in the DRN) or SOM (injected in the CeA) promoters. Axon terminals expressing opsin/DREADD in the CeA or the LHb are stimulated by light/CNO injection through an optic fiber/cannula (Zhou et al., 2019). B. The figure depicts neuronal circuits underlying the comorbidity between pain and depression/anxiety, as dissected using optogenetics/DREADD in the following publications (see main text for details): mPFC-NAc pathway, see Lee et al., 2015; LC-mPFC and LC-spinal cord pathways, see Hirschberg et al., 2017; LC-AMY pathway, see Llorca-Torralba et al., 2019; DRN-AMY-LHb pathways, see Zhou et al., 2019. Abbreviations: 5-HT: serotonin; AMY: amygdala; BLA: basolateral amygdala; CeA: central nucleus of the amygdala; CNO: clozapine-*N*-oxide; DREADD: designer receptor exclusively activated by designer drugs; DRN: dorsal raphe nucleus; LC: locus coeruleus; LHb: lateral habenula; PFC: prefrontal cortex; PSAM: pharmaco-selective actuator module; NA: noradrenaline; NAc: nucleus accumbens; SC: spinal cord; SOM: somatostatin; TH: tyrosine hydroxylase.

While the different studies discussed so far assessed pathways involving two brain structures, circuits underlying pain perception and emotional regulation are likely to be more complex. Recently, Zhou and collaborators (2019) dissected an ascending pathway involved in pain-induced depressive-like behavior that is composed of the dorsal raphe nucleus (DRN), the central nucleus of the amygdala (CeA), and the lateral habenula (LHb), using both chemo- and optogenetics. Their results demonstrated that inhibition of serotonergic DRN neurons terminals in the CeA (Fig. 1A) resulted in depressive-like behavior in naive mice, while activation of this pathway in neuropathic animals produced antidepressant-like and antinociceptive effects. DRN serotonergic neurons preferentially target somatostatin neurons in the CeA, which in turn project to the LHb. Surprisingly, electrophysiological recordings showed that activation of CeA somatostatin fibers elicited excitatory responses in the LHb. Indeed, even though most somatostatin neurons are GABAergic, a small population of them is in fact excitatory. Silencing those fibers in the LHb (Fig. 1A) rescued chronic pain-induced depressive-like behavior, while activation of this pathway in naive animals conversely induced depressive-like behavior. Finally, by combining optogenetics with neuronal tracing and electrophysiology, they assessed the whole DRN-CeA-LHb circuit and showed that CPED in their model stems from the loss of serotonergic inhibition of LHb-projecting neurons of the CeA.

Taken altogether, these results deepen our understanding of the interconnected pathways that differentially modulate affective and sensory components of pain. Nevertheless, more research will be needed to better understand the big picture of CPED. While existing studies have proven the strength of opto- and chemogenetics in combination with pharmacological or electrophysiological approaches, future studies should consider additional levels of analysis. Within this line, cell-type, pathway-specific, or single-cell RNA-sequencing all have the potential to reveal underlying molecular mechanisms. On the other hand, techniques such as fiber photometry or calcium imaging, which are now more and more used *in vivo* and even in freely-moving rodents, give more precise information at cellular and cell-specific levels. Finally, the use of functional magnetic resonance imaging (fMRI) or diffusion tensor imaging could give a more global insight in brain pathway alterations in CPED. Such non-invasive approaches allow for longitudinal studies and offer promising perspectives when considering that temporality is crucial when it comes to chronic pain and its affective consequences.

We believe that the leap forward in novel technics and their combination with more classical approaches will lead to a better understanding of the circuits underlying the anxiodepressive disorders-pain dyad.

Acknowledgments

This work was supported by the Fondation de France (N° Engt: 00081244), the Fondation pour la Recherche Médicale (“Équipe FRM”, EQU201903007809; and ARF20160936006, PEL), the Fondation Deniker (“Bourses 2016”, PEL) and the French National Research Agencythrough the Programme d’Investissement d’Avenir (under the contract “ANR-17-EURE-0022”).

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IV. Circuitry of the ACC in the comorbidity of chronic pain and mood disorders: Special focus on the basolateral amygdala.

Several brain structures play a role in both chronic pain and depression and for some of them, their implication in the comorbidity between these two pathologies has already been demonstrated (Bravo et al., 2020; Humo et al., 2019). Studies in human frequently highlight structural or functional alterations of the PFC, the hippocampus, the amygdala or the NAc in pain (Apkarian et al., 2005; Kregel et al., 2015), depression (Drevets, Price, et al., 2008) and in their comorbidity (Doan et al., 2015). In rodents, similar alterations are found (Bravo et al., 2020; Da Silva & Seminowicz, 2019; Humo et al., 2019; Ménard et al., 2016; Tan & Kuner, 2021; Yalcin et al., 2014) and the recent implementation of techniques such as opto- or chemogenetics brings out the proof of a causal relationship between the activities of these structures and depression- or pain-related behaviors. Among different brain structures, our group (Barthas et al., 2015, 2017; Sellmeijer et al., 2018) and others (Bliss et al., 2016; Koga et al., 2015; Zhuo, 2016) suggested that the ACC can represent one of the critical brain structures for depression and particularly when it is comorbid with chronic pain.

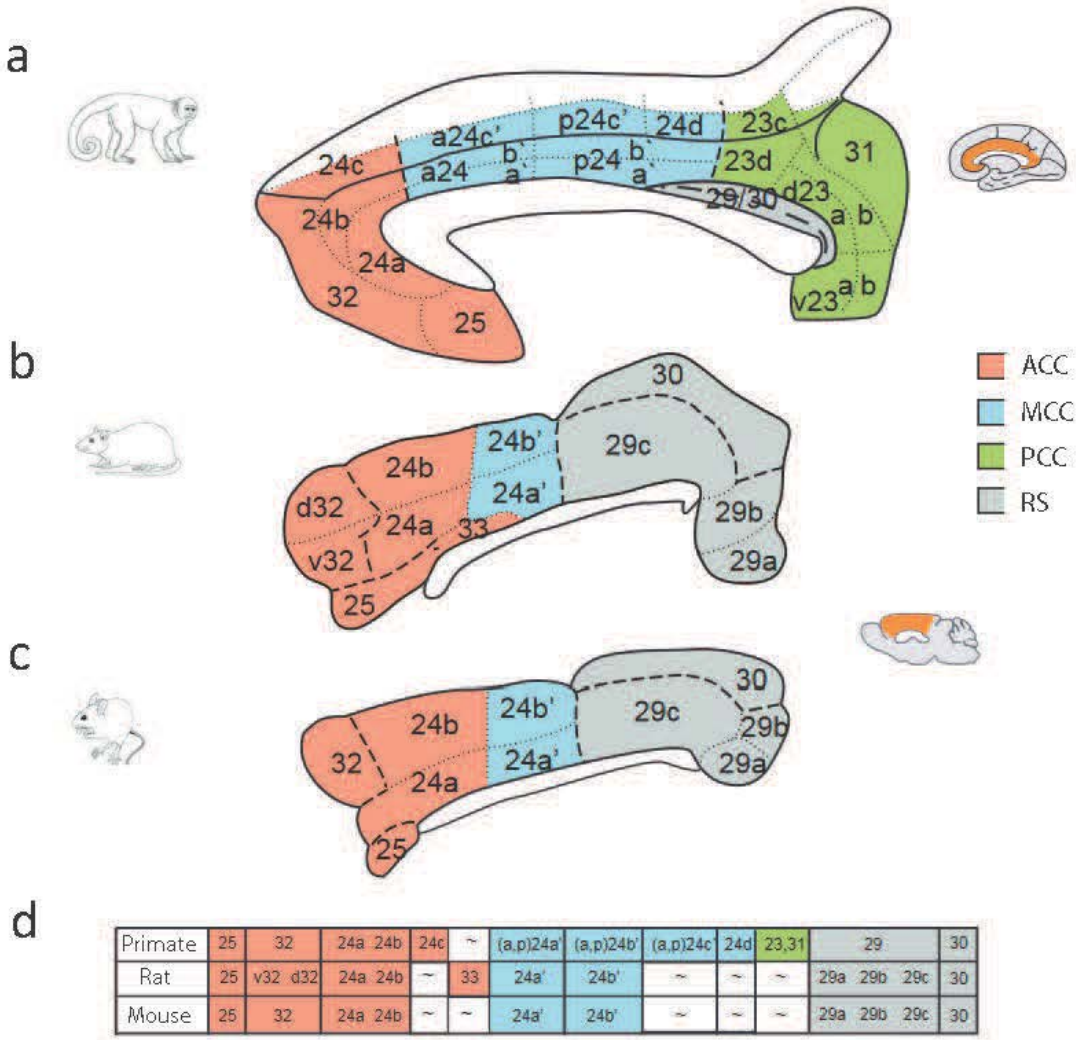
1. The Anterior Cingulate Cortex

i. *Organization*

a. Localization and nomenclature

The ACC corresponds to the most rostral part of the cingulate cortex. Despite being well conserved between rodents and primates, the cingulate area presents minor differences in these two species in terms of organization and nomenclature that can lead to confusion when comparing both species (**Figure 6**).

In primates, the cingulate cortex belongs to the medial wall of the prefrontal cortex, spanning from the supracallosal to the cingulate sulcus. According to the anteroposterior axis, four subregions including the anterior (ACC), the middle (MCC) and the posterior (PCC) cingulate cortices as well as the retrosplenial (RS) cortex compose this sulcus (Vogt, 2005; Vogt & Paxinos, 2014; Vogt BA, 2009). Each region displays a unique pattern of connectivity and is involved in different functions. In rodents, the cingulate cortex is a medial region localized above the corpus callosum. Based on cytoarchitectural and neurochemical observations, similar organization of the primates ACC, MCC and RSC has been described but the PCC is



Primate	25	32	24a 24b	24c	~	(a,p)24a'	(a,p)24b'	(a,p)24c	24d	23,31	29	30
Rat	25	v32 d32	24a 24b	~	33	24a'	24b'	~	~	~	29a 29b 29c	30
Mouse	25	32	24a 24b	~	~	24a'	24b'	~	~	~	29a 29b 29c	30

Figure 6: Organization of the cingulate cortex in primates and rodent

Schematic representation of the areas of the cingulate cortex in primate (a), rat (b), mouse (c) and the corresponding nomenclature (d).

ACC: anterior cingulate cortex, MCC: midcingulate cortex, PCC: posterior cingulate cortex, RS: retrosplenial cortex

Adapted from: Vogt and Paxinos 2014

missing. Regarding the ACC, it is composed of Broadmann's areas 24, 25, 32 and 33 in both primates and rats, mice only displaying areas 24, 25 and 32. The ACC is sometimes divided between the subgenual (sACC) (areas 25, s32 and s24) and pregenual (pACC) parts (areas 32, 24 and 33). This nomenclature is however not common in rodents and mostly used in human neuroimaging studies. Noteworthy, in rodents, the areas 32 and 25 are often referred to as the prelimbic (PrL) and infralimbic (IL) cortices respectively.

Until the 4th edition of the Paxinos and Franklin's the Mouse Brain Stereotaxic Coordinates (2012), the different nomenclatures used to describe the ACC in rodents made the comparison with primates confusing. In the previous stereotaxic atlases of the rat and the mouse brain (Franklin KBJ & Paxinos G, 2007; Paxinos G & Watson A, 2007), areas 24b/24b' and 24a/24a' were respectively labeled cingulate cortex Cg1 (dorsal) and Cg2 (ventral) according to Zilles and Wree (Van De Werd & Uylings, 2014; Zilles K & Wree A, 1995). Thus, the term "anterior cingulate cortex" has been often employed to refer to Cg1 and Cg2, even if areas 24a' and 24b' actually belong to the MCC, not to the ACC (Vogt & Paxinos, 2014). A second major source of confusion is due to the concept of medial prefrontal cortex in rodents (mPFC), constituted by the IL, PrL, "anterior cingulate cortex" (Cg or ACC) and the secondary motor cortex (M2) (Heidbreder & Groenewegen, 2003; Van Eden & Uylings, 1985). Since the IL and the PrL actually correspond to ACC areas 25 and 32 respectively (Vogt & Paxinos, 2014), the concept of four structures constituting the mPFC seems wrong.

To overcome these confusions and to make easier the inter-species comparison, the latest revisions of the rat (Paxinos G & Watson A, 2014) and mouse (Paxinos G & Franklin KBJ, 2012) brain atlases replaced the terms IL, PrL, Cg1 and Cg2 with their corresponding Brodmanns' area and we will employ this latest version in this thesis. Indeed, the ACC that we will describe in this thesis manuscript will refer solely to areas 24a and 24b.

b. Cytoarchitecture and neurochemistry

The ACC has the classical layer organization of a cortical structure but lacks the layer IV and is thus classified as an agranular cortex (**Figure 7**). The region 24 has higher neuronal density and bigger neuronal size compared to its neighboring areas (Van De Werd et al., 2010; Vogt, 2016; Vogt & Paxinos, 2014; Vogt BA, 2015). The areas 24a and 24b also present some differences. Notably, 24b has less dense layer III and a bigger layer V compared to 24a (Vogt

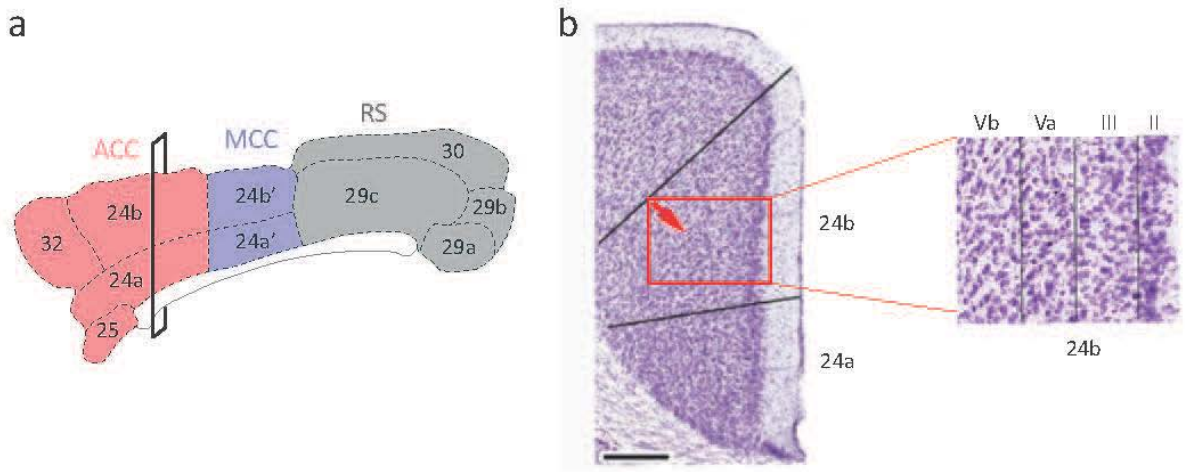


Figure 7: Cytoarchitecture of the mouse ACC

(a) Map showing the level of the section
(b) Nissl section of areas 24a and 24b with a magnified picture of the different layer of 24b. The red arrow indicate the layer Va, containing the largest neurons.

Adapted from Vogt and Paxinos 2014

& Paxinos, 2014; Vogt BA, 2015). As for the other part of the cortex (Gabbott et al., 1997; Markram et al., 2004; Vogt & Peters, 1981; Vogt BA, 2015; Zilles K & Amunts K, 2012), pyramidal cells are the main cells of the ACC. They are found in the totality of the areas 24a and b with a much higher density in layers III, V and VI compared to layers I and II. GABAergic interneurons represent one quarter of the total number of neurons (Zilles K & Amunts K, 2012) and are principally found in layer III and V (Gabbott et al., 1997; Kubota et al., 1994). While several subtypes of GABAergic neurons have been determined in the ACC, the parvalbumine (PV) is the most abundant one. Among the other types of GABAergic interneurons, calretinine, calbindine, VIP or somatostatin (SST) expressing cells have been identified (Ährlund-Richter et al., 2019; Tremblay et al., 2016). These diverse interneurons regulate pyramidal cell firing, participate in cortical rhythms or maintain the excitatory-inhibitory balance. More importantly, they play a crucial role in establishing the feed-forward inhibition. Indeed, excitatory entries generally project on both pyramidal cells and GABAergic cells in the cortex, leading to rapid inhibition of pyramidal neurons following excitatory stimulation and preventing their saturation (Tremblay et al., 2016). The loss of feed-forward inhibition stands as a potential mechanism to explain the hyperactivity observed in mental disorders (Czéh et al., 2018; Saffari et al., 2019). Additionally, the different subtypes of interneurons can trigger disinhibtion of the pyramidal cells through their connections between each other. This disinhibition phenomenon can arise from inhibition of the SST, by PV interneurons (Ährlund-Richter et al., 2019) or by the inhibition of PV, by SST interneurons (**Figure 8**). The latter presumably mediates fear expression (Cummings & Clem, 2020).

c. Connectivity

The anterior cingulate cortex, convergent point in the brain, receives and/or sends projection within almost all divisions of the forebrain and within some regions of the brainstem and the spinal cord (**Figure 9**). The cingulate area forms reciprocal connections with the thalamus and those connections historically served to define the subdivisions of the cingulate area. Indeed, a rostro-caudal organization of thalamic afferents differentiates the anterior from the posterior part. The former being preferentially connected with the mediodorsal nuclei (MD) while the latter connects with the anteroventral and laterodorsal nuclei (Domesick, 1969; Krettek & Price, 1977). This projection from the MD drives feed forward inhibition in the ACC

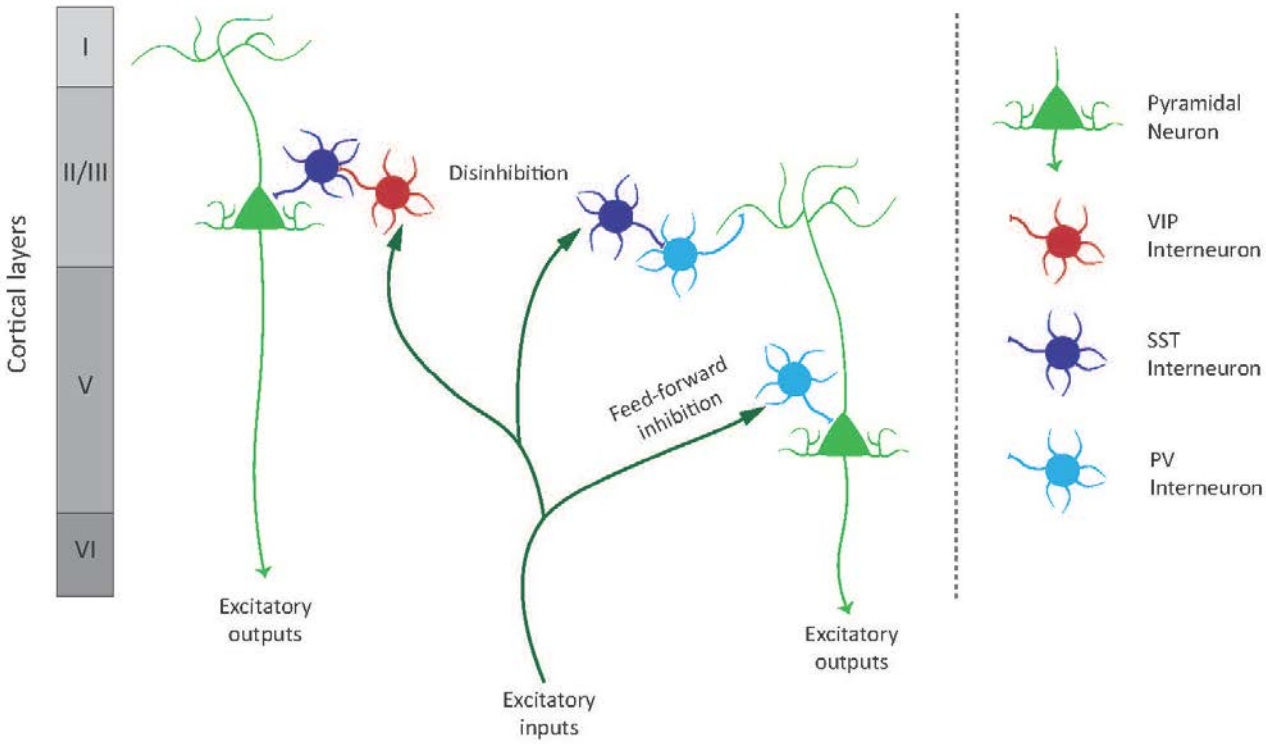


Figure 8: Feed-forward inhibition and disinhibition in the mPFC

In the mPFC, PV interneurons are mainly responsible for feed-forward inhibition while SST and VIP interneurons drive disinhibition of pyramidal cells.

PV: parvalbumine; SST: somatostatin; VIP: vaso-intestinal peptide.

(Delevich et al., 2015) and is involved in chronic pain-induced aversion (Meda et al., 2019). Beside the MD, the ACC also receives projections from lateral, intralaminar and midline (reuniens and rhomboid) nuclei (Hoover & Vertes, 2012; Vertes, 2002). The latter likely acts as a relay between the ACC and the HPC to mediate mnesic functions (Vertes, 2006). Most of these thalamic connections are reciprocal and mainly ipsilateral (Arikuni et al., 1994). Although being dense, the connections with the thalamus do not compose the main inputs and outputs of the ACC since the most important projections are to and come from the other parts of the cingulate cortex (Fillinger et al., 2017, 2018; Jones et al., 2005; Shibata & Naito, 2008). Therefore, the ACC receives afferents from the RS, the area 32 and from the MCC (area 24a' to area 24b). In return, the ACC projects back to the RS, the MCC and massively toward the 32 area (Filinger et al., 2017, 2018). Regarding the connectivity with the rest of the neocortex, the ACC receives major inputs from the orbitofrontal cortex, the associative parietal cortex and the secondary visual cortex. Minor reciprocal connections include the insula, primary visual, sensory and auditory cortices (Heidbreder & Groenewegen, 2003; Hoover & Vertes, 2007; Jones et al., 2005; Zingg et al., 2014). Considering the rest of the central nervous system, the ACC displays reciprocal connection with a large amount of structures among which we can find the claustrum, the hypothalamus, the LC, the ventral tegmental area, the substantia nigra pars compacta or the raphe nucleus (dorsal and medial part). Non-reciprocal connections include afferent from the hippocampus and efferent to the lateral habenula, the PAG and the spinal cord (Fillinger et al., 2017, 2018). Finally, the ACC can be distinguished from the rest of the cingulate complex by its strong and reciprocal connection with the anterior part of the basolateral nucleus of the amygdala (BLA) (Fillinger et al., 2017, 2018) another structure widely involved in emotional processing (J. E. LeDoux, 1995, 2000).

ii. *Physiological function*

Historically, studies in humans focusing on ACC lesions strongly associated the ACC with affect. Indeed, analysis of the behavioral and physiological impact of tumor in the ACC allowed Papez to link the ACC with emotional regulation (Papez, 1995). In addition, subjects treated for anxiety or depression by cingulotomy, could still report negative emotions that did not bother them anymore (Tow & Whitty, 1953). Likewise, in chronic pain, ACC ablation does not alleviate pain, but patients perceive it as less unpleasant (Foltz & White, 1962). Cingulotomy also

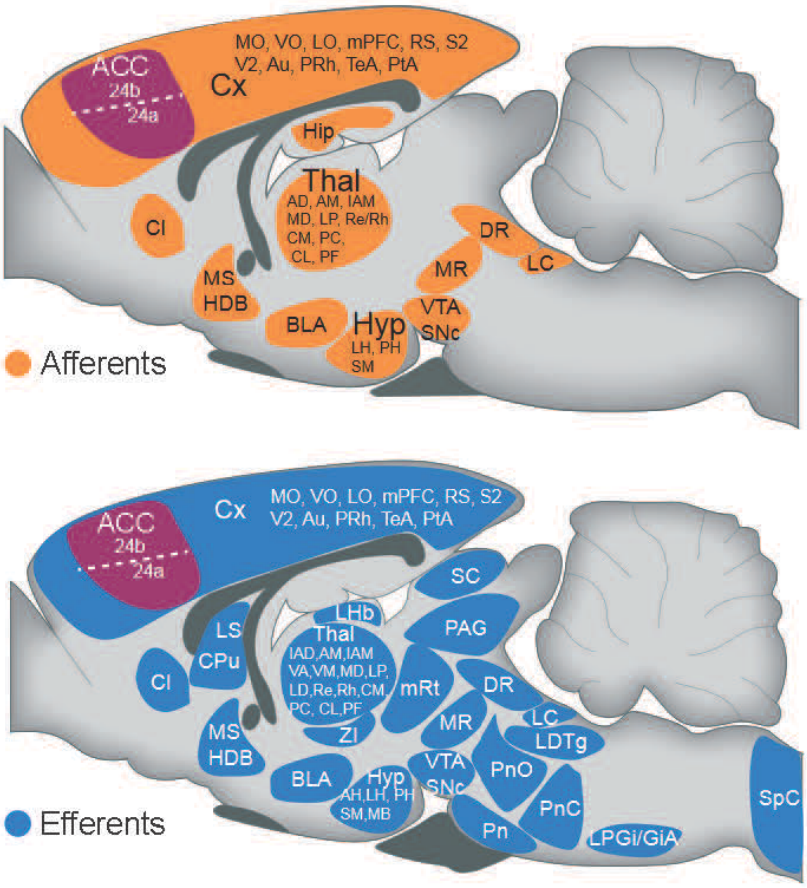


Figure 9: Afferents and efferents of the areas 24a and 24b in mice

Schematic representation of the afferents (orange) and efferents (blue) of the anterior cingulate cortex in mice

ACC: anterior cingulate cortex; AD: anterodorsal thalamic nucleus; AH: anterior hypothalamic area; AM: anteromedial thalamic nucleus; Au: primary auditory cortex; BLA: basolateral amygdala; CL centrolateral thalamic nucleus; Cl: claustrum; CM: central medial thalamic nucleus; Cpu: caudate putamen; Cx: cortex; DR: dorsal raphe nucleus; GiA: gigantocellular reticular nucleus alpha part; HDB: diagonal band of Broca , horizontal limb; Hip: hippocampus; Hyp: hypothalamus; IAD, interanterodorsal thalamic nucleus; IAM: interanteromedial thalamic nucleus, LC: locus coeruleus; LD: laterodorsal thalamic nucleus; LDTg; laterodorsal tegmental nucleus; LH: lateral hypothalamic area; LHb: lateral habenula; LO: lateral orbital cortex; LP: lateral posterior thalamic nucleus; LPGi: lateral paragigantocellular nucleus; LS: lateral spetal nucleus; MB: mammillary bodies; MD: mediodorsal thalamic nucleus; MO: medial orbital cortex; mPFC: medial prefrontal cortex; MR: mesencephalic reticular formation; mRt: mesencephalic reticular formation/ MS: medial septal nucleus; PAG: periaqueductal gray; PC: paracentral thalamic nucleus; PF: parafascicular thalamic nucleus; PH: posterior hypothalamic nucleus; Pn: pontine nucleus; PnC: pontine reticular nucleus; caudal part, PnO: pontine reticular nucleus, oral part; PRh: perirhinal cortex; PtA: parietal associative cortex; Re/Rh: reuniens/rhomboid thalamic nuclei; RS: retrosplenial cortex; S2: secondary somatosensory cortex; SC: superior colliculus; SM: stria medullaris; SNc: substancia nigra, pars compacta; SpC: superior cerebellar peduncle; TeA: temporal association cortex; Thal: thalamus; V2: secondary visual cortex; VA: ventral anterior thalamic nucleus; VM: ventromedial thalamic nucleus; VO: ventral orbital cortex; VTA: ventral tegmental area; ZI: zona incerta.

Fillinger et al., 2017 & 2018

revealed other functions of the ACC such as attentional, mnesic or autonomic functions. Indeed, patients who underwent a cingulotomy encountered attention deficit, urinary disturbance and reported memory lost (Dougherty et al., 2002; Ochsner et al., 2001). More recent studies linked the ACC with empathy (Bernhardt & Singer, 2012; Lockwood, 2016). Therefore, the perception of somebody receiving a reward (Lockwood, 2016) or experiencing pain (Lamm et al., 2011) results in an increased activity of the ACC. This involvement of the ACC in empathy exists in rodents as well, since *in vivo* electrophysiology revealed an increased activity of mirror-like neurons in the ACC in response to the distress of conspecific experiencing pain or fear (Carrillo et al., 2019). The rodent ACC also plays an important role in recall of ancient memory (Aceti et al., 2015; Frankland et al., 2004; Goshen et al., 2011), as well as in associative (Han et al., 2003) and spatial memory (Weible et al., 2009, 2012) but not in working memory (DeCoteau et al., 1997; Ragozzino et al., 1998; St-Laurent et al., 2009). Besides, Zhong and collaborators unraveled the implication of the ACC in decision-making using a social food-foraging paradigm. Animals have the choice between two types of food, one being more palatable than the other, and a resident rat is placed either near the sweet or the standard food. While sham animals tend to prefer foraging next to the resident rat, independently of the palatability of the food, rat with ACC lesion systematically choose to forage away from the resident. These behavioral changes do not seem to be imputable to a deficit in social behaviors since no effect of the lesion was observed in a social interaction test (Zhong et al., 2017). Sustained visual and olfactory attention also depend on the activity of the ACC (Wu et al., 2017) and more particularly rely on fast-spiking parvalbumine neurons (FS-PV). Indeed, those FS-PV neurons exhibit a strong synchronization when attention is successfully directed and their optogenetic inhibition impaired attentional processing (H. Kim et al., 2016).

iii. *Pathological implication in emotions and pain*

Due to its connectivity with other cortical and subcortical regions, the ACC represents a major hub in the neuronal system responsible for mood and emotional regulation. The pACC and sACC were therefore extensively linked with negative mood and psychiatric diseases such as depression, anxiety, PTSD or panic disorder (Drevets, Price, et al., 2008; Drevets, Savitz, et al., 2008; Shin & Liberzon, 2010). Noteworthy, it has been reported that some patients suffering from depression have a smaller sACC volume. Different studies suggest that this volume loss

could be imputed to a loss of glial cells (Cotter et al., 2001; Si et al., 2004) and particularly of oligodendrocytes (Aston et al., 2005; Hamidi et al., 2004; Hercher et al., 2009). Moreover, a similar size reduction has been described in bipolar disorder (Adler et al., 2004; Brambilla et al., 2004; Lochhead et al., 2004; Sassi et al., 2004; Wilke et al., 2004), post-traumatic stress disorder (Kitayama et al., 2006), schizophrenia (Stark et al., 2004) and anxiety disorders with or without MDD comorbidity (van Tol et al., 2010). Those size reductions appear to be more important in patients with a familial history of mood disorders (Hirayasu et al., 1999). In addition to this glial loss, a decreased expression of the connexin forming the gap-junction between astrocytes and OL is associated with depression and suicide, suggesting an impairment of the coupling between those glial cells in the ACC (Tanti et al., 2019). These observations suggest not only an important role of the sACC in emotion and its implication in mental disorders but also that genetic alterations of this brain region might be a risk factor for psychiatric disorders. Furthermore, the majority of neuroimaging studies (MRI, PET) in humans reports hyperactivity of the ACC in depressed patients (Arnone, 2019; Drevets, Savitz, et al., 2008; Mayberg et al., 1997; Morris et al., 2020; Pizzagalli et al., 2001). However, some studies highlight hypoactivity of the ACC (Gonul et al., 2004; Oda et al., 2003). These discrepancies could be due to the heterogenetiy of etiologies of depression, the presence or absence of familial history or the severity of depression (Holtzheimer PE & Mayberg HS, 2009). In the context of chronic pain, the ACC also displays a decrease of volume as well as hyperactivity (Bushnell et al., 2013). These studies confirmed that ACC dysregulation is implicated in psychiatric disorders and chronic pain and made this structure a key target for treatment development. Indeed, deep brain stimulation of the sACC showed antidepressant effect in treatment-resistant patients (Johansen-Berg et al., 2008; Mayberg et al., 2005) and attenuated pain sensation in chronic pain patients (Boccard et al., 2017).

Based on these observations, our team started to investigate the contribution of the ACC in chronic pain-induced depression (CPID) more than five years ago (Barthas et al., 2015). Using lesion approaches, Barthas and collaborators showed that the ACC was necessary for the development of the anxiodepressive consequences of neuropathic pain. Moreover, they were able to induce sustained anxiety- and depressive-like behaviors in naive mice after repeated optogenetic activation of the pyramidal neurons in the ACC. Two years later, using models of

depression induced by chronic mild stress, chronic pain and optogenetic stimulation, they uncovered an overexpression of the mitogen-activated protein kinase phosphatase-1 (MKP-1) in the ACC and showed that silencing of MKP-1 in the ACC was sufficient to attenuate depressive-like behaviors induced by chronic pain (Barthas et al., 2017). Further *in vivo* electrophysiological recording highlighted a hyperactivity of the ACC that followed the course and development of the affective consequences of chronic pain (Sellmeijer et al., 2018). Altogether, the results obtained by our team so far pinpoint the crucial role of the ACC in mood disorders, pain and their comorbidity. Nonetheless, such pathologies most likely rely on the dysfunction of several brain regions and of the circuits that interconnect them. Therefore, the next step was to map the connectome of the ACC in mice. This work, done in the team by Dr. Fillinger, unraveled strong and reciprocal connection between the ACC and the anterior part of the basolateral nucleus of the amygdala (BLA) (Fillinger et al., 2017, 2018), a structure widely involved in anxiety and mood disorders as well as in pain (Janak & Tye, 2015; Kiritoshi & Neugebauer, 2018; J. M. Thompson & Neugebauer, 2019; Tye et al., 2011). In addition, unpublished rs-fMRI recording done in our model of CPID highlighted an alteration in functional connectivity between the ACC and the BLA (Karatas, unpublished).

2. The basolateral amygdala

i. *General organization*

The basolateral amygdala is part of a heterogeneous group of nuclei in the temporal lobe of mammals called the amygdala because of its almond shape (Burdach KF, 1819) (**Figure 10**). It is composed of a dozen of sub-nuclei divided in two categories. The first one or sub-pallial amygdala, encompassing the central and medial nuclei, has a striatal organization, with notably GABAergic projecting cells. Conversely, the second group of nuclei constituting the cortical or pallial amygdala comprises the superficial, lateral, basomedial and basolateral nuclei and has a cortical-like organization (GF Alheid et al., 1995; Olucha-Bordonau FE et al., 2015; Swanson & Petrovich, 1998). The primary type of cells is considered as a pyramidal type with glutamatergic projection neurons. This second group of nuclei also expresses local inhibitory interneurons as diverse as in the cortex, such as PV, calbindin, SST or VIP interneurons (Arruda-Carvalho & Clem, 2015).

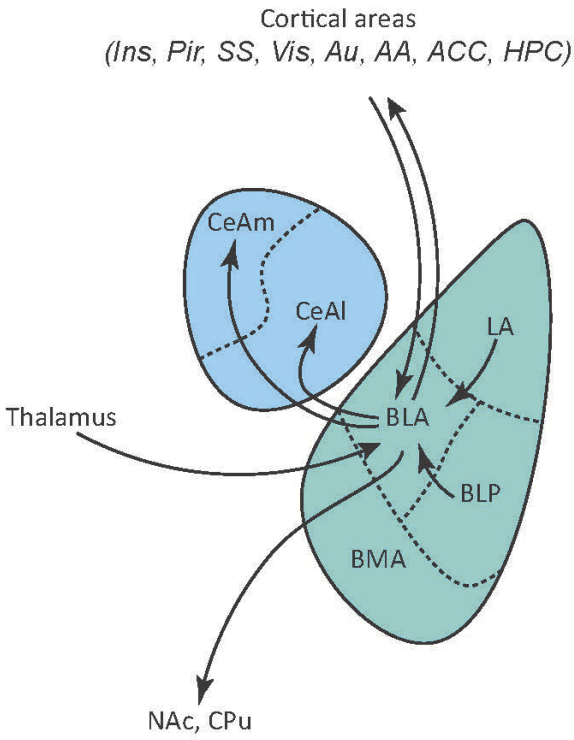


Figure 10: Organization of the amygdala and BLA connectivity

Schematic representation of the main ouputs and inputs of the BLA

AA: associative areas; ACC: anterior cingulate cortex; Au: auditory cortex, BLA: anterior part of the basolateral amygdala; BLP: posterior part of the basolateral amygdala; BMA: basomedial nucleus of the amygdala; CeAl: centrolateral amygdala; CeAm: centromedial amygdala; CPu: caudate putamen; HPC: hippocampus; Ins: insula; LA: lateral nucleus of the amygdala; NAc: nucleus accumbens; Pir: piriform cortex; SS: somatosensory areas; Vis: visual acortex.

The basolateral nucleus is divided into anterior (BLA) and posterior (BLP) part based on different cell-types and connectivity pattern (J. Kim et al., 2017; Olucha-Bordonau FE et al., 2015). The BLA receives inputs from other parts of the cortical amygdala, particularly from the BLP and sends projection to the central and medial amygdala. Due to its cortex-like organization, the BLA receives major inputs from the cortical regions (insular and piriform cortex, somatosensory, visual, auditory and associative areas, hippocampus, orbitofrontal cortex, ACC) and the thalamus and sends projections to the striatum (caude putamen, lateral nucleus accumbens) and the cortex (GF Alheid et al., 1995; McDonald, 1998; Olucha-Bordonau FE et al., 2015; Turner & Herkenham, 1991). Among its cortical projections, the most massive ones target the perirhinal cortex, the insula and the ACC.

ii. *Functional implication*

a. Physiological function

The BLA, and by extension the amygdala, has been extensively studied for its implication in fear acquisition, expression and extinction (Garcia et al., 1999; Sah, 2017; Sun et al., 2020, p. 201). The first observation of amygdala lesions in monkey highlighted loss of emotion of fear and anger (Kluver H & Bucy PC, 1937; Weiskrantz, 1956). These impairments were later observed in human (Adolphs et al., 1994, 2005). In animals, fear is often evaluated through paradigm of fear conditioning in which an unconditioned stimulus (US, usually a foot-shock) is paired with a conditioned stimulus (CS, such as light or sound). Because of its connection with sensory, auditory and visual cortices, the BLA has a crucial role in the integration of the association between US and CS (Tovote et al., 2015). Thus, and not surprisingly, the lesion of the entire BLA compromises the acquisition of the fear memory (Kochli et al., 2015). At the cellular level, *in vivo* electrophysiological recordings highlighted the existence of two distinct populations of projection neurons (PNs) in the BLA, one responding to the CS during the conditioning and the other during the extinction phase (Herry et al., 2008). In addition, PNs activity during fear conditioning is regulated by a circuit of interneurons. Indeed, following auditory CS, PV interneurons of the BLA are activated. This results in an inhibition of neighboring SOM interneurons, leading *in fine* to the disinhibition of PNs and enhancement of US-CS association (Wolff et al., 2014).

The BLA is also important in memory, even though it is not a locus for memory *per se* (McGaugh, 2004). While the pharmacological blockade (lidocaine) of the amygdala did not affect rats' performances in the Morris water paradigm, its activation by an infusion of amphetamine enhanced memory consolidation (Packard & Teather, 1998). The role of BLA in memory is stronger when memories possess an emotional value. Therefore, emotional memory acquisition is linked with synaptic plasticity changes at the level of the BLA, its consolidation involves cholinergic, noradrenergic and serotoninergic release and the extinction phase depends on the loss of excitatory response to CS (Bocchio et al., 2017). More generally, the BLA is involved in the attribution of a negative valence to stimuli (Beyeler et al., 2016; Wassum & Izquierdo, 2015). Indeed, while rewarding stimuli (like water, sugar) activate protein phosphatase 1 regulatory inhibitor subunit 1B neurons (Ppp1r1b+) in the BLP, negative stimuli (like foot shock) activate R-spondin 2 (Rspo+) neurons in the BLA. These two populations also target distinct output regions, the Rspo+ neurons project to the capsular nucleus of the central amygdala and the PrL and Ppp1r1b+ neurons project to the medial and lateral nucleus of the central amygdala and to the IL (H. Kim et al., 2016). The BLA is also involved in reward and appetitive behavior (J. Kim et al., 2017; Wassum & Izquierdo, 2015), notably through its connection with the NAc (Hsu et al., 2020). In addition, circuit from the BLA to the CeA positively or negatively drives appetitive behaviors through the ppp1rb1+ and rspo+ populations (J. Kim et al., 2017).

