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

École Doctorale des Sciences de la Vie et de la Santé

THÈSE

présentée par:

Muris Humo

Soutenue le 27 septembre 2019

Douleur neuropathique et dépression: les modifications moléculaires dans le cortex cingulaire antérieur

Neuropathic pain and depression: molecular alterations in the anterior cingulate cortex

Pour obtenir le grade de: Docteur de l'Université de Strasbourg

Spécialité: Neurosciences

Thèse dirigée par:

Mme Yalcin Ipek	Chargée de recherche, UPR 3212, Strasbourg

Rapporteurs:

Mme Courteix Christine	Professeur, UMR 1107, Clermont-Ferrand
M Krezel Wojciech	Directeur de recherche, UMR 7104, Strasbourg

Examinateur:

M Jeanneteau Freddy	Chargée de recherche, UMR 5203, Montpellier

To my father

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Douleur neuropathique et dépression: les modifications moléculaires dans le cortex cingulaire antérieur

Résumé

Les troubles d'humeur sont fréquemment associées à une douleur chronique et le cortex cingulaire antérieur (CCA) est une région importante dans cette relation. Notre objectif est d'étudier les bases moléculaires de cette comorbidité, tant au niveau de la globalité du CCA (tissus entier) que dans les différents types cellulaires. Dans un modèle murin de douleur chronique présentant des conséquences anxiodépressives et dans plusieurs modèles de dépression, nous avons mis en évidence une surexpression du régulateur négatif de la voie de la protéine kinase activée par des agents mitogènes (MAPK), la MAPK Phosphatase-1 (MKP-1). La diminution de son expression dans le CCA atténue les comportements de type dépressif, ce qui montre que MKP-1 est un facteur clé de la physiopathologie de la dépression. Nous avons également démontré que l'administration aiguë du kétamine normalise la voie MAPK perturbée, tout en produisant un effet analgésique transitoire et un effet antidépresseur prolongé. Enfin, pour étudier la contribution individuelle de différentes populations de cellules dans le développement de la dépression, nous avons isolé les neurones GABAergiques du CCA pour étudier leur expression génomique afin d'établir une liste de gènes candidats plus spécifiques.

Mots-clés: Dépression, Douleur chronique, CCA, MKP-1, Kétamine, Neurones GABAergiques

Abstract

Mood disorders are frequently comorbid with chronic pain and the anterior cingulate cortex (ACC) appears to be an important region in this relationship. We aimed to investigate the molecular basis of this comorbidity, at both the whole structure and the cell type specific level. A genomic analysis of the ACC in a mouse model displaying chronic pain-induced anxiodepressive consequences evidenced an overexpression of the Mitogen Activated Protein Kinase (MAPK) Phosphatase 1 (MKP-1). An upregulated ACC MKP-1 was also observed in other models of depression, while decreasing its expression attenuates depressive-like behaviors, showing that MKP-1 is a key factor in the pathophysiology of depression. This was further validated by showing that acute ketamine administration normalizes the disrupted MAPK pathway, alongside producing a transient analgesic and a prolonged antidepressant effect. Finally, to address the role of different cell populations in this comorbidity, we have isolated GABAergic neurons from animals showing depressive-like behaviors, which will be used for genomic analysis in order to reveal important cell-type specific candidate genes.

Keywords: Depression, Chronic pain, ACC, MKP-1, Ketamine, GABAergic neurons

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Publications et communications

Publications liées aux travaux de thése

Humo M, Hugel S, Waltisperger E, Lutz PE, Yalcin I (2020) The role of ACC GABAergic cells in neuropathic pain-induced depression. En cours

Humo M, Ayazgök B, Waltisperger E, Becker L, Rantamäki T, Yalcin I (2019) Ketamine induces rapid and sustained antidepressant-like effects in chronic pain induced depression: role of MAPK signaling pathway. Soumis

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Barthas F*, **Humo M***, Gilsbach R, Waltisperger E, Karatas M, Leman S, Hein L, Belzung C, Boutillier AL, Barrot M, Yalcin I (2017) Cingulate overexpression of mitogen-activated protein kinase phosphatase-1 as a key factor for depression. Biol Psychiatry 82:370-379 *: co-premier auteur

Publication issue de collaboration

Bollenbach M, Salvat E, Daubeuf F, Wagner P, Yalcin I, **Humo M**, Letellier B, Becker LJ, Bihel F, Bourguignon J, Villa P, Obrecht A, Frossard N, Barrot M, Schmitt M (2018) Phenylpyridine-2-ylguanidines and rigid mimetics as novel inhibitors of TNFα overproduction: Beneficial action in models of neuropathic pain and of acute lung inflammation. Eur J Med Chem 147:163-182

Publication issue du master

Schiweck J, Beauchamp M, **Humo M**, Lelievre V (2015) Old friends, new story: The role of Slit2C signaling through PlexinA1. Cell Adh Migr 9:417-421

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Humo M, Barthas F, Gilsbach R, Waltisperger E, Karatas M, Lehman S, Hein L, Belzung C, Boutillier AL, Barrot M, Yalcin I (2019) Cingulate overexpression of MKP-1 as a key factor for depression. NeuroFrance 2019. Marseille, France

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Communications orales

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CONTENT

Abbreviations

5-HT3aR	serotonin receptor 3a
ACC	anterior cingulate cortex
APA	american psychiatric association
ChR2	channelrhodopsin 2
Dlx5/6	distal-less homeobox genes 5 and 6
DSM	diagnostic and statistical manual of mental disorders
FACS	fluorescence activated cell sorting
FST	forced swimming test
GABA	gamma aminobutyric acid
$GABA_{A/B}$	GABA receptor A/B
GAD65/67	glutamic acid decarboxylase 65/67
GFP	green fluorescent protein
IASP	international association for the study of pain
ICD	international classification of diseases
IN	interneuron
JNK	c-Jun N-terminal kinase
MAPK	mitogen activated protein kinase
MCC	midcingulate cortex
MDD	major depressive disorder
MKP-1	mitogen activated protein kinase phosphatase 1
NMDA	N-methyl-D-aspartate
NP	neuropathic pain
NSF	novelty suppressed feeding
PCC	posterior cingulate cortex
pERK	phosphorylated extracellular signal-regulated kinase
PFC	prefrontal cortex
PV	parvalbumin
qPCR	quantitative polymerase chain reaction
RSC	retrosplenial cortex
SNRI	serotonin noradrenaline reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
SST	somatostatin
TCA	tricyclic antidepressant
TRAP	translating ribosome affinity purification
TST	tail suspension test
VIP	vasointestinal peptide
vTRAP	viral TRAP
WHO	world health organization

Description du projet en français

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Le contexte

La dépression est une maladie invalidante et durable qui, à l'horizon 2030, contribuera de manière décisive à la morbidité mondiale (WHO, 2008). Avec le stress, la douleur chronique est un des facteurs de risque majeur associé à la dépression (Attal et al. 2011). Ainsi, le taux de prévalence est d'environ 50% pour le trouble dépressif majeur chez les patients souffrant de douleur chronique (Bair et al. 2003). Notre équipe utilise un modèle murin préclinique pour étudier les mécanismes neurobiologiques impliqués dans les troubles de l'humeur induits par la douleur chronique (Yalcin et al. 2011a; Barthas et al. 2015; Barthas et al. 2017). Les études précédentes ont montré que la douleur neuropathique, résultant d'une lésion ou d'une maladie du système somatosensoriel, entraîne avec le temps des troubles de type anxiodépressif (Yalcin et al. 2011a).