Because of its implication in memory, reward and emotion, particularly in fear, it is not surprising that pathologies affecting these processes, such as post-traumatic stress disorder, anxiety or depression involve the BLA.

b. Implication of the BLA in emotional disorders and pain

The amygdala plays a major role in emotional disorders. In response to aversive stimuli, the amygdala presents a hyperexcitability in patients suffering from anxiety, which is reversed by cognitive behavioral therapy (Babaev et al., 2018). A disruption in the functional connectivity of the amygdala, notably with the prefrontal cortex, insula and cerebellum, is also reported in adults and adolescents with generalized anxiety disorders (Roy et al., 2013). However, discrepancies between the CeA and BLA still exist since the functional connectivity changes are more related to the mPFC in the case of the BLA and to the midbrain and cerebellum for

the central amygdala (Etkin et al., 2009). In war veteran who developed PTSD, the presentation of trauma-related cues (sounds, smells or pictures) induces an increased activation of the amygdala (Liberzon et al., 1999; Vermetten et al., 2007). Regarding depression, results on amygdala are not so consistent since increase as well as decrease of amygdala volume are observed. One of the hypotheses explaining these discrepancies could be the absence or presence of medication. Indeed, one meta-analysis reported that medicated depressed subjects show an increase in amygdala size, while non-medicated patients present a decreased volume (Hamilton & Gotlib, 2008). Another explanation could arise from the spatial resolution of MRI studies in human. When looking at the entire amygdala no significant changes in volume are associated with depression but more precise analysis suggest that the volumetric changes are restricted to the basolateral nucleus (Tamburo et al., 2009). At the molecular level, patients with a history of depressive disorders present a dysregulation of synaptic plasticity related genes in the BLA. The magnitude of this dysregulation was associated with a greater risk for suicide (Maheu et al., 2013). Additionally, the BLA displays a molecular blueprint of depression in human and is shared with mice subjected to a paradigm of unpredictable chronic mild stress (Sibille et al., 2009). In rats, stressful situations provoke an increase of the glutamate release in the BLA, which is reversed by antidepressant administration (Reznikov et al., 2007). Stress also induces an alteration in the dendritic arborization of BLA neurons (Grillo et al., 2015; Yi et al., 2017). Interestingly, these changes seem to be dependent of the properties of stressors. Thus, pyramidal neurons exhibit dendritic hypertrophy or hypotrophy depending on the acute, repeated, or chronic nature of the stimuli and of its predictability (Wilson et al., 2015). This dendritic remodeling is not specific to anxiety and is encountered in models of depression as well (Yi et al., 2017). As mentioned before, beside stress, chronic pain is a major factor for the emergence of anxiety and mood disorders (Arnow et al., 2006; Gallagher & Verma, 1999). The BLA possesses an ensemble of pyramidal neurons that encode the negative valence of nociceptive stimuli. When inhibiting this ensemble in a model of peripheral neuropathy, animals do not seek to avoid noxious stimulation although they still elicit nociceptive behaviors (Corder et al., 2019). This effect is potentially mediated by the projections from the BLA to the ACC since activation or inhibition of this connection respectively suppresses or enhances the pain-induced aversion in a model of SNI (Meda et al., 2019). In the light of these results, we thus hypothesize that

the input from the BLA to the ACC is a key actor of depression and its comorbidity with chronic pain.

3. The BLA-ACC pathway

There are few studies directly investigating the functional role of the projection from the BLA to the ACC in physiological or pathological conditions. In human, imaging studies only inform us on possible synchronization/desynchronization of activity, but it does not necessarily imply the involvement of the direct projection from the BLA to the ACC. Nonetheless, in physiological conditions, the BLA and the ACC are concomitantly recruited in situation of emotional conflict. Indeed, when asked to determine the emotion displayed in images of sad or happy faces indifferently labeled with the word “sad” or “happy”, healthy subjects display a strong anti-correlation activity of the BLA and the ACC in situations of incongruity (sad face with the word “happy” or the reverse association) (Etkin et al., 2006). In pathological conditions such as emotional disorders, the functional connectivity between the BLA and the ACC is strongly altered. For instance, war veterans suffering from PTSD exhibit an increased functional connectivity between BLA and sACC (Brown et al., 2014) and depression is generally associated with a decoupling of their functional connectivity (Workman et al., 2016). Experiments in rodents allow for more precise targeting of the direct connection between the BLA and the ACC. The inputs from the BLA to the ACC were thus involved in the formation and consolidation of fear memory in mice. Disruption of the activity of this connection, using high-frequency optogenetic stimulation, interferes with acquisition and retention of cue-associated response and facilitates the extinction of the fear-associated memory (Klavir et al., 2017). Besides, integrity of the BLA is necessary for the development of stress-induced changes in the ACC. Indeed, permanent (ibotenic acid) or temporary (lidocaine) inactivation of the BLA prevents the volumetric decrease of layer I and II, the astroglial loss and the increase in glucocorticoid receptors expression within the ACC following chronic immobilization stress (Tripathi et al., 2019a, 2019b, 2019c).

To apprehend how the BLA-ACC pathway can modulate emotion and mood, it is important to understand how these two structures interact with each other. The ACC receives direct and indirect (through the thalamus) inputs from the BLA. Despite the glutamatergic nature of these inputs, in physiological conditions the BLA exerts an inhibitory effect on the ACC. Indeed,

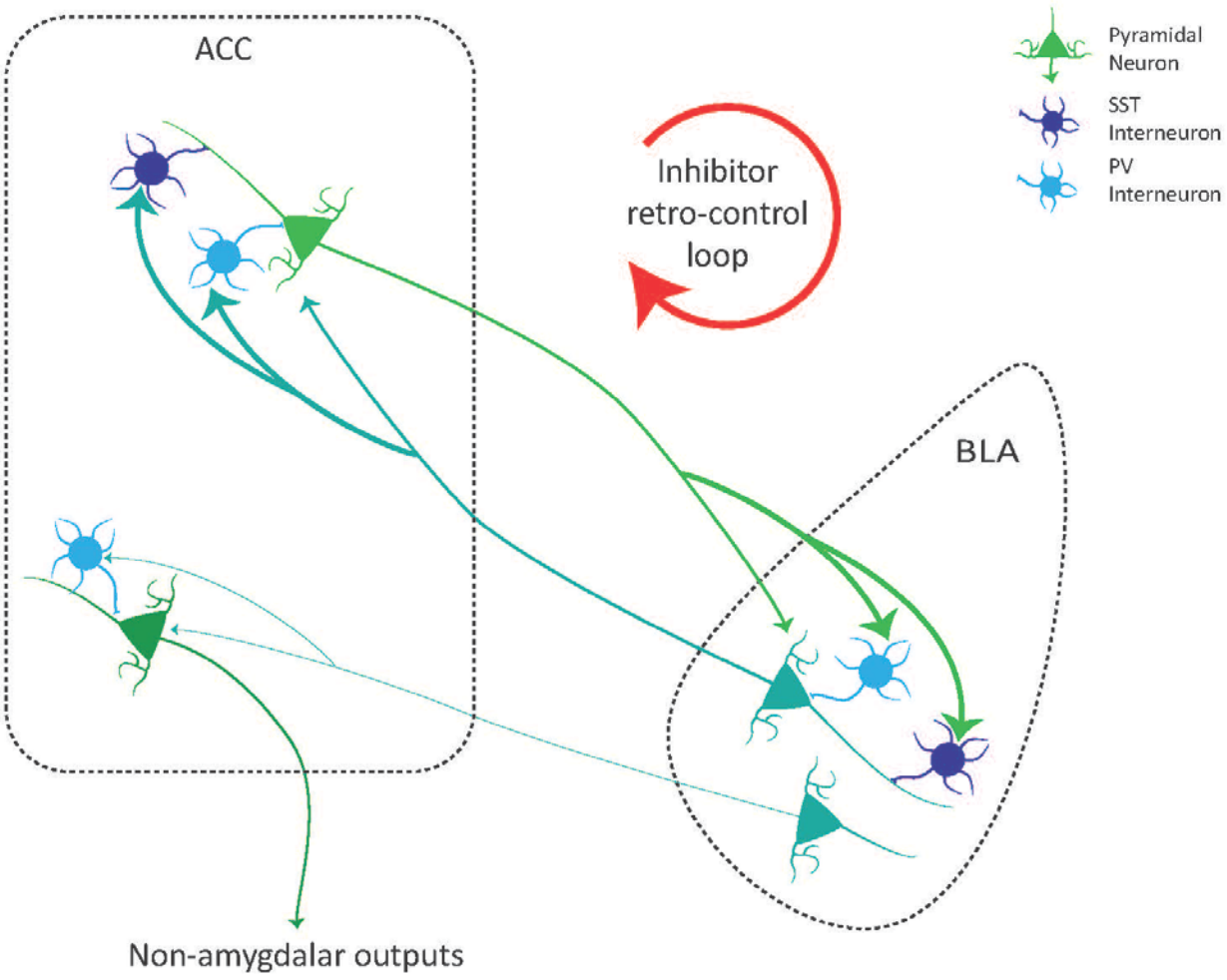


Figure 11: Organization of the connections between the BLA and the ACC

BLA projection neurons preferentially make synapses onto ACC neurons that project back to the BLA and mainly project to GABAergic interneurons which induces a feed-forward inhibition of the ACC by the BLA. Projection from the ACC to the BLA are similarly organized. In the end, in physiological condition the BLA and the ACC form an inhibitor retro-control loop.

ACC: anterior cingulate cortex; BLA: anterior part of the basolateral nucleus of the amygdala; PV: parvalbumine; SST: somatostatin.

even though synapses onto pyramidal neurons exist, the majority targets inhibitory interneurons in the cortex (Ährlund-Richter et al., 2019). *In fine*, the BLA induces a feed-forward inhibition of the ACC notably through PV (Dilgen et al., 2013) and SST interneurons (McGarry & Carter, 2016). The reverse projection from the ACC to the BLA is similarly organized, with pyramidal cells projecting on both interneurons and principal neurons of the BLA, resulting in an overall inhibition of the BLA by the ACC (Rosenkranz & Grace, 2002). Interestingly, BLA inputs present a bias toward ACC pyramidal cells projecting back to the BLA (Little & Carter, 2013; McGarry & Carter, 2016). This is also true for the terminals making synapses onto interneurons that preferentially target the interneurons inhibiting cortico-amygdalar projection neurons (McGarry & Carter, 2016). Therefore, in physiological condition, there is an inhibitor retro-control loop between the BLA and the ACC (**Figure 11**). As outlined by the group of Neugebauer, a loss of this inhibitory control of the BLA over the ACC could be a key mechanism in pathologies such as pain (Kiritoshi & Neugebauer, 2018; J. M. Thompson & Neugebauer, 2019). The same mechanism could be involved in depression as well, considering the already outlined disruption of ACC inhibition in depression (Czéh et al., 2018).

Results

My PhD project was constructed around three objectives:

1) determine the implication of the BLA-ACC pathway in the sensory and affective component of chronic pain

2) assess the impact of manipulating the activity of the BLA-ACC pathway on emotional processing in naïve conditions

3) decipher the underlying molecular mechanisms of depression by comparing transcriptomic data from mice and human.

The following section contains the draft of the scientific paper we are currently working on. It includes the detail of the materials and methods used in this project and all the results obtained so far and pertaining to the aforementioned goals of this project.

Title Page

A bottom-up pathway linking the amygdala to the cingulate cortex contributes to depression and its comorbidity with chronic pain

ABSTRACT

Major depressive disorder (MDD) is a chronic and debilitating disease with poor treatment outcomes. It is often comorbid with chronic pain, resulting in an increased disability and poorer prognosis than either condition alone. Evidence from animal models and clinical studies suggest a crucial role of the anterior cingulate cortex (ACC) and the anterior part of the basolateral amygdala (BLA) in this comorbidity. Here, we demonstrated, using retrograde tracing and the marker of neuronal activity c-fos, as well as rodent brain imaging, that the BLA-ACC pathway is hyperactive when chronic pain triggers depressive-like behaviors. Then, by optogenetically manipulating this circuit, we showed that its hyperactivity is necessary for pain-induced depressive-like behaviors, and is also sufficient to trigger similar deficits in naïve animals (ie, in the absence of chronic pain). We next characterized transcriptomic changes recruited in the ACC when BLA-ACC hyperactivity triggers mood dysregulation, and found that these changes strikingly resembled the molecular blueprint of depression in the human brain. These results, which notably include alterations affecting oligodendrocytes and myelination, establish the translational relevance of our BLA-ACC optogenetic paradigm. Overall, we uncovered a bottom-up pathway that appears critical for chronic pain and MDD comorbidity.

INTRODUCTION

Major depressive disorder (MDD) and chronic pain are long-lasting detrimental conditions that significantly contribute to the worldwide burden of disease (Bair et al., 2003; Rayner et al., 2016). These two pathologies are highly comorbid, which results in increased disability and poorer prognosis compared to each condition alone (Arnow et al., 2006; Gallagher & Verma, 1999). In addition, despite their high prevalence and co-occurrence, available treatments remain poorly efficient, underlining the urgent need for a better understanding of the pathophysiology of this comorbidity.

The frontal cortex, particularly the anterior cingulate cortex (ACC), is at the center of emotional and pain processing (Thompson & Neugebauer, 2019). Accordingly, a large body of evidence documents major alterations in ACC neuronal activity in patients with either chronic pain or MDD, as well as in rodent models for each condition (Apkarian et al., 2005; Drevets et al., 2008; Kummer et al., 2020; Xiao et al., 2021). Among other findings, we previously showed that a lesion (Barthas et al., 2015) or the optogenetic inhibition (Sellmeijer et al., 2018) of the ACC alleviates anxiodepressive-like consequences of neuropathic pain in the mouse, while, conversely, activation of this structure is sufficient to trigger similar emotional dysfunction in naïve animals. These results highlight the critical role of the ACC in pain and MDD comorbidity. However, the mechanisms that lead to such ACC dysfunction remain poorly understood.

MDD originates from alterations affecting both the subcortical processing of external and internal stimuli, and their integration into perceived emotions by higher-level cortical structures (LeDoux & Brown, 2017). It is, therefore, critical to understand how subcortical inputs contribute to the emergence of emotional states in the ACC. Among other afferences, the anterior part of the basolateral nucleus of the amygdala (BLA) shows dense, direct and reciprocal connections with the ACC (Fillinger et al., 2017, 2018), which have been surprisingly poorly studied in animal models of pain and mood. At physiological level, the BLA plays a critical role in emotional processes, since its neurons encode stimuli with a positive valence, such as rewards (Kesner et al., 1989; Namburi et al., 2015), as well as those with a negative valence, including fear (Namburi et al., 2015) or pain states (Veinante et al., 2013). In human, neuroimaging studies have consistently found that depressed patients (Drevets, 2003) and individuals with chronic pain (Simons et al., 2014) exhibit a pathological hyperactivity and increased functional connectivity of the BLA and ACC.

In this context, we postulated in the present work that the neuronal pathway linking the BLA

and the ACC might represent a core substrate of pain and MDD comorbidity. We first demonstrate, using retrograde tracing and the marker of neuronal activity c-fos, as well as rodent brain imaging, that this pathway is hyperactive when chronic pain triggers depressive-like behaviors. Then, by optogenetically manipulating this circuit, we show that its hyperactivity is necessary for pain-induced depressive-like behaviors, and is also sufficient to trigger similar deficits in naïve animals (ie, in the absence of chronic pain). Importantly, we next characterize transcriptomic changes recruited in the ACC when BLA-ACC hyperactivity triggers mood dysfunction, and find that they strikingly resemble the molecular blueprint of depression in the human brain. These results, which notably include alterations affecting oligodendrocytes and myelination, establish the translational relevance of our BLA-ACC optogenetic paradigm.

RESULTS

Chronic neuropathic pain induces hyperactivity in the BLA-ACC pathway. To first confirm and quantify the anatomical connection between the BLA and ACC, we conducted retrograde tracing experiments using the choleric toxin (CTb) injected in the ACC (**Fig. 1a**). Strongly labelled cell bodies were found in the BLA (**Fig. 1b**), consistent with previous reports (Ährlund-Richter et al., 2019; Fillinger et al., 2017; Hintiryan et al., 2021). Next, considering that we, and others (Kummer et al., 2020; Thompson & Neugebauer, 2019; Zhuo, 2016), have found that the ACC is hyperactive when chronic pain triggers depressive-like behaviors, we wondered whether the BLA is similarly affected. To test this, we quantified the immediate early gene, c-fos, in our well-characterized model of chronic pain induced-depression (CPID) (5), in which a peripheral nerve injury leads to immediate and long-lasting mechanical hypersensitivity (**Fig. 1d**), with delayed anxiodepressive-like behaviors (significant at 7 weeks post operation, PO; Splash Test: $p=0.0042$; Novelty suppressed feeding (NSF) test: $p=0.0089$; **Fig. 1e-f**). In our previous study, an increased c-fos immunoreactivity was found in the ACC at 8 weeks PO (Barthas et al., 2017). We conducted a second analysis on the same samples but at the level of the BLA and found an increase c- fos immunoreactivity as well (**Supplementary Fig. 1a-b**; $p<0.05$), indicating concurrent neuronal hyperactivity in the 2 structures when anxiodepressive-like behaviors are present. Next, we investigated whether these activated, c-fos positive cells in the BLA directly innervate the ACC (**Fig. 1g** & **Supplementary Fig.1c**). To do so, the retrograde tracer fluorogold (FG) was injected into the ACC of neuropathic animals, and we quantified its localization, and co-localization with c-fos, in the BLA at 8 weeks PO. Similar numbers of BLA neurons projecting to the ACC

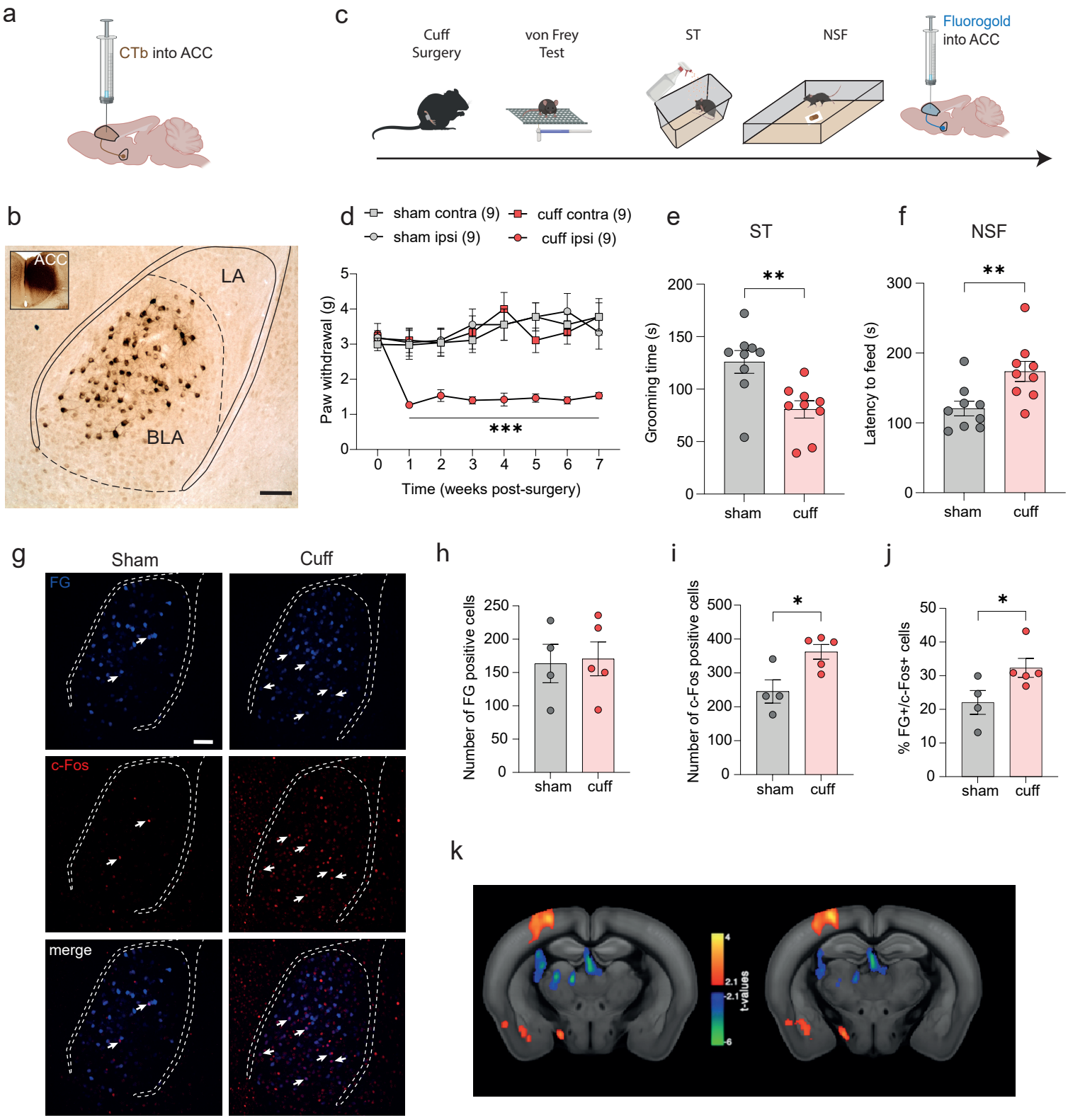


Figure 1. Chronic pain induced-depression (CPID) triggers hyperactivity of BLA neurons projecting to the ACC. a. Illustration of the retrograde tracing strategy, with the injection of the choleric toxin (CTb) in the mouse ACC. b. Representative image of cell bodies labelled in the BLA. c. Experimental strategy for quantification of neuronal hyperactivation of BLA neurons projecting to the ACC in the CPID model. Peripheral nerve injury is induced by implanting cuff around the main branch of sciatic nerve. Mechanical sensitivity is evaluated using von Frey filaments, and anxiodepressive-like behaviors using the splash test (ST) and novelty suppressed feeding test (NSF). Fluorogold is then injected in the ACC. d-f. Peripheral nerve injury induces an ipsilateral long-lasting mechanical hypersensitivity (d; sham: n=9; cuff: n=9; $F(21,224)= 2.710$; $p<0.0001$; post-hoc weeks 1-7 $p<0.05$), decreases grooming behavior in the splash test (e; sham: n=9; 125.90±10.80; cuff: n=9; 80.67±8.22; $p=0.0042$) and increases latency to feed in novelty suppressed feeding test (NSF) (f, sham: n=9; 120.7±10.49; cuff: n=9; 173.7±14.38; $p=0.0089$). g. Representative fluorescence images showing cells that are fluorogold positive (FG+, upper panel), c-fos positive (c-fos+, middle panel), or co-labelled (lower panel). h-j. Quantification of FG+, c-fos+ cells and their co-localization revealed that, 8 weeks after peripheral nerve injury, the number of FG+ cells was not altered (panel h; sham: 163.5±28.85; cuff: 170.6±25.43; $p= 0.3651$), while c-fos+ (panel i; sham: 245.5±34.37; cuff: 362.4±21.62; $p=0.0238$) and FG+/c-Fos+ cells (panel j; sham 22.06%±3.55; cuff: 32.32%±2.81; $p=0.0159$) were increased in the right BLA after fluorogold injection into the ACC (sham: n=4; cuff: n=5), k. At 8 weeks PO, ACC was more connected to amygdala (AMY). FWER corrected at cluster level for $p<0.05$. Data are represented as mean ± SEM. *$p<0.05$; **$p<0.01$. 2-Way ANOVA repeated measures (Time x Surgery) (VF); unpaired t-test (ST and NSF); one-tailed Mann-Whitney test (FG, c-fos quantification). Scale bar = 100µm. PWT, Paw withdrawal threshold.

(FG-positive) were found in both sham and CPID groups, indicating that neuropathic pain does not modify the number of neurons in this pathway (**Fig. 1h** & **Supplementary Fig. 1d**; right BLA p= 0.3651; left BLA: p=0.21). Importantly, an increase in c-fos labelling was observed in the right BLA of CPID mice compared to sham controls (**Fig. 1i**; p=0.0238), with a co-localization of FG+ and c-Fos+ cells stronger in the CPID group (**Fig. 1j**; p=0.0159). This increase is restricted to the BLA ipsilateral to the cuff surgery since no changes in c-fos immunoreactivity or FG co-localization was observed in the left BLA (**Supplementary Fig. 1e-f**). Such lateralization was already reported for the central amygdala in rodent models of chronic pain (Allen et al., 2021; Ji & Neugebauer, 2009). Since the central amygdala receives nociceptive inputs in part through the BLA (Morikawa et al., 2021) it is conceivable that the BLA is also differently affected depending on the considered side. Finally, as a complementary strategy to assess the modified coupling of the ACC and BLA, we took advantage of brain imaging data recently generated by our group using resting-state functional Magnetic Resonance Imaging (fMRI, unpublished). In cuff mice compared to sham controls, we observed enhanced functional connectivity between the BLA and the ACC at 8 weeks PO (**Fig. 1k**), again documenting a synchronized hyperactivity of these 2 structures. Altogether, these results converge to indicate that chronic neuropathic pain induces a hyperactivity of BLA neurons that project to the ACC. Next, we hypothesized that this hyperactivity may be responsible for emotional deficits observed in our CPID model.

***Optogenetic inhibition of the BLA-ACC pathway prevents CPID*.** To decipher the causal role of BLA-ACC hyperactivity, we inhibited this pathway using an optogenetic approach. An adeno-associated virus driving the expression of the archaerhodopsin ArchT3.0 under control of the CaMKIIa promoter (AAV5-CamKIIa-ArchT3.0-EYFP) was injected bilaterally in the BLA. To first characterize neuronal effects of green light illumination, we performed ex vivo electrophysiology 6 weeks following viral injection (**Fig. 2a**). Patch- clamp recordings in coronal sections at the level of the BLA showed that light stimulation drove a strong neuronal inhibition, as shown by outward currents recorded in voltage-clamp mode (**Fig. 2b**). These currents were proportional to the intensity of light stimulation, with a maximal effect reached around 80% of the maximal light intensity (**Fig. 2c**).

To assess behavioral effects of BLA-ACC inhibition in the CPID model, the same viral vector was then injected bilaterally in the BLA, followed by implantation of an optic fiber in the ACC in order to specifically inhibit local BLA axon terminals (**Fig. 2d-e**). We found that acutely, such an inhibition of the BLA-ACC pathway had no effect on mechanical hypersensitivity in

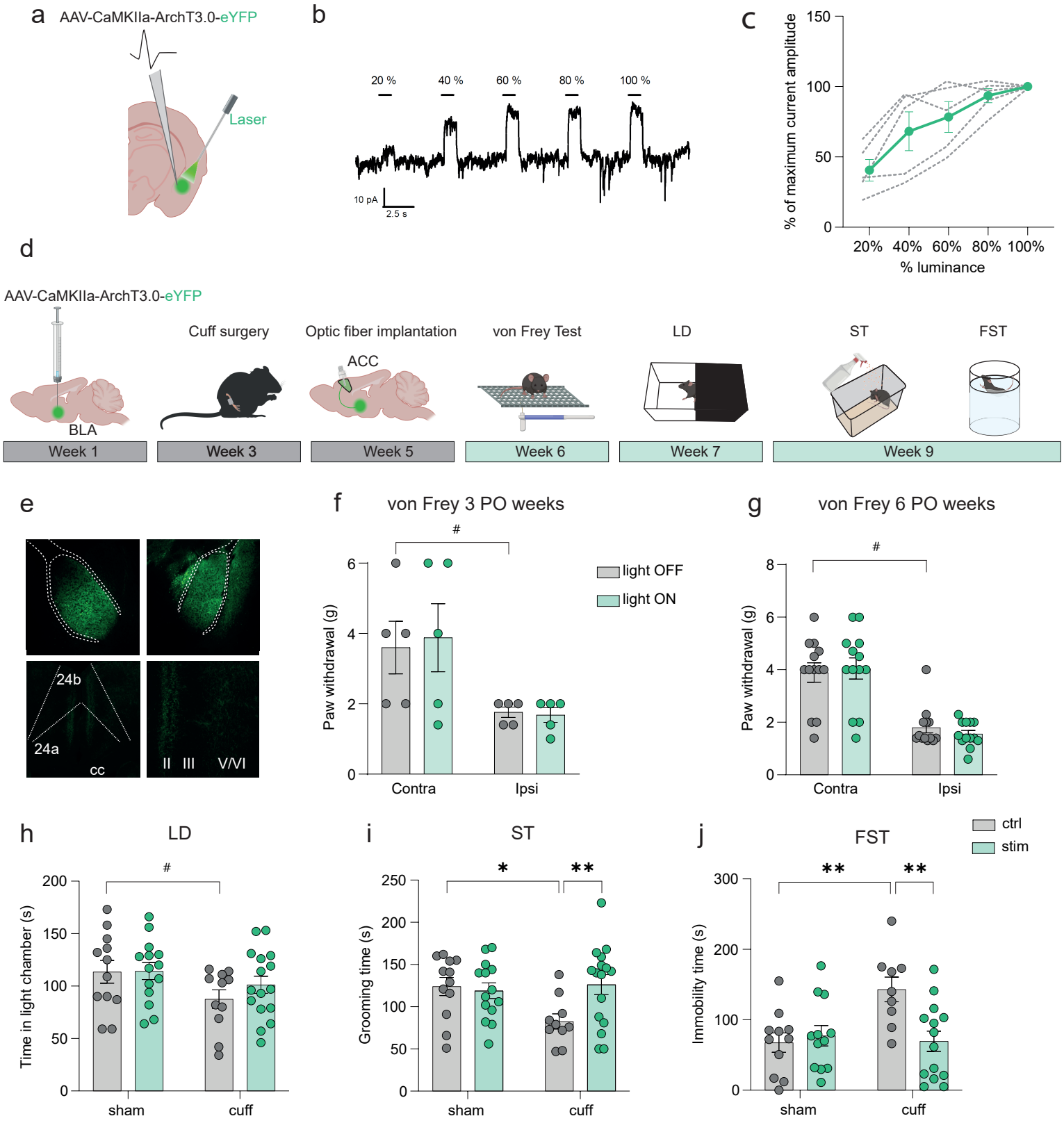
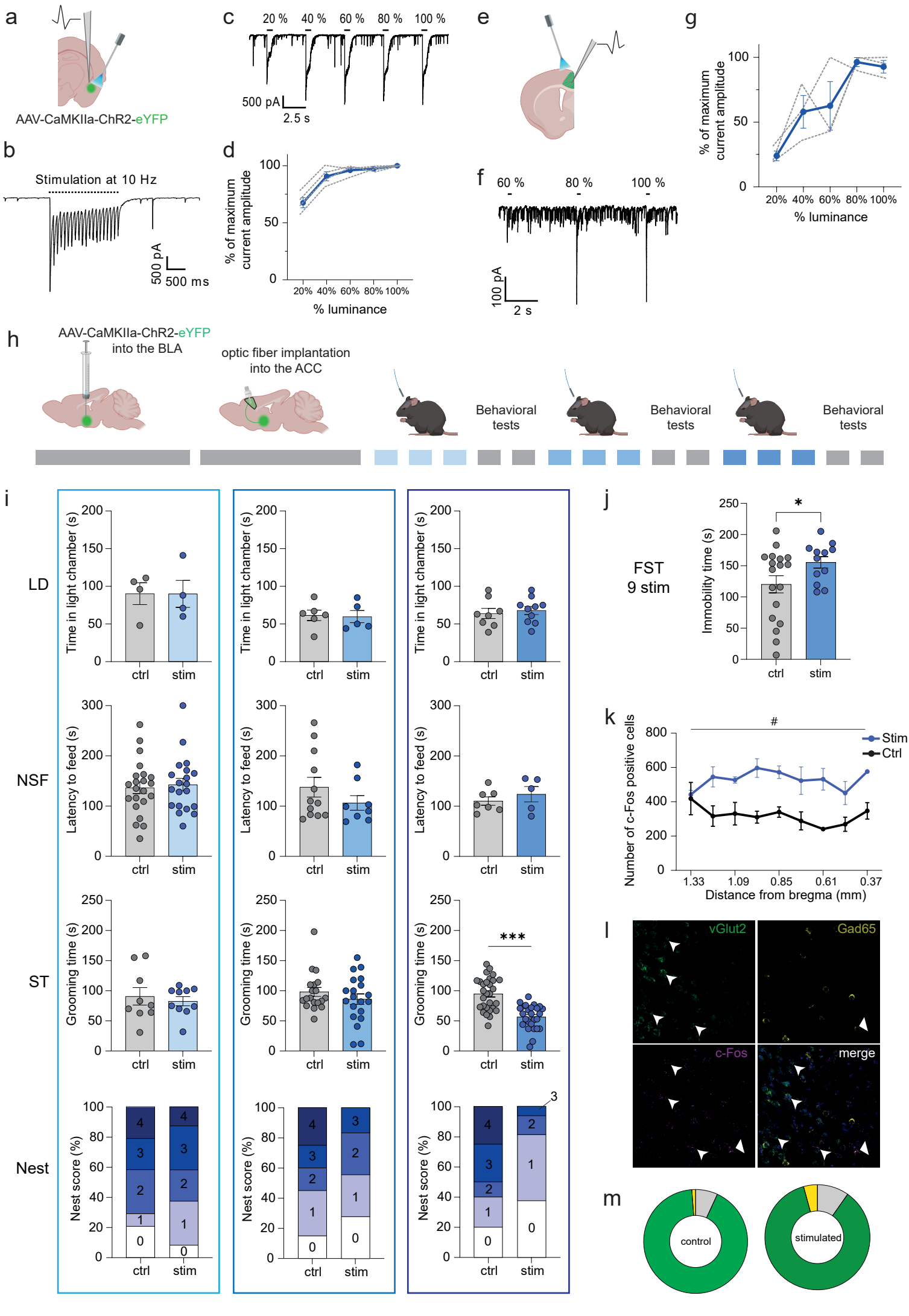


Figure 2. Optogenetic inhibition of the BLA-ACC pathway prevents CPID. a. Graphical representation of inhibitory AAV-CamkIIa-ArchT3.0-eYFP virus delivery to the mouse BLA for in vitro voltage-clamp recordings. b. Representative trace of the outwards currents induced in a BLA neuron by optogenetic stimulation of increased luminance. c. Amplitude of currents induced by optogenetic stimulations of BLA neurons as a function of light stimulation intensities (green trace = mean; grey traces = individual responses). d. Schematic representation of the experimental design for in vivo optogenetic inhibition of the BLA-ACC pathway, including bilateral virus delivery, cuff surgery, fiber implantation, and behavioral testing. e. Representative images of eYFP+ cell bodies in the BLA (upper panels) and eYFP+ axon terminals in the ACC (lower panels). f-g. At 3 (f) or 6 (g) weeks after peripheral nerve injury, mechanical hypersensitivity is not affected by the inhibition of BLA-ACC pathway (ipsi vs contra; 3 PO weeks $F(1,4)=7.752$; $p=0.0496$; 6 PO weeks $F(1,12)=55.80$; $p<0.0001$; light-off vs light-on; 3 PO weeks $F(1,4)=0.6694$; $p=0.4592$ 6 PO weeks $F(1,12)=2.971$; $p=0.1104$). h. At 7 weeks post peripheral nerve injury, optogenetic inhibition of the BLA-ACC pathway 5 minutes before the light-dark test, does not affect the decreased time spent in the light chamber observed in nerve-injured animals (sham vs cuff: $F(1,49) = 4.703$; $p=0.035$; ctrl vs stim: $F(1,49)=0.6342$; $p=0.43$). i. At 8 weeks post peripheral nerve injury, optogenetic inhibition of BLA-ACC pathway during the splash test reverses the decreased grooming behavior observed in nerve-injured non-stimulated animals with no effect on sham animals ($F(1,48)=4.991$; $p=0.03$; post-hoc: sham-ctrl (n=12) > cuff-ctrl (n=10); $p<0.05$; cuff-ctrl (n=10) < cuff-stim(n=16); $p<0.05$ sham-ctrl (n=12) = sham-stim (n=14)). J. At 8 weeks post peripheral nerve injury, optogenetic inhibition of BLA-ACC pathway 5 minutes before the FST, blocks the increased immobility observed in nerve-injured non-stimulated animals with no effect on sham animals ($F(1,42)=7.539$; $p=0.008$, post-hoc: sham-ctrl (n=11) > cuff-ctrl (n=9), $p<0.05$; cuff-ctrl (n=9) > cuff-stim (n=14), $p<0.01$; sham-ctrl (n=11) = sham-stim (n=12)). Data are represented as mean ± SEM. #= main effect; *$p<0.05$; **$p<0.01$. Two-way ANOVA repeated measures (von Frey); Two-way ANOVA (Surgery x Stimulation) (LD, ST and FST). PO, post-operative; PWT, Paw withdrawal threshold.

peripheral nerve-injured animals, as measured in the von Frey test at either 3 (**Fig. 2f**; p=0.73) or 6 weeks PO (**Fig. 2g**; p=0.72). In addition, this inhibition did not alter mechanical thresholds in sham animals (**Supplementary Fig. 2a-b**, 3 weeks PO: $F_{(1, 4)}$=0.1176; p=0.74; 6 weeks PO $F_{(1, 12)}$=0,000; p>0.99). These data are consistent with the notion that the ACC has a modulatory role but is not the essential structure for the expression of somatosensory aspects of neuropathic pain (Barthas et al., 2015; Bliss et al., 2016; Kummer et al., 2020; Vogt, 2005).

We next assessed the effect of inhibiting the BLA-ACC pathway on anxiodepressive-like behaviors. Optogenetic inhibition was applied either just before (for light/dark, LD, and forced swim tests, FST) or during behavioral testing (splash test, ST). As expected, cuff mice showed significantly higher anxiety-like behaviors in the LD at 7 PO weeks (**Fig. 2h**; $F_{(1, 49)}$ = 4.703; p=0.035) and higher depressive-like behaviors at 8 PO weeks (**Fig. 2i-j**) in both the ST ($F_{(1, 48)}$=4.991, p=0.03, post-hoc: sham-ctrl > cuff-ctrl, $p<0.05$) and FST ($F_{(1, 42)}$=7.539, p=0.008, post-hoc: sham-ctrl > cuff-ctrl, $p<0.05$) compared to sham controls, consistent with our previous reports (Sellmeijer et al., 2018; Yalcin et al., 2011). Inhibition of the BLA-ACC pathway had no significant impact in the LD test ($F_{(1, 49)}$=0.6342; p=0.43), suggesting that other neuronal pathways control this specific long-term consequence of neuropathic pain. In contrast, BLA-ACC inhibition completely reversed pain-induced decreased grooming in the ST (**Fig. 2i**; $F_{(1, 48)}$=4.991; p=0.03, post-hoc: cuff-ctrl < cuff-stim, $p<0.05$), and increased immobility in the FST (**Fig. 2j**; $F_{(1,42)}$=7.539; p=0.008, post-hoc: cuff-ctrl > cuff-stim, $p<0.01$), indicating potent antidepressant-like effects. No effect of optogenetic inhibition were observed in sham animals (ST, $F_{(1, 48)}$=4.991; p=0.03, post-hoc: sham-ctrl = sham-stim; FST, $F_{(1,42)}$=7.539; p=0.008, post-hoc: sham -ctrl = sham-stim), implying that these antidepressant-like effects selectively manifest in the context of chronic pain. Overall, these results demonstrate that hyperactivity of the BLA-ACC pathway is necessary for the expression of pain-induced depressive-like behaviors, but not of accompanying anxiety-like responses.

Repeated activation of the BLA-ACC pathway triggers depressive-like behaviors in naive mice. We next sought to determine whether BLA-ACC hyperactivity is sufficient to induce depressive-like behaviors in naïve mice, in the absence of any triggering pain. To do so, we switched to an excitatory opsin, and performed bilateral injection in the BLA of an AAV5-CamKIIa-ChR2-EYFP vector. *Ex vivo* patch-clamp recordings allowed to confirm that


a
AAV-CaMKIIa-ChR2-eYFP
b
Stimulation at 10 Hz
500 pA
500 ms
c
20 %
40 %
60 %
80 %
100 %
500 pA
2.5 s
d
% of maximum current amplitude
% luminance
e
f
60 %
80 %
100 %
100 pA
2 s
g
% of maximum current amplitude
% luminance
h
AAV-CaMKIIa-ChR2-eYFP into the BLA
optic fiber implantation into the ACC
Behavioral tests
Behavioral tests
Behavioral tests
i
LD
NSF
ST
Nest
Time in light chamber (s)
Latency to feed (s)
Grooming time (s)
Nest score (%)
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Number of c-Fos positive cells
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l
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Figure 3. ***Repeated activation of the BLA-ACC pathway triggers depressive-like behaviors in naive mice.*** **a.** Graphical representation of AAV-CamkIIa-ChR2-eYFP virus delivery in the mouse BLA for *in vitro* voltage-clamp recordings. **b.** Representative trace of response of BLA neurons to 10 Hz optogenetic stimulation showing that after an initial decrease in the amplitude of light-induced currents, a plateau is reached. **c.** Representative trace of the outwards currents induced in a BLA neuron by optogenetic stimulation of increased luminance. **d.** Amplitude of currents induced by optogenetic stimulations of BLA neurons as a function of light stimulation intensities (blue trace = mean; grey traces = individual responses). **e.** Graphical representation of AAV-CamkIIa-ChR2-eYFP virus delivered to BLA of mice for *in vitro* voltage-clamp recordings in the ACC. **f.** Representative trace of the inwards currents induced in ACC neuron by optogenetic stimulation of increased luminance of BLA terminals within the ACC. **g.** Amplitude of currents of ACC neurons induced by optogenetic stimulations of BLA terminals as a function of light stimulation intensities (blue trace = mean; grey traces = individual responses). **h.** Schematic representation of the experimental design for *in vivo* optogenetic activation of the BLA-ACC pathway, including bilateral virus delivery, fiber implantation, and behavioral testing. **i.** Repeated activation of the BLA-ACC pathway does not induce anxiety-like behaviors in the LD (3 stim: ctrl: n=4; 90.25±14.42; stim: n=4; 90.0±17.95; p=0.99; 6 stim: ctrl: n=6; 61.50±6.99; stim: n=5; 59.80±8.12; p=0.88; 9 stim: ctrl: n=8; 60.4±6.73; stim: n=10; 68.0±5.34; p=0.64) and the NSF (3 stim: ctrl: n=22; 136.5±11.65; stim: n=20; 142.6±12.44; p=0.72; 6 stim: ctrl: n=12; 137.8±19.52; stim: n=8; 106.4±14.51; p=0.26; 9 stim: ctrl: n=9; 110.3±8.36; stim: n=7; 123.8±15.24; p=0.42). While 3 and 6 sessions of optogenetic activation of the BLA-ACC pathway do not change the grooming behaviour in the splash test (ST) (3 stim: ctrl: n=19; 98.16±7.37; stim: n=20; 86.10±7.52; p=0.62; 6 stim: ctrl : n=9; 90.78±14.42; stim: n=10; 82.70±7.52; p=0.62) and nest scores in nest test (3 stim: ctrl: n=20; stim: n=16; Chi-square=0.012; p=0.91; 6 stim: ctrl: n=20; stim: n=18; Chi-square=2.81; p=0.094), 9 stimulation decreases grooming time (ctrl : n=29; 66.57±4.86; stim: n=26; 56.55±6.22; p<0.0001) and nest quality (ctrl: n=20; stim: n=16; Chi-square=7.35; p=0.0067) in stimulated animals. **j.** 9 stimulations of the BLA-ACC pathway increase the immobility time in the forced swim test (FST; ctrl: n=18; 120.3±13.69; stim: n=12; 155.5±9.15; p=0.033). **k.** Optogenetic activation of the BLA-ACC pathway induces an increase in c-Fos immunoreactivity in the totality of the ACC of stimulated animals (ctrl: n=4; stim: n=4; $F_{1, 6}$=17.24; p=0.006). **l.** Representative images of *vGlut2* (upper-left panel), *Gad65* (upper-right panel), *c-fos* (lower-left panel) mRNA expression and their co-localization (lower-right panel) in the ACC following BLA-ACC activation, measured by RNA scope. **m.** In the ACC, the proportion of *c-fos+/vGlut2+* cells (green) was similar between control (left panel) and stimulated (right panel) animals (ctrl: n=5; mean = 90.71% ± 1.41, stim: n=5; mean = 85.99% ±1.60; p=0.056) and the proportion of *c-Fos+/Gad65+* cells (yellow) was increased in stimulated animals (control: n=5; mean = 1.16% ± 0.17, stimulated: n=5; mean = 4.26% ±0.86; p=0.032). Data are represented as mean ± SEM. #= main effect; *p<0.05; **p<0.01; ***p<0.001. unpaired t-test (LD, NSF, ST); chi-square test for trend (Nest test); Two-Way ANOVA (stimulation x anteroposteriority) (c-Fos immunohistochemistry); Mann-Whitney test (*c-fos/vGlut2*, *c-fos/Gad65* mRNA quantification).

blue light stimulation (wavelength: 475nm, pulse duration: 10ms; frequency: 10Hz) increased basal activity of BLA and post-synaptic ACC neurons (**Fig. 3a-g**). In the BLA, light stimulation induced inward currents with a maximum effect obtained at 40% of the maximum light intensity (**Fig. 3c-d**). In addition, pulsed stimulation at 10Hz produced strong inward currents that remained stable (after a small decrease in amplitude during the two first pulses of the train, **Fig. 3b**). In the ACC, light stimulation of axon terminals coming from the BLA induced strong inward currents, with an effect that reached a plateau at 80% of maximal light intensity (**Fig. 3f-g**), suggesting that our light stimulation protocol is sufficient to potentiate the activity of

ACC neurons.

Having established this optogenetic protocol, we next explored its behavioral impact in naïve mice. An optic fiber was implanted in the ACC, and a blue light pulse stimulation applied during 20min (with parameters validated *ex vivo*). We first showed that a single session of stimulation was not sufficient to trigger any behavioral manifestation in spontaneous locomotor activity (**Supplementary Fig. 3a**) (p=0.16), real-time place preference (**Supplementary Fig. 3b**; $F_{1,14}$ = 0.2537; p=0.62) or anxiodepressive-like assays (**Supplementary Fig. 3c-d**; NSF: p=0.82; ST: p=0.26), suggesting that repeated sessions might be necessary. Thus, we next tested the effect of repeated daily activation, during 3 consecutive days each week, every week during 3 weeks, using the same light stimulation protocol (**Fig. 3h**). Behavioral testing was performed each week, following each block of 3 activating sessions, to evaluate spontaneous locomotor activity, anxiety- and depressive-like behaviors. Repeated stimulations had no effect on locomotor activity (**Supplementary Fig. e**; 3 stim: p=0.52; 6 stim: p=0.81; 9 stim: p=0.33) or anxiety-like behaviors (**Fig.3i**; LD: 3 stim: p=0.99; 6 stim: p=0.88; 9 stim: p=0.64; NSF: 3 stim: p=0.72; 6 stim: p=0.26; 9 stim: p=0.42), consistent with above results indicating that optogenetic inhibition of this pathway did not rescue enhanced anxiety-like behaviors induced by neuropathic pain. Regarding depressive-like behaviors, the effects of repeated stimulations clearly strengthened with time (**Fig. 3i**): no changes were observed after 3 stimulations in any test (ST: p=0.62; Nest: p= 0.91); after 6 stimulations, a tendency for a decrease in the quality of nest building was observed, (p=0.09), with no changes in grooming behavior in the ST (p=0.62); after 9 stimulations, significant deficits emerged, with a significantly poorer nest score in stimulated mice compared to controls, along with a decrease in grooming duration in the ST (ST: p<0.0001; Nest: p=0.007) and an increase immobility in the FST (**Fig. 3j**; p=0.033). Of note, none of these effects remained significant when measured one week after the ninth, last stimulation session (**Supplementary Fig. 3f-g**; ST: p=0.27; Nest: p=0.83).

Importantly, to document this neuronal activation at molecular level and determine which neuronal cell-types are activated in our optogenetic paradigm, we quantified c-fos expression (immunohistochemistry) and its co-localization (RNAscope) with markers of excitatory (glutamatergic, vglut2) and inhibitory neurons (GABAergic, gad65). Immunostainings showed that repeated optogenetic activation induced a strong increase in c-fos levels in the whole 24a and 24b areas of the ACC (**Fig. 3k**: p=0.006).

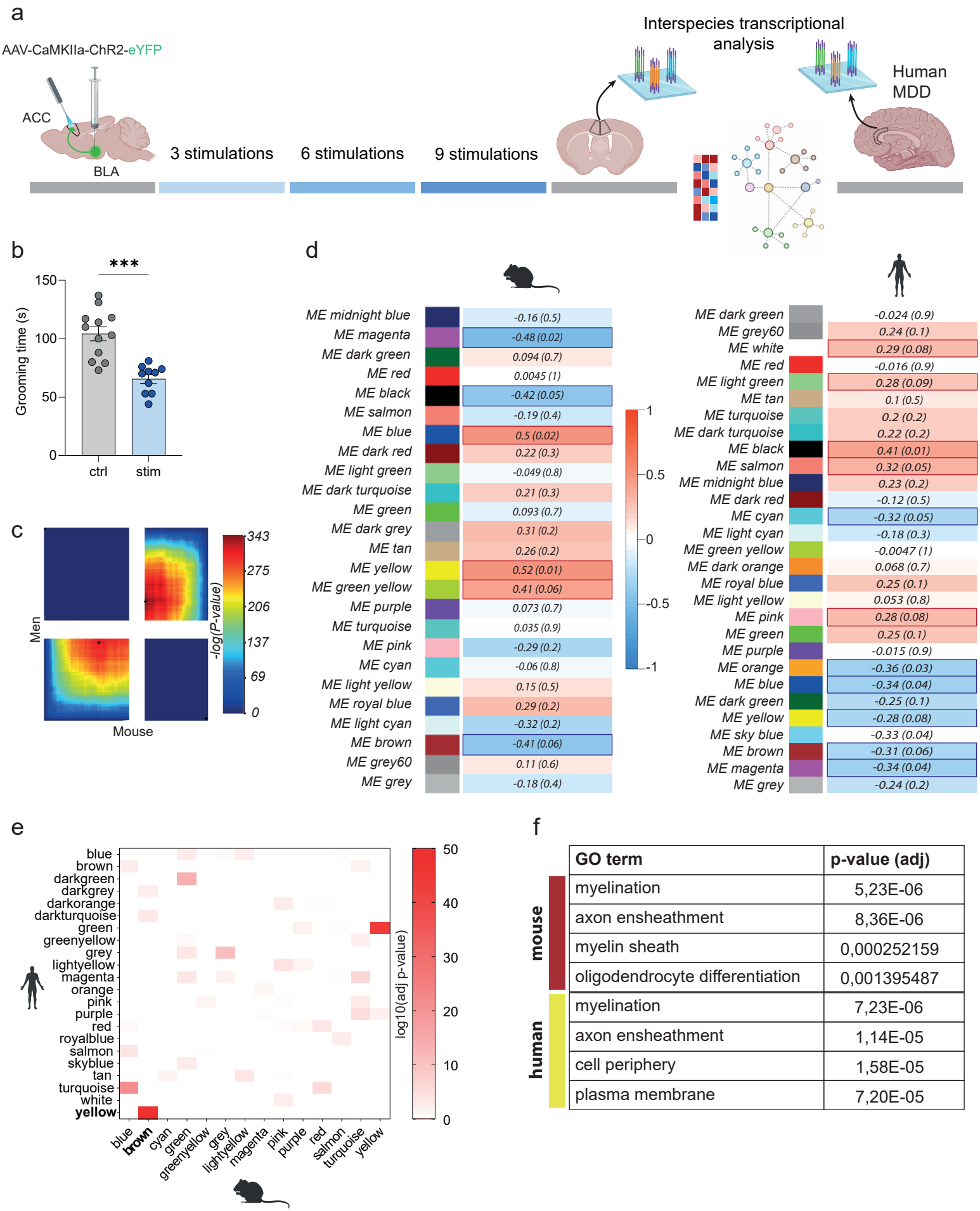
Furthermore, results showed that, in both control and stimulated mice, a large majority of these activated, c-fos+ cells were glutamatergic (control= 90.71% ± 1.41, stimulated= 85.99%

±1.60, p=0.56), with only a small proportion of GABAergic neurons (control= 1.16% ± 0.17, stim= 4.26% ±0.86) that is however significantly higher in stimulated animals (p=0.032) (**Fig. 3l-m**).

Altogether, these results show that repeated activation of the BLA-ACC pathway over 3 weeks is sufficient to trigger the progressive emergence of a depressive-like phenotype, which does not persist when stimulating sessions are interrupted. These behavioral effects primarily rely on a strong and reliable activation of ACC glutamatergic neurons. The need for repeated activation over 3 weeks to produce behavioral effects strongly suggests neuroplastic and transcriptomic alterations within the ACC.

***Repeated activation of the BLA-ACC pathway produces transcriptional alterations similar to those observed in human depressed patients*.** To understand molecular mechanisms underlying behavioral effects of optogenetic stimulations, we next performed RNA-Sequencing of ACC tissue to generate genome-wide gene expression data, 48 hours after the 9th stimulation (3-week time-point), when behavioral deficits are maximal (**Fig. 4a**). We generated 2 animal cohorts (n=12 controls and n=10 stimulated mice in total) and, before harvesting ACC tissue, we confirmed the development of depressive-like behaviors in stimulated mice using ST (**Fig. 4b**). Similar to our previous work (Kremer et al., 2018), analysis of gene expression changes was conducted using DESeq2, by taking into account the animal cohorts and RNA integrity numbers (RIN) as covariates (see material and methods). At the genome-wide level, robust differences were observed across groups (as visualized in the first Principal Components accounting for most variance, see Principal Component Analysis in **Supplementary Fig. 4a**). After correcting for multiple testing (Benjamini-Hochberg procedure), n=54 genes showed evidence of significant dysregulation in stimulated mice as compared to controls (**Supplementary Fig. 4b**), and n=2611 without multiple testing correction (nominal p-value <0.05). Over-Representation Analysis of Biological Processes, performed with WebGestalt on the latter gene set uncovered major alteration in several Gene Ontology term, including intracellular transport, neurogenesis, cytoskeleton organization or myelination.

To proceed further with the analysis of these high-throughput data, we reasoned that while our optogenetic paradigm triggers robust depressive-like behaviors, it is based on the exogenous activation of a single neuronal pathway linking 2 brain structures, the BLA and the ACC. This raises the question of its potential translational value for the understanding of human depression. To address this, we decided to compare our mouse results with human


a
AAV-CaMKIIa-ChR2-eYFP
ACC
BLA
3 stimulations
6 stimulations
9 stimulations
Interspecies transcriptional analysis
Human MDD
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343
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d
ME midnight blue -0.16 (0.5)
ME magenta -0.48 (0.02)
ME dark green 0.094 (0.7)
ME red 0.0045 (1)
ME black -0.42 (0.05)
ME salmon -0.19 (0.4)
ME blue 0.5 (0.02)
ME dark red 0.22 (0.3)
ME light green -0.049 (0.8)
ME dark turquoise 0.21 (0.3)
ME green 0.093 (0.7)
ME dark grey 0.31 (0.2)
ME tan 0.26 (0.2)
ME yellow 0.52 (0.01)
ME green yellow 0.41 (0.06)
ME purple 0.073 (0.7)
ME turquoise 0.035 (0.9)
ME pink -0.29 (0.2)
ME cyan -0.06 (0.8)
ME light yellow 0.15 (0.5)
ME royal blue 0.29 (0.2)
ME light cyan -0.32 (0.2)
ME brown -0.41 (0.06)
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ME red -0.016 (0.9)
ME light green 0.28 (0.09)
ME tan 0.1 (0.5)
ME turquoise 0.2 (0.2)
ME dark turquoise 0.22 (0.2)
ME black 0.41 (0.01)
ME salmon 0.32 (0.05)
ME midnight blue 0.23 (0.2)
ME dark red -0.12 (0.5)
ME cyan -0.32 (0.05)
ME light cyan -0.18 (0.3)
ME green yellow -0.0047 (1)
ME dark orange 0.068 (0.7)
ME royal blue 0.25 (0.1)
ME light yellow 0.053 (0.8)
ME pink 0.28 (0.08)
ME green 0.25 (0.1)
ME purple -0.015 (0.9)
ME orange -0.36 (0.03)
ME blue -0.34 (0.04)
ME dark green -0.25 (0.1)
ME yellow -0.28 (0.08)
ME sky blue -0.33 (0.04)
ME brown -0.31 (0.06)
ME magenta -0.34 (0.04)
ME grey -0.24 (0.2)
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lightyellow
magenta
orange
pink
purple
red
royalblue
salmon
skyblue
tan
turquoise
white
yellow
log10(adj p-value)
50
40
30
20
10
0
f
mouse
human
GO term
p-value (adj)
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axon ensheathment 8,36E-06
myelin sheath 0,000252159
oligodendrocyte differentiation 0,001395487
myelination 7,23E-06
axon ensheathment 1,14E-05
cell periphery 1,58E-05
plasma membrane 7,20E-05

Figure 4. ***Repeated activation of the BLA-ACC pathway produces transcriptional alterations similar to those observed in human depressed patients*****. a**. Graphical representation of experimental design, including virus delivery in the BLA, fiber implantation in the ACC, the 9 sessions of optogenetic stimulation, ACC extraction in mice, and transcriptomic analysis in mice and human. **b**. 9 sessions of optogenetic activation of the BLA-ACC decreases grooming behaviors in the cohort of stimulated animals used for RNA-Sequencing (ctrl: n=12; 104.2±6.0; stim: n=10; 65.60±3.80; p<0.0001). **c.** Rank-Rank Hypergeometric Overlap (RRHO2) unravels shared transcriptomic changes in the ACC across mice and men as a function of optogenetic stimulation (mouse) or a diagnosis of major depressive disorder (MDD). Levels of significance for the rank overlap between men and mice are color-coded, with a maximal Fisher's Exact Test (FET) p=1.26E-153 for up-regulated genes (lower-left panel), and a maximal FET p=1.64E-127 for down-regulated genes (upper-right panel). **d.** WGCNA was used to analyze network and modular gene co-expression in the mouse and men ACC. The tables depict associations between individual gene modules and: i) optogenetic stimulation in mice (left panel), ii) or MDD diagnosis in men (right panel). Each row corresponds to correlations and p-values obtained against each module's eigengene. **e.** Heatmap representing the level of significance of overlaps between mice and men gene modules (measured using the FET). The highest overlap (p= 8.36E-49) was obtained for the human/yellow and mouse/brown modules. **f.** Results of Gene Ontology enrichment analysis for the human/yellow and mouse/brown module, with most significant enrichments corresponding to myelin-related genes in both species.

data, taking advantage of our recent work in the same brain region, the ACC (Lutz et al., 2017). In this later human study, we used a similar RNA-Seq approach to compare individuals with MDD, who died during a major depressive episode (n=26), and healthy individuals with no psychiatric history (n= 24), (**Supplementary Table 1**). Because only male mice are used in our optogenetic paradigm, we reprocessed human data to restrict the analysis of differential expression to men only (adjusting for age and RIN; of note, a similar analysis using human data from both men and women is presented and discussed in **Supplementary Fig. 4**). We then conducted direct comparisons of human and mouse transcriptomes, focusing on 13 572 orthologous genes readily expressed in both species (see Methods).

First, when looking at differentially expressed genes (DEGs, nominal p-value<0.05), 398 genes were found in common in mice and men (**Supplementary Fig. 4c**) representing 29.6% of mice DEGs (and even 34.9% when considering both men and women, likely because of increased statistical power, **Supplementary Fig. 4d**), suggesting a robust overlap between the 2 datasets. Second, to generate a more systematic comparison across the whole genome, we used a 'threshold-free' approach, RRHO2 (for Rank-Rank Hypergeometric Overlap) (Cahill et al., 2018). Results uncovered a clear pattern for dysregulation of gene expression in similar directions across the 2 species as a function of MDD (men) or optogenetic activation of the BLA-ACC pathway (mice). Accordingly, a strong overlap between human and mice up-regulated genes (maximum FET padj 1,26x10-153), as well as down-regulated genes (maximum FET padj 1,64x10-127), was observed (**Fig. 4c**; see **Supplementary Fig. 4e** when including women). Third, to go further in the analysis and

comparison of the modular organization of gene expression, we used WGCNA, (weighted gene co-expression network analysis) which have been proposed to better capture the transcriptomic architecture of psychiatric syndromes (Langfelder & Horvath, 2008). In this approach, pair-wise gene co-expression is measured across biological replicates throughout the genome, and used to construct gene networks. Accordingly, here we constructed gene networks independently for each species, and identified networks composed of twenty-five and twenty-nine gene modules in mice and humans, respectively (**Fig. 4d**). In mice, the eigengene of six modules (a measure that best summarizes each module's expression data) were significantly correlated ($p<0.01$) with exposure to the optogenetic stimulation (**Fig. 4d, left**). In human, the eigengene of twelve modules were associated with the MDD diagnosis (**Fig. 4d, right**). To further identify gene modules that might be conserved and similarly affected in pathological conditions across species, we determined the level of conservation of genes contained in each module using Fischer's exact t-test. Fifty-six percent (14/25) of mice modules were conserved in human, among which 5 of the 6 aforementioned modules that significantly correlated with the optogenetic stimulation (**Fig. 4e**; see **Supplementary Fig. 4g** when including women). Altogether, these findings reinforce the notion that our mouse optogenetic model, based on the repeated activation of the BLA-ACC pathway, recapitulates meaningful transcriptomic features of human MDD, and further points towards the analysis of specific functional modules.

Gene ontology pathways affected in mice and men point towards altered oligodendrocyte function. We then conducted functional enrichment analysis of gene modules significantly associated with optogenetic stimulation and MDD. Results uncover a series of GO terms that notably relate to synaptic activity and structure, mitochondrion or RNA processing (**Supplementary Table 2**), consistent with previous reports on the pathophysiology of MDD in both human post-mortem studies and preclinical paradigms (Bansal & Kuhad, 2016; Howard et al., 2019; Scarpa et al., 2020). Among these, a detailed analysis of the 2 modules most strongly conserved ($p=8.4x10^{-49}$) across human (Yellow) and mouse (Brown) data, and that significantly correlated with MDD and optogenetic stimulation, respectively, showed interesting results. Gene ontology analysis, revealed that genes from these 2 modules were strongly enriched, in both species, in terms related to oligodendrocyte and to myelination (**Fig. 4f**). We ranked the genes within these 2 modules, based on their module membership (MM), which describes the importance of each genes within its module, and the p-value and fold change obtained in differential expression analysis. Among the ten

most central gene of each module, seven were myelin related and five (*mal, mog, aspa, ermn, plp1*) of them displayed a significant decreased expression in both species. Several other myelin and oligodendrocyte related genes (*olig2, erbb3, ugt8, mag*) were found among the genes presenting a MM higher than 0.8 and significantly down-regulated in both mice and human. Beside this drastic down-regulation of myelin components and enzymes for its synthesis, we also noticed an up-regulation of two major myelin inhibitors: *lingo-1* and *sema4A* in our differential expression analysis.

Next, to strengthen these findings and provide experimental validation using an alternate methodology, we generated a new mice cohort that similarly underwent 9 optogenetic stimulations over 3 weeks (n=8 control and 7 stimulated mice; **Fig. 5a**). The behavioral emergence of depressive-like behaviors was confirmed by using ST (24 hours after the 9-th stimulation, $p<0.0001$; **Fig. 5b**), and ACC tissue collected 48 hours after the 9- th stimulation, similar to RNA sequencing experiments. Gene expression of myelin-related genes was then analyzed using microfluidic qPCR (Fluidigm Biomark HD system), similar to our previous study (Y. Zhou et al., 2018) (**Fig. 5c-d**). We showed that results obtained from a new cohort and analyzed by Fluidigm were strongly coherent with RNA sequencing results (**Fig. 5c**; linear regression: $r^2=0.65$; $p=0.0086$), with similar fold changes observed with the 2 techniques. At the level of individual genes (**Fig. 5d**), a down-regulation of myelin components or synthesis enzyme (*mbp, plp1, mog, mal, aspa)* and an up-regulation of myelination inhibitors (*sema4A and lingo-1*) were found.

Taken together, our results uncover shared transcriptomic signatures between depression in human and the repeated activation of the BLA-ACC pathway in the mouse. In both species, a strong and reproducible down-regulation of genes encoding myelin essential components, as well as an up-regulation of genes known to inhibit myelination processes, was observed. In the past, associations between myelin, oligodendrocytes and depressive states have already been documented (Boda, 2021). However, these previous studies were mostly correlative, and a causal link between depression and myelination deficits is still missing. Since our results showed strong up-regulation of the myelination inhibitor lingo-1, for which the increased expression in the ACC has been previously implicated in the relationship between mood regulation and demyelination in a rat study (Ma et al., 2019), we hypothesized that this specific gene may be essential in the progressive induction of depressive-like behaviors. Next experiments were designed to directly test that hypothesis and are currently ongoing.

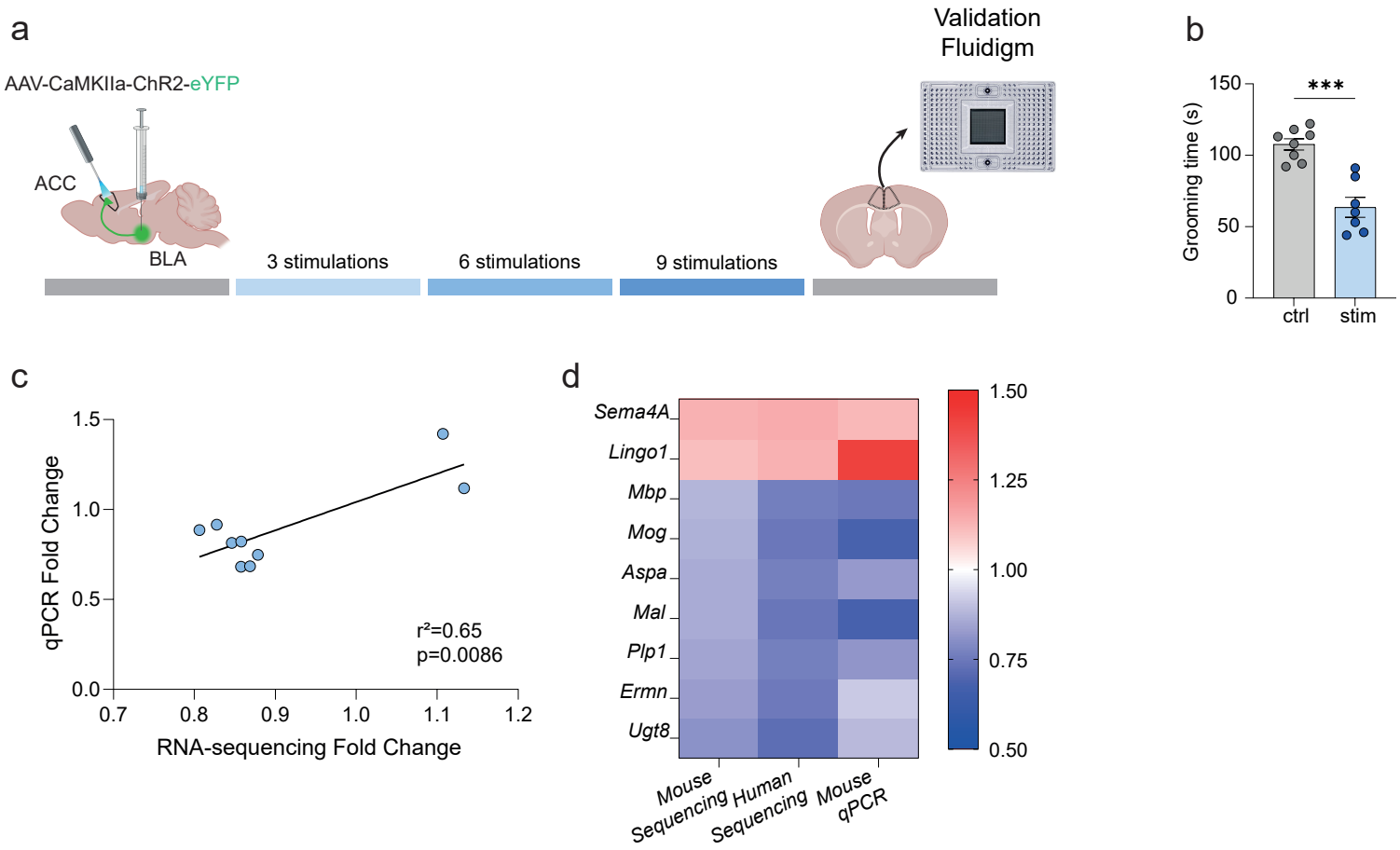

Figure 5. BLA-ACC repeated activation triggers major alterations in myelination. a. Graphical representation of experimental design, including virus delivery in the BLA, fiber implantation in the ACC, the 9 sessions of optogenetic stimulation, ACC extraction and RT-qPCR. b. Repeated activation of the BLA-ACC decreases grooming time in stimulated animals (ctrl n=8; 107.6±3.65; stim n=7; 63.57±6.96; p<0.0001). c. Linear regression of fold changes measured by RNA sequencing (x-axis) and qPCR (y-axis), showing a significant positive correlation between the two methods (r^2=0.65, p=0.0086) d. Down-regulation of the myelin-related genes (mbp, mog, aspa, mal, plp1, ermn and ugt8) and up-regulation of known inhibitors of the myelination process (lingo1 and Sema4A) is consistently found across mice and men by RNA-Sequencing, and is validated by qPCR in mouse after 9 optogenetic stimulations.

DISCUSSION

The ACC is deeply involved in higher brain functions that are altered in psychiatric conditions and pain. Although neuroimaging studies have already identified the connectome of the ACC and yielded correlations with emotional processing, pain and aversion, the specific function of its circuitry have remained elusive due to the lack of direct experimental manipulations. Here, by using optogenetics, we demonstrate that ACC inputs coming from the BLA are necessary for the expression of emotional deficits during chronic pain, and that repeated activation of this pathway is sufficient for the emergence of depressive-like behaviors in naïve mice, in the absence of any physical pain. Importantly, through a combination of bioinformatics analyses, our results reveal that, from a molecular point-of-view, the hyperactivation of the BLA-ACC pathway in the mouse induces global transcriptional effects that strikingly resemble those previously documented in the ACC of patients MDD. Our network-based approach further allowed us to identify gene modules that are conserved across mice and men, and that show consistent alterations as a function of emotional dysfunction, affecting in particular myelination and oligodendroglial cells.

Human fMRI studies have shown that the ventral part of the ACC, which is involved in emotional processing (Kanske and Kotz, 2012), is hyperactive in depressed patients (Mayberg et al., 1999; Yoshimura et al., 2010) and the ablative (Shields et al., 2008) or deep brain stimulation (Drobisz & Damborská, 2019) of the ACC can clinically alleviate depressive symptoms. This role of the ACC in depression is further supported by animal studies showing increased ACC activity accompanying depressive-like behaviors across several paradigms, including social-defeat (Yu et al., 2011), chronic variable stress (unpublished data) and the chronic pain model used in the present study (Barthas et al., 2017; Sellmeijer et al., 2018). In the latter model, we hypothesized that such hyperactivity might be mediatedby long-term alterations of functional connections onto pyramidal neurons (Koga et al., 2015) and participate in the long-lasting presence of affective symptoms. Since one of the major inputs of the ACC originates from the BLA, we first focused on the BLA-ACC pathway. Indeed, in addition to our fMRI data showing increased functional connectivity between the BLA and ACC in CPID animals, we also demonstrated using tract tracing and c-fos immunochemistry that BLA neurons projecting to the ACC are hyperactive when chronic pain induces anxiodepressive deficits. Considering that BLA projection neurons are known to be

glutamatergic, this suggests that in chronic pain conditions the excitatory transmission between the BLA and its down-stream target is enhanced. Therefore, the increase activity of the BLA observed here could act as a trigger for the ACC hyperactivity we previously reported (Barthas et al., 2017; Sellmeijer et al., 2018).

To study the functional role of the BLA-ACC pathway and a causal link between its hyperactivity and the behavioral outputs, we performed optogenetic inhibition of the terminals of the BLA neurons within the ACC. We showed that the inhibition of the BLA-ACC pathway suppress the depressive-like consequences of neuropathic pain without affecting the mechanical hypersensitivity and anxiety-like behaviors. These results support the idea that discrete cortical pathways encode distinct components of chronic pain (Huang et al., 2019; Zhu et al., 2021). We previously showed that the ACC was mediating both anxiety and depressive consequences of neuropathic pain (Barthas et al., 2015; Sellmeijer et al., 2018). Our current results indicate that the BLA inputs to the ACC are only responsible for the depressive consequences of neuropathic pain. Therefore, chronic pain-induced anxiety presumably rely on other afferents to the ACC. The projection from the locus coeruleus (LC) might be a one of the candidate structure, since activating the LC-ACC pathway in a rat model of peripheral neuropathy was shown to worsen anxiety-like behaviors (Hirschberg et al., 2017). Interestingly, Zhu and collaborators uncover two distinct thalamic projections, both mediating the somatosensory component of chronic pain, but recruited by different pain etiologies. Indeed, the projection from the posterior thalamic nucleus to the primary sensory cortex, regulates tissue injury-induced allodynia, whereas the projection from the parafascicular thalamic nucleus to the ACC, modulates depression-induced allodynia (Zhu et al., 2021). Altogether, this suggests that discrete corticopetal pathways underlie the diverse sensory and affective deficits induced by chronic pain and that the recruitment of these pathway may differ depending on chronic pain etiology.

We further showed that, the exogenous activation of the BLA-ACC pathway in a repeated fashion triggers depressive-like behaviors in naïve mice. While the neuroimaging findings in human support an alteration of the functional connectivity between the BLA and ACC in depressed patients without a history of chronic pain (Workman et al., 2016), this is the first time that the proficiency of the hyperactivity for depression have been demonstrated. Surprisingly, the activation of the BLA-ACC pathway did not induce anxiety-like behaviors, strengthening the aforementioned notion that this pathway specifically modulates depressive-like behaviors. This also implies that the anxiogenic effect of the ACC stimulation (Barthas et

al., 2015) does not rely on ACC neurons receiving inputs from the BLA. To our knowledge there are no evidences linking a specific afferent of the ACC to the development of anxiety behaviors. Therefore, the pathways targeting the ACC and underlying anxious responses remain to be determined. We also demonstrated that a single activation of the BLA-ACC pathway was not sufficient to induce behavioral changes. This is in line with our previous study showing that chronic, but not acute, activation of the ACC induce anxiodepressive-like behaviors (Barthas et al., 2015). Moreover, the behavioral deficits induced by chronic ACC activation, were accompanied by major alterations of the mitogen- activated protein kinase (MAPK) pathway and by epigenetic alteration at the level of *c-fos* and *MAPK phosphatase-1* (*mkp-1*) promoters (Barthas et al., 2017). Altogether, this suggests that the development of a depressive state following the activation of the BLA-ACC requires long-term molecular and neural plasticity. In order to identify these long term molecular alterations, we characterized gene expression changes that might occur in the ACC when optogenetic modulation of the BLA-ACC pathway triggers depressive-like behaviors. Our results showed that optogenetically-induced depression was associated with strong transcriptomic alterations within the ACC. More precisely, strong enrichment for intracellular transport, neurogenesis, cytoskeleton organization or myelination were found in our DEGs list. Therefore, the pro-depressant effect of BLA-ACC activation might be underpinned by the dysregulation of these different processes. Our results supported by studies conducted in human or preclinical models of depression which report alterations in axonal transport (Bakhtiarzadeh et al., 2018), microtubule organization (Marchisella et al., 2016), neurogenesis (Tochigi et al., 2008) or myelination (Liu et al., 2012; Nagy et al., 2020) in the PFC.

Even though enormous efforts have been devoted to the identification of single genes that individually exhibit genetic variations in expression that associate with the disease, these approaches have had limited success, leading to the hypothesis that systems-based approaches may potentially be more powerful. In particular, gene ‘networks’ have proven a valuable method to better understand the molecular architecture of autism, Alzheimer’s disease, and depression. In this respect, beside individual gene alterations, we also studied the gene modules reorganization in the ACC. Our analysis highlighted that BLA-ACC activation strongly alters modules enriched for mitochondrion, ion channel activity, RNA processing, chromatine remodeling and myelin related genes. Dysregulation of all these functions are known to be associated with depression in clinic and were also reported in rodent models of depression (Bansal & Kuhad, 2016; Howard et al., 2019; Nestler et al., 2016; Scarpa et

al., 2020). On the other hand, these results reinforce our idea that manipulating pathway activity using optogenetic might constitute a valuable tool to uncover the molecular mechanisms underlying MDD.

While optogenetic approaches allowed great advances in physiology and neuroscience during the last decade, they have been comparatively less frequently used to develop animal models of chronic psychiatric conditions such as MDD. To strengthen the translational value of our optogenetic model for the understanding of the human condition (Lutz et al., 2017), we compared genes and gene modules previously identified in human with our optogenetic model. Interestingly, our results highlighted a strong similarity in gene expression alteration between our optogenetic-induced model of depression and the human pathology. For instance, mitochondrial function are consistently found to be dysregulated in the PFC of mice and human. This is also corroborated by studies describing impairment in mitochondrial activity in depressed patients and animal models (Rezin et al., 2009). Further work will be needed to determine the causal relationship between depression and mitochondrial function. In the present work, the strongest impairment was observed in the expression of genes related to myelination. Importantly, this goes along with the conclusion of Lutz and collaborators obtained for the human data used in this study and adds up with other studies reporting deficits in myelination (Tham et al., 2011), white matter tract organization (Bae et al., 2006; Bhatia et al., 2018; Hyett et al., 2018) or oligodendrocytes integrity (B. Zhou et al., 2021) in the ACC of MDD diagnosed patients. This potential crucial role of oligodendrocyte and myelin in the pathophysiology of depression is also supported by several preclinical studies describing myelin impairments in rodent model of depression (Birey et al., 2015; Liu et al., 2012). In addition, strategies aiming at restoring myelination in the PFC already proved to exert an antidepressant effect in mice (Liu et al., 2016). Altogether, this highlights a crucial role of myelination and oligodendrocyte in the pathophysiology of depression.

In conclusion, our results indicate that the BLA-ACC pathway has a strong and selective modulatory role in depression and its comorbidity with chronic pain. These findings strengthen our knowledge about the brain circuity underlying emotional processing, and assert the idea that distinct aspects of negative emotions are regulated through discrete pathways of the nervous system. Based on the combination of rodent and human data, our study provides a cross-species translational study on the behavioral functions of this circuitry, and uncovers the essential role of oligodendrocytes and myelination in the development of depression. Targeting those cells, particularly within the ACC, may unveil new cellular

mechanisms and therapeutic opportunities in relation to depression.**MATERIAL AND METHODS**

Animals

Experiments were conducted using male adult C57BL/6J (RRID:IMSR JAX: 000664) mice (Charles River), 8 weeks at the beginning of experimental procedures, group-housed with a maximum of 5 animals per cage and kept under a reversed 12 h light/dark cycle. After the optic fiber implantation, animals were single housed to avoid possible damage to the implant. We conducted all the behavioral tests during the dark phase, under red light. Our animal facility (Chronobiotron) is registered for animal experimentation (Agreement A67-2018-38), and protocols were approved by the local ethical committee of the University of Strasbourg (CREMEAS, APAFIS8183-2016121317103584).

Surgical procedures

Surgical procedures were performed under zoletil/xylazine anesthesia (zoletil 50 mg/ml, xylazine 2.5 mg/ml; ip, 4 ml/kg, Centravet). For stereotaxic surgery, a local anaesthetic was delivered subcutaneously at incision site (bupivacaine, 2 mg/kg).

Neuropathic pain induction: cuff surgery

For the BLA-ACC inhibition study, we used our well-characterized chronic pain-induced depression model (Sellmeijer et al., 2018; Yalcin et al., 2011)*. Before surgery, mice were assigned to experimental groups so that these groups did not initially differ in mechanical nociceptive threshold or body-weight. Chronic neuropathic pain was induced by placing a 2-mm polyethylene tubing (Cuff, Harvard Apparatus, Les Ulis, France) around the right common branch of sciatic nerve* (Yalcin et al., 2014)*. The Sham group underwent the same procedure without cuff implantation.*

Virus injection

After anesthesia, mice were placed in a stereotaxic frame (Kopf Instruments). The 0.5 µl of AAV5-CaMKIIa-ChR2(H134R)-EYFP or AAV5CaMKIIa-eArchT3.0-EYFP (UNC Vector core) were injected bilaterally into the BLA using a 5 µl Hamilton syringe (0.05 µl/min, coordinates for the BLA, anteroposterior (AP): -1.4 mm from bregma, lateral (L): +/-3.2 mm, dorsoventral (DV): 4 mm from the brain surface). After injection, the 32-gauge needle remained in place for 10 min and then the skin was sutured. Following surgery, animals were left undisturbed

for at least two weeks before cannula implantation. To check viral injection localization at the end of the experiment, animals were anesthetized with Euthasol (182 mg/kg) and perfused with 30mL of 0.1M phosphate buffer (PB, pH 7.4) followed by 100mL of 4% paraformaldehyde solution (PFA) in 0.1M PB. Brains were extracted and post fixed overnightand kept at 4°C in 0.1M PB saline (PBS) until cutting. Coronal sections (40 µm) were obtained using a vibratome (VT 1000S, Leica, Deerfield, IL) and were serially collected in PBS. Sections were serially mounted with Vectashield medium (Vector laboratories) and localization of the fluorescence was checked using an epifluorescence microscope (Nikon 80i, FITC filter). Only animals well-injected bilaterally in the BLA were kept for further analyses.

Tracer injection

Analysis of the afferents from BLA to ACC was performed by injecting the retrograde tracer Hydroxystilbamidine methanesulfonate (FluoroGold®,0.5 µl) bilaterally in the ACC, using a microsyringe pump controller (UMC4, World precision instruments) and a 5 µl Hamilton syringe (100 nl/min, coordinates for the ACC, AP: +0.7 mm from bregma, L: 0.2 mm, DV: 2 mm from the bregma). 7 days after the tracer injection, mice were anesthetized with Euthasol (182 mg/kg) and perfused with 30 ml of 0.1 M phosphate buffer (PB, pH 7.4) followed by 150 ml of 4 % paraformaldehyde solution (PFA) in 0.1 M PB. Brains were removed, postfixed overnight in PFA at 4 °C, and then kept at 4° C in 0.1 M PB saline (PBS, pH 7.4) until cutting. Coronal sections (40 µm) were obtained with a Vibratome (VT 1000S, Leica, Deerfield, IL) and serially collected in PBS.

Optic fiber cannula implantation

At least two weeks after virus injection, the animals underwent a unilateral optic fiber cannula implantation into the ACC. The optic fiber cannula was 1.7 mm long and 220µm in diameter. The cannula was inserted 1.5 mm deep in the brain at the following coordinates: AP: +0.7mm L: +/- 0.2mm (MFC 220/250–0.66 1.7mm RM3 FLT, Doric Lenses) (2). For behavioral experiments, cannulas were implanted in the left hemisphere in half of each experimental group, whereas the other half received the implant in the right hemisphere. For rs-fMRI protocol, all mice were implanted in the left hemisphere.

Optogenetic stimulation procedure

After 3 to 7 days of recovery, the BLA-ACC pathway was activated or inhibited by a blue light emitting diode (LED) with a peak wavelength of 463 nm (LEDFRJ-B FC, Doric Lenses) or a

green light emitting laser with a peak wavelength of 520 nm (Miniature Fiber Coupled Laser Diode Module, Doric Lenses) respectively. From the light source, the light travelled through the fiber optic patch cable (MFP 240/250/900-0.63 0.75m FC CM3, Doric Lenses) to the implant cannula. Blue light was delivered by pulses generated through a universal serial bus connected transistor-transistor logic pulse generator (OPTG 4, Doric Lenses) connected to a LED driver (LEDRV 2CH v.2, Doric Lenses). Transistor-transistor logic pulses were generated by an open-source software developed by Doric Lenses (USBTTL V1.9). Stimulated animals received repetitive stimulation sequences of 3 seconds consisting of 2 seconds at 10 Hz with 10 milliseconds pulses and 1 second without stimulation. The whole sequence is repeated during 20 minutes each day for 3 consecutive days during 3 weeks. Light intensity was measured before implantation at the fiber tip using a photodetector (UNO, Gentec, Quebec, Canada) and was set between 3 mW and 5 mW. Green light was delivered in a continuous manner during 5 min prior (Forced Swim Test and Dark/light test) or during behavioral testing (Splash test, Novelty Suppressed Feeding Test and von Frey test). The onset and end of stimulation were manually directed. Light intensity was measured as described above and set at 16 mW. All control animals underwent the same procedure but the light remained switched off.