Parmi les régions cérébrales impliquées dans la dépression, le cortex cingulaire antérieur (CCA) est essentiel car il présente des modifications fonctionnelles et morphologiques chez les patients souffrant de dépressions sévères (Matthews et al. 2008; Pizzagalli 2011). L'ablation chirurgicale du CCA (Shields et al. 2008) ou la stimulation cérébrale profonde du CCA (Lipsman et al. 2013) peuvent atténuer les symptômes dépressifs. Dans le domaine préclinique, notre équipe a mis en évidence que l'activation optogénétique des neurones pyramidaux du CCA était suffisante pour induire des comportements de type anxiodépressif chez des animaux naïfs, tandis qu'une lésion du CCA empêchait la dépression induite par la douleur chronique (Barthas et al. 2015). Ainsi, le CCA apparaît comme une structure cérébrale clé dans la dépression mais également dans les troubles de l'humeur résultant de la douleur chronique.

Objectifs

Sur la base des données publiées et des résultats préliminaires, mon projet de thèse s'articule autour de trois objectifs principaux: 1) étudier les modifications moléculaires dans le

CCA entier à l'aide de modèles animaux bien validés et se concentrer sur une cible moléculaire spécifique, 2) étudier l'effet thérapeutique de la kétamine sur les propriétés comportementales et moléculaires associées à la comorbidité entre douleur neuropathique et dépression, 3) caractériser le rôle fonctionnel et moléculaire des neurones libérant l'acide gamma-aminobutyrique (neurones GABAergiques) du CCA dans cette comorbidité.

Résultats concernant le premier objectif

Les approches ouvertes portant sur l'ensemble du génome peuvent être puissantes pour identifier les données moléculaires de la dépression. Pour le premier objectif, nous avons effectué une analyse par micropuce à ADN dans le CCA chez des animaux témoins et des animaux présentant des comportements de type dépressif après l'induction d'une douleur neuropathique (DN). Sur la base de cette analyse d'expression génomique, nous nous sommes concentrés sur le régulateur négatif de la voie de la protéine kinase activée par des agents mitogènes (MAPK), la MAPK Phosphatase-1 (MKP-1), dont l'expression est fortement augmentée chez les animaux avec DN. Nous avons généralisé nos résultats à d'autres modèles de dépression tels que le stress léger chronique ou la stimulation optogénétique du CCA, ce qui suggère l'existence d'une corrélation entre la dépression et l'augmentation du niveau de MKP-1 dans le CCA. En utilisant l'immunohistochimie, le Western blot et l'immunoprécipitation de la chromatine, nous avons également montré que l'expression prolongée de MKP-1 pouvait être associée dans le CCA à une augmentation de l'expression de c-Fos, des niveaux de p-CREB et de p-ATF, ainsi qu'à une augmentation de H3K9/14acétylation dans les régions promotrices de *Mkp-1*. Afin de passer des analyses corrélatives à des analyses causales, nous avons combiné plusieurs approches pour manipuler MKP-1 et avons montré qu'une délétion totale du gène *Mkp-1* (knock-out), un antagoniste ou un blocage local de MKP-1 dans le CCA atténuaient les comportements de type dépressif. De plus, un antidépresseur classiquement utilisé, la fluoxétine, supprime les niveaux élevés de MKP-1 dans le CCA. Ces résultats liés au premier objectif ont été publiés dans Biological Psychiatry (Barthas et al. 2017), dont je suis le co-premier auteur.

Résultats concernant le deuxième objectif

A la suite des résultats issus du premier objectif, nous avons également étudié les effets comportementaux et moléculaires du traitement à la kétamine dans notre modèle murin. La kétamine est un antagoniste non compétitif du récepteur du N-méthyl-D-aspartate (NMDA), qui empêche le glutamate de se lier à son récepteur et crée une inhibition des

neurones glutamatergiques (Bergman, 1999). Bien qu'initialement utilisée uniquement comme anesthésique dissociatif, la kétamine s'est révélée efficace au fil des ans pour traiter à la fois la dépression et la douleur (Persson 2013; Abdallah et al. 2015). Par conséquent, nous avons recherché les effets de l'administration systémique aiguë de kétamine sur l'hypersensibilité mécanique et les conséquences affectives de la douleur neuropathique, ainsi que sur la voie MAPK. Nos principaux résultats ont montré qu'une seule injection de kétamine par voie intraperitonéale (15 mg/kg en i.p.) entraînait une réduction immédiate des comportements de type dépressif durant plusieurs jours, alors que hypersensibilité mécanique n'était réduite que de manière transitoire. Ce phénotype lié à la kétamine était accompagné de changements de la voie MAPK du CCA des animaux neuropathiques. Notamment, la kétamine a réduit l'augmentation de l'expression protéique de MKP-1, ainsi que la diminution de la forme phosphorylée de la kinase régulée par le signal extracellulaire (pERK) dans le CCA des souris présentant des comportements de type anxiodépressif induits par la neuropathie. Ces résultats ont mis en lumière les modifications moléculaires du CCA associées à l'administration systémique de kétamine dans la dépression induite par la neuropathie.

Résultats concernant le troisième objectif

Une des difficultés rencontrées dans les études de neuroscience provient du fait que les structures cérébrales comprennent souvent une variété de types cellulaires. C'est pourquoi l'attention des scientifiques se déplace depuis quelques années vers l'étude des implications fonctionnelles de l'hétérogénéité neuronale dans les troubles psychiatriques (Oldham, et al. 2008). Pour le deuxième objectif de notre étude, nous nous intéressons à la caractérisation des traits fonctionnels et moléculaires de populations neuronales spécifiques du CCA. Notre étude cible en particulier les neurones corticaux exprimant les gènes *Dlx5* et *Dlx6* de l'homéoboite, qui se sont révélés être des marqueurs des cellules GABAergiques (Stühmer et al. 2000; Batista-Brito et al. 2008; Taniguchi 2011).

En combinant une lignée de souris transgéniques exprimant une *Cre-recombinase* dans les cellules *Dlx 5* et *Dlx 6* (Dlx5/6-Cre) avec un virus porteur de la chanelrhodopsine-2 dépendant de la Cre nous avons pu montrer que l'activation optogénétique locale des cellules GABAergiques dans le CCA était suffisante pour réduire les comportements dépressifs induits par la DN, sans affecter la sensibilité mécanique. Ces résultats suggèrent que la population de cellules GABAergiques exprimant Dlx5/6 dans le CCA joue effectivement un

rôle dans les comportements affectifs liés à la neuropathie et constitue une cible intéressante pour une analyse moléculaire plus précise.

L'analyse génomique de ces cellules pourrait permettre d'identifier de nouveaux mécanismes indétectables dans le tissu entier. Nous avons donc cherché à identifier les adaptations génomiques spécifiques des neurones GABAergiques du CCA chez les animaux avec DN. Cela nous permettra éventuellement de manipuler de manière sélective les gènes cibles de cette population neuronale et d'examiner leur effet sur le comportement. Pour identifier les adaptations transcriptionnelles spécifiques des cellules GABAergiques du CCA, nous utilisons la technique de purification des ribosomes par affinité appelée TRAP (Translating Ribosome Affinity Purification). Ceci est obtenu par la transfection dans le CCA de souris transgéniques Dlx5/6-Cre d'un virus adéno-associé (AAV) porteur des protéines ribosomales L10 associées à la protéine fluorescente verte (EGFP) et dont l'expression est dépendante de Cre. Trois semaines après l'injection du virus, le CCA est disséqué et incubé avec des billes magnétiques couplées à des anticorps anti-EGFP qui reconnaitront les ribosomes marqués dans le type cellulaire d'intérêt (neurones GABergiques du CCA). Le complexe billes-ribosomes est précipité et l'acide ribonucléique messager (ARNm) attaché aux ribosomes est purifié pour le séquençage de l'ARN.