Behavioral assessment

Nociception-related behavior. The mechanical threshold of hind paw withdrawal was evaluated using von Frey hairs (Bioseb, Chaville, France) (Yalcin et al., 2011)*. Mice were placed in clear Plexiglas® boxes (7 x 9 x 7 cm) on an elevated mesh screen* (Yalcin et al., 2011)*. Filaments were applied to the plantar surface of each hind paw in a series of ascending forces (0.4 to 8 grams). Each filament was tested five times per paw, being applied until it just bent, and the threshold was defined as 3 or more withdrawals observed out of the 5 trials. All animals were tested before the cuff surgery to determine the basal threshold, every week after cuff surgery to ensure the development of mechanical allodynia and during optogenetic stimulation to assess the effect of the inhibition of the BLA-ACC on mechanical hypersensitivity.*

Locomotor activity. Spontaneous locomotor activity was monitored for each experimental group. Mice were individually placed in activity cages (32 x 20 cm floor area, 15 cm high) with 7 photocell beams. The number of beam breaks was recorded over 30 min using Polyplace software (Imetronic, Pessac, France).

Real time place avoidance (RTA) and Conditioned Place Preference (CPP). The apparatus consists of 2 connected Plexiglas chambers (size 20 cm x 20 cm x 30 cm) distinguished by the wall patterns. On the first day (pre-test), animals are free to explore the apparatus during 5 minutes (CPP) or 10 min (RTA), and the time spent in each chamber is measured to control for the lack of spontaneous preference for one compartment. Animals spending more than 75% or less than 25% of the total time in one chamber were excluded from the study. For RTA, the second day (test), animals are plugged to the light source placed between the 2 chambers and let free to explore for 10 min. Light is turned on when the mice enter its head and forepaws in the stimulation paired chamber and turned off when it quit thecompartment. Total time spent in the stimulation-paired chamber is measured. For CPP, on the second and third days (conditioning), animals are maintained during 5 minutes in one chamber, where optogenetic stimulation occurs, and 4 h later placed during 5 min in other chamber, without optogenetic stimulation. On the 4th day (test), the time spent in each chamber is recorded during 5 min.

Anxiodepressive-related behaviors

Behavioral testing was performed during the dark phase, under red light. While each mouse went through different tests, they were never submitted to more than 2 tests per week, and never went through the same test twice. The forced swimming test (FST) was always considered as terminal. Body weights were measured weekly.

Dark-Light Box Test. The apparatus consisted of light and dark boxes (18 x 18 x 14.5 cm each). The lit compartment was brightly illuminated (1000 lux). This test evaluates the conflict between the exploratory behavior of the rodent and the aversion created by bright light. Mice were placed in the dark compartment in the beginning of the test, and the time spent in the lit compartment was recorded during 5 min (Barthas et al., 2015)*. For inhibition experiment, the test was performed immediately after the light stimulation.*

Novelty suppressed feeding test. The apparatus consisted of a 40 x 40 x 30 cm plastic box with the floor covered with 2 cm of sawdust. Twenty-four hours prior to the test, food was removed from the home cage. At the time of testing, a single pellet of food was placed on a paper in the center of the box. The animal was then placed in a corner of the box and the latency to eat the pellet was recorded within a 5-minute period. This test induces a conflict between the drive to eat the pellet and the fear of venturing in the center of the box (Santarelli et al., 2003)*. For inhibition experiments, optogenetic stimulation was conducted*

during the test.

Splash Test. This test, based on grooming behavior, was performed as previously described (Santarelli et al., 2003; Yalcin et al., 2011)*. Grooming duration was measured during 5 minutes after spraying a 20% sucrose solution on the dorsal coat of the mice. Grooming is an important aspect of rodent behavior and decreased grooming in this test is considered related to the loss of interest in performing self-oriented minor tasks* (Yalcin et al., 2008)*. For inhibition experiments, optogenetic stimulation was conducted during the test.*

Nest Test. This test, based on a rodent innate behavior, was performed in the cages identical to the home cages of animals. Each mouse was placed in a new cage with cotton square in the center. Water and food were provided ad libitum. After 5 h, mice were placed back in their original cages and pictures of the constructed nest were taken. A score wasgiven blindly to each nest as follows: 0 corresponds to an untouched cotton square, 1 to a cotton square partially shredded, 2 if the cotton is totally shredded but not organized, 3 if cotton is totally shredded and organized in the center of the cage, 4 if the cotton is totally shredded and shows a well-organized shape in the corner of the cage, like a nest (Deacon, 2006; Otabi et al., 2017)*.*

Forced Swim Test. FST (Porsolt et al., 1977) *was conducted by gently lowering the mouse into a glass cylinder (height 17.5 cm, diameter 12.5 cm) containing 11.5 cm of water (23-25C). Test duration was 6 minutes. The mouse was considered immobile when it floated in the water, in an upright position, and made only small movements to keep its head above water. Since little immobility was observed during the first 2 minutes, the duration of immobility was quantified over the last 4 minutes of the 6-minute test. Concerning inhibition experiments, the test was performed just after the stimulation.*

MRI data acquisition

Scans were performed with a 7T Bruker BioSpec 70/30 USR animal scanner, room temperature surface coil for the acquisition of the MRI signal and ParaVision software version 6.1 (Bruker, Ettlingen, Germany) at baseline and 8 weeks after peripheral nerve injury. Animals were prepared and placed in the scanner under 2% isoflurane; a bolus of medetomidine (0.15 mg/kg body weight (bw) s.c.) was administered during preparation. 10 minutes after the bolus injection, isoflurane was stopped, and animal bed was placed in the scanner. Right before the rs-fMRI scans the medetomidine infusion was started (0.3 mg/kg bw/h, s.c.). Respiration and body temperature were monitored throughout the imaging session.

Acquisition parameters for rs-fMRI were: single shot GE-EPI sequence, 31 axial slices of 0.5 mm thickness, FOV=2.12×1.8 cm, matrix=147×59, TE/TR= 15 ms/2000 ms, 500 image volumes, 0.14× 0.23× 0.5 mm³ resolution. Acquisition time was 16 minutes. Morphological T2-weighted brain images (resolution of 0.08×0.08×0.4 mm^3) were acquired with a RARE sequence using the following parameters: TE/TR =40 ms/4591 ms; 48 slices,
0.4 mm slice thickness, interlaced sampling, RARE factor of 8, 4 averages; an acquisition matrix of 256 × 256 and FOV of 2.12×2 cm2.

MRI data processing

Rs-fMRI images were spatially normalized into a template using Advanced Normalization Tools (ANTs) software (Avants et al., 2011) *using SyN algorithm and smoothed (FWHM=0.28×0.46×1 mm3) with SPM8. Seed-based functional connectivity analysis was performed with a MATLAB tool developed in-house. Regions of interest (ROI) were extracted from Allen Mouse Brain Atlas* (Lein et al., 2007) *which were later normalized into the template space. Resting-state time series were de-trended, band-pass filtered (0.01-0.1Hz) and regressed for cerebrospinal fluid signal from the ventricles. Principal component analysis (PCA) of the BOLD time courses across voxels within a given ROI was performed and first principal component accounting for the largest variability was selected as the representative time course for further analysis. Partial correlation (PC) between the representative time courses of selected ROIs were computed to construct individual connectivity matrices for each mouse (76 pre-selected regions comprising limbic, cortical, reward, and nociceptive areas and covering the entire isocortex and major subcortical areas). Fisher's r-to-z transformation was applied to individual matrices and average PC matrices were computed by pooling the two groups at baseline, and for each group at 8 PO weeks. Connections surviving p<0.001 (uncorrected) threshold for one sample t-test were selected for graph theoretical analysis using NetworkX software package for Python (https://networkx.github.io). A ranking of hub regions (nodes) are reported. For statistical comparison between the two groups, individual baseline matrices were subtracted from those belonging to 8 PO weeks, and two sample t-test was applied for subtraction matrices. Most changed connections (edges, expressed as degrees) and most changed nodes (expressed as Stouffer coefficients) were ranked among connections surviving p<0.05 (uncorrected) threshold. Briefly, Stouffer method uses a single p-value computed for each region based on the combination of the p-values derived from the statistical tests made on the correlations with all other regions, highlighting the regions with major changes in the inter-group comparison (Stouffer et al., 1949).*

Furthermore, Spearman correlations between the PCA time course of single ROIs and each voxel of the brain was computed at the group and individual levels and r values were converted to z using Fisher's r-to-z transformation. Individual connectivity maps for baseline rs-fMRI acquisitions were subtracted from 8 PO weeks counterparts for each subject. Baseline subtracted connectivity maps were subsequently used for two sample t-test with SPM8 to perform group comparison. Family- wise error rate (FWER) correction was applied at the cluster level ($p<0.05$) for each statistical image.

Immunohistochemistry

c-Fos immunoperoxydase: Animals were stimulated once with the same procedure as described before (for BLA-ACC activation). 90 minutes later, animals were anesthetized with Euthasol (182 mg/kg) and perfused with 30 ml of 0.1 M phosphate buffer (PB, pH 7.4) followed by 100 ml of 4% paraformaldehyde solution (PFA) in 0.1 M PB. Brains were removed, post fixed overnight and kept at 4°C in 0.1 M PBS (PBS, pH 7.4) until cutting. Coronal sections (40 µm) were obtained using a vibratome (VT 1000S, Leica, Deerfield, IL) and were serially collected in PBS. Sections were incubated 15 minutes in a 1% H2O2/50% ethanol solution and washed in PBS (3 x10 minutes). Sections were then pre-incubated in PBS containing Triton X-100 (0.3%) and donkey serum (5%) for 45 minutes. Sections were then incubated overnight at room temperature in PBS containing Triton X-100 (0.3%), donkey serum (1%) and rabbit anti-c-Fos (1:10000, Santa Cruz Biotechnology, E1008). Sections were then washed in PBS (3 x 10 minutes), incubated with biotinylated donkey anti-rabbit secondary antibody (1:300) in PBS containing Triton X-100 (0.3%), donkey serum (1%) for 2 hours and washed in PBS (3 x 10 minutes). Sections were incubated with PBS containing the avidin-biotin-peroxidase complex (ABC kit; 0.2% A and 0.2% B; Vector laboratories) for 90 minutes. After being washed in Tris-HCl buffer, sections were incubated in 3,3'diaminobenzidine tetrahydrochloride (DAB) and H2O2 in Tris-HCl for approximately 4 minutes and washed again. Sections were serially mounted on gelatin-coated slides, air dried, dehydrated in graded alcohols, cleared in Roti-Histol (Carl Roth, Karlsruhe, Germany) and coverslipped with Eukitt. c-Fos immunohistochemistry then allowed controling for both the implant location and the activation of the ACC by the optogenetic procedure. Animals having c-Fos induction outside of the ACC, for instance in the motor cortex, were excluded from analysis.

c-Fos immunofluorescence: Animals were anesthetized with Euthasol (182 mg/kg) and perfused with 30 ml of 0.1 M phosphate buffer (PB, pH 7.4) followed by 100 ml of 4%

paraformaldehyde solution (PFA) in 0.1 M PB. Brains were removed, post fixed overnight and kept at 4°C in 0.1 M PBS (PBS, pH 7.4) until cutting. Coronal sections (40 µm) were obtained using a vibratome (VT 1000S, Leica, Deerfield, IL) and were serially collected in PBS. Sections were washed in PBS (3 x 10 minutes) and pre-incubated in PBS containing Triton X-100 (0.3%) and donkey serum (5%) for 45 minutes. Sections were then incubated overnight at room temperature in PBS containing Triton X-100 (0.3%), donkey serum (1%) and rabbit anti-c-Fos (1:1000, Synaptic System, 226-003). Sections were then washed in PBS (3 x 10 minutes), incubated with Alexa fluor 594 donkey anti-rabbit secondary antibody (1:400) in PBS containing Triton X-100 (0.3%), donkey serum (1%) for 2 hours and washed in PBS (3 x 10 minutes). Sections were finally serially mounted with vectashield medium (Vector laboratories).

Fluorogold and cFos quantification

Single-layer images were acquired using a laser-scanning microscope (confocal Leica SP5 Leica Microsystems CMS GmbH) equipped with x20 objective. Excitation wavelengths were sequentially diode 405nm, argon laser 488nm and diode 561nm. Emission bandwidths are 550-665 nm for fluorogold fluorescence and 710-760 nm for Alexa594 signal. Quantification of c-Fos and fluorogold positive cells was performed from 3 sections for each animal using the StarDist 2D model. The StarDist 2D model was trained from scratch for 400 epochs on 6 paired image patches (image dimensions: (176,176), patch size: (176,176)) with a batch size of 2 and a mae loss function, using the StarDist 2D ZeroCostDL4Micnotebook (v 1.11) (10). Key python packages used include tensorflow (v 0.1.12), Keras (v2.3.1), csbdeep (v 0.6.1), numpy (v 1.19.4), cuda (v 10.1.243). The training was accelerated using a Tesla T4 GPU.

RNAscope

Brain samples were immersed in isopentane and immediately placed at -80°C. Frozen samples were embedded in OCT compound and 14µm thick sections were performed on cryostat, mounted on slides and put back in -80°C freezer. Sections were fixed, dehydrated and pre-treated using the « RNAscope Sample Preparation and Pre-treatment Guide for Fresh Frozen Tissue using RNAscope Fluorescent Multiplex Assay » protocol (Advanced Cell Diagnostics). Hybridation of Slc17a7 (ACD, 416631), Gad65 (ACD, 415071-C2) and cFos (ACD, 316921) probes and development of the different signals with Opal 520, 590 and 690 fluorophores were performed in accordance with the « RNAscope Multiplex Fluorescent Reagent Kit v2 Assay » instructions (Advanced Cell Diagnostics).

Single-layer images were acquired using a laser-scanning nanozoomer (S60; Hamamatsu Photonics) at 10X magnification and adjusted for brightness and contrast using Image J 1.52p software. Quantification of c-Fos, Vglut1 or Gad65-positive cells was performed from two sections for each animal. Using a plugin, identification and counting of positive cells were performed as follow: detection of nucleus, determination of ROI (ACC), detection of cFos positive cells, Vglut1 positive cells and Gad65 positive cells.

RNA extraction

Two different batches of animals were generated for RNA-sequencing, with a third one for Fluidigm validation of RNA-sequencing results. Bilateral ACC was freshly dissected from animals killed by cervical dislocation and tissues were stored at -80C. Total RNA was extracted from ACC tissue with the Qiagen RNeasy Mini Kit (Hilden Germany). Around 20 mg of ACC tissue was disrupted and homogenized with a Kinematica Polytron 1600E in 1.2 ml QIAzol Lysis reagent, for 30 s, and then left at room temperature for 5 min. Next, 240 ul of chloroform was added and mixed before centrifugation for 15 min at 12.000 rpm at 4°C. The aqueous phase (600 ul) was transferred to a new collection tube and mixed with 600 ul of 70% ethanol. The mix was transferred into a RNeasy spin column in a 2 ml collection tube, and centrifuged at 10.000 rpm for 15 s. Next, 350 ul of RW1 buffer was added and centrifuged at
10.000 rpm, for 15 s, before adding 10 ul of DNAse and 70 ul of RDD buffer. The mix was left at room temperature for 15 min and 350 ul of RW1 buffer was added and centrifuged at 10.000 rpm for 15s. The column was then transferred to a new 2 ml collection tube and washed with 5000 ul of RPE buffer, before being centrifuged at 10.000 rpm. Finally, the column was dry centrifuged at 10.000 rpm for 5 min, and transferred to a new 1.5 ml collection tube to which 18 ul of RNase-free water was added. Finally, the RNA was eluted by centrifugation for 1 min at 10.000 rpm. Samples were kept at -80°C until use.

Mouse RNA-sequencing

RNA-sequencing was performed by the Genomeast platform at IGBMC. Full length cDNAs were generated from 5 ng of total RNA using the Clontech SMARTSeq v4 Ultra Low Input RNA kit for Sequencing (PN 091817, Takara Bio Europe, Saint-Germain-en-Laye, France) according to manufacturer's instructions, with 10 cycles of PCR for cDNA amplification by Seq-Amp polymerase. Six hundred pg of pre-amplified cDNA were then used as input for Tn5 transposon tagmentation by the Nextera XT DNA Library Preparation Kit (PN 15031942,

Illumina, San Diego, CA), followed by 12 PCR cycles of library amplification. Following purification with Agencourt AMPure XP beads (BeckmanCoulter, Villepinte, France), the size and concentration of libraries were assessed by capillary electrophoresis. Libraries were then sequenced using an Illumina HiSeq 4000 system using single-end 50 bp reads. Reads were mapped onto the mm10 assembly of the Mus musculus genome, using STAR version 2.5.3a (Dobin et al., 2013)*. Gene expression quantification was performed from uniquely aligned reads using htseq-count* (Anders et al., 2015) *version 0.6.1p1, with annotations from Ensembl version 95. Read counts were then normalized across samples with the median-of-ratios method proposed by Anders and Huber* (Anders & Huber, 2010)*, to make these counts comparable between samples. Principal Component Analysis was computed on regularized logarithm transformed data calculated with the method proposed by Love and collaborators* (Love et al., 2014)*. Differential expression analysis was performed using R and the Bioconductor package DESeq2 version 1.22.1* (Love et al., 2014)*, using RIN values and batch as covariates. Because we generated two batches of mice, the lfcShrink function was used instead of betaPrior in order to calculate p-values from the log2 Fold-changes unshrinked and to perform the shrinkage afterwards.*

Human RNA-Sequencing data

Human gene expression data, obtained from our previous publication (Lutz et al., 2017)*, were generated initially using post-mortem ACC tissue from the Douglas-Bell Canada Brain Bank. This cohort was composed of 26 subjects who died by suicide during a major depressive episode, and 24 psychiatrically healthy controls. Groups were matched for age, post-mortem interval and brain pH, and include both male (19 in control group and 19 in MDD group) and female (5 in control group, 7 in MDD group) subjects. Demographics for the cohort can be found in Supplementary Table 1. While differential expression analysis for the whole cohort (both males and females) was reported previously* (Lutz et al., 2017)*, during the present work we reprocessed raw gene counts from male individuals only, and conducted a new differential expression analysis to compare men with MDD and men healthy control (taking into account RIN, age, and ethnic origin, as in* Lutz et al., 2017*)*

Rank-rank hypergeometric overlap (RRHO) analysis

In order to compare mouse and human RNA-Sequencing data, we used the Rank-rank hypergeometric overlap (RRHO2) procedure, as described by (Cahill et al., 2018)*, using the R package available at: https://github.com/Caleb-Huo/RRHO2. Mice-human orthologous genes*

were first obtained using the R package BioMart, leaving a total of 13572 genes. Genes in each data set were ranked based on the following metric: -log10(p-value) x sign(log2 Fold Change). Then, the RRHO2 function was applied to the 2 gene lists at default parameters (with stepsize equal to the square root of the list length). Significance of hypergeometric overlaps between human and mouse gene expression changes are reported as log10 p-values, corrected using the Benjamini–Yekutieli procedure.

Weighted Gene Coexpression Network Analysis (WGCNA)

WGCNA (Langfelder & Horvath, 2008) *was used to construct gene networks in mice and human using RNA-seq expression data and then identify conserved gene modules between the two species. The RNA-sequencing expression data were normalized for batch and RIN in mice; and for age, ethnic origin and RIN in human (with sex included when analyzing the whole cohort). First, a soft-threshold power was defined (mouse: 4, human: 8) to reach a degree of independence superior to 0.8 and thus ensure the scale-free topology of the network. To construct the network and detect modules, the blockwiseModules function of the WGCNA algorithm was used, with the minimum size of modules set at 30 genes. Then, the eigengene of each module was correlated with our traits of interest (optogenetic stimulation of the BLA-ACC pathway in mice, or MDD in human) and gene significance (GS), defined as the correlation between each individual gene and trait, was calculated. Inside each module a measure of the correlation between the module eigengene and the gene expression profile, or module membership (MM), was also assessed. Conservation of WGCNA module across mice and human was assessed by Fisher's exact test. Modules were considered as significantly overlapping, and therefore conserved, when Padj<0.05. Among the modules displaying a significant overlap between human and mice, only those with a significant (padj<0.1) association between the module eigengene and trait, in both species, were kept for further analysis.*

Gene Ontology

Enrichment for functional terms in DEGs in human MDD and mice was performed using WEBGSTALT for biological process, cellular component and molecular function. Analysis was restricted to the genes differentially expressed at p<0.05. The same procedure was applied to the list of genes changed in the same direction in mice and human obtained by RRHO without regards on the p-value. The gene ontology$

Fluidigm

cDNA were generated by subjecting 50ng of RNA from each sample to reverse transcriptase reaction (Reverse Transcription Master Mix Kit Fluidigm P/N-100-6297). Then, 1.25 µL of each cDNA solution was used to generate a preamp mix containing a pooled of the 26 primers pairs and the PreAmp Master Mix Kit (Fluidigm P/N 100-5744). Preamp mixes were run for 14 cycles and the remaining primers were digested with Exonuclease I (New England BIOLAB. P/N M0293l. LOT 0191410). Preamp samples were analyzed for the expression of 22 genes of interest (for primer sequences see Supplementaty Table 3) using the BioMark qPCR platform (Fluidigm, San Francisco, CA, USA). Data were normalized to Gadph, B2m, Actb and Gusb (from the same animal) and fold changes were calculated using the 2-ΔΔCt method (Schmittgen & Livak, 2008).

Statistical analyses

*Statistical analyses were performed in GraphPad Prism v9.0 software. Data are expressed as mean ± SEM, with statistical significance set as *p<0.05, **p<0.01, ***p<0.001. Student's t-test (paired and unpaired), One-Way Analysis of Variance (ANOVA), One-Way Repeated Measures ANOVA, and Two-way ANOVA followed by Newman-Keuls post hoc test were used when appropriate. If data failed the Shapiro-Wilk normality test, Mann-Whitney non-parametric (one- or two-tailed) analysis was used.*

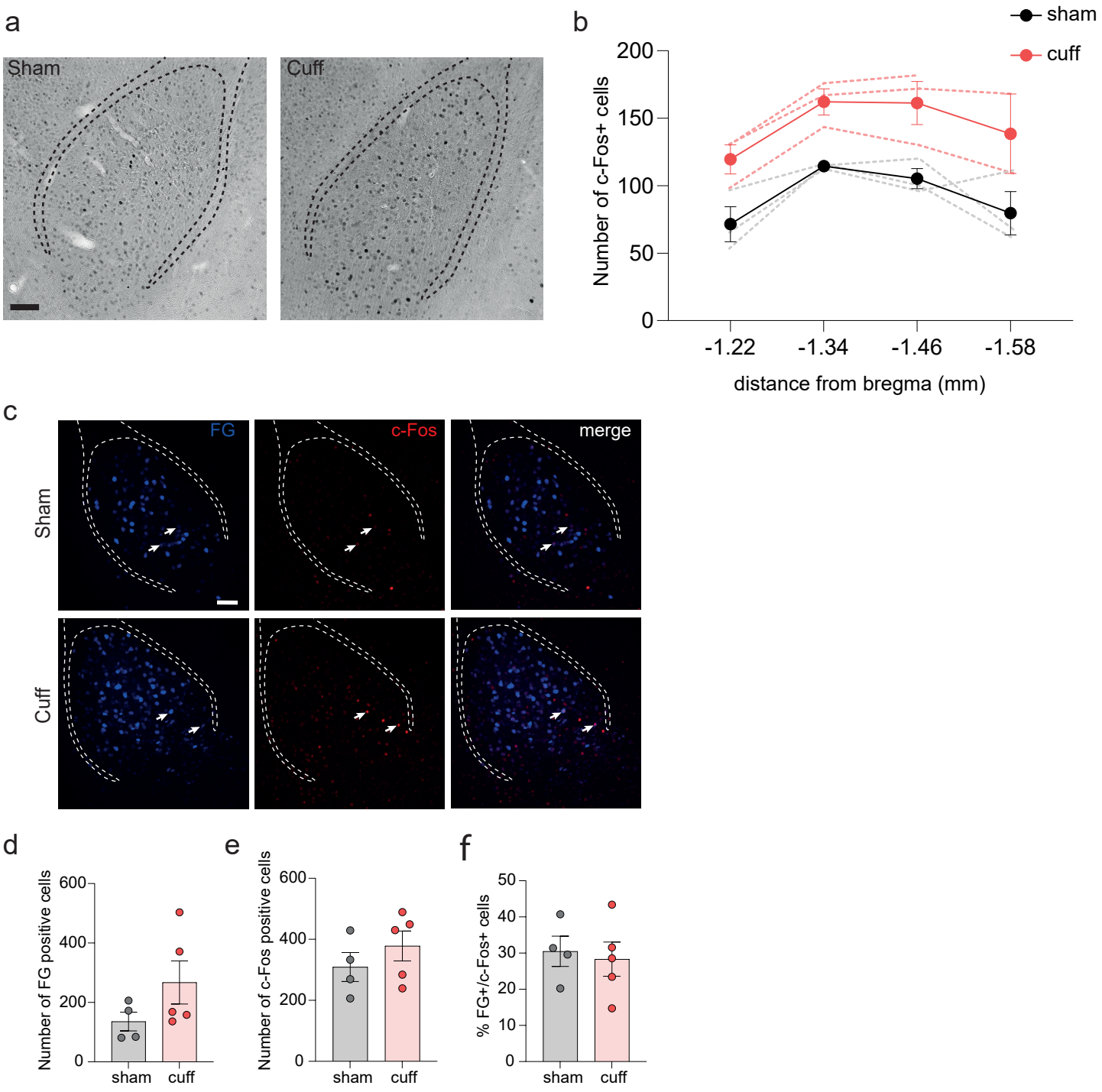

Figure S1.

a. Representative micrographs showing c-Fos immunoreactivity in the BLA of sham (left panel) and cuff (right panel) animals 8 weeks PO. b. At 8 weeks PO, the number of c-Fos positive cells in the totality of the BLA is increased in cuff animals (ctrl: n=3; stim: n=3; $F(1,4)=15.44$; $p=0.017$). c. Representative fluorescence micrographs showing fluorogold positive (FG+, upper panel), c-fos positive (c-fos+, middle panel) and their co-localization (lower panel) in the left BLA after Fluorogold injection into the ACC. d-f. Quantification of FG+, c-fos+ cells and their co-localization revealed that 8 weeks after cuff surgery, the number of FG+ (d; sham: 135.8±31.52; cuff: 267.2±72.58; $p=0.21$), c-fos+ (e; sham: 309.3±47.60; cuff: 378.2±49.10; $p=0.14$) and FG+/c-fos+ cells (f; sham 30.50±4.20; cuff: 28.33±4.72; $p=0.45$) was not altered in the left BLA (sham: n=4; cuff: n=5).

Data are represented as mean ± SEM. *$p<0.05$; **$p<0.01$. 2-Way ANOVA (anterioposteriority x Surgery) (c-fos quantification); one-tailed Mann-Whitney test (FG, c-Fos quantification). Scale bar = 100µm. PO, post-operative

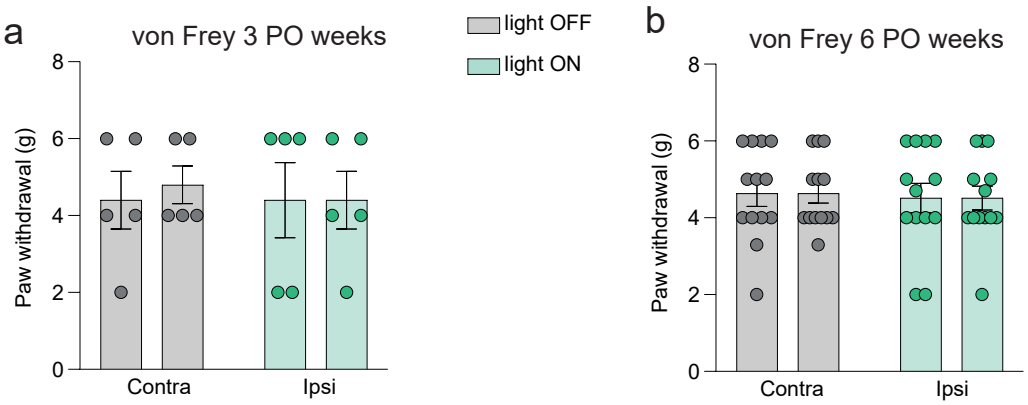

Figure S2.
At 3 (a) or 6 (b) weeks after peripheral nerve injury, sham animals do not display mechanical hypersensitivity and their mechanical nociceptive threshold was not altered by optogenetic inhibition of the BLA-ACC pathway (ipsi vs contra; 3 PO weeks $F(1,4)=0.12$; $p=0.75$; 6 PO weeks $F(1,12)=0.19$; $p=0.67$; light-off vs light-on; 3 PO weeks $F(1,4)=0.12$; $p=0.75$; 6 PO weeks $F(1,12)=0.00$; $p>0.99$).
Data are represented as mean ± SEM. #= main effect; *$p<0.05$; **$p<0.01$. Two-way ANOVA repeated measures (von Frey).

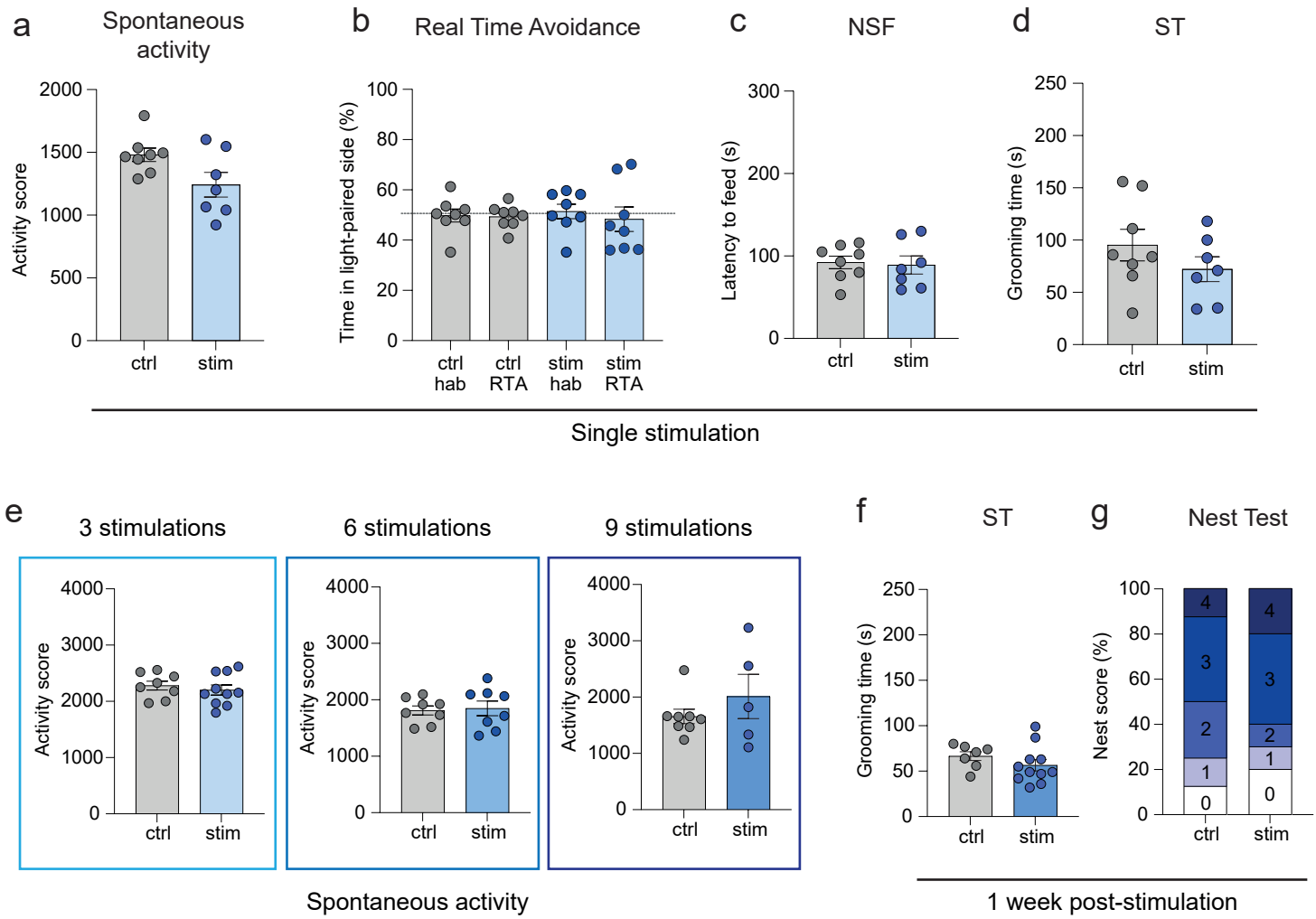

Figure S3.

a-b. Single activation of the BLA-ACC pathway does not alter spontaneous locomotor activity (a; ctrl n=8; 1480±53.37; stim n=7; 1329±85.36; p=0.15), or induce avoidance in the real time avoidance test (RTA, ctrl: n=8; stim: n=8; F(1, 14)=0.006; p=0.94). c-d. Single activation of the BLA-ACC pathway does not change anxiety-like behaviors in the NSF (c; ctrl n=8; 92.25±7.57; stim n=7; 89.14±10.97; p=0.82), or the grooming behavior in the ST (d; ctrl n=8; 95.25±15.13; stim n=7; 72.14±11.85; p=0.26). e. Repeated activation of the BLA-ACC pathway does not change spontaneous locomotor activity (3 stim: ctrl: n=8; 2281±78.89; stim: n=10; 2202±88.73; p=0.53; 6 stim: ctrl: n=8; 1812±80.24; stim: n=8; 1849±129.3; p=0.81; 9 stim: ctrl: n=8; 1656±128.0; stim: n=5; 2011±393.4; p=0.33). f-g. One week after the ninth stimulation no further deficits in grooming (f; ctrl: n=7; 66.57±4.86; stim: n=11; 56.55±6.22; p=0.27) or nesting behaviors (g; ctrl: n=7; stim: n=11; Chi-square=1.469; p=0.83) were observed in stimulated animals. h. Representative image of AAV-CamkIIa-ChR2-eYFP injection in bilateral BLA (upper panels) and eYFP fibers in the ACC (lower panels).

Data are represented as mean ± SEM. unpaired t-test (Spontaneous activity, NSF, ST); chi-square test for trend (Nest test); Two-Way ANOVA repeated measures (Time point x Stimulation) (RTA).

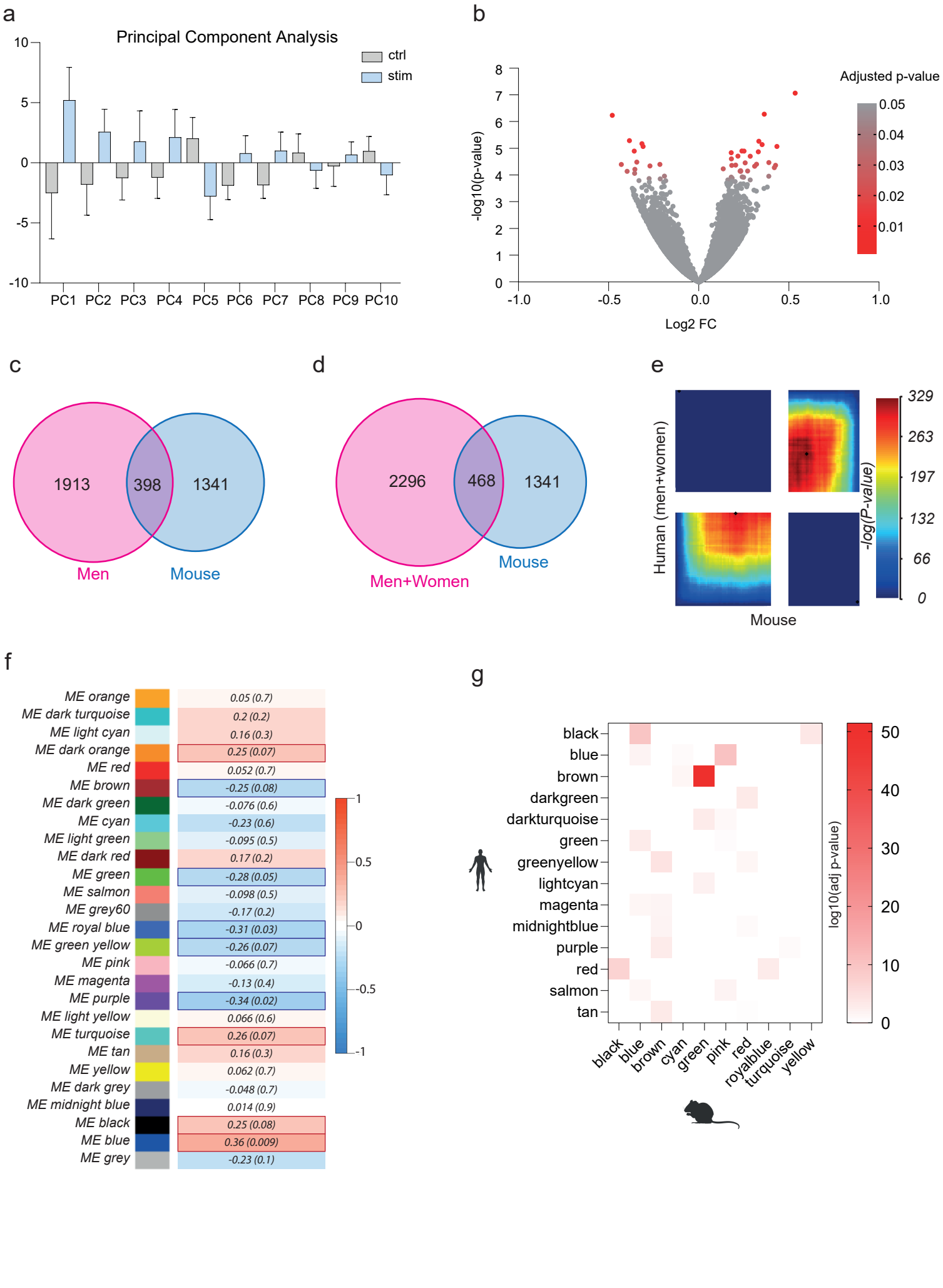
a
Principal Component Analysis
ctrl
stim
10
5
0
-5
-10
PC1 PC2 PC3 PC4 PC5 PC6 PC7 PC8 PC9 PC10
b
Adjusted p-value
0.05
0.04
0.03
0.02
0.01
-log10(p-value)
8
7
6
5
4
3
2
1
0
-1.0
-0.5
0.0
0.5
1.0
Log2 FC
c
1913
398
1341
Men
Mouse
d
2296
468
1341
Men+Women
Mouse
e
Human (men+women)
Mouse
-log(P-value)
329
263
197
132
66
0
f
ME orange 0.05 (0.7)
ME dark turquoise 0.2 (0.2)
ME light cyan 0.16 (0.3)
ME dark orange 0.25 (0.07)
ME red 0.052 (0.7)
ME brown -0.25 (0.08)
ME dark green -0.076 (0.6)
ME cyan -0.23 (0.6)
ME light green -0.095 (0.5)
ME dark red 0.17 (0.2)
ME green -0.28 (0.05)
ME salmon -0.098 (0.5)
ME grey60 -0.17 (0.2)
ME royal blue -0.31 (0.03)
ME green yellow -0.26 (0.07)
ME pink -0.066 (0.7)
ME magenta -0.13 (0.4)
ME purple -0.34 (0.02)
ME light yellow 0.066 (0.6)
ME turquoise 0.26 (0.07)
ME tan 0.16 (0.3)
ME yellow 0.062 (0.7)
ME dark grey -0.048 (0.7)
ME midnight blue 0.014 (0.9)
ME black 0.25 (0.08)
ME blue 0.36 (0.009)
ME grey -0.23 (0.1)
1
0.5
0
-0.5
-1
g
black
blue
brown
darkgreen
darkturquoise
green
greenyellow
lightcyan
magenta
midnightblue
purple
red
salmon
tan
black blue brown cyan green pink red royalblue turquoise yellow
log10(adj p-value)
50
40
30
20
10
0

Figure S4

a. Analysis of the first Principal Components show robust differences between control and stimulated animals at the whole genome level. **b.** Volcano plot showing the bidirectional distribution of the 2611 (6.9% of all genes) genes differentially expressed between stimulated (n=10) and control animals (n=12): red circles depict the 54 genes that showed a significant deregulation after correction for multiple testing (p_{adj}<0.05). **c-d.** Among the 1341 orthologous genes differentially expressed in mice, 398 (29.6%; **c.**) were also differentially expressed in men and 468 (34.9%; **d.**) when including women. **e.** Rank-Rank Hypergeometric Overlap (RRHO2) unravel shared transcriptomic changes in the ACC across mice and human (men and women included) as a function of optogenetic stimulation (mouse) or a diagnosis of major depressive disorder (MDD). Levels of significance for the rank overlap between human and mice are color-coded, with a maximal Fisher's Exact Test p=4,69E-140 for up-regulated gene (bottom-left panel) and maximal FET p=1,62E-130 for down-regulated genes (upper-right panel). **f.** WGCNA was used to analyze network and modular gene co-expression in the human ACC. The tables depict associations between individual gene modules and MDD diagnosis in human. Each row corresponds to correlations and p-values obtained against each module's eigengene. **g.** Heatmap representing the level of significance of overlaps between mice and men gene modules (measured using the FET). The highest overlap (p= 3.52E-52) was obtained for the human/brown and mouse/green modules.

Supplementary Table 1: Human and Mouse cohort metrics

Human	**MDD** (n=26)	**Controls** (n=24)
Age (Years)	42.2 ± 2.85	46.2 ± 4.4
Sex (M/F)	19/7	19/5
RIN	6.83 ± 0.15	6.86 ± 0.12
Mouse	**Stimulated** (n=10)	**Controls** (n=12)
Batch (1/2)	4/6	6/6
RIN	9.40 ± 0.28	9.44 ± 0.15

Supplementary Table 2: GO term enriched in the 5mouse modules significantly associated with optogenetic stimulation and conserved in human

module	enrichmentP	BonferoniP	termOntology	termName
blue	1.49E-08	0.000269653	CC	preribosome
blue	1.55E-06	0.028138839	BP	RNA processing
blue	3.32E-06	0.060160709	CC	cytoplasmic vesicle
blue	3.82E-06	0.069182237	CC	intracellular vesicle
blue	8.64E-06	0.156507228	CC	clathrin-coated vesicle membrane
blue	9.72E-06	0.175981124	CC	clathrin-coated vesicle
blue	9.94E-06	0.179982376	CC	protein-containing complex
blue	1.08E-05	0.195746883	BP	mRNA processing
blue	1.19E-05	0.215232033	MF	RNA binding
blue	1.25E-05	0.226475177	MF	ATP-dependent protein binding
brown	2.89E-10	5.23E-06	BP	myelination
brown	4.61E-10	8.36E-06	BP	axon ensheathment
brown	1.39E-08	0.000252159	CC	myelin sheath
brown	7.71E-08	0.001395487	BP	oligodendrocyte differentiation
brown	8.93E-08	0.001616898	BP	glial cell differentiation
brown	9.37E-08	0.001697005	BP	gliogenesis
brown	1.80E-07	0.003260951	BP	nervous system development
brown	1.87E-07	0.003391081	CC	cell periphery
brown	3.49E-07	0.006325485	BP	neurogenesis
brown	1.21E-06	0.02197177	CC	plasma membrane
greenyellow	0.00015471	1	BP	neuronal action potential
greenyellow	0.00036253	1	MF	ion channel activity
greenyellow	0.00059938	1	MF	channel activity
greenyellow	0.000614	1	MF	passive transmembrane transporter activity
greenyellow	0.00069401	1	BP	action potential
greenyellow	0.00084187	1	MF	ion transmembrane transporter activity
greenyellow	0.00094943	1	BP	trigeminal nerve structural organization
greenyellow	0.00103757	1	MF	cation channel activity

greenyellow	0.00126358	1	CC	calyx of Held
greenyellow	0.00141493	1	BP	trigeminal nerve development
magenta	0.0002393	1	MF	ubiquitin-like protein-specific protease activity
magenta	0.00082264	1	CC	nucleoplasm
magenta	0.00118341	1	MF	nucleic acid binding
magenta	0.00218795	1	BP	protein desumoylation
magenta	0.00228823	1	MF	cysteine-type peptidase activity
magenta	0.00255046	1	CC	cytoplasmic stress granule
magenta	0.00349927	1	CC	nucleus
magenta	0.0038129	1	CC	nuclear lumen
magenta	0.0045933	1	BP	double-strand break repair via break-induced replication
magenta	0.00480606	1	BP	lung alveolus development
yellow	1.90E-15	3.44E-11	BP	mitochondrial translational termination
yellow	5.13E-15	9.28E-11	BP	translational elongation
yellow	5.24E-15	9.49E-11	CC	mitochondrial inner membrane
yellow	1.05E-14	1.90E-10	BP	mitochondrial translational elongation
yellow	1.51E-13	2.74E-09	BP	translational termination
yellow	2.07E-13	3.74E-09	BP	mitochondrial translation
yellow	2.58E-13	4.67E-09	CC	mitochondrial envelope
yellow	3.71E-13	6.72E-09	CC	organelle inner membrane
yellow	9.12E-13	1.65E-08	CC	mitochondrial membrane
yellow	1.54E-12	2.79E-08	BP	regulation of cellular amino acid metabolic process

Supplementary Table 3: Primers sequences for RT-qPCR

Gene name	Sequence
B2m	F TGCTACGTAACACAGTTCCACC R: TCTGCAGGCGTATGTATCAGTC
Gapdh	F: TGGCCTCCAAGGAGTAAGAAAC R: TGGGATGGAAATTGTGAGGGAG
Actb	F: ATCAGCAAGCAGGAGTACGATG R: GGTGTAAAACGCAGCTCAGTAAC
Gusb	F: TACCGACATGAGAGTGGTGTTG R: TAATGTCAGCCTCAAAGGGGAG
Plp1	F: AGCAAAGTCAGCCGCAAAAC R: TGAGAGCTTCATGTCCACATCC
Ermn	F: AGAGAACCTCTTCGTTGTTCACC R: TTGCTGGGCAGTTCTTTCCTTC
Aspa	F: CACTTCTAACATGGGTTGCACTC R: AAACAGAGCAGGGTAATGGAGC
Ugt8	F: TGAAGGAGAGCTGTATGATGCC R: TCCGTCATGGCGAAGAATGTAG
Mal	F: ATTACCATGAAAACATCGCCGC R: TTAATGGGGAAGATGGGCTGAC
Mog	F: CATAAAGATGGCCTGTTTGTGGAG R: CCCTGGTCCTATCACTCTGAATTG
Mbp	F: ATCGGCTCACAAGGGATTCAAG R: TATATTAAGAAGCCGAGGGCAGG
Lingo1	F: CAACAAGACCTTCGCCTTCATC R: TGCTTTGTGTTGCCTTTGCC
Sema4a	F: AGCCATGTGGTCATGTATCTGG R: TGAATCTCCTCCACGAGATAAGC

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General Discussion

Chronic pain and MDD are complex conditions, involving deficits in diverse modalities such as somatosensory and nociceptive perception, motivation, memory or emotional regulation (Hart et al., 2003; Pan et al., 2019; Zambito Marsala et al., 2015). Because of its wide connections with structures involved in all those modalities (Fillinger et al., 2017, 2018), the ACC appears as an ideal structure to integrate the polymodal information involved in pain and emotional regulation. It is therefore not surprising that the ACC is one of the strongly altered brain area in chronic pain and mood disorders patients (Apkarian et al., 2005; Bushnell et al., 2013; Drevets, Savitz, et al., 2008) as well as in animal models of pain and depression (Barthas et al., 2017; Bliss et al., 2016; Koga et al., 2015; Sellmeijer et al., 2018; Zhuo, 2016). In addition, strategies aimed at reversing functional alterations within the ACC proved to be useful in the treatment of those pathologies (Boccard et al., 2017; Mayberg et al., 2005; Peyron et al., 2019). Yet, especially in the case of depression, the clinical picture can be very different from patient to patient complicating the choice of an appropriate therapeutic strategy (Goldberg, 2011). To overcome this issue, a better understanding of the pathophysiology of chronic pain and mood disorders as well as the mechanisms underlying already existing treatments is needed. To that end, linking alterations in discrete pathways with precise symptoms could be a big step forward (Young et al., 2017; Zotev et al., 2020). Indeed, these advances might help developing personalized medicine according to the clinical picture of each patient such as such as rTMS or DBS (*i.e.,* refining stimulation protocol or electrode placement) (Fabbri & Serretti, 2020; Saltiel & Silvershein, 2015).

The following section will discuss the main findings of this work and put them into perspective with the current knowledge in the field. First, we will discuss how neuropathic pain affects the BLA-ACC pathway. An emphasis will then be made on the importance of distinct circuits for the regulation of the different components of chronic pain and emotional processing. We will also tackle the possible mechanisms occurring in the ACC and discuss the benefit of conducting transcriptomic investigations by combining human and preclinical data. We will also discuss different hypotheses concerning the role of oligodendrocytes in depression. Finally, we will

replace the BLA-ACC pathway in the pain and emotion networks and conclude on the perspectives that might be implemented in order to complete our characterization of the BLA-ACC pathway in emotional regulation.

I. Activation of the BLA neuron projecting to the ACC is crucial for chronic pain-induced depression

Clinical investigations report major alterations of the ACC in chronic pain and depression patients. Indeed, increased activity (Drevets, Savitz, et al., 2008; Mayberg et al., 1997), volumetric and functional connectivity changes (Cotter et al., 2001; Drevets, Price, et al., 2008; Si et al., 2004) or transcriptomic alterations (Lutz et al., 2017; Nagy et al., 2020) are frequently observed in the ACC of depressed patients. These observations are strengthened by preclinical studies highlighting morphological and functional alterations in the ACC following chronic stress or chronic pain exposure (Sellmeijer et al., 2018; Zhuo, 2016). Several years ago, our team showed that the ACC was a crucial hub for anxiety, depression and their comorbidity with chronic pain. Indeed, while activation of the ACC promotes anxiodepressive-like behaviors in naïve mice, its lesion or optogenetic inhibition alleviates the affective consequences of neuropathic pain (Barthas et al., 2015; Sellmeijer et al., 2018). Then, using a tract-tracing approach, we mapped the connectome of the ACC and uncovered a strong connection with the BLA, another structure strongly altered in chronic pain and depressed patients (Bushnell et al., 2013; Maheu et al., 2013; Sibille et al., 2009; Tamburo et al., 2009; Vachon-Presseau et al., 2016). Surprisingly, very few studies have investigated the role of the pathway between the BLA and the ACC in depression or in its comorbidity with chronic pain. Indeed, at the clinical level, one study reported altered synchronization between these two structures in depressed patients (Workman et al., 2016) and in rodent, it was shown that lesion or inactivation of the BLA prevents the morphological alteration of the ACC induced by chronic immobilization stress (Tripathi et al., 2019a, 2019b, 2019c). Therefore, we were interested in deciphering the effect of chronic pain induced-depression on the BLA-ACC pathway activity and the impact of manipulating this pathway on pain-, anxiety- and depressive-like consequences.

1. Neuropathic pain alters the BLA, the ACC and their communication

In this present study, we showed that eight weeks following neuropathic pain induction, when negative affective consequences are present, an increased c-fos immunoreactivity is observed in the BLA suggesting a hyperactivity of the structure in our CPID model. Surprisingly, this increase was found only in the BLA ipsilateral to the peripheral nerve lesion. This is inconsistent with a study showing that in a rat model of chronic constriction injury, only the contralateral part of the BLA is activated by noxious stimulation (Seno et al., 2018). A possible explanation would be the difference between the experimental designs. Indeed, in the study of Seno et al., animals were perfused ninety minutes after the noxious stimulation while in our case, no prior stimulation was performed. Therefore, the increase we observed suggests the presence of a latent hyperactivity, while the study of Seno et al., reflects the changes directly related to mechanical hypersensitivity. Nevertheless, in both cases a lateralization of BLA response is observed. Such lateralization of the amygdala activity in pain is also shown for the central nucleus of amygdala in patients (CeA) (Allen et al., 2021). In rodent, while the left and right CeA display similar activity during nociceptive stimulation in naïve animals, only the neurons of the right CeA show an increased activity in a model of arthritic pain (Ji & Neugebauer, 2009). Considering that the CeA receives polymodal nociceptive information through the BLA (Allen et al., 2021; Veinante et al., 2013), it is easily conceivable that a similar hemispheric lateralization might also occur in the BLA when pain becomes chronic. This effect could rely on a difference in interneuron number between the left and right BLA. Indeed, the left BLA intrinsically contains a bigger and denser population of PV interneurons than the right BLA (Butler et al., 2018). Therefore, in basal condition the right BLA is under an attenuated inhibitory control compared to the left BLA. In turn, this could lead to an enhanced activity of the right side compared to the left.

We further showed that the increase neuronal activation induced by chronic pain particularly affects neurons projecting to the ACC. Previous work from our team, done in the same model and at the same timepoint, highlighted a similar increase in c-fos immunoreactivity in the ACC (Barthas et al., 2017). While c-fos immunoreactivity is a useful and easily accessible tool to assess neuronal activity, one drawback is its inability to capture the dynamic of the changes. However, in our case the increase that we showed is more likely reflecting a long-lasting

hyperactivity since no prior stimulation was applied. In addition, in the work of Barthas and collaborators, the increase in c-fos was accompanied by epigenetic changes. Notably, the H3K9/14Ac mark for transcriptionally active chromatin was increased at the c-fos promotor (Barthas et al., 2017). Finally, the fMRI study conducted by Dr. Karatas in our team (data unpublished), highlighted an increase in the functional connectivity between the BLA and the ACC at the same time point (8 weeks post neuropathic pain induction). Interestingly, this increase was not found at two weeks post pain induction, suggesting that BLA and ACC alterations only emerge when the affective consequences of chronic pain emerge. This is further supported by the work of Sellmeijer and collaborators showing that the ACC is hyperactive at eight but not two weeks following cuff surgery (Sellmeijer et al., 2018). Altogether, this indicates that a sustained hyperactivity of the direct projection from the BLA to the ACC is induced when the depressive-consequences of chronic pain are present.

2. The BLA-ACC pathway modulates emotional processing but not somatosensory component of pain

Having established that CPID indeed increases the BLA-ACC pathway activity, we aimed at determining if this hyperactivity was driving the affective consequences of neuropathic pain. Accordingly, we inhibited this pathway using an optogenetic approach and evaluated nociception, anxiety, and depressive-like behaviors in the cuff model. We found that acute inhibition of the BLA-ACC pathway reverses the depressive phenotype without affecting mechanical hypersensitivity. This is consistent with our previous study showing that the lesioning or optogenetic activation/inhibition of the ACC does not modulate mechanical thresholds (Barthas et al., 2015; Sellmeijer et al., 2018). Noteworthy, the optogenetic activation or inhibition of BLA-PrL pathway was shown to respectively enhance or alleviate mechanical hypersensitivity in SNI model (J. Huang et al., 2019). Therefore, sensory and affective components of chronic pain appear to rely on different pathways between the BLA and the mPFC. Now regarding the affective consequences of neuropathic pain, we demonstrated here that the inhibition of the BLA-ACC pathway completely alleviates depressive-like behaviors without affecting anxiety. This led us to the conclusion that while the hyperactivity of the whole ACC is crucial for the expression of anxiety and depression induced by chronic pain, the inputs from the BLA are only underlying the depressive-like behaviors. Among other possibilities, the modulation of anxiety by the ACC could be mediated

by the noradrenergic projection from the LC to prefrontal cortex. Indeed, activating this projection in a rat model of peripheral neuropathy worsen the chronic pain-induced anxiety (Hirschberg et al., 2017). While it might seem surprising that in our study the BLA is not involved in anxiety modulation, considering the large body of evidence linking the BLA with those behaviors, it is possible that this modulation pass through other pathways. For instance, activation of the BLA to CeA inputs (Cai et al., 2018) or silencing of the LC-BLA projection (Llorca-Torralba et al., 2019) abolished pain-induced anxiety.

II. BLA to ACC inputs are crucial for the emergence of depression

To determine if the increased activity of the BLA-ACC pathway could trigger depressive-like behaviors outside of the context of chronic pain, we proceeded to the optogenetic activation of this pathway in naïve animals. Again, we saw that the projection from the BLA to the ACC only modulates depressive- but not anxiety-like behaviors. Interestingly, PrL/IL-projecting neurons in the BLA are known to drive anxiety-like behaviors in naïve condition (Burgos-Robles et al., 2017). Therefore, anxious, and depressive behaviors are probably encoded by distinct cell populations in the BLA, one targeting the most rostral parts of the cingulate cortex and the other one targeting the ACC.

Conversely to what we saw in our inhibition experiment, the effect of BLA-ACC activation was not immediate. Indeed, depressive-like behaviors only emerge after 9 stimulations of the BLA-ACC pathway. This suggests that plastic changes within the ACC are needed to trigger behavioral deficits. One possibility could be the alteration of synaptic plasticity in the ACC, like it was described in chronic pain. For instance, we previously showed that neuropathic pain is associated with a facilitation of excitatory transmission through pre- and post-synaptic changes (Sellmeijer et al., 2018). This is further supported by a study showing that two forms of LTP are occurring in the ACC (Koga et al., 2015). One is post-synaptic, mediated by NMDA and AMPA receptors and is recruited in chronic pain condition. The other one is pre-synaptic, involves kainate receptors and underlies chronic pain-induced anxiety (Zhuo, 2016). Therefore, it is possible that similar mechanisms occur at the level of the synapses between BLA terminals and ACC pyramidal neurons and participate in the behavioral outcomes observed in this work.

III. A common ground for depression in human and mice

To gain insight into the molecular mechanisms underlying the effect of BLA-ACC activation on depressive-like behaviors, we performed an analysis of the transcriptomic changes in the ACC following 9 stimulations of the pathway. As a result, several gene expression dysregulations already described in depressed patients were found in the ACC of mice after the stimulation of the BLA-ACC pathway. For instance, alteration in mitochondrion (Bansal & Kuhad, 2016; Scarpa et al., 2020), RNA processing (Scarpa et al., 2020), synaptic activity and structure (Howard et al., 2019), cytoskeleton (Fabbri et al., 2019), cell proliferation (Tochigi et al., 2008), chromatin modeling (Peña & Nestler, 2018) or myelination (Lutz et al., 2017; Nagy et al., 2020) are known to be associated with MDD diagnosis and were highly enriched in our differential expression analysis. In addition, to avoid possible alterations irrelevant to human MDD, we conducted most of our transcriptomic analysis based on sequencing results obtained in the ACC of MDD diagnosed patients (Lutz et al., 2017). Such analysis is emergent in the field but already proved to be valuable to determine molecular targets for further manipulation in models of depression. For instance, a study following a similar design of analysis as ours, showed that a strong sexual dimorphism in the transcriptomic alteration associated with depression exists in both human and mice (Labonté et al., 2017). Indeed, they showed that gene-network analysis in mice displaying depressive-like behaviors uncovered two hub genes, *Dusp6* in female and *EMX1* in male, strongly dysregulated in the PFC of depressed patients. A genetic manipulation in a mice model of depression showed that these two genes trigger stress susceptibility in a sex-dependent manner. Therefore, conducting molecular analysis by comparing human and animal data seems to be a promising tool for deciphering depression pathophysiology and potentially unveil new therapeutical targets. In our case, our results suggest that our optogenetic model inducing depressive-like behaviors in animals can induce similar molecular blueprint as MDD patient. Indeed, we found that 20 % of the differentially expressed genes are conserved between species, with a similar enrichment in down- and up-regulated genes as well as a gene network conservation superior to 50% between depressed patients and mice exposed to BLA-ACC activation. These findings are very similar with another study using the same type of comparison between human and animals submitted to chronic variable stress (CVS), social isolation (SI) or chronic social defeat stress (CSDS) (Scarpa et al., 2020). Indeed, as in the work of Scarpa and collaborators we uncovered dysregulation in

synaptic function, mitochondrion, and translation regulation. Thus, these alterations might constitute a common ground for depression across human and various model of depression in rodents. Interestingly, the most striking alterations induced by the optogenetic activation of the BLA-ACC pathway was observed in myelin and oligodendrocyte related genes. Indeed, the most highly conserved module between human and mice was enriched in genes related to myelination and oligodendrocyte functions. More precisely, we observed an overall down-regulation of myelin constituents (*plp1*, *mal*, *mog*, *mbp*) and enzyme of synthesis (*aspa*, *ugt8*), as well as an up-regulation of myelination inhibitors (*lingo-1*, *sema4A*) in the ACC of animals displaying depressive-like behaviors. As a follow up experience, we recently studied the number of Olig2 positive cells in the ACC after repeated optogenetic activation and the preliminary results showed a decrease in the number of Olig2 positive cells in stimulated animals displaying depressive-like behaviors. Such deficits are extensively described in clinical study of MDD. For instance, the reduction of volume reported in the ACC is frequently associated with a decrease in number, size, and density of oligodendrocytes (Antontseva et al., 2020; Edgar & Sibille, 2012; Nagy et al., 2020; Rajkowska et al., 2015) and important decrease in myelin constituents or synthesis enzymes are observed in depressed patients (Aston et al., 2005; Miguel-Hidalgo et al., 2018). Altogether, we can suggest that our optogenetic manipulation recapitulates some of the aspect of MDD pathophysiology and therefore be a useful tool for uncovering the mechanisms underlying this disorder.

IV. Oligodendrocyte as the fundamental actor of mood regulation

Myelination is an important regulator of neuronal activity and is strongly impaired in depressed patients. Therefore, it is essential to decipher the mechanisms which lead to myelin impairment and its potential consequences in order to better understand the pathophysiology of MDD. Oligodendrocytes are the myelin synthetizing cells of the CNS. By ensheathing axons, myelin mediates the conduction speed of action potential, maintains axonal integrity, and participates to the formation and stabilization of complex neuronal circuits (Antontseva et al., 2020). While myelin sheaths were for long seen as stable structures, more recent studies highlighted dynamic remodeling and adaptations that are still present at adult stage and particularly in the PFC (Kato & Wake, 2019; Nave & Ehrenreich, 2014). Through modulation of myelin thickness, or nodal/internodal distance, oligodendrocytes can regulate the fine

communication between neurons (Kato & Wake, 2019; Saab & Nave, 2017). Therefore, even minor alterations in myelin organization can lead to a cascade of events disturbing the activity of local circuits. For instance, a hypomyelination can reduce the conduction speed and have dramatic impact on synaptic plasticity. Indeed, it is known that the delay between pre- and post-synaptic spiking is determinant in the generation of long-term potentiation of long-term depression (Dan & Poo, 2004). Thus, a modification of the conduction speed, even of few milliseconds, has the potential to switch the synaptic plasticity from potentiation to depression and vice versa. In structures like the ACC where the precision of inputs timing is crucial for the integration of polymodal information, such small changes in myelination and synaptic plasticity could act as underlying mechanism for behavioral deficits (Nave & Ehrenreich, 2014). Interestingly, hypomyelination appears to be a common feature for several psychiatric disorders like attention deficit hyperactivity disorder, schizophrenia, bipolar disorder or depression (Edgar & Sibille, 2012). In rodent, a decrease in myelin thickness as well as in the expression of oligodendrocyte genes (*mbp*, *cnp1*, *olig2*, *mag*, *mog*, *mal*, *ermn*) are reliably observed in various model of depression (UCMS, CSDS, SI, CRS), particularly in the mPFC (Antontseva et al., 2020; Edgar & Sibille, 2012; Miguel-Hidalgo et al., 2018; Surget et al., 2009). Additionally, strategies aiming at altering myelination by pharmacological agents (cuprizone) or genetic manipulation (CNP1 KO, 2',3'-Cyclic-nucleotide 3'-phosphodiesterase, expressed uniquely in oligodendrocytes) tend to exacerbate depressive-like behaviors in mice (Edgar & Sibille, 2012). Conversely, administration of clemastine (a pro-myelinating agent) in mice socially isolated (J. Liu et al., 2016) or subjected to social defeat (Shimizu et al., 2020) alleviates depressive-like behaviors and myelin deficits in the PFC. It is thus clear that myelination and oligodendroglial function should be accounted in the pathophysiology of MDD. Yet, the mechanisms triggering myelin deficits in depression remain to be clarify.

Because of the nature of our optogenetic model and the results we obtained in our transcriptomic analysis, three hypotheses can be proposed. (1) Oligodendrocytes express all three types of ionotropic glutamate receptors and are therefore sensible to glutamate release (Spitzer et al., 2016). In physiological conditions, glutamate signaling is involved in the fine regulation of myelination. For instance, activation of NMDA receptors on oligodendrocyte membrane activates myelin synthesis (Rinholm et al., 2011, 2016) and promotes the

stabilization of myelin sheath (Hines et al., 2015). However, if glutamate levels are too high it becomes noxious and leads to sheath impairment or even to cell apoptosis (Habermacher et al., 2019; Spitzer et al., 2016). In our paradigm, the depressive-like behaviors are only observed after a repeated activation of the BLA-ACC pathway. Since the projection neurons of the BLA are glutamatergic, the stimulation of this pathway can induce an increase in glutamate release in the ACC and therefore might lead to myelin impairments. (2) Another possibility would be the increased expression of myelination inhibitors. Several proteins are known to regulate positively or negatively the differentiation, proliferation, and survival of oligodendrocytes. Our sequencing data highlighted the up-regulation of two inhibitors, *sema4A* and *lingo-1*. Sema4A is part of the semaphorin family, which are secreted membrane proteins expressed by neurons, notably on the axonal part. Depending on the semaphorin class, they can promote or inhibit axon ensheathment and in the case of sema4A its presence on the axon impedes myelination (Chiou et al., 2019). Lingo-1 belongs to the leucine rich repeat and Immunoglobin-like domain-containing protein family. Through interaction with the NgR1/p75 factors, lingo-1 triggers a RhoA cascade leading *in fine* to the inhibition of myelination and oligodendrocyte proliferation (Mi et al., 2004). For now, a causal link between sema4A or lingo-1 and depression is unknown, but an increase expression of lingo-1 was recently associated with the comorbidity between epilepsy and depression in rat (Ma et al., 2019). (3) Finally, the impairment in myelination could result from epigenetic modulation. Indeed, our sequencing results highlighted enrichment in genes mediating chromatin remodeling. Therefore, it is possible that a modification of chromatin compaction takes place at the level of the genes that we found dysregulated. Such changes were described in the work done on the human sequencing data by Lutz and colleagues (Lutz et al., 2017). Indeed, a decrease in methylation level at the *lingo-3* and *pou3f1* loci (both known to regulate myelination) was observed specifically in the oligodendrocyte cells. Similar alterations could thus explain the downregulation of myelin constituents or the up-regulation of *ligno-1* and *sema4A*.

V. Replacing the BLA-ACC pathway in pain and emotional processing at network level.

Pain and emotions are multifactorial processes that require the integration of polymodal stimuli in order to modulate the responses of the individual and generate adapted reactions. This integration of pain and affective information relies on similar brain areas and circuits and therefore explains the similarity of mechanisms found in chronic pain and emotional disorders (Goesling et al., 2013; Tappe-Theodor & Kuner, 2019). Among the different structures involved, the BLA and the ACC seem to have a central place in the pain/emotion network. We demonstrated in this project that the BLA-ACC pathway is activated in chronic pain state and that mimicking this activity in naïve condition leads to depressive-like behaviors and transcriptomic changes in the ACC. Now, how can we integrate these findings in the rest of the network?

The BLA receives inputs from structures conveying nociceptive (PAG, PB, MD, somatosensory and insular cortices), or contextual information (hippocampus, subiculum, entorhinal cortex), involved in stress and affective responses (nucleus incertus, DRN, LC) or motivation (VTA) and also from higher order areas carrying already integrated information (somatosensory associative areas, IL, PL and ACC) (Olucha-Bordonau FE et al., 2015). Accordingly, the pathways linking these up-stream structures and the BLA were already shown to drive pain-, anxiety- and depressive-like behaviors but also other modalities altered in chronic pain and depression such as memory, attention or motivation (Janak & Tye 2015). In the scope of my project, the hyperactivity of the BLA neurons projecting to the ACC could arise from the inputs mediating nociception. In order to verify this hypothesis we could perform a trans-synaptic tract-tracing experiment to determine the structures projecting to BLA-ACC neuron that are also activated in our CPID model.

The BLA, in turn, sends projections back to most of the aforementioned structures and again, these efferents modulate all the behaviors listed above (Janak & Tye, 2015). Interestingly, the projection neurons of the BLA receive diverse array of afferents but mostly project to other parts of the brain in a non-overlapping way. Indeed, while the BLA neurons projecting to the BNST, NAc, vHPC or mPFC constitute distinct cell populations, they all receive similar brain-wide afferents (L. Huang et al., 2021). This suggests that information are integrated and sorted

out within the BLA and then targeted to different part of emotion and pain networks to modulate discrete behavioral response. Therefore, manipulation of distinct BLA projecting neurons should have different behavioral outcomes. For instance, we demonstrated that manipulating the BLA-ACC pathway only modulates depressive-like behaviors and studies conducted by the Kay Tye group showed that BLA to IL/PrL or to vHPC respectively mediates anxiety or social behaviors (Felix-Ortiz et al., 2013; Felix-Ortiz & Tye, 2014).

During this project, we observed that activating the BLA inputs to the ACC only triggers depressive-like behaviors after repeated but not acute stimulation. Therefore, the emergence of depressive-like behaviors depends on plastic changes within the ACC. These changes are most likely very diverse and probably include synaptic plasticity, chromatin remodeling, mitochondrial function, transcription regulation and myelination deficits. For instance, impairment in long-term synaptic plasticity or imbalance in glutamatergic and GABAergic transmission were already described in the PFC of depressed patients (Lener et al., 2016). In our case a deficit in myelination and oligodendroglial cells seems to be central. Considering our RNA-sequencing results, we hypothesize that myelin and oligodendrocyte impairments induced by BLA-ACC pathway activation bring into play actors such as lingo-1 and sema4A. However, these observations remain correlative and further experiments will be needed to confirm our hypothesis.

VI. Perspectives and future directions

My PhD project is dedicated to the first extensive characterization of the role of the BLA-ACC pathway in the regulation of emotions in chronic pain and healthy conditions at the behavioral and molecular levels. It also revealed that the use of optogenetic approach to induce depression in mice can be a powerful tool to unveil transcriptomic alterations that have a potential translational value. This project also raised several questions that I want to discuss in this section, as well as experiments that could be considered in order to complete this study.

One of the points which might need further behavioral characterization, is the distinct role of the BLA-ACC pathway in anxiety and depression. Since the neuropathic pain model that we used in this present study has been shown to develop anxiety and depressive like behavior in a time dependent manner and our c-fos and fMRI experiments revealed alterations in BLA-ACC pathway activity only at late stages of chronic pain (when the depressive consequences

of neuropathic pain emerge), more precise follow up of the activity of the BLA neurons projecting to the ACC would be of interest. For this purpose, in the experiments in which we checked the c-fos/fluorogold co-localization, the staining could be performed early stages of the chronic pain, for instance at five weeks post-surgery (when the anxiety-like behaviors emerge). Alternatively, we could use fiberphotometry, which was recently developed in our team, to follow the calcium activity of BLA neurons projecting to the ACC over the course of CPID development. Such experiments would inform us more precisely on the dynamic leading to hyperactivity of the BLA-ACC pathway.

The need for repeated stimulations in order to induce depressive-like behaviors in naïve animals raises a lot of questions concerning the mechanisms taking place in the ACC. A growing body of evidence suggests that, in depression, there is a dysregulation of the excitatory/inhibitory balance in the PFC. It is well accepted that an overall increase in excitatory transmission occurs in the ACC and it is in line with our observation that, after activation of the BLA fibers, the majority of c-fos positive cells in the ACC are glutamatergic. Therefore, the repetition of the optogenetic stimulation could increase the synaptic strength between BLA fibers and pyramidal neurons of the ACC. Yet, the BLA primarily targets GABAergic cells of the ACC and in physiological condition induces an inhibition of the ACC. Therefore, it is also possible that the repetition of the stimulation decreases the synaptic strength between BLA terminals and GABAergic neurons, resulting in a loss of inhibitory control. Such plastic changes where already described in models of chronic pain at the level of BLA to IL/PrL synapses (Kiritoshi & Neugebauer, 2018; J. M. Thompson & Neugebauer, 2019).Finally, a third option would be an enhancement of the synaptic transmission between the BLA and GABAergic cells (like VIP or somatostatin interneurons) that drive disinhibition of ACC pyramidal cells. Since our RNAscope experiment revealed a slight increase in the number of *Gad65/c-fos* positive cells after BLA-ACC activation it might be interesting to go further and determine which type of interneurons are affected by our optogenetic manipulation. This could be achieved by performing co-labelling of c-fos and different GABAergic markers (*i.e.* VIP, somatostatin, parvalbumin). More globally, it would be exciting to carry out electrophysiological experiments in order to determine if and how the responses of ACC neurons to photostimulation of BLA fibers are affected by our optogenetic protocol.

Finally, our transcriptomic analysis revealed a strong down-regulation of oligodendrocyte genes and one preliminary experiment suggests that the number of olig2 positive cells is decreased after 9 stimulations of the BLA-ACC pathway. We hypothesize that this impairment is due to the increase in *sema4A* and *lingo-1* expression. In order to leap from correlation to causality, we are currently designing sh-RNA to knock-down these two genes in the ACC of mice subjected to the 9 stimulations of the BLA-ACC pathway. With that experiment, we aim to determine if blocking the up-regulation of either *sema4A* or *lingo-1* could prevent the development of depressive-like behaviors but also the down-regulation of oligodendrocyte related genes. If it is indeed the case, it would then be interesting to determine how the up-regulation of *sema4A* and *lingo-1* happen in the first place. To that end, studying the methylation or histone modification at the level of the promoters of these two genes could be interesting.

Altogether, this project shed light on the role of the BLA -ACC pathway in the modulation of depressive-like behaviors in mice. With the completion of the knock-down experiment, we would provide a proof of the causal relationship between impairment in myelination and the emergence of a depressed-like phenotype. Therefore, this could be a first step to uncover potential preclinical targets that could open new therapeutic leads in the future.

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Appendix

Résumé de Thèse en Français

Introduction

L'épisode dépressif majeur (EDM) est une maladie psychiatrique chronique et invalidante dont les retombées économiques et l'impact sur la vie des patients et de leur famille sont considérables. L'EDM est caractérisé par la persistance pendant au moins deux semaines d'une anhédonie et/ou d'une humeur dépressive accompagnée d'autres symptômes tels que des troubles de l'appétit, du sommeil, une baisse d'énergie ou encore des pensées de mort récurrentes (DSM-V). L'Organisation Mondiale de la Santé estime que cette pathologie sera l'une des principales causes de handicap au monde d'ici 2030. Malheureusement, notre compréhension de la physiopathologie de l'EDM reste limitée. Ces lacunes peuvent en partie s'expliquer par le caractère hétérogène de l'EDM. En effet, les facteurs prédisposant au développement de cette pathologie sont nombreux et variés, qu'il s'agisse de prédispositions génétiques ou de facteurs environnementaux. Si le stress chronique constitue la première cause de développement d'un EDM, la douleur chronique se place en deuxième position. Une douleur est considérée comme chronique lorsqu'elle persiste pendant plus de trois mois. Elle devient alors invalidante, détériore significativement la qualité de vie des patients et conduit à une comorbidité avec l'EDM dans 50 % des cas. Malheureusement, sur le plan thérapeutique, l'efficacité des traitements disponibles face à la douleur chronique et à l'EDM reste limitée, et cette comorbidité représente un facteur pronostic péjoratif majeur. Ainsi, dans l'optique de dégager de nouvelles pistes thérapeutiques, il apparait nécessaire de mieux comprendre les mécanismes neurobiologiques mis en jeu dans la douleur, la dépression et leur comorbidité.

De multiples approches (imagerie cérébrale chez l'Homme, modélisation animale) démontrent l'implication de plusieurs structures cérébrales dans la composante affective de la douleur chronique, notamment l'hippocampe, l'amygdale, le cortex préfrontal (PFC) et plus particulièrement le cortex cingulaire antérieur (CCA). Sur la base de la littérature et de nos propres résultats, le CCA et ses connexions semblent au centre du traitement des émotions. Cette structure présente des altérations fonctionnelles et morphologiques chez les patients déprimés, et son ablation chirurgicale ou sa stimulation cérébrale profonde peuvent atténuer les symptômes dépressifs chez des patients présentant des formes sévères et résistantes. Dans le contexte spécifique de la douleur chronique, le CCA présente une hyperactivité neuronale et sa stimulation cérébrale profonde pourrait également s'avérer bénéfique. Outre ce rôle central du CCA, les études de neuroimagerie mettent également en évidence une activation excessive de la partie antérieure du noyau basolatéral de l'amygdale (BLA), dans la dépression et lors de stimulations nociceptives chez des patients atteints de douleur chronique. De plus, une forte connectivité fonctionnelle a été documentée entre l'amygdale et le CCA, au repos, chez des patients dépressifs non traités. Chez des patients douloureux chronique, la connectivité fonctionnelle de la BLA, notamment au sein du « default mode network », est également altérée.

Dans les modèles précliniques chez le rongeur, une désynchronisation de la BLA et du CCA après exposition au stress a été mise en évidence par électrophysiologie. De plus, notre équipe a récemment montré que l'activation par optogénétique des neurones pyramidaux du CCA suffit à provoquer des comportements de type anxio-dépressif chez des souris naïves, alors que la lésion de cette structure bloque le développement

de ces comportements après une douleur chronique. De façon concordante, chez le rat, l'inactivation pharmacologique de la BLA dans un modèle de neuropathie périphérique permet l'atténuation des comportements de type nociceptif, anxieux et dépressif.

En résumé, l'ensemble de ces études chez l'Homme et chez l'animal suggèrent que le CCA et la BLA sont deux régions clés dans la régulation de l'humeur, en particulier lors d'une douleur chronique. Bien que les études se focalisant sur des régions cérébrales considérées isolément aient permis des avancées majeures dans la compréhension de la physiopathologie de la comorbidité entre douleur chronique et EDM, il est maintenant nécessaire de définir les circuits cérébraux sous-jacents. En d'autres termes, de comprendre comment ces deux structures, le CCA et la BLA, interagissent lors d'une douleur chronique. Sur le plan neuro-anatomique, notre équipe a mis en évidence une connexion forte et réciproque entre la BLA et le CCA. Dans notre modèle de dépression induite par une douleur neuropathique, nos données d'IRM fonctionnelle chez la souris (non publiées) dévoilent également une augmentation de la connectivité fonctionnelle entre le CCA et la BLA.

Sur la base de ces données, ce projet de thèse, vise à caractériser le rôle fonctionnel de cette voie neuro-anatomique et à déterminer les mécanismes moléculaires qui sous-tendent son contrôle des réponses émotionnelles. Ce projet s'articule autour de 3 axes:

1) l'étude de l'activation et de l'inhibition de la voie BLA-CCA sur les réponses émotionnelles chez des souris naïves et dans un modèle de dépression induite par une douleur chronique, respectivement ;

2) la description des mécanismes transcriptomiques recrutés dans le CCA lorsque l'activation de la voie provoque des comportements de type dépressif ;

3) l'évaluation de l'impact de la manipulation de gènes cibles sur les processus émotionnels en présence et en absence de douleur chronique.

Régulation des réponses émotionnelles de la souris par la voie BLA-CCA

Pour répondre à ce premier objectif nous avons utilisé un modèle de douleur chronique, de type neuropathique conçu et caractérisé par notre équipe. En plus d'induire des comportements nociceptifs chez les animaux, ce modèle permet le développement de comportements de type anxieux et dépressif 4 à 6 semaines suite à la mise en place de la douleur chronique. En couplant des approches de traçage neuronal et d'immunofluorescence nous avons pu montrer que les neurones du BLA et du CCA sont activés lorsque les animaux présentent une dépression induite par une douleur chronique et que les neurones activés dans le BLA sont ceux projetant au CCA. Nous avons ensuite effectué des tests comportementaux tout en inhibant la voie BLA-CCA grâce à des outils optogénétiques. Nos résultats montrent que cette inhibition permet d'atténuer sélectivement les comportements de type dépressif induits par la douleur chronique sans modifier les comportements de type anxieux et nociceptif. Afin de déterminer si cet effet était spécifique au contexte d'une douleur chronique, nous avons également cherché à activer la voie BLA-CCA chez des animaux naïfs. Tout d'abord nous avons observé qu'une activation aiguë n'avait aucun effet sur les comportements de type anxieux et dépressif. En revanche, l'activation chronique de la voie BLA-CCA induit un développement progressif de comportements de type dépressif, sans affecter les comportements de

type anxieux. Ainsi, la voie BLA-CCA semble contribuer sélectivement aux comportements apparentés à la dépression chez la souris, indépendamment de la présence d'une douleur chronique. Au niveau cellulaire, nos résultats pointent vers l'implication des neurones glutamatergiques du CCA dans ce phénomène. Cependant, la nécessité de répéter notre stimulation pour déclencher des comportements dépressifs sous-entends la mise en place de mécanismes de plasticité et notamment via des modifications transcriptomiques.

Mécanismes moléculaires sous-jacent à la dépression chez la souris et chez l'Homme

Le second objectif de ce travail de doctorat a donc été de caractériser les mécanismes moléculaires sous-tendant les réponses comportementales provoquées par l'activation chronique de la voie BLA-CCA. Pour ce faire nous avons réalisé un séquençage des ARN du CCA après activation de la voie BLA-CCA, lorsque les comportements de type dépressif sont présents. Afin de faire ressortir des altérations transcriptomiques pertinentes au regard de la pathologie humaine, nos résultats ont été comparés avec ceux obtenus dans des tissus humains de patients déprimés (générés récemment par notre équipe en utilisant des échantillons post-mortem de la banque de cerveaux Douglas-Bell Canada Brain Bank). Grâce à l'utilisation d'une approche de biologie des systèmes, c'est-à-dire une analyse des « réseaux de co-expression de gènes » nous avons fait ressortir des gènes centraux conservés chez la souris et chez l'Homme et dont l'expression est corrélée à la présence de symptômes dépressifs. Parmi ces gènes nous retrouvons des composants de la myéline (*mbp*, *mog*, *mal*, *plp1*) et leur enzyme de synthèse (*aspa*) présentant une forte sous-expression, ainsi que des inhibiteurs de la production de myéline ou de la maturation des oligodendrocytes (*sema4A*, *lingo-1*), étant eux surexprimés. Ces résultats ont ensuite été confirmés par PCR et nous ont amenés à poser l'hypothèse que la mise en place de comportements de type dépressif par l'activation de la voie BLA-CCA induisait un déficit de production de la myéline via l'augmentation de l'expression de lingo-1.

Caractérisation du rôle de *lingo-1* dans la dépression et dans les déficits de myélinisation associés

Nos résultats de séquençage soulignent une altération de la myélinisation tant dans notre modèle murin de dépression que chez le patient dépressif. En parallèle, nous observons une augmentation d'un régulateur négatif de la myélinisation et de la maturation des oligodendrocytes, *lingo-1*. Cependant ces résultats restent corrélatifs. Pour pallier à ce manquement nous venons de démarrer une étude de manipulation du gène *lingo-1* dans nos modèles de dépression induite par optogénétique et par une douleur chronique. Nous planifions d'évaluer les comportements de type anxieux et dépressif suite à l'inactivation par ARN interférence de *lingo-1* dans le CCA de nos souris. Ces travaux, qui devraient être achevés d'ici la soutenance de cette thèse, nous permettront d'apporter une réponse quant à la causalité de la relation entre la régulation de la myélinisation et les comportements apparentés à la dépression.