Conclusion et Perspectives

Après avoir déterminé les propriétés génomiques de la population de cellules GABAergiques du CCA dans la dépression induite par la DN, les résultats seront comparés à ceux obtenus sur CCA entier. Pour déterminer l'importance d'un gène donné sur le comportement affectif, nous prévoyons de le manipuler dans le CCA de souris naïves et de souris exprimant des comportements de type dépressif induits par la DN. Cette stratégie est similaire à celle que nous avons récemment mise en œuvre avec succès pour le gène *Mkp-1*, identifié à partir d'une analyse de tissu entier.

L'aboutissement de ce projet, nous permettra d'acquérir des informations détaillées sur l'impact moléculaire de la dépression induite par la douleur chronique dans les neurones GABAergiques corticaux du CCA. Cela constituera une première avancée vers la validation de la cible préclinique qui pourrait permettre d'établir un lien de causalité entre une expression génique altérée et la comorbidité de la douleur chronique et de la dépression. Ces résultats pourraient certainement conduire à de nouvelles perspectives de traitements plus ciblés.

Preface

Chronic pain, a persistent pain lasting more than 3 months (Treede et al. 2015), is a detrimental condition affecting 25-30% of Europeans (Leadley et al. 2012), while neuropathic pain, a type of chronic pain resulting from a disease or a lesion affecting the somatosensory system (Jensen et al. 2011), is estimated to affect 7-8% of Europe (Torrance 2006; Bouhassira 2008). Alarmingly, around one third of these patients also develop mood disorders such as anxiety and major depressive disorder (MDD) (Radat et al. 2013) which have additional negative effects on their overall quality of life (Attal et al. 2011).

Both anxiety and depressive disorder are highly debilitating psychiatric conditions with strong repercussions on one's personal, social, educational, occupational, and other important domains of everyday functioning. The most widely accepted criteria used in clinical settings for the diagnosis of MDD and anxiety come from the Diagnostic and Statistical Manual of Mental Disorders (DSM), published by the American Psychiatric Association (APA), and from the International Classification of Diseases (ICD), proposed by the World Health Organization (WHO). While the terminology might be slightly different in certain cases, both manuals tend to conform on the diagnosis procedures (Sadock et al. 2003). Hence, depression is characterized by cardinal symptoms such as depressed mood and anhedonia, which are accompanied by additional experiences such as feelings of worthlessness, hopelessness, appetite and/or sleep disturbances, fatigue, suicidal thoughts etc., lasting for at least two weeks (APA 2013; WHO 2018). On the other hand, anxiety is described as distress resulting from excess worry or fear even when there is no actual threat present, which is also associated with additional feelings such as fatigue, irritability, disturbed sleep and other similar physical and cognitive symptoms (APA 2013; WHO 2018).

However, the biological underpinnings of the comorbid relationship between neuropathic pain and anxiodepressive symptoms are still largely unknown. Therefore, we utilized a murine model of persistent moderate neuropathic pain, which develops anxiodepressive-like behaviors in a time-dependent manner (Yalcin et al. 2011a; Barthas et al. 2015). Furthermore, in order to study the consequences of this comorbidity, we focused on a neuroanatomical substrate known as the anterior cingulate cortex (ACC), which has repeatedly been shown to undergo morphological and functional alterations in both neuropathic pain and depression (Davis et al. 1997; Mayberg et al. 2005).

Our research employed an open-approach where we used genomic analyses to explore ACC gene expression of mice displaying neuropathic pain-induced anxiodepressive-like behaviors compared to their healthy control littermates. Next, we plan to go even further by

assessing the transcriptomic expression specifically in inhibitory γ-aminobutyric acid (GABAergic) neurons under the same circumstances, which has a potential to identify additional mechanisms that may be unforeseeable at the level of the whole tissue. Both approaches have the aim of yielding specific molecular targets which might be crucial factors in the development and persistence of comorbid pain and depression. These can then be further manipulated and modified with various additional techniques to assess their significance in affecting the comorbid phenotype, and establish whether specific candidates might constitute viable targets for future treatment strategies.

The following introductory section will describe in more detail chronic pain, notably neuropathic pain, and its relationship to anxiety and depression. Next, our recently published review will shed more light about the molecular alterations associated with this comorbidity. Then, the role of the ACC in the co-existence of chronic pain and mood disorders will be discussed, before the attention is shifted to the role of GABAergic cells in pain and depression. Finally, the introduction will be completed with a brief overview of pharmacological treatment strategies employed in dealing with this comorbidity. After laying down the general research objectives, the results section will present our published and unpublished work in the format of individually completed peer-reviewed publications. At last, a general discussion will address the interpretation, implications and limitations of the completed work.

Introduction

Pain

The definition put forth by the International Association for the Study of Pain (IASP) characterizes pain as:

„*An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*.“ (IASP, 1979)

This definition has been formulated based on the work of Harold Merskey (1964) and has reached wide consensus among clinicians and researchers over the past decades (Cohen et al. 2018). Such description clearly identified pain as a conscious concept, an experience, distinguishing it from nociception, which was first described by Sherrington (1906) in terms of nociceptor-mediated detection of potentially tissue-damaging extraneous stimuli, which further activate the peripheral and central nervous system. Consequently, nociception can elicit brain activity without pain perception (Lee et al. 2009), and, on the other hand, pain can be experienced without the nociceptive component (Nikolajsen and Jensen, 2001; Barthas et al. 2015).

While the work by Merskey was the first to focus on pain as an experience which encompassed both the sensory and emotional experience, Melzack and Casey (1968) proposed a conceptual neural mechanism of stimuli processing, involving the sensory, affective and cognitive determinants. This was based on the gate control theory (Melzack and Wall, 1965) which proposed that the spinal cord acts as a „gate“ which either propagates or blocks peripheral signals on their way to the brain, thus proposing a physiological mechanism while incorporating a psychological role in pain perception. Although it drew a substantial amount of criticism and revision due to its uncomplete and undetailed nature, over time, the gate control theory proved to be one of the most influential theories in studying pain, paving the way for future studies focusing on various physiological and psychological components of pain perception.

With consequent research, it became more evident how mechanical, thermal or chemical nociceptive stimuli are processed into a physical reaction and pain sensation. In general, a peripheral noxious stimulus gets detected by primary afferent nociceptors, which are subpopulations of fast-conducting, lightly myelinated (A∂) and slow-conducting, unmyelinated (C) fibers (Urch 2007). Activation of these nociceptors leads to the transmission of the signal to the second order neurons located in the dorsal horn of the spinal cord. Here, an intricate system of excitatory and inhibitory neuronal circuits processes the input and conveys it via projection neurons to supraspinal structures (Almeida et al. 2004; Garland et al. 2012), which is known as the ascending modulatory pain pathway. On the other hand, brain

structures responsible for higher order pain processing can also modulate the nociceptive input in the dorsal horn via the descending pain pathway, by sending signals to the brainstem and further down to the spinal cord (Fig. 1) (Heinricher et al. 2009).

Moreover, with the technological advancements in the past several decades that fostered the development of non-invasive brain imaging techniques, nociception and pain research was able to directly associate a given stimulus with specific neural activity responses (Morton et al. 2016). Before functional neuroimaging, our understanding about the role of the brain in pain processing was based primarily on animal studies, while now, pain can be studied directly in human subjects, which allows for better translational knowledge between species. By defining neuroanatomical substrates that functionally respond to various noxious stimuli, human neuroimaging studies have yielded a concept known as the pain „neuromatrix", a network of brain regions accounting for different components of the overall pain perception (Melzack 1999; Price 2000; Garcia-Larrea and Peyron 2013). Hence, we now know that the stimuli transferred from the spinal cord are further „relayed" by the thalamus to various cortical and subcortical structures including the somatosensory cortices, amygdala, hypothalamus, prefrontal cortex (PFC), insula and ACC, which results in sensory-discriminative, affective, motivational, cognitive and social interpretations of the pain experience (Apkarian et al. 2005; Tracey and Mantyh 2007).