Conclusion

Ce travail de thèse a permis de mettre en évidence le rôle central de la voie BLA-CCA dans la dépression, que cela s'inscrive dans le contexte d'une douleur chronique ou non. De façon intéressante cette action de la voie BLA-CCA est sélective des comportements dépressifs et n'intervient pas dans les comportements anxieux. Or, des travaux provenant d'autres groupes et s'intéressant aux projections de la BLA vers des aires plus antérieures du cortex cingulaire démontrent des effets anxiogènes et anxiolytiques. Ainsi la BLA

semble pouvoir moduler de façon fine les comportements dépressifs et anxieux, via des populations neuronales ciblant des aires cérébrales différentes. Bien que les neurones de la BLA fassent majoritairement synapse sur les interneurones inhibiteurs du CCA nous observons une activation majoritaire des neurones excitateurs suite à la mise en place de comportements dépressifs. Des phénomènes de plasticité entrent probablement en jeu et des expériences complémentaires, notamment d'électrophysiologie, pourraient apporter des réponses et enrichir notre connaissance sur les mécanismes impliqués dans la désinhibition du CCA, phénomène fréquemment relevé en condition pathologique. Enfin, notre dernière expérience apportera un lien de causalité entre la dépression et les déficits de myélinisation ainsi qu'une proposition mécanistique quant au développement de comportements apparentés à la dépression suite à une hyperactivité de la voie BLA-CCA.

Ce projet aura été l'occasion de caractériser pour la première fois et de façon intégrée, la contribution de la voie BLA-CCA dans la dépression, la douleur chronique et leur comorbidité. Ainsi, nous sommes persuadés que la complétion de ce projet de thèse permettra la publication d'un article scientifique dans un journal prestigieux. Cet article est actuellement en cours d'écriture et sera soumis dans un premier temps au journal *Nature Neuroscience* d'ici la fin de l'été. Nous espérons ainsi que nos travaux apporteront de nouveaux éléments de compréhension des réseaux de neurones régulant les réponses émotionnelles et permettront à terme, d'ouvrir la voie vers de nouvelles stratégies thérapeutiques des troubles de l'humeur.

Research Paper

PAIN

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Action of mefloquine/amitriptyline THN101 combination on neuropathic mechanical hypersensitivity in mice

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Abstract

Tricyclic antidepressants that inhibit serotonin and noradrenaline reuptake, such as amitriptyline, are among the first-line treatments for neuropathic pain, which is caused by a lesion or disease affecting the somatosensory nervous system. These treatments are, however, partially efficient to alleviate neuropathic pain symptoms, and better treatments are still highly required. Interactions between neurons and glial cells participate in neuropathic pain processes, and importantly, connexins—transmembrane proteins involved in cell–cell communication—contribute to these interactions. In a neuropathic pain model in rats, mefloquine, a connexin inhibitor, has been shown to potentiate the antihyperalgesic effect of amitriptyline, a widely used antidepressant. In this study, we further investigated this improvement of amitriptyline action by mefloquine, using the cuff model of neuropathic pain in mice. We first observed that oral mefloquine co-treatment prolonged the effect of amitriptyline on mechanical hypersensitivity by 12 hours after administration. In addition, we showed that this potentiation was not due to pharmacokinetic interactions between the 2 drugs. Besides, lesional and pharmacological approaches showed that the prolonged effect was induced through noradrenergic descending pathways and the recruitment of α_2 adrenoceptors. Another connexin blocker, carbenoxolone, also improved amitriptyline action. Additional in vitro studies suggested that mefloquine may also directly act on serotonin transporters and on adenosine A_1 and A_{2A} receptors, but drugs acting on these other targets failed to amplify amitriptyline action. Together, our data indicate that pharmacological blockade of connexins potentiates the therapeutic effect of amitriptyline in neuropathic pain.

Keywords: Neuropathic pain, Amitriptyline, Mefloquine

1. Introduction

Neuropathic pain is defined as pain arising as a consequence of a lesion or disease affecting the somatosensory nervous system.[40,47] Like all forms of chronic pain, it is a major public health issue.[15] Although some antidepressant drugs are one of the best therapeutic options, their efficacy remains limited, with a number needed to treat around 3.6 to 6.4[13,37] and a pain relief that is usually partial. Enhancing the efficacy of antidepressants on neuropathic pain might thus be of therapeutic interest.

From the periphery to the central nervous system, interactions between neurons and nonneuronal cells, including glial cells, contribute to the encoding and modulation of sensory information.[4,10,17,21,27] Connexins may partly mediate some of these interactions, particularly through astrocyte networking activity.[4] These transmembrane proteins form hexa-protein complexes to form hemichannels or gap junctions, facilitating the passage of ions or small molecules such as adenosine triphosphate (ATP) or glutamate.[25] In nociceptive pathways, this interaction can occur in the dorsal root ganglia, the spinal cord, and the brain. In various models of neuropathic pain (including the chronic constriction injury [CCI] of the sciatic nerve, CCI of the infraorbital nerve, spinal nerve ligation, partial sciatic nerve ligation, spared nerve injury, spinal cord injury, and oxaliplatin-induced neuropathy), dynamic changes have been shown in the expression of connexins within these structures (for review: Refs. 31,43), particularly concerning Cx43, which is the most abundant isoform in the nervous system. With mostly intrathecal delivery, drugs or molecular tools used to modulate gap junctions and connexins have shown some efficacy to reduce mechanical hypersensitivity in animal models of neuropathic pain,[5,6,26,32,46,52] suggesting that it may be of interest to target neuroglial mechanisms through connexin manipulation. One of these drugs, mefloquine, displays inhibitory properties on gap junctions and connexins[19,35,36] at doses below its classical use in malaria.[42,45] With intracerebroventricular delivery in the spared nerve injury model and intracisternal delivery in the model of infraorbital nerve CCI, mefloquine

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.painjournalonline.com).

PAIN 00 (2021) 1–13

http://dx.doi.org/10.1097/j.pain.0000000000002276

has been shown to reduce nociceptive symptoms.[5,34] In addition, systemic injection at a dose much lower than those used for malaria potentiates the antihyperalgesic effect of the antidepressant amitriptyline in the CCI model in rats.[19]

In this work, we characterized and studied the potentiating action of systemic mefloquine/amitriptyline combination, designed as THN101, on mechanical hypersensitivity, using the cuff model of neuropathic pain in mice.[2,53] To advance mechanistic understanding, we aimed here to identify the targets of THN101 components that were critical to the potentiating effect. For the amitriptyline component of THN101, we thus tested noradrenergic contribution at anatomical and receptor level. Concerning mefloquine, it has been suggested that it may have multiple targets besides connexins, such as an action on serotonin-related and adenosine-related factors.[16,18,44,51] We thus tested the relevance of mefloquine action on a large set of targets, and we evaluated whether the confirmed ones may potentiate amitriptyline action.

2. Material and methods

2.1. Animals

C57BL/6 J male mice (Charles River, L'Arbresle, France), 8 to 16 weeks old, were used for this study. They were habituated to the animal facility and to the testing environment before procedures. Mice were group housed with food and water ad libitum, in facilities with controlled temperature and hygrometry, under a 12-hour light/dark cycle (lights on at 7 AM). Animal killing at the end of the protocols was performed using either controlled carbon dioxide exposure (automatic TEM SEGA system, 20% per min) or after an overdose of anesthetic (ketamine (Imalgene1000, 300 mg/kg)/xylazine (Rompun 2%, 20 mg/kg, Centravet, Tadden, France). The Chronobiotron (UMS3415) animal facility has an agreement for animal housing and experimentation, delivered by the French veterinary services (D-67-2018-38). Protocols were performed following the European ethical guidelines (EU 2010/63) and approved by the local ethical committee "Comité d'Ethique en Matière d'Expérimentation Animale de Strasbourg" (CREMEAS, CEEA35). Chronograms detailing the experiments are presented in the Supplemental Figure 1 (available at http://links.lww.com/PAIN/B343).

2.2. Cuff surgery

Surgeries were performed under either ketamine (Imalgene 1000, 100 mg/kg)/xylazine (Rompun 2%, 10 mg/kg, Centravet) anesthesia or under tiletamine–zolazepam (Zoletil 50, 80 mg/kg, Virbac Sante Animale, France)/xylazine (Rompun 2%, 20 mg/kg, Centravet) anesthesia, with atipamezole (Antisedan, 0.2 mg/kg, Vetoquinol, France) delivered for anesthesia reversal at least 1 hour after the induction. Anesthesia compounds were delivered intraperitoneally (i.p.; 5 mL/kg). After having exposed the common branch of the right sciatic nerve (sham group), a hemisectioned 2-mm long PE-20 polyethylene cuff (Harvard Apparatus, Les Ulis, France) was placed around the nerve to induce a compression (cuff group) according to previously published procedures.[2,53] The skin was closed with one or 2 stitches along the length of the incision.[2,53]

2.3. Treatments

To test the combination of amitriptyline and mefloquine, independent groups of mice were treated per os (oral gavage, p.o.) with either amitriptyline hydrochloride (Sigma Aldrich, St Quentin Fallavier, France, PubChem Substance ID 24278073) from 5 to 20 mg/kg or mefloquine hydrochloride (Sigma Aldrich, PubChem Substance ID 24724536), 0.05 or 0.1 mg/kg, or with their combination (designed, regardless of doses, as THN101 throughout the article). The drugs were dissolved in sterile 0.9% NaCl (B. Braun, Dublin, Ireland) with DMSO from 0.02% to 1% (Sigma Aldrich, PubChem Substance ID 329757302). For most experiments, mice received the treatments either (1) in the morning for tests at 1, 2, 4, 7, and 30 hours after administration or (2) at the end of the day for tests at 12, 16, and 20 hours after administration. Experimental design is presented in Supplemental Figure 1A (available at http://links.lww.com/PAIN/B343).

To test the central effect of mefloquine, alone or in combination with amitriptyline, mice were first treated with amitriptyline 10 mg/kg p.o. or received a vehicle solution (p.o., 10 mL/kg). Then, under 3% isoflurane anesthesia, they received a single dose of 3.6 μg of mefloquine dissolved in 10 μL of vehicle solution (DMSO, 1% in 0.9% NaCl) or 10 μL of vehicle solution, delivered intrathecally (i.t.) at the lumbar level through a 27-gauge needle connected to a 50-μL Hamilton syringe. Experimental design is presented in Supplemental Figure 1B (available at http://links.lww.com/PAIN/B343).

To study the adrenergic component of THN101 and of mefloquine action at a high dose, mice were treated with yohimbine hydrochloride (α_2-adrenoceptor antagonist, Tocris, Abingdon, United Kingdom, PubChem ID 6169), either i.p. (0.5 mg/kg) or p.o. dissolved in the drinking water (20 μg/mL), or with propranolol hydrochloride (β-adrenoceptor antagonist; Tocris, PubChem ID 66366), given p.o. dissolved in the drinking water (50 μg/mL).[22] Antagonist treatments in the drinking water started 5 days before THN101 administration. In another experiment, 5 μg of atipamezole (α_2-adrenoceptor antagonist, Antisedan; Vétoquinol, Lure, France, PubChem ID 71310) or 3 μg of sotalol hydrochloride (β-adrenoceptor antagonist, Sigma-Aldrich, PubChem ID 66245) were delivered i.t. (10 μL) at the lumbar level in 0.9% NaCl. Experimental designs are presented in Supplemental Figures 1B and 1C (available at http://links.lww.com/PAIN/B343).

To evaluate the relevance of potential mefloquine pharmacological targets, mice were treated with either (1) fluoxetine hydrochloride (serotonin reuptake inhibitor; Sigma Aldrich, PubChem Substance ID 24278096), 10 mg/kg p.o.; (2) DPCPX, 8-cyclopentyl-1,3-dipropylxanthine (A_1 adenosine receptor antagonist; Tocris, PubChem ID 1329), 1 mg/kg p.o.; (3) SCH58261, 2-(2-furanyl)-7-(2-phenylethyl)-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (A_{2A} adenosine receptor antagonist; Tocris, PubChem ID 176408), 0.1 mg/kg p.o.; or (4) carbenoxolone, 3β-hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate (blocker of gap junctions; Sigma Aldrich, PubChem Substance ID 57654021), 5 nmol injected i.t. (10 μL in 1% DMSO, 0.9% NaCl). These drugs were administered alone or in combination with amitriptyline, 10 mg/kg p.o. (10 mL/kg). Experimental design is presented in Supplemental Figure 1A (available at http://links.lww.com/PAIN/B343).

2.4. Behavioral testing

Mechanical sensitivity was assessed using the von Frey test. The investigators performing the test were blinded to the surgery and drug treatment. Animals were placed into clear Plexiglas boxes (7 × 9 × 7 cm) on an elevated metallic grid. After habituation, ascending calibers of von Frey filaments (Aesthesio; DanMic Global LLC, San Jose, CA) were applied to the plantar surface of

hind paws until they just bent, as previously described.[2,3,53] The paw withdrawal threshold (PWT), expressed in grams, corresponds to the first filament that resulted in at least 3 paw withdrawals of 5 applications.[2,3,53] For some experiments, areas under the curve (AUC) were also calculated over the duration of the considered test. For each animal, these AUC were calculated as the surface (g.h) above its PWT value at time 0.

2.5. Mass spectrometry

The concentrations of amitriptyline, its metabolite nortriptyline, and of mefloquine were measured by mass spectrometry in blood, plasma, brain samples, and spinal cord lumbar sections taken from mice treated orally with amitriptyline (10 mg/kg) or THN101 (amitriptyline 10 mg/kg and mefloquine 0.1 mg/kg). Experimental design is presented in Supplemental Figure 1A (available at http://links.lww.com/PAIN/B343).

2.5.1. Blood, plasma, and tissue collection

Blood was collected from the tip of the tail (sectioned at its soft nonbone level with scissors) using 20-μL heparinized capillaries (Minicap Capillary Pipette, Hirschmann Instruments, ref 11772533). Blood was recovered 1, 2, 4, and 7 hours after p.o. administration of amitriptyline or THN101. Immediately after collection, the blood was mixed with 10 μL of 5000 IU/mL sodium heparin (Sanofi, France) and stored at −80°C. After the last blood collection, mice were anesthetized with ketamine/xylazine (100/10 mg/kg) and killed by cardiac exsanguination. The blood collected in vacuumed lithium-heparinized tubes (BD Vacutainer LH PST II REF 367374) was centrifuged at 425*g* for 10 minutes at 4°C, and plasma was frozen at −80°C. In parallel, the brain and spinal cord were recovered in Eppendorf tubes on dry ice and stored at −80°C.

2.5.2. Plasma, blood, and tissue preparation

Plasma (50 μL) and blood (20 μL) were spiked with 20 pmol of D3-amitriptyline, D3-nortriptyline, and D10-mefloquine (Merck, Molsheim, France and Alsachim, Illkirch, France) to perform a quantification using the isotopic dilution method,[50] which allows absolute quantification of the compounds. In brief, a known amount of a stable isotope-containing target compound (ie, 20 pmol of D3-amitriptyline, D3-nortriptyline, and D10-mefloquine) is added to the sample. An MS analysis allows determination of the intensity ratio between the studied compound and its heavy counterpart. As the studied compound and its heavy counterpart behave the same way, such a method overcomes problems of degradation and loss due to extraction steps as well as matrix impact. Finally, it permits analysis of the presence of different compounds using a unique protocol even with low recovery yields. Recovery yields calculated from standard curves (n = 3) are reported in the Supplemental Table 1A (available at http://links.lww.com/PAIN/B343). Volumes were adjusted to 100 μL with ascorbic acid (50 μM final) and samples submitted to a solid-phase extraction (SPE). Samples were loaded on Phree SPE-cartridges (1 cc, Phenomenex, Le Pecq, France), and elution was performed with 300 μL of 99% acetonitrile (ACN)/1% formic acid (FA, vol/vol). Eluted fractions were dried under vacuum and suspended in 10 μL of 20% ACN/0.1% FA before MS analysis.

Brains and spinal cords were homogenized with a tissue mixer in 0.1 mM ascorbic acid. Homogenates were sonicated (2x5s, 100 W) and centrifuged (20,000*g*, 20 minutes, 4°C). One hundred microliter of the supernatant was spiked with 20 pmol of D3-amitriptyline, D3-nortriptyline, and D10-mefloquine and extracted on Phree SPE-cartridges as described above. Eluted fractions were dried under vacuum and suspended in 10 μL of 20% ACN/0.1% FA before MS analysis.

2.5.3. Liquid chromatography coupled to tandem mass spectrometry instrumentation and analytical conditions

Quantifications were performed using the multiple reaction monitoring (MRM) mode and the isotopic dilution method. Analyses were performed on a Dionex Ultimate 3000 HPLC system (Thermo Scientific, San Jose, CA) coupled with an Endura triple quadrupole mass spectrometer (Thermo Electron). Dried samples were suspended in 10 or 5 μL of 99.9% H_2O/0.1% FA (vol/vol), and 1 μL of the solution was loaded into a Luna Omega Polar reverse-phase capillary column (#00D-4760-AF; 100 x 0.5 mm 3 μm, Phenomenex). Elutions were performed by applying a linear gradient of buffers described in Supplemental Table 1B (available at http://links.lww.com/PAIN/B343). The MRM mode was completed at 3500 V (positive mode) of liquid junction voltage and 276°C capillary temperature. 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. The selection of the monitored transitions and the optimization of the collision energy were manually determined. The transitions and the corresponding collision energies used for MRM are reported in the Supplemental Table 1C (available at http://links.lww.com/PAIN/B343).

2.6. Noradrenergic lesions

The lesion of noradrenergic fibers from the descending pathways was performed with intrathecal (i.t.) administration of 6-hydroxydopamine (6-OHDA; 20 μg per mouse, in 5 μL of 0.9% NaCl solution containing 100 μg/mL ascorbic acid) on 8-week-old mice under 3% isoflurane anesthesia. For this i.t. injection, an incision was made at the T9 to T11 level so that a 27-gauge needle connected to a 50-μL Hamilton syringe could be inserted into the space between the thoracic T12 and lumbar L1 vertebrae.[24] For the peripheral pharmacological lesions affecting sympathetic peripheral sprouting,[24] 5 daily intraperitoneal injections of guanethidine monosulfate (30 mg/kg, Sigma Aldrich) were given in a volume of 5 mL/kg in 8-week-old mice.

Mice underwent sham or cuff surgery 2 weeks after the above procedures, and 2 weeks later, they were treated with THN101 (amitriptyline 10 mg/kg and mefloquine 0.1 mg/kg) or with mefloquine alone (0.4 mg/kg) for the central lesions, in a single p.o. administration. The control groups of nonlesioned mice (solvent injection) underwent the same nerve cuffing procedure as the lesioned groups. Experimental design is presented in Supplemental Figure 1D (available at http://links.lww.com/PAIN/B343).

2.7. Immunohistochemistry

Mice with and without 6-OHDA lesion were anesthetized under tiletamine–zolazepam (Zoletil50, 80 mg/kg, Virbac Sante Animale, France)/xylazine (Rompun 2%, 20 mg/kg, Centravet) and perfused with 30 mL of 0.1 M phosphate buffer (PB, pH 7.4), followed by 100 mL of 4% paraformaldehyde solution (PFA) in PB (10 mL/min). Brain and spinal cord were dissected. Brains were postfixed overnight in PFA, included in 2% agar, and cut into 40-μm thick sections (Vibratome VT1000S, Leica). The spinal cord was postfixed in PFA for 1 hour and cryoprotected (4 hours in

15% sucrose in phosphate buffered saline (PBS) followed by 24 hours in 30% sucrose in PBS), lumbar area was embedded in optimal cutting temperature compound (Sakura Finetek), frozen, and cut into 10-μm thick sections (Cryostat CM3050S, Leica) that were mounted on Superfrost Plus slides (Thermo Scientific).

Brain sections were processed for chromogenic immunostaining, as previously described.[12] All steps were performed on free-floating sections on a rotary shaker at room temperature. Sections were washed 3 × 5 minutes in PBS, incubated 15 minutes in a 1% H_2O_2/50% ethanol solution for peroxidase extinction, washed 3 × 5 minutes in PBS, incubated 45 minutes in PBS-Triton X100 (0.3%) with 5% goat serum, and then incubated overnight in PBS-Triton X100 (0.3%) with 1% goat serum and the primary chicken antibody against TH (1:2500, #ab76442; Abcam, Cambridge, United Kingdom). Sections were then washed 3 × 5 minutes in PBS, incubated for 2 hours with a biotinylated goat anti-chicken secondary antibody (1:400, #BA9010, Vector Laboratories, Burlingame, CA) in PBS, washed 3 × 5 minutes in PBS, incubated 90 minutes with the avidin–biotin–peroxidase complex (#PK4000, ABC Elite, 0.2% A and 0.2% B, Vector Laboratories) in PBS, washed 3 × 10 minutes in 0.05 M Tris-HCl buffer (TB; pH 7.5), and the chromogenic signal was revealed by incubation for 10 minutes in 0.025% 3.3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.0006% H_2O_2 (Sigma) in TB. After washes 2 × 10 minutes in TB and 3 × 5 minutes in PBS, sections were serially mounted on Superfrost Plus slides (Thermo Scientific), air-dried, dehydrated in graded alcohols baths (1 × 70%, 1 × 90%, and 2 × 100%), cleared in Roti-Histol (Carl Roth, Karlsruhe, Germany), and coverslipped with Eukitt. Evaluation of TH+ cells was performed using a Nikon Eclipse 80i microscope and pictures were taken with a digital camera (CX 9000, MBF biosciences). For each animal, all TH+ cells were bilaterally counted every other section over the whole anteroposterior extent of the considered cell group: A7 from −4.64 to −5.04 mm (distance from bregma), locus coeruleus from −5.36 to −5.84 mm, and A5 from −5.12 to −5.92 mm.

Spinal cord sections were processed for fluorescence immunostaining. All steps were performed on sections mounted on Superfrost slides. Sections were washed 3 × 5 minutes in PBS, incubated 45 minutes in PBS-Triton X100 (0.5%) with 10% goat serum, and incubated overnight (4°C) in PBS-Triton X100 (0.5%) with 1% goat serum and the primary chicken antibody against TH (1:100, #ab76442, Abcam). Sections were then washed 3 × 5 minutes in PBS-Tween20 (0.1%), incubated for 1 hour with a secondary Alexa Fluor 488-conjugated goat antibody (1:400, #A11039; Invitrogen, Carlsbad, CA) in PBS, washed 3 × 5 minutes in PBS-Tween20 (0.1%), and mounted with ProLong Gold (#P36930, Invitrogen). Images were acquired with a Nikon E80i microscope with the 20x objective and a digital camera (CX 9000, MBF biosciences, Williston, VT) using Neurolucida acquisition software. Evaluation of TH-positive fiber length was performed using ImageJ software. The total TH+ fiber length was measured within given zones (mean zone surface per section: 230066 ± 3171 μm^2) in the dorsal horn of 3 sections per animal at the lumbar L4-L5 level.

The analysis of the sections was performed by an investigator blinded to the lesion status of the animals.

2.8. In vitro pharmacology

To determine possible pharmacological targets for mefloquine, an in vitro pharmacological study was performed through the Contract Research Organization Eurofins CEREP (Le Bois l'Evêque, France), which consisted in identifying mefloquine activity on various transmembrane and soluble receptors. Radioligand binding and functional assays were performed with 1 μM of mefloquine, diluted in DMSO (10^{-2} M). EC_{50} and IC_{50} of mefloquine on A_1 and A_{2A} adenosine receptors, and on serotonin uptake transporters, were also measured using mefloquine at several concentrations. Detailed method information, as provided by the contractor, are given in the supplemental section (Supplemental Table 2, available at http://links.lww.com/PAIN/B343).

2.9. Statistics

Statistical analyses were performed with Statistica (Dell software, France). Student *t* tests (plasma, spinal cord, or brain drug levels; immunohistochemistry experiment) and analysis of variance (other figures) were performed. The surgery procedure (sham or cuff), peripheral lesion procedure (guanethidine or saline), central lesion procedure (6-OHDA or saline), and the various treatments when conducted on independent groups of mice were considered as between-group factors. When needed, the time of measurement, or the treatments when conducted at a week delay on same animals (drug tests in 6-OHDA-lesioned animals and their controls), was considered as within-subject factor. The Duncan test was used for post hoc analyses.

3. Results

3.1. Amitriptyline/mefloquine THN101 effect on mechanical hypersensitivity

To evaluate the potential synergy between amitriptyline (AMI) and mefloquine (MEF), we first performed dose–response studies. Mice from the vehicle-treated (VEH) cuff group displayed mechanical hypersensitivity (sham > cuff, $F1,137 = 223.0$, $P < 10^{-5}$). Oral amitriptyline (**Fig. 1A**) dose-dependently relieved mechanical hypersensitivity (group effect, $F4,47 = 21.19$, $P < 10^{-5}$; post hoc: sham>all other groups at $P < 0.043$), with no significant effect at 5 mg/kg, a partial and transient recovery at 10 mg/kg ($F4,188 = 5.51$, $P < 0.001$; post hoc AMI10>cuff VEH, at 2 hours $P = 0.013$), and a more complete and transient recovery at 20 mg/kg ($F4,188 = 5.51$, $P < 0.001$; post hoc: AMI20>cuff VEH, at 2 hours, $P < 0.001$). The 2 doses tested for oral mefloquine (**Fig. 1B**) had no significant action per se on the hypersensitivity (group effect, $F3,38 = 64.02$, $P < 10^{-5}$; post hoc: sham>all other groups at $P < 4*10^{-6}$ and cuff VEH-treated group similar to cuff MEF-treated groups at $P > 0.274$). The 2 lowest doses of amitriptyline (5 and 10 mg/kg) were then combined with mefloquine. When combined with mefloquine, amitriptyline 5 mg/kg led to a partial relief of hypersensitivity, limited within 4 hours when combined with mefloquine 0.05 mg/kg (Group*Time, $F4,64 = 3.47$, $P = 0.013$; post hoc: AMI5 + MEF0.05 > VEH, at 4 hours $P = 0.047$ post hoc) (**Fig. 1C**) and present at 7 hours when combined with mefloquine 0.1 mg/kg (Group*Time, $F4,68 = 6.99$, $P = 9*10^{-5}$; post hoc: AMI5+ MEF0.1>VEH, at 7 hours $P = 9*10^{-5}$) (**Fig. 1D**). Mefloquine further enhanced the action of amitriptyline 10 mg/kg, with a partial and transitory improvement with mefloquine 0.05 mg/kg (Group*Time, $F4,64 = 2.95$, $P = 0.026$; post hoc: AMI10+ MEF0.05>VEH, at 2 hours $P = 0.032$, at 7 hours $P = 0.040$ post hoc) (**Fig. 1E**) and a better and sustained effect with mefloquine 0.1 mg/kg (**Fig. 1F**, left), for which the relief of mechanical hypersensitivity was stably maintained for at least 7 hours (Group*Time, $F4,118 = 4.30$, $P = 1.6*10^{-4}$; post hoc: AMI10 + MEF0.1 > VEH, at 2 hours $P = 0.004$, at 4 hours $P = 1.8*10^{-5}$, at 7 hours $P = 1.1*10^{-5}$). We also observed that THN101 at this dose combination had no effect per se on PWTs in

the sham surgery group (**Fig. 1F**, left) (post hoc: $P > 0.51$ for SHAM-THN101 at 1-7 hours against value at 0 hours) or on the contralateral PWTs in the cuff group (**Fig. 1F**, right) (Group*Time, $F4,118 = 0.46$, $P = 0.88$). This prolonged benefit of the THN101 combination compared with individual treatments is further highlighted by the AUC presentation of data (**Fig. 1G**) (Group effect, $F4,51 = 6.02$, $P = 4.79*10^{-4}$; post hoc: THN101 > VEH, MEF0.1 and AMI10-treated groups at $P < 0.0396$). All the following experiments were thus conducted using amitriptyline 10 mg/kg + mefloquine 0.1 mg/kg as THN101 combination.

The duration of THN101 effect was then evaluated beyond 7 hours (**Fig. 2A**). This experiment allowed showing that the action of a single THN101 administration partially lasted at least 16 hours and was no more present after 20 hours (Group*Time, $F6,82 = 9.07$, $P < 10^{-6}$; post hoc: THN101>all others groups at 12 hours $P < 7*10^{-5}$ and at 16 hours $P < 4*10^{-4}$). Repeating the evening injection procedure in a separate set of mice allowed showing that relief of mechanical hypersensitivity can be repeatedly observed over 5 days, 12 hours after each THN101 administration (Group*Time, $F1,14 = 15.55$; $P = 0.0015$; post hoc: THN101>VEH at day 1 $P = 0.0011$, day 2 $P = 4*10^{-4}$, day 3 $P = 0.011$, day 4 $P = 0.028$, and day 5 $P = 0.029$) (Supplemental Figure 1E and **Fig. 2B**, available at http://links.lww.com/PAIN/B343).

3.2. Pharmacokinetic profile of THN101

We then tested whether mefloquine may affect the antidepressant pharmacokinetic profile. Blood levels of mefloquine, amitriptyline, and its metabolite nortriptyline were monitored 1, 2, 4, and 7 hours after administration of amitriptyline alone or THN101. Amitriptyline, nortriptyline, and mefloquine levels were also measured in plasma, brain, and spinal cord 7 hours after administration.

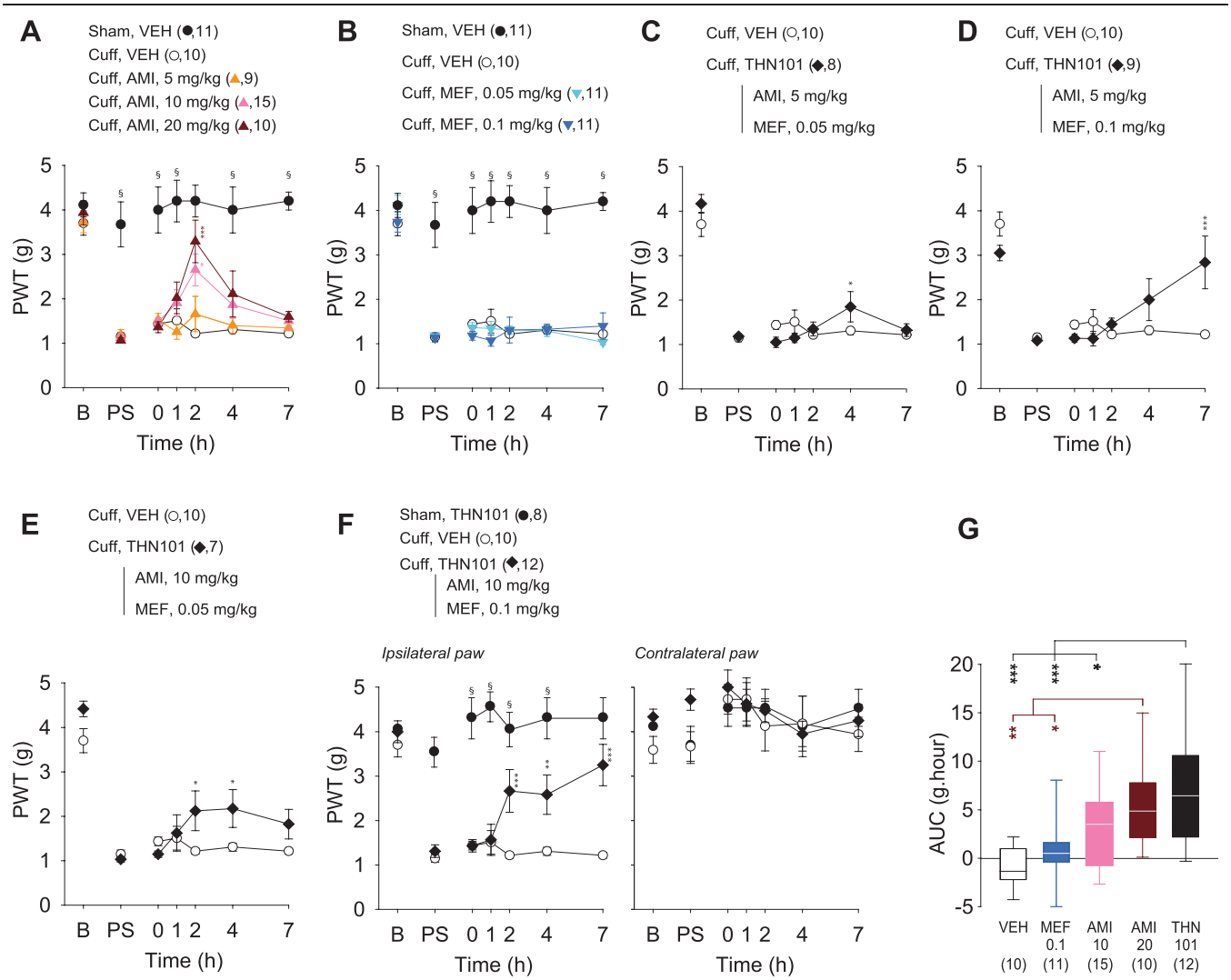


Figure 1. Effect of amitriptyline and mefloquine combination on mechanical hypersensitivity in mice with neuropathic pain. The mechanical right hind paw withdrawal threshold (PWT) was assessed with von Frey filaments before surgery (B: baseline), the mean of postsurgical values before treatment is displayed as PS (postsurgery baseline), and the oral drug treatment was performed 2 to 3 weeks after surgery. On the drug treatment day, sham (n=8-11) and cuff (n=7-15 per group) mice were tested before and from 1 to 7 hours after drug administration. Mice PWT was assessed after amitriptyline (A) or mefloquine (B) treatment at different doses and after treatment with amitriptyline and mefloquine combination (C-F). Comparison of areas under the curve (AUC) between cuff VEH, mefloquine, amitriptyline, and THN101 (AMI 10 mg/kg/MEF 0.1 mg/kg)-treated groups are shown with whisker boxes (G). Vehicle solution (NaCl 0.9%, DMSO 0.02% 10 mL/kg p.o., once after time 0 test) was used as negative control (A to G). Results are expressed as mean ± SEM. Symbols for some of the post hoc comparisons: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs cuff VEH (or vs the designated group in (G)); §$P < 0.05$ vs cuff AMI 20 mg/kg (A) or vs cuff MEF 0.1 mg/kg (B) or vs cuff THN101 (F).

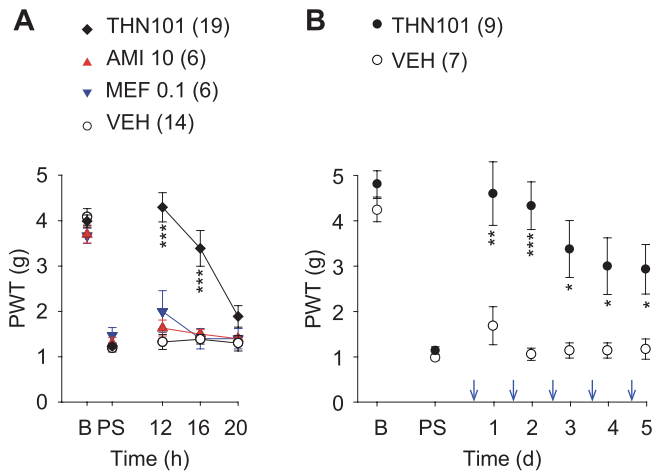


Figure 2. THN101 action on mechanical hypersensitivity. Experiments were conducted in cuff mice. The mechanical right hind paw withdrawal threshold (PWT) was assessed with von Frey filaments before surgery (B: baseline), the mean of postsurgical values before treatment is displayed as PS (postsurgery baseline), and the drug treatment was performed or started 2 to 3 weeks after surgery. Treatments were given orally in the evening, and mice tested on the following day. In a first experiment, time course of THN101 (amitriptyline 10 mg/kg/mefloquine 0.1 mg/kg) action was compared with control, amitriptyline, and mefloquine treatments (A). In a second experiment, THN101 was orally given each evening over 5 consecutive days, and mice tested each time 12 hours postadministration (B). Results are expressed as mean ± SEM. Symbols for some of the post hoc comparisons: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs cuff VEH.

Blood levels of mefloquine were found to gradually increase up to 7 hours after oral administration (**Fig. 3A**) (Time effect, F3,42 = 21.52, $P < 10^{-6}$), reaching 292.1 ± 18.9 μM. At 7 hours postadministration, plasma levels were at 92.6 ± 8.4 μM (**Fig. 3B**), and central concentrations were of 44.2 ± 9.1 pmol/mg of protein in the spinal cord (**Fig. 3C**) and 1.0 ± 0.1 pmol/mg of protein in the brain (**Fig. 3D**).

Conversely, blood amitriptyline and nortriptyline concentrations (**Fig. 3A**) were highest 1 hour after oral drug delivery and decreased similarly in the amitriptyline-treated and THN101-treated groups (Group*Time, amitriptyline: F3,42 = 0.55, P = 0,65, nortriptyline: F1,14 = 4.13, P = 0.061) (**Fig. 3A**). Similarly, no significant difference was found between amitriptyline alone and THN101 concerning the plasma (**Fig. 3B**), spinal cord (**Fig. 3C**), and brain (**Fig. 3D**) levels of amitriptyline (P = 0.41, 0.24 and 0.30, respectively) and nortriptyline (P = 0.32, 0.67 and 0.11, respectively). It should be noted that one of the mice displayed much higher plasma amitriptyline concentrations than the others; however, statistical conclusions remained the same whether this animal was included (see above) or no (without the potential outlier: plasma: P = 0.83; spinal cord: P = 0.37; brain: P = 0.66) (Supplemental Fig. 2, available at http://links.lww.com/PAIN/B343).

3.3. Contribution of descending aminergic pathways in THN101 and mefloquine action

We next investigated the mechanism of THN101 by studying the role of aminergic pathways.[24] To test the involvement of the sympathetic system, we injected guanethidine i.p. for 5 days.[3] This peripheral lesion did not impact on the THN101-sustained relief of mechanical hypersensitivity, as tested 12 hours after oral administration (treatment effect, F1,16 = 36.73, $P < 2^{*}10^{-5}$; Lesion*Treatment, F1,16 = 0.002, P = 0.96; post hoc: THN101 action similar between both groups at P = 0.80) (**Fig. 4A**).

To test the involvement of the noradrenergic-descending controls of pain, i.t. injections of 6-OHDA into the T12-L1 area were performed 2 weeks before the cuff surgery. Using immunohistochemistry, we completed our previous characterization[24] of this lesion procedure by studying its impact on 3 noradrenergic nuclei and on lumbar spinal cord TH+ fibers (Supplemental Fig. 3, available at http://links.lww.com/PAIN/B343). The i.t. 6-OHDA did not significantly change the total number of TH+ cells in the A7 cell group (P = 0.29), in the locus coeruleus (P = 0.56), and in the A5 cell group (P = 0.11). However, a nonsignificant trend toward some loss (A7: −17.1%; locus coeruleus: −11.5%; A5: −15.7%) was present in each group of cells, which may suggest that only a small subset of cells might have been affected. In the dorsal horn of the lumbar spinal cord, a significant 55.7% decrease in TH+ fibers was observed ($P < 7^{*}10^{-6}$), in line with a previous qualitative report.[24] In agreement with a previous report,[24] this lesion procedure did not change the mechanical PWTs of the mice and did not change either the development of the mechanical hypersensitivity after the cuff surgery (Lesion*Treatment*Time, F4,96 = 0.26, P = 0.90) (Supplemental Fig. 4, available at http://links.lww.com/PAIN/B343).

Oral VEH solution did not affect the time course of the PWTs (Group*Time effect, F4,56 = 1.22, P = 0.315) (**Fig. 4B**, left). A week later, we tested THN101 and observed that its action was significantly more potent in the nonlesioned mice (saline i.t.) than in the lesioned ones (6-OHDA i.t.) from 4 to 7 hours after administration (Group*Time effect, F4,56 = 4.69, P = 0.0024; post hoc: saline>6-OHDA at 4 hours P = 0.050 and at 7 hours P = 0.0001) (**Fig. 4B**, right). As observed above, neither vehicle, THN101, nor 6-OHDA changed the PWTs in mice from the sham group (Lesion*Time, F4,252 = 0.19, P = 0.94; Treatment*Time, F8,252 = 1.29, P = 0.25; Lesion*Treatment*Time, F8,252 = 0.77, P = 0.62) (**Fig. 4C**, left and center).

The above data were supportive of a critical role of descending noradrenergic pathways in the action of THN101. We then tested whether mefloquine alone at a higher dose may affect mechanical sensitivity and, if so, whether noradrenergic descending pathway functionality contributed to the effect observed. We thus administered 0.4 mg/kg mefloquine per os to cuff animals whose descending noradrenergic pathways were or not lesioned. We observed that mefloquine relief of hypersensitivity was lost after lesion (Lesion*Treatment*Time effect, F4,92 = 17.53, $P <$ 10-6, post hoc: saline/MEF>6-OHDA/MEF, at 1 hour P = 0.010, at 2 hours $P < 10^{-5}$) (**Fig. 4D**). Importantly, although it relieved the mechanical hypersensitivity in cuff mice, 0.4 mg/kg mefloquine had no effect on PWTs in the sham group (**Fig. 4C**, right).

To further support a potential spinal action of mefloquine in THN101 combination, we administered mefloquine i.t. after an oral administration of 10 mg/kg amitriptyline. Two hours after this procedure, the combination provided a significant improvement in PWTs compared with mice that received one only of the treatments (Group*Time, F6,62 = 3.26, P = 0.007; post hoc: MEF/AMI > all other groups at 2 hours $P < 0.002$) (**Fig. 5**).

3.4. Adrenergic mechanism of action of THN101 combination

Beside the lesion experiments, we also used a pharmacological approach to study the role of the noradrenergic system in an independent set of experiments. As downstream actors after noradrenaline recruitment, the respective contribution of both α and β adrenoceptors[24] in THN101 response was tested.