Chronic pain

Unlike acute pain, which is a protective evolutionary asset, signaling potential harm and inflicting a range of possible responses, from spinally-mediated reflexes to adaptive voluntary action, serving to avoid and limit tissue damage (Morrison et al. 2013), chronic pain seems to have no biological benefits. Chronic pain is generally characterized as pain lasting more than 3 to 6 months (Merskey and Bogduk 1994), which is usually well beyond the normal healing process (Bonica 1953). Whether it is persistent or recurrent, chronic pain has a serious debilitating effect on the health and daily life of those affected (Palermo 2000). In addition, with an estimated 20% of people suffering from it worldwide (Breivik et al. 2006; Goldberg et al. 2011), chronic pain represents a serious socio-economic burden (Phillips 2009).

Although they show converging characteristics over time, broadly speaking, the two major categories of chronic pain are inflammatory and neuropathic pain (Xu and Yaksh 2012; St John 2018). However, due to a wide range of symptom etiology and severity, chronic pain can be further classified into a variety of categories including primary pain (i.e. fibromyalgia,

nonspecific back pain, etc.), cancer pain, postsurgical and posttraumatic pain, headache and orofacial pain, visceral pain, musculoskeletal pain and neuropathic pain (Treede et al. 2015).

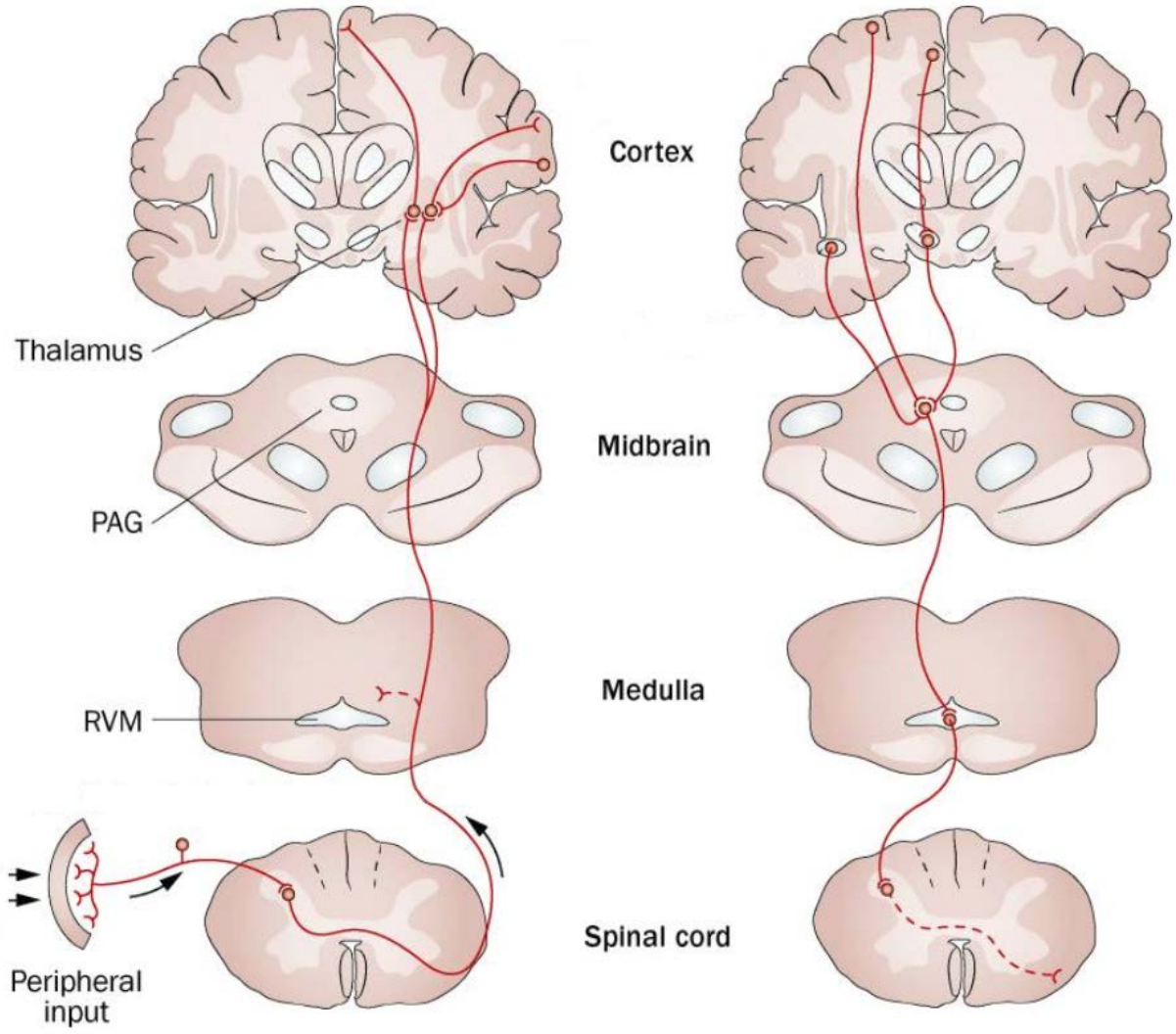

Figure 1: Simplified schematic representation of the principal pain-modulatory pathways. Ascending (left) and descending (right) pain pathways (red lines) that transmit signals from the periphery to the brain and from the brain to the spinal cord, respectively. Abbreviations: PAG, periaqueductal grey; RVM, rostroventral medulla. (Adapted from: Zhou and Verne 2014)

Neuropathic pain

Neuropathic pain is characterized as pain resulting from a lesion or disease of the somatosensory nervous system (Treede et al. 2008; Jensen et al. 2011). It is different from nociceptive pain, which pertains to the activation of nociceptors due to actual or threatened damage of non-neural tissues where the somatosensory system is not permanently compromised (Gierthmühlen and Baron 2016; Spahr et al. 2017). Neural damage might affect the peripheral nervous system, caused by conditions like post-herpetic neuralgia, nerve lesions, trigeminal neuralgia and amputation, or it can be related to the central nervous system, resulting from stroke, spinal cord injury, multiple sclerosis and other debilitating states (Colloca et al. 2017). Due to the wide range of characteristics pertaining to the anatomical location, origin and severity of the harm, neuropathic pain patients generally have a complex array of positive and negative symptoms, which can be a result of abnormal neuronal excitability and axon/neuron loss, respectively (Jensen and Baron 2003; Woolf 2004; von Hehn et al. 2012).

However, despite the high diversity in the pathophysiology which stems from an interaction between etiological, genotypic and environmental factors, there are many common symptoms in a wide range of patients. Namely, 15-50% of neuropathic pain patients report having allodynia and/or hyperalgesia, which are classified based on the sensory modality (touch, pressure, temperature etc.) used to evoke the sensation (Jensen and Finnerup 2014). According to the definitions by the IASP, allodynia is a painful response to a non-nociceptive stimulus, while hyperalgesia stand for an increased pain sensitivity (Loeaser and Treede 2008). Other prominent symptoms associated with neuropathic pain include paresthesia, which is a spontaneous or evoked abnormal sensation (i.e. „pins and needles“, tingling, prickling) and dysesthesia which is also an abnormal sensation, but which is considered unpleasant (Beran 2015). Curiously, an absence of nociceptive response can also be an indicator of neuropathic pain, such as hypoesthesia and hypoalgesia, negative symptoms which stand for reduced sensation to nonpainful and painful stimuli, respectively (Arning and Baron 2009).