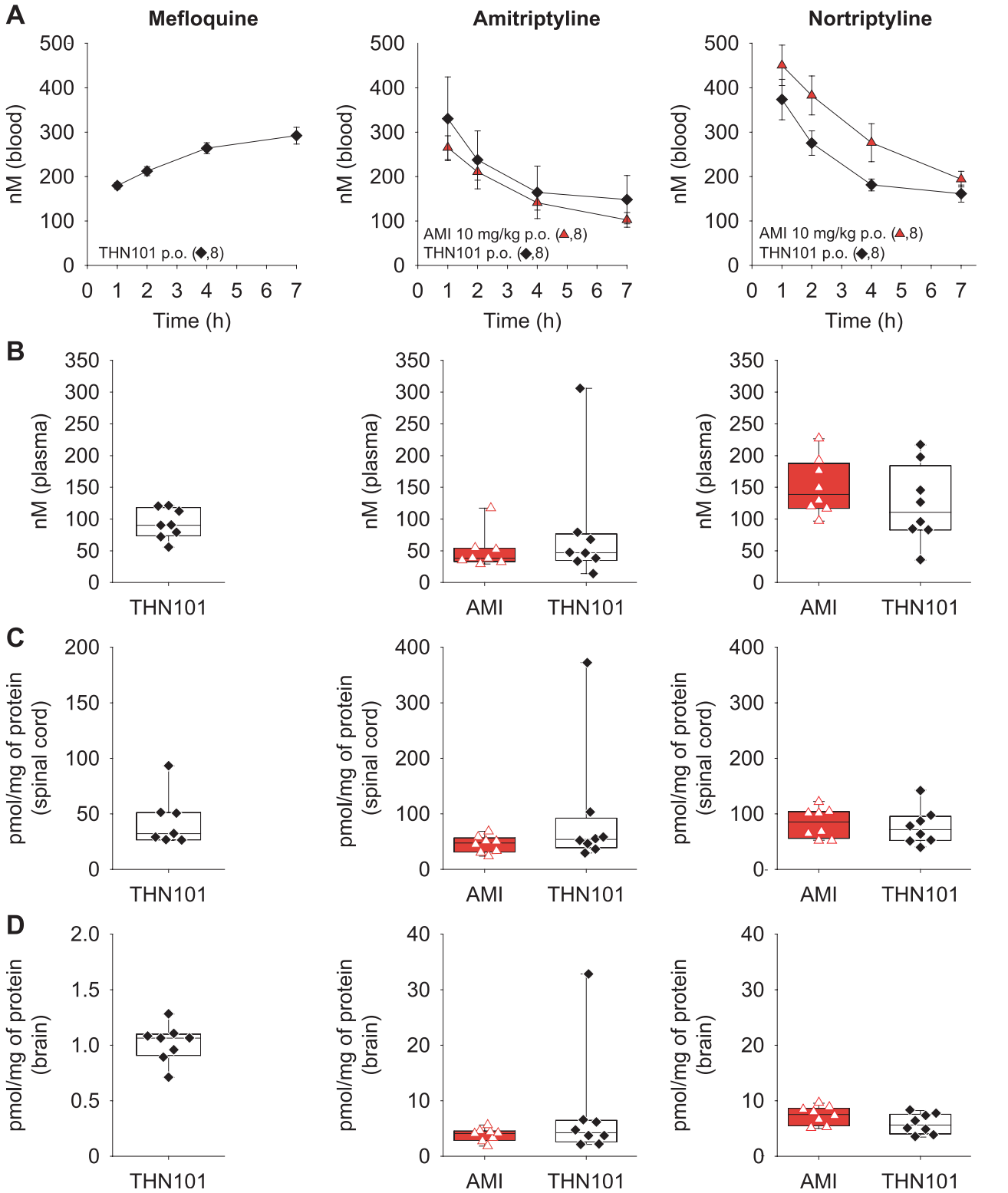


Figure 3. Evaluation of mefloquine, amitriptyline, and nortriptyline concentrations by mass spectrometry after amitriptyline or THN101 treatment. Measures were performed only in cuff mice. Mefloquine, amitriptyline, and nortriptyline concentrations (nM) were measured in blood from 1 to 7 hours after amitriptyline (10 mg/kg) or THN101 (AMI 10 mg/kg/MEF 0.1 mg/kg) per os treatment. Results are expressed as mean ± SEM (A). Mefloquine, amitriptyline, and nortriptyline plasma concentrations (nM) (B), and spinal cord (C) and brain (D) concentrations (pmol/mg of protein) were also assessed 7 hours after amitriptyline or THN101 per os treatment (whisker boxes).

Yohimbine and propranolol were delivered through drinking water, which had no effect per se on mechanical sensitivity thresholds (PS, for postsurgery baseline, vs time point "0" on **Fig. 6A**), as previously reported.[24] We thus tested the impact of these antagonists on THN101 action and observed that its lasting activity on mechanical hypersensitivity remained present under propranolol treatment but was suppressed under yohimbine treatment (**Fig. 6A**) (Group*Time, $F_{12,104} = 3.90$, $P < 6*10^{-5}$;

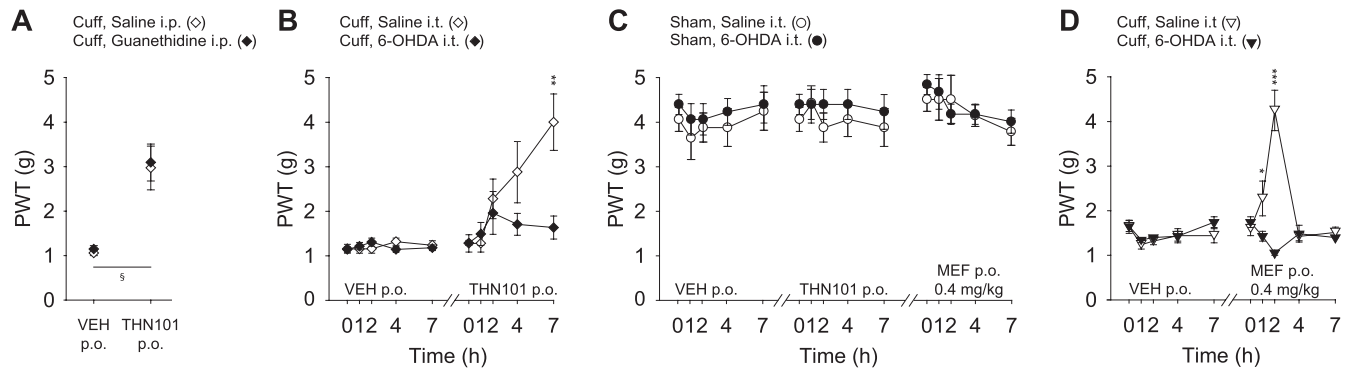


Figure 4. Central descending aminergic pathways are necessary to the action of THN101 and mefloquine. The mechanical hind paw withdrawal thresholds (PWTs) were assessed using von Frey filaments in mice 2 to 4 weeks after cuff or sham surgery. Peripheral (A) and central (B–D) lesions were performed before surgery. In separate sets of experiments, cohorts of saline-treated and guanethidine-treated cuff mice were tested for response to vehicle and THN101 a week apart (A), cohorts of saline-treated and 6-OHDA-treated cuff mice were tested for response to vehicle and THN101 a week apart (B), cohorts of saline-treated and 6-OHDA-treated sham mice were tested for response to vehicle, THN101, and mefloquine (0.4 mg/kg) a week apart (C), and cohorts of saline-treated and 6-OHDA-treated cuff mice were tested for response to vehicle and mefloquine a week apart (D). Mice were tested before (0) and from 1 to 7 hours after drug administration. Results are expressed as mean ± SEM. Symbols for some of the post hoc comparisons: §$P < 0.001$ for cuff THN101 vs cuff VEH (A); *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs cuff VEH and vs cuff 6-OHDA (B to D).

post hoc: (THN101 = SHAM THN101)>all others groups at 12 hours $P < 0.05$, at 16 hours THN101 < SHAM THN101 at $P < 0.005$ and THN101 > all others groups except PRO-THN101 at $P < 0.05$). This finding was further illustrated by the area under the curve (calculated for each mouse above its time point "0" threshold) (**Fig. 6A**, right) (Group effect, $F3,35 = 8.31$, $P = 2*10^{-4}$; post hoc: THN101>YOH-THN101 and VEH at $P < 5*10^{-4}$ and PRO-THN101>YOH-THN101 and VEH at $P < 0.02$). Finally, the THN101/yohimbine combination had no effect on PWTs in mice from the sham group (**Fig. 6A**).

This experiment suggested a preferential role of α_2 adrenoceptors in THN101 action. As tests were conducted on the day following THN101 administration while adrenergic receptor blockade was continuous, we then tested whether α_2 adrenoceptors were more specifically involved in the induction or the maintenance of THN101 action. Yohimbine was thus delivered i.p. either at the same time as THN101 administration (induction) or 30 minutes before testing time (expression). When yohimbine was given at the same time as THN101, THN101 action was no longer observed 12 hours later (**Fig. 6B**, left), whereas its effect was still present when yohimbine was administered 30 minutes before the final test (**Fig. 6B**, right) (YOH0: Group*Time effect, $F4,38 = 5.94$, $P < 8*10^{-4}$; post hoc: THN101>YOH0-THN101 at $P < 0.005$ for 12hours and 13hours; YOH12: Group*Time effect, $F4,38 = 7.13$, $P < 2*10^{-4}$; post hoc: THN101=YOH12-THN101 > VEH at $P < 0.05$ for 12hours and 13hours). These data supported a role of α_2 adrenoceptors in the induction of THN101 long-lasting action.

To more specifically test the role of spinal adrenergic receptors in this induction process, we used i.t. delivery of antagonists just after the saline or THN101 per os administration (**Fig. 6C**). As the i.t. procedure may lead to shorter effect of the antagonist than oral or i.p. delivery, PWTs were tested at 4 hours. The α_2-adrenoceptor antagonist atipamezole fully suppressed THN101 action, whereas the β-adrenoceptor antagonist sotalol partially suppressed it (Antagonist*Treatment, $F1,51 = 15.61$, $P < 0.001$; post hoc: (cuff saline THN101 = all sham groups) > all others groups at $P < 0.05$, cuff sotalol THN101 > (all cuff saline groups and cuff atipamezole THN101) at $P < 0.05$). This suppression was present while these antagonists had no effect per se in mice from the cuff group and had no effect in THN101-treated mice from the sham group (**Fig. 6C**). Interestingly, this partial suppression observed with local (i.t.) sotalol at 4 hours is in line with the partial effect observed with chronic oral propranolol at the time point 16 hours (**Fig. 6A**). Together it suggests that spinal α_2 adrenoceptors are essential to THN101 action and that β adrenoceptor may also contribute (although perhaps to a lesser extent) to this action.

3.5. Contribution of mefloquine in the mechanisms of action of THN101

We then further explored the mefloquine component of THN101 action, by analyzing potential targets (**Fig. 7A and B**) besides its

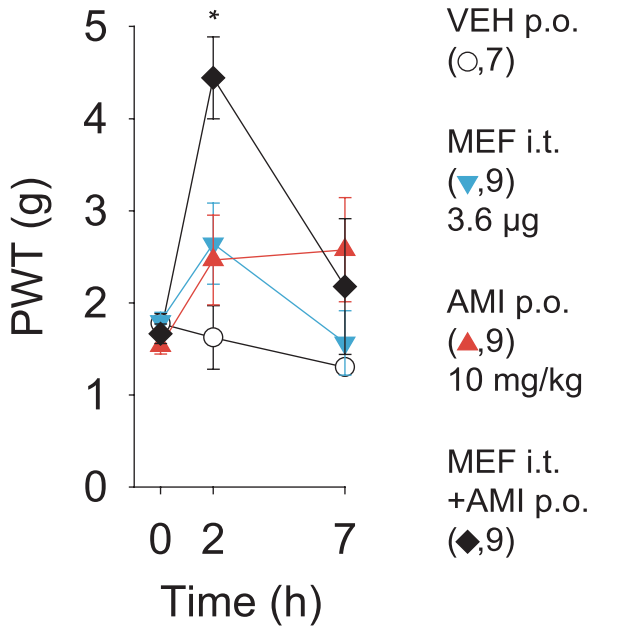


Figure 5. Spinal action of mefloquine in THN101 combination. The experiment was conducted in cuff mice. The mechanical hind paw withdrawal thresholds (PWTs) were assessed using von Frey filaments in mice 2 to 3 weeks after cuff surgery. Central (i.t.) administration of mefloquine combined or not with oral administration of amitriptyline (10 mg/kg) was tested and monitored since 7hours after drug administration. Results are expressed as mean ± SEM. Symbols for some of the post hoc comparisons: *$P < 0.05$ vs cuff VEH.

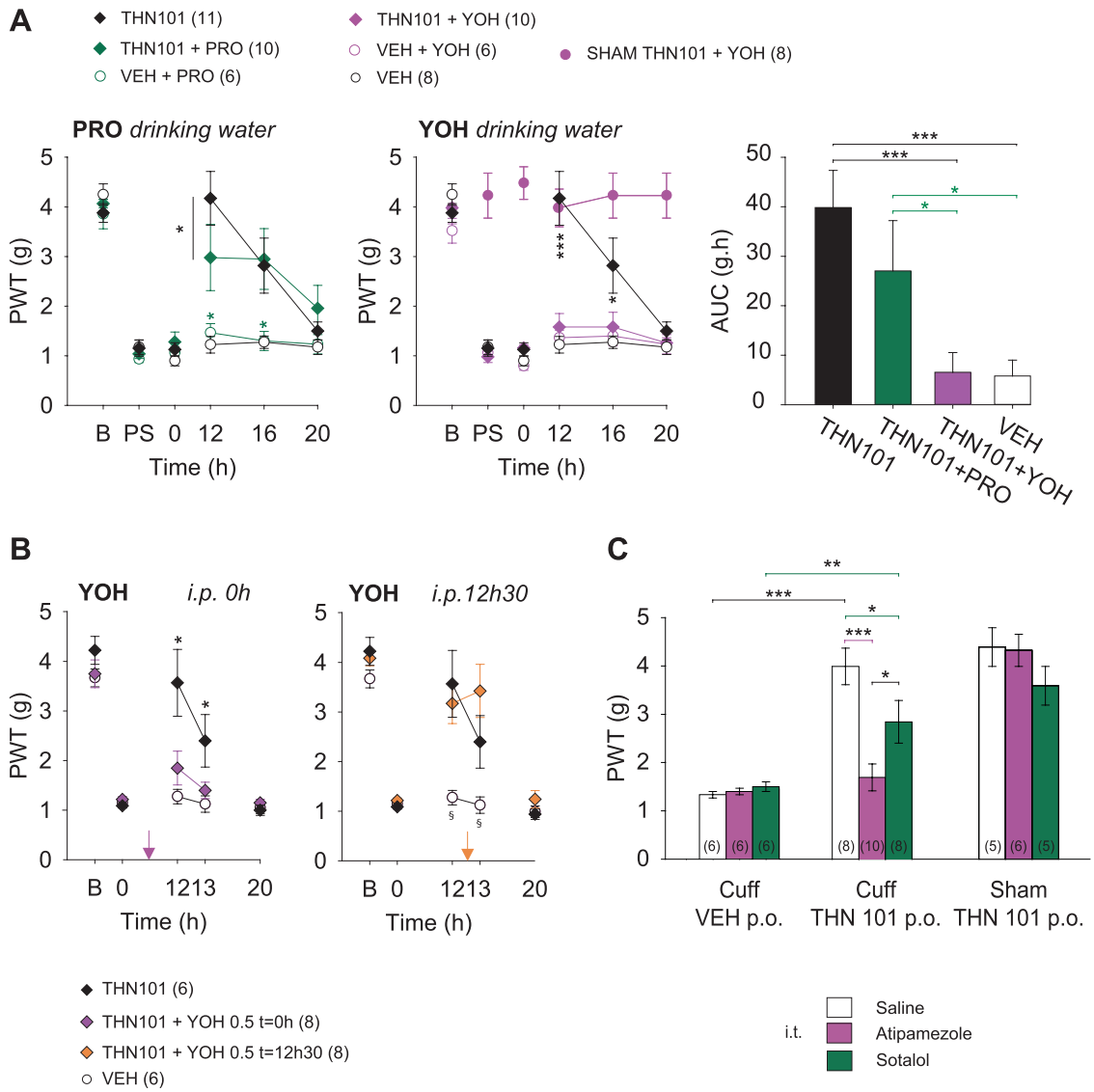


Figure 6. THN101 antiallodynic action and adrenoceptors. The mechanical hind paw withdrawal thresholds (PWTs) were assessed using von Frey filaments in mice 2 to 3 weeks after cuff or sham surgery. In a first set of experiments (A), the β_2 (propranolol, PRO)-adrenoceptor and the α_2 (yohimbine, YOH)-adrenoceptor antagonists were delivered through the drinking water. The treatment with these antagonists started 5 days before the THN101 acute administration (time course: $^*P < 0.05$ and $^{***}P < 0.001$ indicate post hoc significance for cuff THN101 vs (cuff THN101 + PRO or YOH); bar graph: $^*P < 0.05$ for (cuff THN101 + PRO) vs (cuff THN101 + YOH) and vs cuff VEH and $^{***}P < 0.001$ for cuff THN101 vs (cuff THN101 + YOH) and vs cuff VEH). In the second set of experiments (B), yohimbine was injected i.p. either at the same time as THN101 (left graph) or 30 minutes before the final test (right graph) ($^*P < 0.05$ for cuff THN101 vs (cuff THN101 + YOH); §$P <$ 0.001 for cuff VEH vs cuff THN101 and vs cuff THN101 + YOH). (C) Central (i.t.) administration of saline, α_2-adrenoceptor antagonist atipamezole or β-adrenoceptor antagonist sotalol combined with oral administration of vehicle or THN101 treatment was tested 4hours after drug administration ($^*P < 0.05$, $^{**}P <$ 0.01, and $^{***}P < 0.001$ for the indicated comparisons). Results are expressed as mean ± SEM. B, baseline before surgery; PS, postsurgery baseline before antagonist oral delivery in drinking water.

known action on connexins.[7] The in vitro binding assays showed that 1 μM mefloquine does not directly target noradrenaline and dopamine uptake sites or α and β adrenoceptors (**Fig. 7A**), although yohimbine was able to antagonize the action of mefloquine alone at a high dose (Group*Time, F4,40 = 11.16, $P < 5^*10^{-6}$; post hoc: MEF/saccharine>MEF/yohimbine at 2 hours $P < 2^*10^{-5}$, 4 hours $P < 2^*10^{-4}$ and 7 hours $P = 0.019$) (**Fig. 8A**). The analyses also reported a lack of direct binding to a wide range of other tested targets (**Fig. 7A** and Supplemental Fig. 5, available at http://links.lww.com/PAIN/B343). This analysis, however, showed a potential antagonistic action (ie, at least 50% binding displacement at 1 μM) for (1) the adenosine receptors A1 and A2A, (**Figs. 7A and B**) and (2) the serotonin transporter pathway (**Figs. 7A and B**).[18]

In agreement with lesion data showing that descending noradrenergic pathways are important to mefloquine action at a high dose (**Fig. 4C**), we observed that yohimbine blocked this action (Group*Time, F4,40 = 11.16, $P < 5^*10$-6; post hoc: MEF > MEF/yohimbine at 2 hours $P < 2^*10$-5, 4 hours $P < 2^*10$-4 and 7 hours $P = 0.019$) (**Fig. 8A**). However, in vitro analysis (**Fig. 7**) showed that mefloquine does not directly target these receptors. We then tested in vivo whether the selective manipulation of the mefloquine targets that were identified in vitro may also modulate amitriptyline action. Interestingly, we observed no potentiating effect of the serotonin uptake inhibitor fluoxetine (Group*Time, F8,72 = 6.18, $P = 0.52$) (**Fig. 8B**), the adenosine A1 antagonist DPCPX (Group*Time, F8,60 = 1.32, $P = 0.24$) (**Fig. 8C**), or the A2A antagonist SCH58261 (Group*Time, F16,52 = 0.59, $P =$

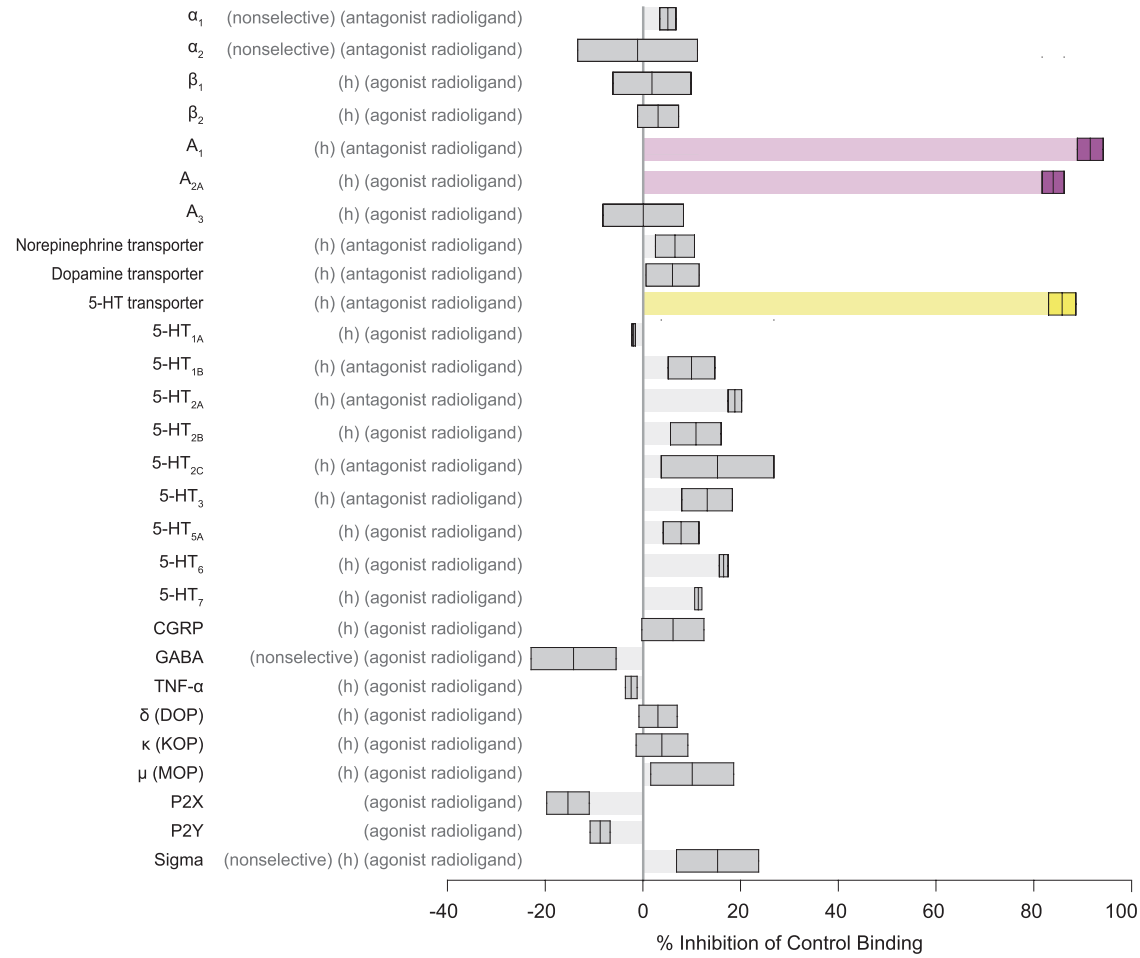
0.88) (**Fig. 8D**) on amitriptyline action. However, the concomitant i.t. blockade of gap junctions by carbenoxolone led to potentiate the action of oral amitriptyline (Group*Time, F6,40 = 5.65, P = 2.52*10-4; post hoc: AMI + CBX>all the other groups at 2 hours $P > 0.0048$) (**Fig. 8E**). These data suggested that mefloquine may preferentially improve amitriptyline effect through targeting of gap junctions.

4. Discussion

Modulators of connexins have been suggested to modulate the therapeutic action of drugs acting on the central nervous system, both in sleep disorders[9,38,49] and in neuropathic pain.[19,20] In this study, we showed that mefloquine enhanced the effect of the tricyclic antidepressant amitriptyline on mechanical hypersensitivity in a mouse model of neuropathic pain. Pharmacokinetic and pharmacological approaches allowed us to highlight some mechanistic features of this potentiation, pointing towards the role of descending noradrenergic pathways, α_2 adrenoceptors, and gap junctions.

A single THN101 (amitriptyline/mefloquine combination) administration led to a long-lasting (around 16 hours) effect on the mechanical hypersensitivity of mice with sciatic nerve compression. This action was likely synergistic and not a simple additive effect. Indeed, it was present for a dose of mefloquine that was ineffective, and it lasted for hours after the acute action of

A **Mefloquine 1 µM - duplicates**



B

Binding assay	Radioligand	IC50	Ki	nh
A_1(h) (antagonist radioligand)	[3H]DPCPX	2.0E-07 M	1.3E-07 M	1.1
A_{2A}(h) (agonist radioligand)	[3H]CGS21680	1.1E-07 M	9.0E-08 M	0.9
5-HT transporter (h) (antagonist radioligand)	[3H]imipramine	1.9E-07 M	8.6E-08 M	1.1

Functionnal assay	Referent	EC50	IC50	KB
A1(h) (agonist effect)	CPA	n.c.		
A2A(h) (agonist effect)	NECA	n.c.		
A1 (h) (antagonist effect)	DPCPX		1.1E-06 M	2.6E-07 M
A2A(h) (antagonist effect)	ZM 241385		1.6E-06 M	1.7E-07 M
5-HT uptake	imipramine		7.7E-07 M	

Figure 7. In vitro evaluation of mefloquine pharmacological potential targets. In vitro determination of pharmacological targets of mefloquine at 1 µM (A). Binding and functional properties of mefloquine on relevant adenosine and serotonin targets (B). α1, alpha 1 adrenoceptor; α2, alpha 2 adrenoceptor; β1, beta 1 adrenoceptor; β2, beta 2 adrenoceptor; A1, adenosine receptor A1; A2A, adenosine receptor A2A; A3, adenosine receptor A3; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; CPA, N6-cyclopentyladenosine; NECA, 59-N-ethylcarboxamidoadenosine.

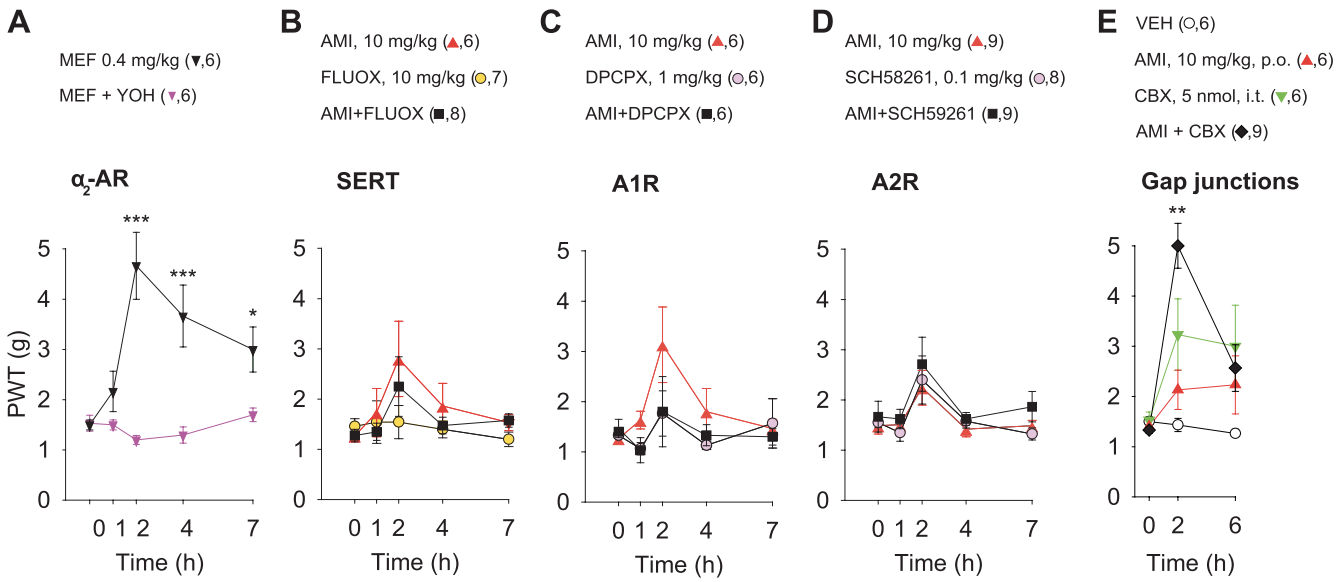


Figure 8. In vivo evaluation of mefloquine pharmacological potential targets. All experiments were conducted in cuff mice. The mechanical right hind paw withdrawal threshold (PWT) was assessed with von Frey filaments. We tested the role of α_2 adrenoceptors in mefloquine action using yohimbine (YOH) (A) (*$P <$ 0.05 ***$P < 0.001$ for MEF vs MEF + YOH). We also tested whether the serotonin uptake (SERT) inhibitor fluoxetine (B), the adenosine A1 receptor antagonist DPCPX (C), the adenosine receptor A2 antagonist SCH59261 (D), and the gap junction blocker carbenoxolone (CBX) (E) may potentiate oral amitriptyline action (**$P < 0.01$ for AMI + CBX vs VEH). Results are expressed as mean ± SEM.

amitriptyline alone stopped. This long-lasting effect with a single injection had not been previously detected when, at another dose combination and with another route of administration, amitriptyline/mefloquine had been first tested in rats with CCI.[19] In this previous study, the benefit of the combination was anyway present after a few days of co-administration. The interest of amitriptyline/mefloquine association is thus illustrated in 2 species and 2 different neuropathic pain models.

A prolonged action of the amitriptyline/mefloquine combination could be the basis of the faster and enhanced recovery observed in this previous rat study, but the detailed time course of hyperalgesia relief after each mefloquine administration had not then been tested. The present time course study highlighted that THN101 kinetic on mechanical hypersensitivity differs from the kinetic of its individual component. Although, besides the spinal noradrenergic and gap junction components, we cannot yet provide mechanistic insights explaining such prolonged effect, it should be noted that the kinetic of mefloquine blood levels strikingly differs from the one of amitriptyline. Although the antidepressant levels peak rapidly in the organism and decrease regularly, mefloquine bioavailability is slower and levels are high 7 hours after administration. This feature may contribute to the lasting potentiation observed in THN101.

An interaction of mefloquine with amitriptyline pharmacokinetics could also have explained the potentiating effect of THN101. The previous study conducted in rat with chronic delivery of amitriptyline and a higher dose of mefloquine showed that brain and serum levels of amitriptyline were overall not affected by mefloquine co-treatment.[19] Moreover, using mass spectrometry, we showed that under our procedures in mice the THN101 combination did not alter the time course of blood levels of mefloquine, of amitriptyline, and importantly, of its active metabolite nortriptyline, and we further showed that it did not alter either the plasma, brain, or spinal cord concentrations of these drugs. This finding is consistent with the fact that amitriptyline and mefloquine are mostly metabolized by different enzymes. Indeed, amitriptyline is mainly metabolized by the cytochrome P450 CYP2C19, leading to nortriptyline by demethylation.[33] By contrast, mefloquine is metabolized by CYP3A4, leading to the formation of a major nonactive metabolite in malaria, carboxymefloquine, and to a lesser extent to hydroxymefloquine.[14] The benefit of THN101 combination is thus not metabolism based.

To explore the action of THN101, we then focused on the target mechanisms of its drug components, amitriptyline and mefloquine. We first tested 2 mechanisms known to contribute to amitriptyline-mediated relief of mechanical hypersensitivity, a peripheral mechanism and a central one.[23,24] The peripheral component of antidepressant drugs relies on the recruitment of noradrenergic fibers in the dorsal root ganglia.[3,24] The downstream mechanism is then mediated by the recruitment of β_2 adrenoceptors present on nonneuronal cells and leading to a relief of the neuroimmune activation induced by the nerve lesion.[3,24] The guanethidine experiment, leading to a sympathectomy, showed that this peripheral component was not critical to THN101 prolonged action. This finding is in agreement with data from the rat study showing that amitriptyline/mefloquine combination had no synergistic impact on markers associated with neuroinflammation.[19] However, the pharmacological study, particularly the i.t. delivery of sotalol that partially blocked THN101 action, suggested that β adrenoceptors can nevertheless contribute to THN101 action with a potential central component.

A central mechanism for antidepressant drug action on neuropathic pain has also been described.[1,24] It is mostly based on the recruitment of noradrenergic descending inhibitory controls of pain[30] and on spinal α_2 adrenoceptors. We thus tested the role of these descending pathways by using i.t. 6-OHDA and observed that it suppressed THN101 action. 6-OHDA is a toxin targeting both noradrenergic and dopaminergic systems, and it is for example classically used to model Parkinson disease loss of midbrain dopamine cells and forebrain dopamine fibers.[11,48] Because of its homology with dopamine and noradrenaline, 6-OHDA enters cells through the transporters of dopamine (DAT) and noradrenaline (NET). However, dopamine projections to the spinal cord arise from hypothalamic A11 and

A13 cell groups, which have the peculiarity of not (or poorly) expressing the DAT.[22,41] High doses of i.t. 6-OHDA can still affect spinal dopamine,[28] but the dose that we used was shown (using mass spectrometry) to induce a 69% decrease in the spinal noradrenaline content without changing the spinal dopamine content[24] and was shown (using immunohistochemistry) to decrease the presence of TH-positive fibers in the lumbar dorsal horn of the spinal cord.[24] Completing the lesion characterization, we showed here that it had no significant impact on the total number of noradrenergic cells in A5, locus coeruleus, and A7, although a trend toward 11% to 17% loss was observed in each group of cells. It may be hypothetized that this trend could reflect the loss of a subset of noradrenergic cells corresponding to cells projecting to the spinal cord but masked by the overall population of the other noradrenegic neurons. The impact of the lesion in the spinal cord was anyway supported by the significant decrease (−55.7%) in TH-positive fibers at the lumbar level. This 6-OHDA experiment together with the use of α_2-adrenoceptor antagonists, particularly atipamezole with intrathecal delivery, highlighted that a spinal noradrenergic component was critical to THN101 mechanism. Interestingly, beyond amitriptyline, this central component seemed to be also present for mefloquine itself at a high dose, which supports a convergence of anatomical targets between the 2 drug components of THN101. Of course, this spinal action does not exclude the contribution of important supraspinal actors in THN101 action.

Mefloquine is known to target connexins, both in vitro[20,35,36] and in vivo,[8] but this drug could also act on other targets. Contract Research Organizations, such as CEREP/Eurofins, are mostly working for pharmaceutical companies and provide professional assessment of binding or activity of drugs on potential targets and off-targets. However, these profiles are rarely communicated to the scientific community, which constitutes a major loss of important information. The literature[16,18,44,51] and the present screening of 80 putative targets suggested that mefloquine may act below μM range on serotonin reuptake and around μM range on adenosine receptors A_1 and A_{2A}, in each case with blocker or antagonist properties. We thus tested the potential implication of these targets by using selective drugs in combination with amitriptyline. The selective serotonin reuptake inhibitor fluoxetine did not improve amitriptyline action (which already has a serotonergic component[23,29]). It suggests that although mefloquine may act on serotonin reuptake, this action alone could not explain the potentiation observed with THN101 combination. The manipulation of adenosine receptors is also known to significantly modulate nociceptive symptoms.[39] However, blocking adenosine A_1 and A_{2A} receptors did not improve amitriptyline action, which suggested that these targets may not be critical either in THN101-potentiating effect. Finally, results showing that the intrathecal delivery of another gap junction blocker, carbenoxolone, can amplify amitriptyline effect support the hypothesis that gap junctions are a relevant target for potentiating antidepressant drug action in neuropathic pain.

Together, our data indicate that amitriptyline action can be potentiated by the antimalaria drug mefloquine at a low dose. This potentiation seems to be mediated by blocking gap junctions and we show that it requires descending noradrenergic controls and α_2 adrenoceptors. The present data now support the interest to assess at the clinical level the effect of the TNH101 drug combination in patients with neuropathic pain, as well as support further exploration of the detailed mechanism that would link gap junctions and the noradrenergic system and lead to potentiate the action on neuropathic pain.

Conflict of interest statement

The authors have no conflicts of interest to declare.

Acknowledgments

This work was supported by Theranexus, Agence Nationale de la Recherche et de la Technologie (Convention Industrielle de Formation par la Recherche 2016/0621), Centre National de la Recherche Scientifique (UPR3212), Université de Strasbourg, and Agence Nationale de la Recherche (Euridol ANR-17-EURE-0022). The authors thank the staff of the Chronobiotron UMS3415 for animal care and Dr Jennifer Kaufling for her help in neuroanatomical figure design. This work was co-funded by a collaboration between the Theranexus Company and the Centre National de la Recherche Scientifique. B. Letellier, F. Mouthon, M. Droguerre, and M. Charvériat are employees of Theranexus.

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B343.

Article history:
Received 14 May 2020
Received in revised form 19 February 2021
Accepted 22 February 2021
Available online 24 March 2021

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Progress in Neuropsychopharmacology & Biological Psychiatry 100 (2020) 109898

Contents lists available at ScienceDirect

Progress in Neuropsychopharmacology & Biological Psychiatry

journal homepage: www.elsevier.com/locate/pnp

Ketamine induces rapid and sustained antidepressant-like effects in chronic pain induced depression: Role of MAPK signaling pathway

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ARTICLE INFO

Keywords:
Ketamine
Neuropathic pain-induced depression
Comorbidity
Anterior cingulate cortex
MKP-1
pERK

ABSTRACT

Chronic pain produces psychologic distress, which often leads to mood disorders such as depression. Co-existing chronic pain and depression pose a serious socio-economic burden and result in disability affecting millions of individuals, which urges the development of treatment strategies targeting this comorbidity. Ketamine, a non-competitive antagonist of the *N*-methyl-D-aspartate (NMDA) receptor, is shown to be efficient in treating both pain and depression-related symptoms. However, the molecular characteristics of its role in chronic pain-induced depression remain largely unexplored. Hence, we studied the behavioral and molecular effects of a single systemic administration of ketamine (15 mg/kg, i.p.) on mechanical hypersensitivity and depressive-like consequences of chronic neuropathic pain. We showed that ketamine transiently alleviated mechanical hypersensitivity (lasting < 24 h), while its antidepressant effect was observed even 72 h after administration. In addition, ketamine normalized the upregulated expression of the mitogen activated protein kinase (MAPK) phosphatase 1 (MKP-1) and the downregulated phosphorylation of extracellular signal-regulated kinase (pERK) in the anterior cingulate cortex (ACC) of mice displaying neuropathic pain-induced depressive-like behaviors. This effect of ketamine on the MKP-1 was first detected 30 min after the ketamine administration and persisted until up to 72 h. Altogether, these findings provide insight into the behavioral and molecular changes associated with single ketamine administration in the comorbidity of chronic pain and depression.

1. Introduction

Chronic pain and depression are detrimental conditions affecting an increasing number of people around the world (Bair et al., 2003; Rayner et al., 2016). Moreover, the co-existence of these conditions represents a serious socio-economic burden and results in a more pronounced disability and a poorer prognosis than either condition alone (Arnow et al., 2006; Gallagher and Verma, 1999). Preclinical data suggests that the comorbid relationship of chronic pain and depressive-like behaviors can be modeled in murine models (Humo et al., 2019; Yalcin et al., 2014a), which allows studying molecular characteristics and treatment strategies in more depth.

Ketamine is a versatile pharmacological agent described in 1965 (Domino et al., 1965) and extensively used in clinical practice since 1970 (Aroni et al., 2009). It primarily acts as a noncompetitive antagonist of the glutamatergic *N*-methyl-D-aspartate (NMDA) receptors (Bergman, 1999). Although initially used as a dissociative anesthetic, ketamine has been later shown efficient in treating both depression and pain (Abdallah et al., 2015; Persson, 2013).

However, while there is widespread evidence of ketamine use for the individual treatment of chronic pain and depression in humans (Aan Het Rot et al., 2012; Hocking and Cousins, 2003), evidence for its beneficial use in the comorbidity of these two conditions is highly limited (Bigman et al., 2017; Weber et al., 2018). Moreover, despite the increasing number of evidence accumulated over the past several decades about the antidepressant and antinociceptive activity of ketamine

Abbreviations: ACC, anterior cingulate cortex; ATF1, transcription factor 1; CREB, cyclic AMP (adenosine monophosphate) response element binding protein; ERK, extracellular signal regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; MKP-1, MAPK phosphatase-1; NMDA, N-Methyl-D-Aspartate; pERK, phosphorylated ERK; GABA, Gamma aminobutyric acid.

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https://doi.org/10.1016/j.pnpbp.2020.109898
Received 11 February 2020; Accepted 24 February 2020
Available online 25 February 2020
0278-5846/

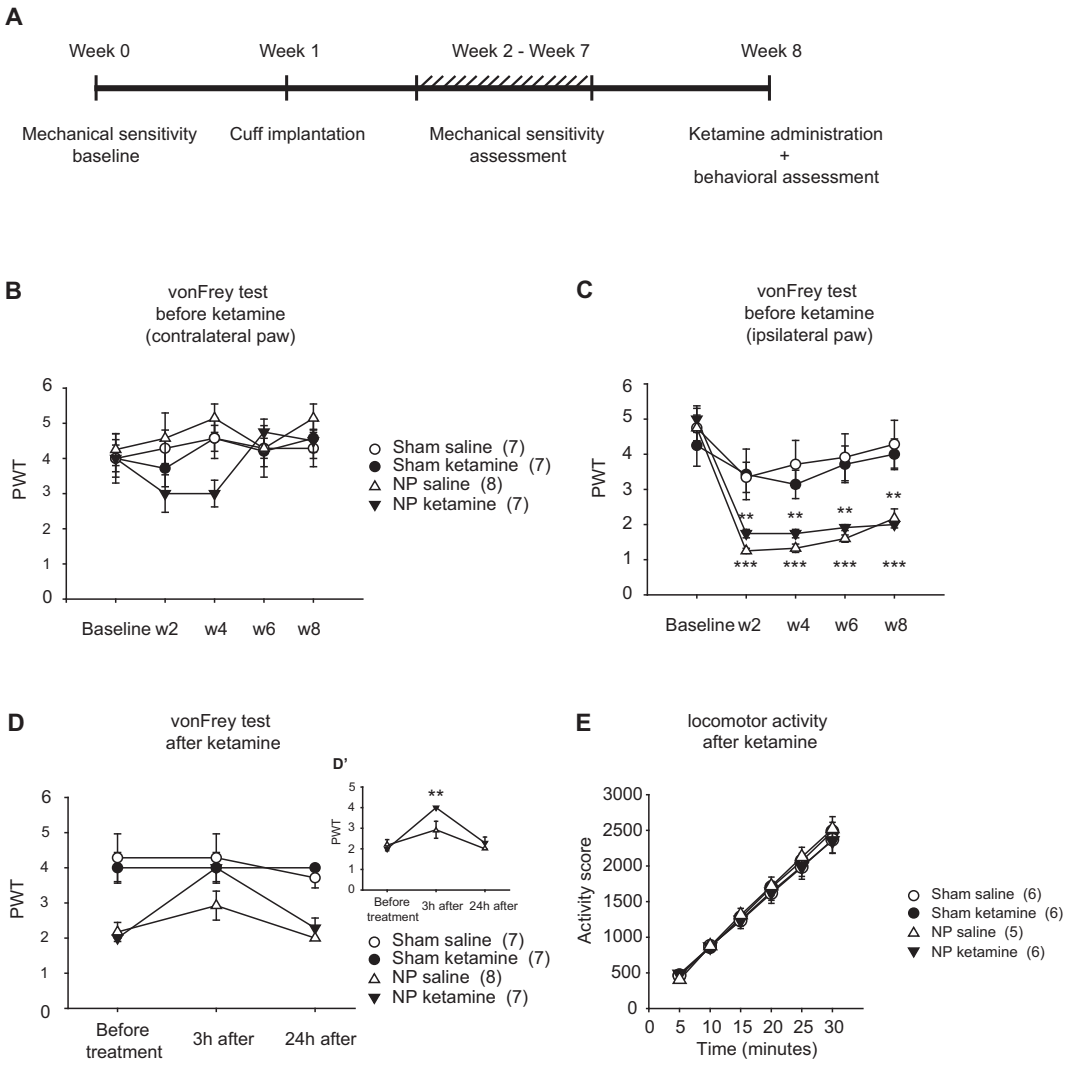

Fig. 1. Antinociceptive effects of single systemic ketamine administration. A) Timeline of surgical and behavioral procedures. B) Pre-ketamine treatment mechanical sensitivity showing no difference in the post-surgery threshold of the contralateral paw of sham and neuropathic (NP) mice as assessed by von Frey test. C) von Frey test results from different time points during the post-surgery period showing a decreased mechanical sensitivity threshold of the ipsilateral paw of NP mice compared to sham-operated controls. D) von Frey test results after single ketamine, showing an increase in the mechanical sensitivity in the ipsilateral paw of NP mice at 3 h (D'), but not 24 h, after administration. E) Neither the peripheral nerve injury nor ketamine treatment affects the spontaneous locomotor activity.

(Cohen et al., 2018; Sleigh et al., 2014; Zanos and Gould, 2018a, 2018b), the underlying mechanism of ketamine action in the chronic pain induced depression has not yet been elucidated.

We have recently shown that neuropathic pain-induced depressive like behaviors in mice are associated with the upregulation of the mitogen activated protein kinase (MAPK) phosphatase-1 (MKP-1) in the anterior cingulate cortex (ACC) (Barthas et al., 2017), a brain structure implicated in both pain- and mood- related processing (Barthas et al., 2015). MKP-1 dephosphorylates both threonine and tyrosine residues of MAPKs thereby acting as an important negative regulator of the extracellular signal-regulated kinase (ERK) signaling cascades (Jeffrey et al., 2007). The relation between MKP-1 and ERK is bidirectional as ERK signaling can also regulate transcription factors such as the cyclic-adenosine monophosphate (cAMP) response element binding protein (CREB) and transcription factor 1 (ATF1) found on the *Mkp-1* promoter region (Rastogi et al., 2013). The present study aimed to determine if neuropathic pain-induced depressive-like behaviors are associated with a disruption of this feedback loop and whether ketamine exerts its activity by targeting different members of the MAPK signaling pathway.

Specifically, the present study evaluated the effect of systemic ketamine administration on the mechanical hypersensitivity and depressive-like consequences of neuropathic pain as well as its effect on the MAPK signaling pathway. Our main results showed that a single intraperitoneal (i.p.) injection of ketamine (15 mg/kg) results in rapid reduction of depressive-like behaviors lasting several days, while only transiently alleviating mechanical hypersensitivity. This ketamine-mediated phenotype was accompanied by a decrease in MKP-1, starting from 30 min and lasting at least until 72 h, and by an increase in phosphorylated ERK (pERK) in the ACC of neuropathic animals. The current findings shed light on the molecular alterations accompanying ketamine administration in the comorbidity of chronic pain and depression.

2. Materials and methods

2.1. Animals

110 adult male C57BL/6 J mice (Charles River, L'Arbresle, France) were used. The mice were 8 weeks old at the beginning of the experiments, housed 4 per cage and kept under a 12 h light/dark cycle (lights on: 7 p.m. and off: 7 a.m.) with food/water availability ad libitum. Results were obtained from a total of four independent cohorts of

animals, wherein two were used for behavioral testing and two were used for western blot analyses. Protocols were approved by the University of Strasbourg ethics committee and performed according to animal care and use guidelines of the European Community Council Directive (EU 2010/63).

2.2. Neuropathic pain model and nociception assessment

Animals were anaesthetized with a combination of zoletil (25 mg/kg tiletamine and 25 mg/kg zolazepam) and xylazine (10 mg/kg) (Centravet, Taden, France) i.p. before neuropathy was induced by unilateral implantation of a 2 mm polyethylene tube (cuff) around the main branch of the right sciatic nerve (Yalcin et al., 2014b). Control (sham) mice received the same surgery without placing the cuff. The mechanical threshold was assessed before surgery (baseline) and on a weekly basis in the postoperative period with the von Frey test. During each session, the animals were individually habituated (10 min) in transparent, bottomless plastic boxes which were placed on a mesh platform. Next, filaments of different pressure (0.4–8.0 g; Bioseb, Chaville, France) were applied to the ventral surface of each hindpaw in an ascending fashion. A positive response for a given pressure was recorded when 3 out of 5 applications resulted in withdrawal or licking of the stimulated hindpaw. The mechanical sensitivity threshold was characterized as a response to 2 consecutive filaments.

2.3. Pharmacological agents

Ketamine hydrochloride (Yliopiston Apteekki, Helsinki, Finland) was dissolved in 0.9% sodium chloride (NaCl) to a 3 mg/ml concentration and injected 0.10–0.15 ml i.p., depending on the weight of the animal, to achieve a 15 mg/kg of ketamine dose per animal. Control animals received single i.p. injections of 0.9% NaCl.

2.4. Behavioral tests

The presence of anxiodepressive-like behaviors was assessed during the 8th week after neuropathy induction. All the tests were performed during the animals' active phase (dark cycle), under red light and as previously described (Yalcin et al., 2011). The experimental design is detailed in Figs. 1A and 2A.

2.4.1. Novelty-suppressed feeding test

Food deprived (24 h) mice were placed in the corner of an open plastic box (40 cm × 40 cm × 30 cm) containing 2 cm of sawdust and a food pellet in the center. The latency to first contact and start eating the pellet was recorded within a time frame of 5 min after being placed in the box. The test measures the animal's motivation to approach the open space of the center of the arena where the food is located.

2.4.2. Splash test

This test involved spraying a 20% sucrose solution onto the dorsal coat and recording of animal's total grooming activity over the next 5 min. A reduced grooming rate indicates a loss of motivation for self-hygiene, parallel to human apathy.

2.4.3. Forced swimming test

Each animal was slowly lowered into a glass cylinder (17.5 cm height x 12.5 cm diameter) with 12 cm of water (24 °C). Two minutes after, the immobility time, which involved floating on the surface without active swimming movements, was recorded over the next 4 min. Due to the stressful circumstances, this test was always performed last. The test measures the animal's helplessness-like behavior.

2.5. Tissue collection and protein analysis

For the molecular analyses, animals were sacrificed 30 min or 72 h after ketamine injection by cervical dislocation and the ACC was dissected and stored at −80 °C. Next, protein extraction, followed by Western blot was performed. Protein extraction was started by mechanical dissociation of the tissues in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 15% EDTA, 10% glycerol, 1% NP40) and centrifugation of the lysate (15,000 g/4 °C/10 min). Next, the supernatant was recuperated (100 µl) and a fraction of it used to determine its concentration with a protein assay (Quick Start Bradford, Bio-Rad, Munich, Germany) and spectrophotometry (Mitrhras LB940, Berthold Technologies). After adjusting the concentration of each sample to 1 µg/µl with lysis and Laemmli buffers, SDS-PAGE gel electrophoresis was done by separating 15 µl of the denatured proteins on 8% polyacrylamide gels and electroblotting them onto a polyvinylidene fluoride (PVDF) membrane (Millipore). Finally, the membrane was incubated over night at 4 °C in the primary antibody (anti-MKP-1, ab195261 lot GR239206-8 rabbit monoclonal, 1:60000; Abcam or pERK Phospho-p44/42 MAPK (Erk1/2) ref. 9101 lot 28 rabbbit monoclonal; 1:600 Cell signaling), washed with TBST, and then incubated in the secondary antibody for 1 h under agitation (AP370P Milipore lot 2899737; goat anti-rabbit; 1:10000 or 1:7500, respectively). Imaging was performed with the enhanced chemiluminescence detection system (ECL Amersham) using the Amersham Imager 680 system. The relative protein expression was calculated with the densitometry tool of Adobe Photoshop CS3 software.

2.6. Statistical analysis

All graphical results are expressed as mean ± SEM (standard error of mean). Statistical analyses were performed with STATISTICA 7.1 (Statsoft, Tulsa, Oklahoma) by using multifactor analysis of variance (ANOVA), with independent (Two-way ANOVA) or repeated measures and Duncan post hoc analyses. The significance level was set at $p \leq .05$. For detailed information see Supplementary table S1.

3. Results

3.1. Single ketamine administration transiently relieves neuropathy-induced mechanical hypersensitivity

Prior to the sciatic nerve cuffing surgery, we evaluated the mechanical threshold for nociceptive sensitivity using the von Frey filaments and organized the groups in such a way that their baseline sensitivity would be equal (Fig. 1B and C). After neuropathy induction, both sham operated and neuropathic mice were divided into a group which later received ketamine and one which received saline, resulting in a total of 4 different groups. Before ketamine administration, cuff-implanted animals consistently showed mechanical hypersensitivity in the ipsilateral paw, which lasted more than eight weeks after the surgery (Fig. 1C; $F_{(4,\ 100)} = 6.86$, $p \leq .001$). At 8 weeks, we first established the time-response curve of single dose of ketamine (15 mg/kg, i.p.) on mechanical sensitivity. We observed that ketamine alleviated the decreased mechanical threshold observed in neuropathic animals 3 h after the administration but this effect was no longer present at 24 h post-treatment (Fig. 1D and D', $p \leq .001$). This rapid but transitory allodynia relief was not observed in neither sham nor neuropathic animals administered with saline (Fig. 1D and D'). Since the motor performance is an important cofounder for behavioral tests, we assessed the locomotor activity of animals 8 weeks after the surgery. The results confirmed our previous observation that the cuff surgery does not affect spontaneous activity (Fig. 1E, (Barthas et al., 2017; Barthas et al., 2015; Sellmeijer et al., 2018; Yalcin et al., 2011)) and also demonstrated for the first time that ketamine does not alter the locomotor activity at 1 h after its administration, the time point at which we performed our first behavioral test (Fig. 1E).

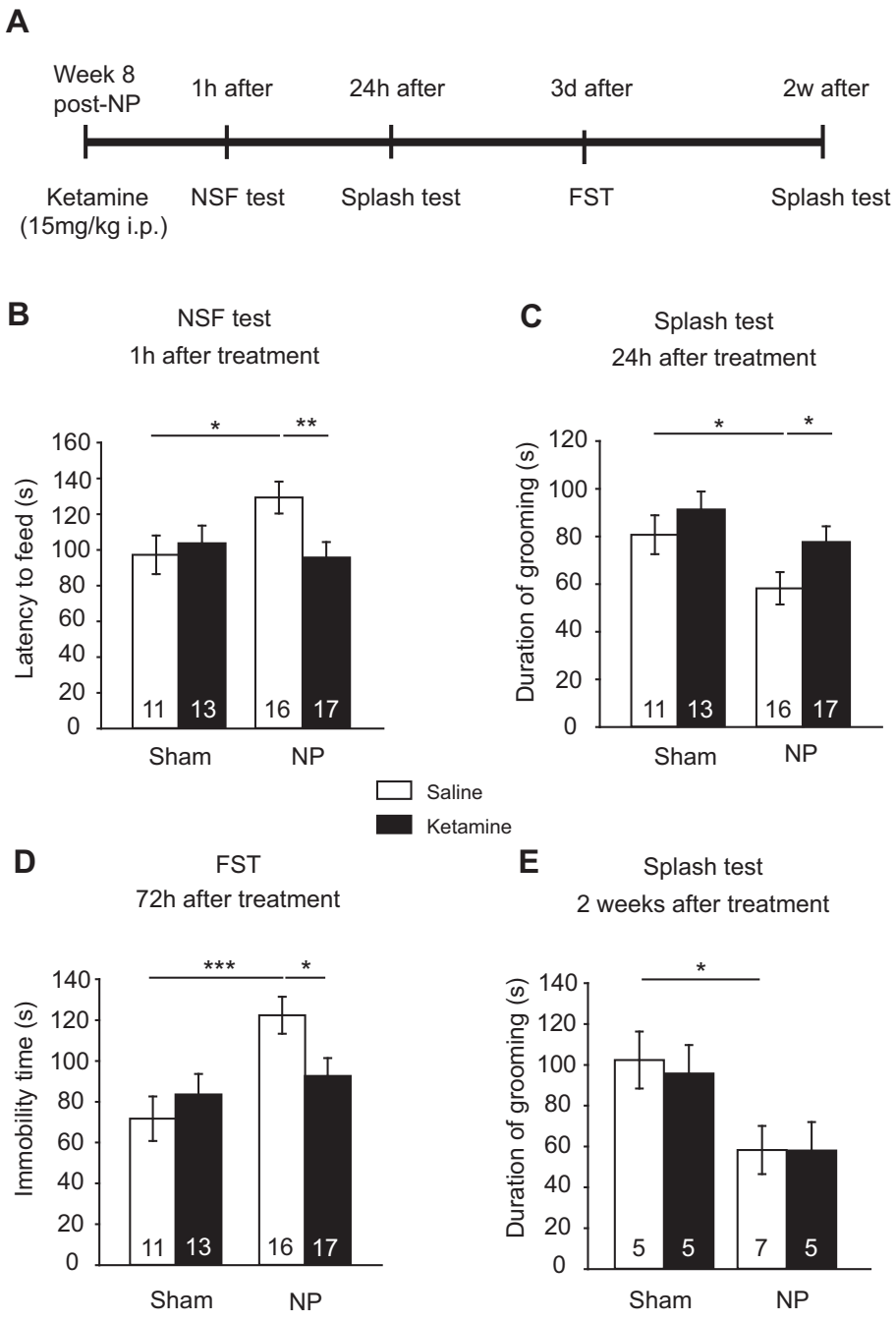

Fig. 2. Antidepressant-like effect of single systemic ketamine administration. A) Timeline of surgical and behavioral procedures. B) NSF test showing a decrease in the latency to feed of NP mice 1 h after ketamine injection. C) Acute ketamine treatment resulted in an increased grooming duration of NP animals in the splash test 24 h later. D) Ketamine-treated NP mice showed a decrease in immobility time during FST 72 h after administration. E) Two weeks after ketamine injection, there was no difference in the grooming duration in the splash test between treated and non-treated NP mice, suggesting that the antidepressant-like effect of acute ketamine was no longer present. Sample sizes are presented in brackets next to experimental groups.

3.2. Single ketamine administration ameliorates depressive-like behaviors in neuropathic mice

Compared to the control animals which received a sham surgery, peripheral nerve injury resulted in depressive behaviors 8 weeks after the surgery, as displayed by an increased latency to feed in the NSF test (Fig. 2B; $p \leq .05$), a decreased grooming duration in the Splash test (Fig. 2C; $p \leq .05$) and an increased immobility in the FST (Fig. 2D; $p \leq .001$). While the anti-allodynic effect was transient, a single injection of a subanesthetic dose of ketamine was sufficient to reduce neuropathy-induced depressive-like behaviors for a prolonged period of time (Fig. 2B–E). Specifically, the neuropathic animals showed a decrease in depression-related behaviors 1 h after ketamine administration in the NSF test (Fig. 2B; $p \leq .01$), 24 h after in the Splash test (Fig. 2C; $p \leq .05$) and 72 h after in the FST (Fig. 2D; $p \leq .05$). However, the antidepressant-like effect of ketamine ceased 2 weeks after the treatment since we observed no differences between saline and ketamine treated cuff animals (Fig. 2E; $p > .05$).

3.3. Single ketamine administration restores the disrupted MAPK signaling pathway

The level of the MKP-1 in the ACC was evaluated 30 min and 72 h after the ketamine administration using two different batches. Western blot analysis showed that MKP-1 protein level was increased in the ACC of saline injected animals displaying neuropathic pain-induced anxio-depressive-like behaviors both at 30 min (Fig. 3A; $F_{(1,\ 19)} = 4.08$, $p \leq .05$; post hoc: sham saline < NP saline, $p \leq .01$) and 72 h after (Fig. 3C; $F_{(1,\ 18)} = 8.69$, $p \leq .01$; post hoc: sham saline < NP saline, $p \leq .01$). This increase was diminished 30 min and 72 h after single

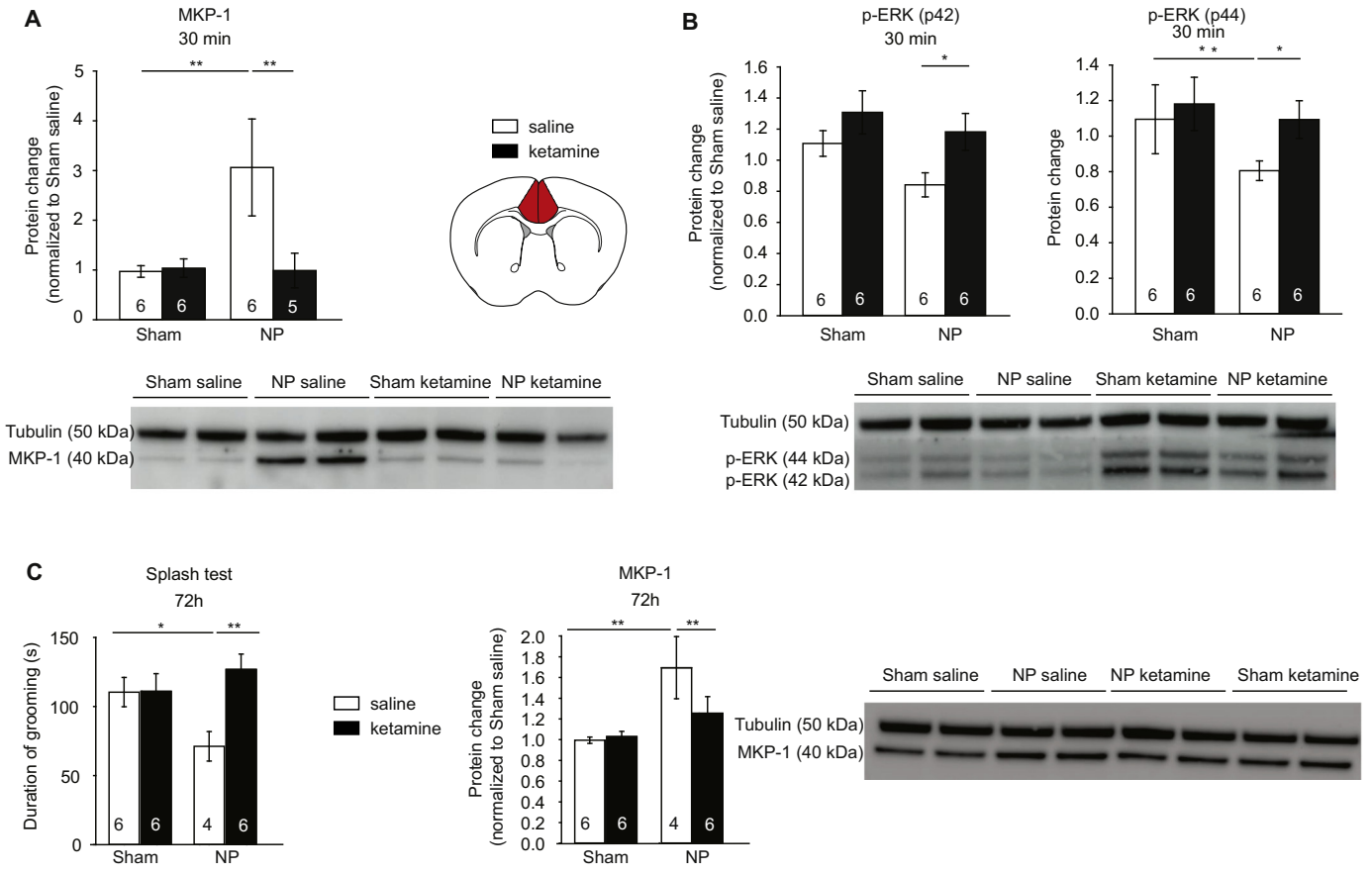

Fig. 3. The effect of ketamine on the MAPK pathway in the ACC. A) Western blot analysis showing an increase in MKP-1 protein level in the ACC of vehicle treated NP mice, and a decrease at 30 min after acute systemic ketamine treatment. B) Western blot results showing decreased pERK in the ACC of NP mice, which is restored 30 min after ketamine administration. C) Compared to the control group, the NP animals administered with ketamine still showed an increase in grooming duration in the splash test and a decrease in upregulated ACC MKP-1 at 72 h after treatment. Sample sizes are presented in brackets next to experimental groups.

ketamine administration (Fig. 3A; $p \leq .01$; Fig. 3C; $p \leq .01$). Similarly, neuropathic animals displayed a decreased p-ERK protein level in the ACC (Fig. 3B; $F_{(1,\ 20)} = 0.42$, $p \leq .05$; post hoc: sham saline > NP saline, $p \leq .01$) which was restored 30 min after single ketamine administration (Fig. 3B; NP saline < NP ketamine, $p \leq .05$).

4. Discussion

By using a mouse model of the comorbidity of neuropathic pain and depression, the present study demonstrated that systemic administration of a single sub-anesthetic dose of ketamine (15 mg/kg) is sufficient to: i) transiently alleviate mechanical hypersensitivity; ii) decrease depressive-like behaviors induced by chronic pain for up to 72 h; iii) restore the increased MKP-1 and the decreased p-ERK protein levels in the ACC.

Treating chronic pain and depression in an independent manner is challenging, but the comorbidity of these disorders is far more difficult (Bair et al., 2003). Subclasses of classical antidepressant drugs such as tricyclic antidepressants, serotonin noradrenaline reuptake inhibitors and anticonvulsants such as gabapentin and pregabaline (Gilron et al., 2015) remain among the first line treatments for many chronic pain conditions as they have direct analgesic effects. Additionally, other treatments such as topical lidocaine, cannabinoids and opioids are used (Attal et al., 2010; Dworkin et al., 2010). Even with these possibilities, there is still a lack of efficiency, and a considerable amount of patients experiencing side effects (Chou et al., 2015; Finnerup et al., 2010), which poses an urgent need for alternative pain remedies. In the current study, we demonstrated that a single systemic administration of ketamine (15 mg/kg) alleviated mechanical allodynia in nerve injured mice 3 h after the administration, but the hypersensitivity was restored already at 24 h post-injection. This transient anti-nociceptive effect of a single ketamine administration is in accordance with previous results (Koizuka et al., 2005; Qian et al., 1996).

One of the main targets of ketamine are the NMDA receptors which are crucial in the development and maintenance of central sensitization (Petrenko et al., 2003), characterized by an increase in dorsal horn excitability, resulting in hypersensitivity, hyperalgesia and allodynia (Woolf, 2011). Thus, by inhibiting NMDA receptors, ketamine has been shown effective in the alleviation of pain in patients suffering from various conditions, including post-surgical pain (Stubhaug et al., 1997), fibromyalgia (Graven-Nielsen et al., 2000), complex regional pain syndrome (Schwartzman et al., 2009) and neuropathic pain (Jorum et al., 2003). However, it is necessary to utilize higher cumulative doses, as well as prolonged serial infusions to achieve a longer lasting effect following the treatment (Niesters et al., 2014; Sigtermans et al., 2009). Curiously, a recent study using the same neuropathy model as here showed that a prolonged ketamine treatment (twice a day for 10 days) with the same dose (15 mg/kg i.p.) had different results depending on the time period of the drug administration (Salvat et al., 2018). Indeed, when ketamine was administered before neuropathic pain surgery, progressive recovery of mechanical allodynia was observed until 2 months after the cessation of the treatment. However, starting ketamine administration with a 25-day delay after neuropathy induction produced only partially recovery during the 10-day treatment, and allodynia completely returned 2 weeks post-treatment. These results suggest that ketamine efficiency is not only dependent on the

dose and frequency of administration, but also on the temporal progression of chronic pain (i.e. development vs. maintenance phase).

Besides its effect on the somatosensory component of chronic pain, here we showed that a single injection of ketamine at a sub-anesthetic dose is sufficient to relieve neuropathic pain-induced depressive-like behaviors for at least 72 h, well beyond the acute pharmacological effects (elimination t1/2–10–15 min). The current study is the first to use a mouse model of comorbid neuropathic pain and depressive-like behaviors to show the antidepressant-like effect of acute ketamine administration, which has previously been shown only in rats (Wang et al., 2011) and in some clinical case studies (Bigman et al., 2017; Weber et al., 2018). These results are also in accordance with previous data from stress-related rodent models of depressive-like behaviors (Autry et al., 2011; Li et al., 2010) and human patients with major depressive disorder (Ballard et al., 2014; Berman et al., 2000; Zarate Jr. et al., 2006) showing that ketamine is a rapid-acting, long-lasting antidepressant agent whose therapeutic effects are manifested within hours and sustain for several days. This is in contrast to the generally prescribed, monoaminergic-related drugs which take several weeks or even months to manifest their benefits (Insel and Wang, 2009; Machado-Vieira et al., 2010). Thus, it is of great interest in the field of psychiatric disorders, notably depression, to understand the physiochemical mechanisms behind the fast-acting, long-term antidepressant properties of ketamine (Abdallah et al., 2015; Kavalali and Monteggia, 2015).

We then studied where and how ketamine acts. The transient antinociceptive and prolonged antidepressant effect of acute ketamine administration observed in the current study highlights the distinct sensory and affective responses to chronic pain. The fact that the duration of ketamine's antidepressant benefits surpasses its anti-nociceptive action suggests that the analgesic properties of ketamine might be mediated at the spinal and peripheral level (Koizuka et al., 2005; Oatway et al., 2003; Sawynok and Reid, 2002), whereas its antidepressant effects require the recruitment of cortical and limbic areas such as the ACC, hippocampus and amygdala (Li et al., 2017; Moghaddam et al., 1997; Niesters et al., 2012). Among these brain structures, the ACC seems to be critical for chronic pain induced depression (Barthas et al., 2017; Barthas et al., 2015) as well as for the antidepressant action of ketamine. For instance, Perrine et al. (2014) showed that ketamine administration resulted in an increase of GABA levels in the ACC of rats subjected to chronic unpredictable stress. This is in line with previous studies showing a decreased level of GABA in the ACC of depressed patients (Bhagwagar et al., 2008), as well as an overall depression-associated hyperactivity of the ACC in both rodents (Sellmeijer et al., 2018) and humans (Drevets et al., 2002). Interestingly, it has been recently shown that acute ketamine reduces hyperactivity of ACC neurons induced by chronic pain in rats (Zhou et al., 2018), which might point to a potential mechanism through which it also exerts its antidepressant activity. Accordingly, we performed all the mechanistic studies in the ACC.

Since the activation of NMDA receptors is related to an increase in intracellular MAPKs, including ERK, p38 and the c-Jun N-terminal kinase (JNK) (Crown et al., 2006; Ji et al., 2009; Waxman and Lynch, 2005) and our neuropathic pain induced depression model induces alterations in this pathway in the ACC as well, we decided to focus on the impact of ketamine on the MAPK pathway in the ACC. Our results showed that an acutely administered ketamine attenuates the disrupted MAPK signaling pathway in the ACC of mice displaying neuropathic pain-induced depressive-like behaviors. Specifically, ketamine lowered the elevated ACC MKP-1 protein level in neuropathic mice at 30 min and 72 h after the drug administration, while it had no effect on the expression of MKP-1 in the ACC of control mice. In addition, ketamine restored the decreased p-ERK in the ACC of mice with comorbid neuropathic pain and depressive-like behaviors. These results are supported by recent evidences from pain field suggesting that ketamine's analgesic activity might be partly mediated through the modulation of several members of the MAPK pathways (Choi et al., 2009; Kwon et al., 2014; Mei et al., 2011). Specifically, it was shown that ketamine's analgesic effect is associated with an inhibition of both spinal astrocyte JNK activation (Mei et al., 2011) and the increased expression of p38 and phospho-p38 (Kwon et al., 2014) as well as with a decreased upregulated ERK (Choi et al., 2009) in the spinal cord of rats with neuropathic pain. In accordance with these findings, it has been shown that a single dose of subanesthetic ketamine which recruits the MAPK signaling cascade (Kohtala et al., 2019; Li et al., 2010) also ameliorates chronic stress-induced deficits in spine number and function (Li et al., 2011), a common characteristic in depression (Banasr et al., 2011). Moreover, Réus et al. (Reus et al., 2014) found that acute blockade of MAPK signaling is sufficient to induce depressive-like behaviors and prevent the antidepressant response to ketamine. Therefore, ketamine might exert its effect by altering the sustained negative regulation of MAPKs through MKP-1, which is thought to contribute to the neuronal atrophy and volume loss in limbic brain areas associated with depression (Sheline et al., 1996; Stockmeier et al., 2004). This has already been suggested as a potential mechanism of other pharmacological agents which alter the MAPK pathway in pain and depression-associated brain regions (Duman and Voleti, 2012). For instance, evidences suggest that antidepressants such as fluoxetine and imipramine might act by restoring the dysregulated expression of MKP-1 and ERK in the hippocampus and ACC (Barthas et al., 2017; Duric et al., 2010; Yasuda et al., 2014).

Besides the ACC, ketamine has been shown to alter the expression of *Mkp-1* in brain regions like the striatum or hippocampus (Ficek et al., 2016). The reason we focused solely on the ACC is the absence of MKP-1 alterations in other brain regions in our mouse model of chronic pain-induced depression, including the hippocampus and the somatosensory cortex (Barthas et al., 2017).

While our results suggest that upregulated MKP-1 contributes to increased dephosphorylation of ERK, which, in turn, fosters the development of depression, the relationship of MKP-1 and ERK does not seem to follow a linear direction, and this pattern of altered expression does not always seem to be the case. In fact, some previous studies show that both chronic stress and neuropathic pain are associated with an increase in ERK activation in the ACC (Kuipers et al., 2003; Wei and Zhuo, 2008), and that this activation contributes to the induction of affective pain, including aversion in response to painful stimuli (Cao et al., 2009; Dai et al., 2011). Additionally, by combining chronic constriction injury and chronic mild stress, Bravo et al. (2012) showed that rats with comorbid chronic pain and depressive-like behaviors show a robust increase of ERK in the ACC. Moreover, Yasuda et al. (2014) demonstrated that chronic constriction injury induces an up-regulation of pERK1/2 in the ACC of rats, while treatment with the tricyclic antidepressant imipramine successfully reduced this overexpression. The observed discrepancies might stem from the intricate bi-directional relationship between MKP-1 and ERK, which might be differently regulated depending on the condition and specific cells and networks. Namely, although MKP-1 is the negative regulator of ERK (Boutros et al., 2008; Sun et al., 1993), it has been demonstrated that ERK can induce *mkp-1* gene expression at the transcriptional level (Brondello et al., 1997), as well as enhance its phosphatase activity (Slack et al., 2001), reflecting its role in a negative feedback control. On the other hand, it is also known that activated ERK can trigger MKP-1 proteolysis via the ubiquitin-proteasome pathway, hence achieving a positive feedback loop by decreasing its phosphatase activity (Lin et al., 2003). These findings suggest that, depending on whether kinase activity needs to be sustained or inhibited, ERK has a dual function in regulating MKP-1 stability, which is achieved through docking to its different domains (Lin and Yang, 2006).

In addition to NMDA receptor antagonism and MAPK inhibition, ketamine produces indirect opioid system activation, demonstrated first in clinical studies (Williams et al., 2019; Williams et al., 2018). Recently Malinow's group showed that ketamine does not act as an opiate,

but its effects require both NMDA and opiate receptor signaling, suggesting that interactions between these two neurotransmitter systems are necessary to achieve an antidepressant effect (Klein et al., 2020). Besides its antidepressant effect, the implication of opioid receptors has also been shown in the analgesic effect of ketamine. Indeed, the antagonists of μ- and δ- but not kappa-opioid receptors block ketamine-induced central antinociception (Pacheco Dda et al., 2014).

In conclusion, this study clearly showed that ketamine could serve as alternative treatment for neuropathic pain patients with major depressive disorder as it has a dual effect on both the somatosensory and emotional consequences of chronic pain. While our study shows the sustained effect of ketamine on MAPK pathway, the present study could not directly test the causal link between ketamine-induced changes in MKP-1 and the relief of depressive-like symptoms. Indeed, since the pharmacological blockade or genetic suppression of MPK-1 being sufficient per se to suppress or prevent depressive-like behaviors in animal models (Barthas et al., 2017; Duric et al., 2010), the direct testing of the role of MKP-1 downregulation in ketamine action becomes very difficult.

Funding information

This work was supported by the Centre National de la Recherche Scientifique (contract UPR3212), the University of Strasbourg, NARSAD Young Investigator Grant from the Brain & Behavior Research Foundation (24736), Fondation pour la Recherche Médicale (FRM FDT201805005527), French National Research Agency (ANR) through the Programme d'Investissement d'Avenir under the contract ANR-17-EURE-0022. This study was also supported by The Scientific and Technological Research Council of Turkey (TUBITAK) through the 2219 international post-doctoral research fellowship program and the Academy of Finland (grant no. 276333). We would like to thank Chronobiotron for animal care.

Ethical statement

Protocols were approved by the University of Strasbourg ethics committee and performed according to animal care and use guidelines of the European Community Council Directive (EU 2010/63).

Declaration of Competing Interest

None. The authors declare that they have no conflict of interest. The funding sources had no role in the study design, in the interpretation of data and in the writing of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pnpbp.2020.109898.

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Neurobiology of Disease 155 (2021) 105363

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Enhanced analgesic cholinergic tone in the spinal cord in a mouse model of neuropathic pain

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A B S T R A C T

Endogenous acetylcholine (ACh) is an important modulator of nociceptive sensory processing in the spinal cord. An increased level of spinal ACh induces analgesia both in humans and rodents while interfering with cholinergic signaling is allodynic, demonstrating that a basal tone of spinal ACh modulates nociceptive responses in naïve animals. The plasticity undergone by this cholinergic system in chronic pain situation is unknown, and the mere presence of this tone in neuropathic animals is controversial. We have addressed these issues in mice through behavioral experiments, histology, electrophysiology and molecular biology, in the cuff model of peripheral neuropathy. Our behavior experiments demonstrate the persistence, and even increased impact of the analgesic cholinergic tone acting through nicotinic receptors in cuff animals. The neuropathy does not affect the number or membrane properties of dorsal horn cholinergic neurons, nor specifically the frequency of their synaptic inputs. The alterations thus appear to be in the neurons receiving the cholinergic signaling, which is confirmed by the fact that subthreshold doses of acetylcholinesterase (AChE) inhibitors in sham animals become anti-allodynic in cuff mice and by the altered expression of the β2 nicotinic receptor subunit. Our results demonstrate that endogenous cholinergic signaling can be manipulated to relieve mechanical allodynia in animal models of peripheral neuropathy. Until now, AChE inhibitors have mainly been used in the clinics in situations of acute pain (parturition, post-operative). The fact that lower doses (thus with fewer side effects) could be efficient in chronic pain conditions opens new avenues for the treatment of neuropathic pain.

Significance statement: Chronic pain continues to be the most common cause of disability that impairs the quality of life, accruing enormous and escalating socio-economic costs. A better understanding of the plasticity of spinal neuronal networks, crucially involved in nociceptive processing, could help designing new therapeutic avenues. We here demonstrate that chronic pain modifies the spinal nociceptive network in such a way that it becomes more sensitive to cholinergic modulations. The spinal cholinergic system is responsible for an analgesic tone that can be exacerbated by acetylcholinesterase inhibitors, a property used in the clinic to relief acute pain (child birth, post-op). Our results suggest that lower doses of acetylcholinesterases, with even fewer side effects, could be efficient to relieve chronic pain.

1. Introduction

Endogenous acetylcholine (ACh) is an important modulator of nociceptive sensory processing in the spinal cord. Epidural administration of neostigmine, an acetylcholinesterase (AChE) inhibitor that induces an increase in ACh spinal level by preventing its degradation, produces pain relief for child birth and post-operation pain in clinics (Eisenach, 2009). In rodents, local spinal injection of AChE inhibitors (by intrathecal injections, or i.t.) similarly produces analgesia to thermal or chemical noxious stimulation (Chen and Pan, 2003; Chiari et al., 1999; Hartvig et al., 1989; Miranda et al., 2002; Naguib and Yaksh, 1994). The analgesic effect of endogenous ACh is not only observed when its level is artificially increased (by AChE antagonists), but also with its physiological levels. Indeed, impairment of cholinergic signaling by locally antagonizing ACh receptors (nicotinic or muscarinic) or by knocking-down the β2 subunit of nAChRs induces hyperalgesia and/or allodynia in rodents (Hama and Menzaghi, 2001; Rashid and Ueda, 2002; Yalcin et al., 2011b; Zhuo and Gebhart, 1991). This suggests that a basal "tone" of spinal ACh modulates the nociceptive threshold.

A dense plexus of cholinergic processes (composed of both dendrites and axons) lies in laminae (L) II-III of the dorsal horn (DH) of rodents (Barber et al., 1984; Mesnage et al., 2011). Within this plexus, cholinergic dendrites receive synaptic contacts from primary afferents, and

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https://doi.org/10.1016/j.nbd.2021.105363
Received 10 November 2020; Received in revised form 12 March 2021; Accepted 31 March 2021
Available online 18 April 2021

European Journal of Medicinal Chemistry 147 (2018) 163–182

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Review article

Phenylpyridine-2-ylguanidines and rigid mimetics as novel inhibitors of TNFα overproduction: Beneficial action in models of neuropathic pain and of acute lung inflammation

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ARTICLE INFO

Article history:
Received 8 August 2017
Received in revised form
13 January 2018
Accepted 16 January 2018
Available online 31 January 2018

Keywords:
Pyridine-2-yl guanidine
TNFα inhibitor
Phenotypic screening
Inflammation
Neuropathic pain

ABSTRACT

4-phenylpyridin-2-yl-guanidine (5b): a new inhibitor of the overproduction of pro-inflammatory cytokines (TNFα and Il1β) was identified from a high-throughput screening of a chemical library on human peripheral blood mononuclear cells (PBMCs) after LPS stimulation. Derivatives, homologues and rigid mimetics of **5b** were designed and synthesized, and their cytotoxicity and ability to inhibit TNFα overproduction were evaluated. Among them, compound **5b** and its mimetic **12** (2-aminodihydroquinazoline) showed similar inhibitory activities, and were evaluated *in vivo* in models of lung inflammation and neuropathic pain in mice. In particular, compound **12** proved to be active (5 mg/kg, ip) in both models.

1. Introduction

Tumor necrosis factor-alpha (TNFα) is a cytokine that is overproduced in inflammatory disease states, mimicked in animal models by LPS (lipopolysaccharide) administration, for instance in the airways [1–3]. The clinical efficacy and FDA (Food and Drug Administration) approval of anti-TNFα biologics such as etarnercept, infliximab, adalimumab, certolizumab pegol and golimumab confirm its critical role in inflammatory diseases [4–8].

Proof of concept with anti-TNFα biologics was also obtained in animal models for the treatment of neuropathic pain [9–12], which arises from a lesion or disease affecting the somatosensory system. The contribution of cytokines originating from immune and/or glial cells, including TNFα, to the pathophysiology of neuropathic pain is supported by experimental and clinical evidence [9,12–19]. Some of the first line treatments of neuropathic pain, namely antidepressant drugs targeting aminergic uptake sites (such as nortriptyline and venlafaxine) and gabapentinoids targeting calcium channels (such as pregabalin), suppress the TNFα overexpression observed in experimental neuropathic pain [12,20]. While existing immunotherapies could be an option to block TNFα in these diseases, their potent action in blocking TNFα is associated with major adverse effects. This limitation might be overcome by the development of new chemicals exerting a milder action on TNFα.

Small molecules inhibiting TNFα production would be expected to be effective disease-modifying treatments for inflammation that recruits this cytokine. For example, thalidomide (and its derivatives), xanthine derivatives (such as pentoxifylline), the antidepressant bupropion and the natural compound curcumin have been shown to induce the inhibition of TNFα expression [21–24].

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https://doi.org/10.1016/j.ejmech.2018.01.049
0223-5234/