A great portion of research has focused on the underlying mechanisms of neuropathic pain symptoms at the level of the periphery and the spinal cord (Basbaum et al. 2009). For instance, a decrease in the activation threshold and an increase in the responsiveness of peripheral nociceptors due to inflammation or tissue damage is known as peripheral sensitization (Spiegel et al. 2017). On the other hand, when maladaptive changes result in the increased responsiveness of neurons found in the dorsal horn of the spinal cord, then it is

referred to as central sensitization (Woolf 1983). This can result from a variety of pathophysiological alterations such as disrupted inhibitory control in the dorsal horn (Coull et al. 2003), modifications in the descending modulatory pathway coming from the brain (Lin et al. 1996), or changes in plasticity of neuronal populations in the dorsal horn (Sandkühler 2007).

Nevertheless, since pain generation is not only a sensory mechanism, but also involves complex emotional and cognitive components (Apkarian et al. 2004a), recent studies have shifted their focus on studying the structural and functional alterations of higher brain centers in neuropathic pain.

Structural changes

DaSilva et al. (2008) used magnetic resonance imaging on patients with trigeminal neuropathy to study gray matter thickness in regions associated with sensory (e.g. sensory and motor cortices) and emotional (e.g. ACC, dorsolateral PFC, Insula) pain processing, and found cortical thickening and thinning in sensorimotor regions, and mainly thinning in emotional regions, which was correlated with allodynic activation. This is in line with previous data suggesting that neuropathic pain patients display grey matter atrophy and white matter connectivity reorganization in the insula, PFC and nucleus accumbens, regions known to be involved in emotional, autonomic, and pain perception (Geha et al. 2008). Grey matter atrophy is also observed in the right thalamus of chronic back pain patients, implicating the thalamocortical processes in the pathophysiology of neuropathic pain (Apkarian et al. 2004b). Similarly, it has been reported that patients suffering from diabetic peripheral neuropathy display gray matter volume reductions in the primary somatosensory cortex, supramarginal gyrus, and cingulate cortex (Selvarajah et al. 2014), which is in line with previous findings concerning diabetic patients (Musen et al. 2006; McCrimmon et al. 2012). Jutzeler et al. (2016) also reported a variety of structural changes in the brains of neuropathic patients with traumatic spinal cord injury including grey matter increase in the right motor cortex, and decreased in the right primary somatosensory cortex and thalamus. Magnetic resonance imaging results also showed that patients with the same neuropathic condition displayed a grey matter volume decrease in the left dorsolateral PFC and in bilateral anterior insulae and subgenual ACC, all associated with pain modulation and corresponding affective and cognitive processes (Yoon et al. 2013). In addition, Yoon et al. (2013) showed that these patients exhibit white matter changes in the corticospinal and the thalamocortical tract, as suggested by an altered mean diffusivity, a feature of diffusion tensor imaging that can be

used to examine differences of brain structural integrity (Clark et al. 2011). These results suggest that neuropathic pain is associated with a disrupted pain modulation at both the level of signal transmission and integration.

Functional changes

At the level of the whole brain, it has been demonstrated that neuropathic pain patients display an altered resting state brain activity and functional connectivity during spontaneous pain (Cauda et al. 2009). Among the most prominent markers of neuropathic pain is the thalamic hypoactivity/hypometabolism contralateral to the pain (Di Piero et al. 1991; Garcia-Larrea and Peyron 2013). However, there is also a hyperexcitability of wide dynamic range and nociceptor-specific neurons in the ventral posterior thalamus of rats after spinal nerve ligation (Patel et al. 2016), illustrating the complex functional fingerprint of neuropathic pain within one structure. Ikeda et al. (2007) demonstrated an increase in synaptic plasticity and neuronal excitability in the central amygdala in rats with neuropathic pain. This might be related to an increase in the vesicular glutamate transporter vGlut2, which has been reported in the amygdala, as well as the thalamus and periaqueductal grey in mice which underwent spared nerve injury (Wang et al. 2015). Additionally, vGlut2 heterozygotes show reduced neuropathic pain, suggesting that this transporter is an important player in the development and maintenance of neuropathic pain (Moechars et al. 2006). The same brain regions display an increased activation of microglia in rodents after chronic constriction injury, which might play a role in the affective components of pain and the descending pain modulation (Ni et al. 2016; Taylor et al. 2017). Microglia activation has also been reported in the hippocampus, which might account for pain-induced memory deficits by affecting neuronal processes such as long term potentiation (Liu et al. 2017), and neurogenesis (Tyrtyshnaia et al. 2017). Next, Hubbard et al. (2015) used functional magnetic resonance imaging to show that neuropathic pain induces widespread and functionally diverse changes within the somatosensory and cingulate cortices of rats. Furthermore, the ACC, a brain region considered to play an important role in pain perception and modulation, shows increased activity during neuropathic pain (Hsieh et al. 1995; Vogt 2005; Zhuo 2008). Shen et al. (2015) demonstrated that this increased activity might be in part due to the neuropathy-induced up-regulation of voltage-gated calcium channels, thus inhibiting them has an effect in alleviating allodynia. Similarly, rats administered with the chemotherapy agent paclitaxel show an activation of astrocytes and a differential upregulation of glutamate and sodium receptor subunits in the ACC, pointing to

another possible mechanism contributing to ACC hyperactivity in neuropathic pain (Masocha 2015, 2016).

Together, these results show that a damage to the somatosensory system results in a disrupted signal transmission within the nervous system and can lead to a wide range of molecular and cellular changes in both the brain and spinal cord, which in turn might result in various debilitating health states (Colloca et al. 2017). ***Finally, it is known that chronic pain clusters with other somatic symptoms such as fatigue, sleep disruption and mood disturbances, hence the present work will more closely examine the comorbid relationship of neuropathic pain and mood disorders, specifically anxiety and MDD***.

Chronic pain and mood disorder comorbidity

An important aspect responsible for a diminished health-related life quality, as well as an increased financial burden and treatment failure is the existence of comorbid conditions (Kirsh 2010; Attal et al. 2011; Dominick et al. 2012). One of the prevalent conditions co-occurring with chronic pain is depression (Means-Christensen et al. 2008; Woo 2010; Yalcin et al. 2014). According to the fifth edition of the DSM and the latest version of the ICD, MDD is characterized by a combination of emotional, somatic and functional impairments which adversely affect the patients' social, occupational and educational aspects of life (APA, 2013; WHO 2018). Its prevalence, recurrence and detrimental effects are some of the features that make depression one of the leading causes of disability worldwide (WHO, 2008) and secure its place among the top contributors to the global disease burden (Whiteford et al. 2013). This coexistence of chronic pain and depression is frequently present in clinical settings. It has been estimated that around 50-60% of patients diagnosed with depression report chronic pain (von Knorring et al. 1983; Lee et al. 2009; Agüera-Ortiz et al. 2011), while patients treated for chronic pain show a prevalence between 18% and 85% for major depression (Bair et al. 2003; Williams et al. 2003). The intricate relationship between these two debilitating conditions is also mirrored in the fact that chronic pain is a strong risk factor for the onset of depression (Hilderink et al. 2012), and vice versa (Gureje et al. 2001; Caroll et al. 2004).

Alongside depression, anxiety is among the most frequent mental disorders observed in the general medical practice (Ormel et al. 1994; Leon et al. 1995; Olfson et al. 2000; Ansseau et al. 2004). It is characterized by excessive worry, including a wide spectrum of behavioral and emotional characteristics with varying degrees of severity (APA, 2013; WHO 2018). These can range from uncertainty, to anticipation of future threat, to constant fearfulness and panic attacks (Stein and Sareen 2015). As with acute pain, the evolutionary benefit of anxiety in life- and well-being-threatening situations is lost once it becomes

chronic. Clinical and pre-clinical data suggest that, regardless of depression, pathological anxiety, persisting beyond developmental advantages such as danger awareness and motivation, leads to detrimental physical, social and cognitive consequences (de Beurs et al. 1999; Slattery et al. 2012).

The comorbid interaction of chronic pain, depression and anxiety has a substantial effect on the course and severity of each individual disorder, which results in more pronounced disability, increased treatment difficulty and delays in remission (Frank et al. 1991; Means-Christensen et al. 2008; Hooten 2016). Patients suffering from post-traumatic chronic pain have been shown to experience high levels of pain intensity, anxiety and depression (Stålnacke 2011). Moreover, several studies have identified depression as one of the strongest predictors of chronic low back pain, and found that this interaction strengthens with the increase in pain intensity and depression severity (Reid et al. 2003; Meyer et al. 2007). Similarly, anxiety has also been strongly associated with chronic pain, with prevalence rates as much as double of what is observed in the general population (McWilliams et al. 2003). Highly elevated anxiety levels have been reported in children with abdominal pain (Di Lorenzo et al. 2001), noncardiac chest pain (Lipsitz et al. 2005) and fibromyalgia (Kashikar-Zuck et al. 2008).

Consequently, growing evidence suggests that chronic pain and mood disorders are among the leading factors responsible for an increase in suicidality (Racine et al. 2018). With suicide accounting for 1.4% of all deaths in 2015, the World Health Organization ranked it as the 17th leading cause of mortality worldwide, and the second leading cause of death in those aged 15-29 (WHO 2017). Moreover, chronic pain patients are estimated to be at least twice as likely as healthy subjects to engage in suicidal behaviors (i.e. thoughts, plans and attempts) or to commit suicide (Fishbain 1999; Tang and Crane 2006). Additionally, depression accounted for around 30% of mortality due to unnatural causes at the beginning of the current century, placing it among the leading global causes of suicide (Bartolote et al. 2003; Bartolote et al. 2004). Hence, given that chronic pain is highly comorbid with depression (Fishbain et al. 1997; Arnow et al. 2006) and depression is closely linked to suicide (Nock et al. 2008; Kessler et al. 1999; WHO 2014; Moller, 2003), it is evident that studying the comorbid relationship of chronic pain and depression is necessary to decrease the detrimental living conditions and fatality of patients worldwide.

Interestingly, large-scale epidemiological studies focusing on co-existing pathological conditions provide evidence about the etiological relationship of chronic pain and depression. A study which assessed more than 40,000 individuals demonstrated that chronic widespread

pain showed comorbidity with other conditions such as chronic fatigue and depressive symptoms (Kato et al. 2006). Similarly, a large twin study involving almost 4,000 twins, focusing on comorbid existence of several chronic conditions including, among others, chronic back pain, chronic headache, panic attacks and major depression, found that the association between these debilitating states was exceeding expected occurrence predicted by chance alone (Schur et al. 2007). These studies suggest that comorbid states, including that of chronic pain and depression, may have common etiology and even share common genetic risk factors.

Therefore, the existence of comorbid conditions such as chronic pain, anxiety and depression requires more attention as a whole in both pre-clinical and clinical settings. Tackling them individually leads to multiple diagnoses, which are frequently established and treated separately, by different practitioners, in a given patient. This can result in frequent testing that adversely affects anxiety and depression, and also brings about multiple treatments which may interact and produce undesirable effects (Caughey et al. 2010). Thus, it is important to address comorbidities as separate, unique pathologies, where the interaction of different conditions produces an outcome which is not simply the sum of pathophysiological changes induced by the individual disorders, but rather a unique state characterized by distinguishable behavioral, cellular and molecular characteristics.

An invaluable advantage in studying the pain-depression dyad is the existence of animal models which mimic the features of both conditions, thus providing a venue for in-depth physiological and molecular analyses (Liu and Chen 2014; Li 2015). Currently, there are models which successfully achieve construct and face validity by imitating human pain and psychiatric conditions due to various circumstances (e.g., nerve injury, inflammation, cancer pain, chronic environmental stress, social stress etc.) and predictive validity by responding to analgesics and antidepressants in behavioral paradigms (e.g., hot/cold plate test, von Frey test, forced swimming test [FST], tail suspension test [TST], learned helplessness etc.) (Burma et al. 2017; Belzung and Lemoine 2011; Krishnan and Nestler 2011; Xu et al. 2012). Moreover, these models are also useful in deciphering the affective and cognitive impairments associated with pain and depression (Liu and Chen 2014). However, using animal models to study predominantly human pathologies is not without challenges, particularly because a large portion of the pain and mood disorder experience is subjective and relies on personal description (Mogil 2009; Le Bars et al. 2001). ***The following section will introduce in more depth preclinical rodent models used to study specifically the comorbidity of neuropathic pain and anxiodepressive-like behaviors.***

The molecular neurobiology of chronic pain-induced depression (Review)

The following section is a recently published review concerning past research focusing to molecular alterations associated with the co-presence of chronic pain and mood disorders. It is a collection of pre-clinical and clinical findings, obtained during the past several decades, pertaining to the comorbid relationship of chronic pain, predominantly neuropathic, and anxiety and depression. It summarizes data from animal and human studies, including specific genetic, epigenetic, neurotransmitter, neuroendocrine, neuroinflammatory, and other molecular and cellular changes associated with this comorbidity.

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REVIEW

The molecular neurobiology of chronic pain–induced depression

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Abstract

The increasing number of individuals with comorbidities poses an urgent need to improve the management of patients with multiple co-existing diseases. Among these comorbidities, chronic pain and mood disorders, two long-lasting disabling conditions that significantly reduce the quality of life, could be cited first. The recent development of animal models accelerated the studies focusing on the underlying mechanisms of the chronic pain and depression/anxiety comorbidity. This review provides an overview of clinical and pre-clinical studies performed over the past two decades addressing the molecular aspects of the comorbid relationship of chronic pain and depression. We thus focused on the studies that investigated the molecular characteristics of the comorbid relationship between chronic pain and mood disorders, especially major depressive disorders, from the genetic and epigenetic point of view to key neuromodulators which have been shown to play an important role in this comorbidity.

Keywords Chronic pain · Depression · Molecular characteristics · Behavior · Neuropathic pain

Introduction

Depressive disorders affect around 16% of the population at some point over their lifespan (Bromet et al. 2011) and result in personal suffering and notable economic burden (Simon 2003). They comprise disabling and long-lasting conditions, which are estimated to become foremost contributors to the worldwide burden of disease by 2030 (WHO 2008). While chronic stress is a relevant etiology (Pittenger and Duman 2008), chronic pain is also among the first determinants of mood disorders (Attal et al. 2011). Indeed, a mean prevalence rate around 50% for major depressive disorder has been reported in patients with chronic pain (Bair et al. 2003). Therefore, it is imperative to closely focus on understanding the causes and effects of the relationship of chronic pain and mood disorder in order to come up with more effective treatment strategies.

A strong asset of pre-clinical studies is the use of animal models for mimicking various aspects of chronic pain and mood disorder characteristics. The most utilized animal models of chronic pain, which will be mentioned throughout this review, are those imitating common chronic pain pathologies such as neuropathic pain, which results from lesions or disease affecting the somatosensory system, or chronic joint inflammation observed in arthritic pain, but also dysfunctional pain syndromes such as fibromyalgia and irritable bowel syndrome (IBS) (Leite-Almeida et al. 2015). Models of neuropathic pain in rodents can be based on peripheral nerve injuries, central injuries, trigeminal neuralgia, diabetic neuropathies, chemo-induced neuropathies, postherpetic neuralgia, and so forth (see reviews of Barrot 2012; Sorkin and Yaksh 2009). Almost all of the pre-clinical studies on the anxiodepressive consequences of neuropathic pain were performed on models related to sciatic nerve manipulation, using either nerve compression or section (see Table 1). Inflammatory models are based on injection of inflammatory agents such as formalin, capsaicin, carrageenan, and complete Freund's adjuvant (CFA) (Duric and McCarson 2006, 2007; Zhao et al. 2007; Li et al. 2009b; Kim et al. 2012). The most frequently used tests for assessing depression-related behaviors in rodents involve exposure to stressful situations and the measure of time spent in active versus passive stress

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Table 1 Traumatic neuropathy models used to induce chronic neuropathic pain and concomitant anxiodepressive-like behaviors

Phenotype onset (post-op)				
Model	Behavioral test	Anxiety-like	Depressive-like	References
CCI	EPM, OF, FST, SPT, TST	2–4 weeks	2–6 weeks	Caspani et al. 2014; Roeska et al. 2008; Li et al. 2014; Urban et al. 2011; Gregoire et al. 2012; Hu et al. 2009; Fukuhara et al. 2012; Alba-Delgado et al. 2013; Zhao et al. 2014a, b; Zeng et al. 2008; Dellarole et al. 2014
PSNL	EPM, OF, DLB, FST, SPT, TST	2–4 weeks	4 weeks	Narita et al. 2006a, b; Matsuzawa-Yanagida et al. 2007; Roeska et al. 2008; Sawada et al. 2014; Wallace et al. 2007; Bura et al. 2013; Gai et al. 2014
SNC	EPM, OF, DLB, FST, SPT, NSF	4–6 weeks	6–9 weeks	Benbouzid et al. 2008; Yalcin et al. 2011; Dimitrov et al. 2014
SCI	OF, SPT, TST	No phenotype at 4–7 weeks	8 weeks	Galan-Arriero et al. 2014; Wu et al. 2014
SNI	EPM, OF, DLB, FST, SPT	3–4 weeks	1–2 weeks	Leite-Almeida et al. 2009, 2012; Urban et al. 2011; Mutso et al. 2012; Avila-Martin et al. 2015; Gonçalves et al. 2008; Norman et al. 2010b; Goffer et al. 2013; Stratinaki et al. 2013
SNL	EPM, OF, DLB, FST	2–4 weeks	4–8 weeks	Suzuki et al. 2007
SNT	OF, FST	4 weeks	4 weeks	Hu et al. 2010; Hasnie et al. 2007

CCI, chronic constriction injury; *PSNL*, partial sciatic nerve ligation; SNC, sciatic nerve cuffing; *SCI*, spinal cord injury; *SNI*, spared nerve injury; *SNL*, spinal nerve ligation; *SNT*, spinal nerve transection; *DLB*, dark-light box; *EPM*, elevated plus-maze; *FST*, forced swimming test; *NSF*, novelty suppressed feeding; *OF*, open field; *SPT*, sucrose preference test; *TST*, tail suspension test

coping, such as the forced swimming test or the tail suspension test. Another symptom, frequently examined, is the animal's interest in pleasurable activities such as the preference for sucrose solution or engaging in social interactions (see reviews of Yalcin et al. 2014; Leite-Almeida et al. 2015). Since the late 1990s, several research groups worked on modeling the anxiodepressive consequences of chronic pain in animals. The first studies (Kontinen et al. 1999) and some recent ones (Kodama et al. 2011; Urban et al. 2011; Pitzer et al. 2019) failed to show the comorbidity of chronic pain and depression, while other studies reported anxiety and/or depressive-like consequences in rodent models (see review of Yalcin et al. 2014). Temporal parameter (time after the surgery), species, strains of animals, chronic pain models, and the time of the day-night cycle when the animals are tested may all influence the results.

Hence, this review summarizes a portion of clinical and pre-clinical research performed over the past two decades pertaining to molecular aspects of the comorbid relationship of chronic pain and depression. The main hypothesis is that the close reciprocal relationship of these two pathologies can be due to common underlying biological mechanisms linking these pathologies (Liu and Chen 2014). We thus focused on the studies that investigated the molecular characteristics of the comorbid relationship between chronic pain and mood disorders, especially major depressive disorders, from the genetic and epigenetic point of view to key neuromodulators which have been shown to play an important role in this comorbidity.

The contribution of genetic and epigenetic modifications

Genetic modifications

In the past decades, several studies highlighted the individual differences in response to chronic pain and susceptibility to mood disorders which suggest that genetic factors play a significant role in the comorbidity of pain and depression (Magni et al. 1987; Reichborn-Kjennerud et al. 2002; Lembo et al. 2007; McIntosh et al. 2016). A common approach to study the effect of genetics on physical and mental conditions is by looking at monozygotic and dizygotic twins, who share 100% and 50% of their genes, respectively. A study by Lembo et al. (2007) looked at a total of 986 twin pairs and found that there is a genetic contribution to chronic pain associated with IBS, which may be mediated by the hereditability of anxiety and depression. Indeed, they estimated 22% of genetic variance for IBS which became non-significant when adjusted for anxiety and depression, suggesting that mood disorders such as depression and chronic pain disorders possibly follow a similar causal genetic pathway. Similarly, Pinheiro et al. (2018) found that 64% of genetic factors covariate between chronic low back pain and symptoms of depression and anxiety, supporting a potential role of common biological pathways.

Besides studies suggesting that common genetic factors might mediate the heritability of chronic pain and depression (Reyes-Gibby et al. 2013; Burri et al. 2015; Pinheiro et al. 2015; Gasperi et al. 2017), there is also evidence that

environmental factors such as early shared environment or sleep quality might also play a substantial role in the vulnerability to chronic pain and mood disorders (Pinheiro et al. 2015; Gasperi et al. 2017). Interestingly, McIntosh et al. (2016) showed that presence of chronic pain and genetic predisposition in a spouse has a significant contribution to their partner's risk of developing a comorbidity of chronic pain and mood disorders. It is also intriguing that patients who suffer from chronic pain generally have more first-degree relatives who suffer from depression compared to general population, even if they personally do not have a history of depression (Lepine and Briley 2004; Magni et al. 1987).

However, besides heritability studies, which use statistics to estimates the degree of variation in a phenotypic trait due to genetics, there are many other which took a more in-depth approach and looked at single-nucleotide polymorphisms (SNPs) of specific genes in order to further delineate the comorbidity of chronic pain and mood disorders (Lee et al. 2012). Some of the extensively studied polymorphisms are those of the brain-derived neurotrophic factor (BDNF), which is the most widespread neurotrophin in the central nervous system (CNS) (Hofer et al. 1990) and also highly abundant in non-neuronal cells and tissues such as platelets and blood vessels (Donovan et al. 2000). In particular, the BDNF Val66Met SNP seems to be closely related to the pain-depression comorbidity since it has been shown to moderate the relationship between life stress and depression (Hosang et al. 2014), life stress and chronic multi-site musculoskeletal pain (Generaal et al. 2016), and coronary artery disease and depression (Bozzini et al. 2009). Other frequent targets of SNP studies are receptors of various ligands which play a role in the relationship of pain and depression. For instance, although inconclusive, Max et al. (2006) showed that the galanin-2 receptors are potential candidates to mediate the affective consequences of pain, since two of its allele copies show a pain-gene interaction and are associated with post-surgical mood disorder protection. Moreover, Lebe et al. (2013) found that women, but not men, harboring the promoter polymorphisms of serotonin receptor-1A (5HTR1A) and serotonin receptor-2A (5HTR2A) showed significantly higher depression scores due to chronic pain. Taken together, these studies highlight the pivotal role of genetic variations (see Table 2) in the development of chronic pain and mood disorders and point to the need of further understanding how individual genetic characteristics shape the comorbid relationship of pain and depression.

Epigenetic modifications

Epigenetic mechanisms characterized by structural chromatin modifications that allow or prevent transcription factors to access promoter regions on the DNA through several processes such as DNA and histone methylation, histone acetylation, phosphorylation, and ubiquitination (Berger 2007) play a crucial role in this comorbidity. Tran et al. (2013) showed that rats experiencing stress-induced visceral hypersensitivity had an increase in DNA methylation at the glucocorticoid receptor (GR) promoter and a decrease at the corticotropin-releasing factor (CRF) genes in the amygdala, which resulted in a decrease of the GR and an increase in CRF, respectively. This suggests that methylation related to the HPA-axis might play an important role in the pain-depression comorbidity. In fact, based on a recent study, a potential candidate which might modulate this pattern of methylation might be DNA methyltransferase 3a (Dnmt3a). Wang et al. (2017) suggested that mice vulnerable to mood disorder development after peripheral nerve ligation (PNL) had a reduced protein level of Dnmt3a in the central nucleus of the amygdala. Besides methylation, histone acetylation also seems to be involved in orchestrating the interaction and development of chronic pain and depression. Among the most obvious evidence for this is the notion that the use of histone deacetylase (HDAC) inhibitors has already been considered as a therapeutic strategy for treating both chronic pain and depression (Descalzi et al. 2015; Schroeder et al. 2010). Indeed, Descalzi et al. (2017) showed that HDAC5 was elevated in the nucleus accumbens (NAc) and the periaqueductal gray (PAG) of mice experiencing anxiodepressive-like behaviors due to a spared nerve injury (SNI). In addition, they suggest that knocking out HDAC5 was sufficient to significantly reduce SNI-induced depressive-like behaviors. In accordance with this, Tran et al. (2014) demonstrated that pharmacological deacetylation of H3K9 in the central amygdala of rats after elevated corticosteroid exposure was sufficient to attenuate the anxiety-like behavior, as well as the somatic and visceral hypersensitivity. Indeed, the infusions of histone deacetylase inhibitors into the central amygdala caused a decrease in GR expression and led to a disinhibition of CRF. However, increased deacetylation might be region-specific, since it was found that histone acetylation is increased in the anterior cingulate cortex (ACC), a brain structure known to process both pain- and depression-related stimuli, in mice which express neuropathy-induced depressive-like behaviors (Barthas et al. 2017). Specifically, there was an increased histone H3 lysine 9/lysine 14 (H3K9/K14) acetylation at the promoter regions of *c-Fos* and *Mkp-1*, a gene coding for a critical phosphatase in the mitogen-activated protein kinase pathway. These results (see Table 2) point out the need to further investigate histone modifications in the comorbid relationship of pain and depression since they could constitute a promising target for future treatment strategies.

Although it is still debatable whether microRNAs (miRNAs) should be considered as members of the epigenetic machinery, it is undeniable that they play a significant role in regulating gene expression. They serve as negative posttranscriptional regulators of their target genes and have also been

Table 2 Single-nucleotide polymorphisms and epigenetic factors involved in neuropathic pain and concomitant anxiodepressive-like behaviors

	Expression	Species	Sample	Models	Pain type	Mood disorder	Reference
Single-nucleotide polymorphisms							
[3H] Imipramine sites	Decreased	Human	Platelets	Mianserin treatment	Various	MDD	Magni et al. (1987)
GALR2 SNP	Modulatory	Human	Lymphoblastoid cells	Discectomy	Neuropathic	MDD	Max et al. (2006)
5HTR1A/5HTR2A SNPs	Modulatory	Human	Blood	Lumbar disk surgery	Neuropathic	MDD	Lebe et al. (2013)
BDNF Val66Met SNP	Modulatory	Human	Blood	Genotyping, interview	Various	MDD	Generaal et al. (2016)
Glra3, Gpr88	Increased	Mouse	IC	Endometrios	Visceral	D-L	Li et al. (2018)
Serpina3n	Decreased	Mouse	IC	Endometrios	Visceral	D-L	Li et al. (2018)
Chrnb4, Npas4	Increased	Mouse	HPC	Endometrios	Visceral	D-L	Li et al. (2018)
Lcn2	Increased	Mouse	AMY	Endometrios	Visceral	D-L	Li et al. (2018)
Nptx2	Decreased	Mouse	AMY	Endometrios	Visceral	D-L	Li et al. (2018)
Epigenetics and transcription factors							
GR methylation	Increased	Rat	AMY	Water avoidance stress	Visceral	A-L	Tran et al. (2013)
CRF methylation	Decreased	Rat	AMY	Water avoidance stress	Visceral	A-L	Tran et al. (2013)
H3K9 deacetylation	Increased	Rat	CeA	CORT infusion in CeA	Hyperalgesia	A-L	Tran et al. (2014)
Dnmt3a	Decreased	Mouse	CeA	PNL	Neuropathic	A-L	Wang et al. (2017)
HDAC5	Increased	Mouse	NAc and PAG	SNI	Neuropathic	AD-L	Descalzi et al. (2017)
Lmx1b	Decreased	Mouse	Nervous system	Formalin/carrageenan	Inflammatory	AD-L	Zhao et al. (2007)
p-CREB	Decreased	Mouse	Cortex and HPC	SNI	Neuropathic	AD-L	Li et al. (2017); Zhao et al. (2017)
c-Fos	Increased	Mouse	PFC, ACC, IC, AMY	Zymosan-induced IBS	Spontaneous	A-L	Zhang et al. (2014)
	Increased	Rat	LHb	CMS, formalin injections	Inflammatory	AD-L	Li et al. (2016)
	Increased	Mouse	ACC	SNC	Neuropathic	AD-L	Barthas et al. (2017)

5HTR1A, serotonin 1A receptor; *5HTR2A*, serotonin 2A receptor; *ACC*, anterior cingulate cortex; *AD-L*, anxiodepressive-like; *A-L*, anxiety like; *AMY*, amygdala; *BDNF*, brain-derived neurotrophic factor; *CeA*, central amygdala; *Chrnb4*, cholinergic receptor nicotinic beta 4 subunit; *CMS*, chronic mild stress; *CORT*, corticosteroids; *CRF*, corticotropin-releasing factor; *D-L*, depressive-like; *Dnmt3a*, DNA methyltransferase; *GALR2*, galanin-2 receptor; *Glra3*, glycine receptor alpha 3; *Gpr88*, G protein–coupled receptor 88; *GR*, glucocorticoid receptor; *HDAC5*, histone deacetylase 5; *HPC*, hippocampus; *IBS*, irritable bowel syndrome; *IC*, insular cortex; *IL-6*, interleukin 6; *Lcn2*, lipocalin-2; *LHb*, lateral habenula; *Lmx1b*, LIM homeobox transcription factor 1 beta; *MD*, maternal deprivation; *MDD*, major depressive disorder; *NAc*, nucleus accumbens; *Npas4*, neuronal PAS domain protein 4; *Nptx2*, neuronal pentraxin-2; *PAG*, periaqueductal gray; *p-CREB*, phospho-c-AMP-response element binding; *PFC*, prefrontal cortex; *PNL*, partial sciatic nerve ligation; *Serpina3n*, serpin peptidase inhibitor; *SNC*, sciatic nerve cuffing; *SNI*, spared nerve injury; *SNP*, single-nucleotide polymorphism

shown to target members of the HDAC family in both neuronal and non-neuronal cells (Liu and Liu 2016; Li et al. 2009a). It is thus valuable to consider the role of miRNAs in the comorbidity of chronic pain and depression, alongside the classical epigenetic factors (Descalzi et al. 2015). Masotti et al. (2017) observed a significant negative correlation of circulating serum microRNA miR-320b and depressive symptoms in fibromyalgia patients, which might point to a functional involvement of this miRNA in pain-depression comorbidity. This specific miRNA has been previously associated with neural development, regeneration, and neurite outgrowth (White and Giffard 2012), and it was shown that its expression is disrupted in the blood of patients suffering from complex regional pain syndrome, a condition characterized by neuropathic pain (Orlova et al. 2011).

Transcription factors

However, once the histones unwrap, DNA becomes accessible to transcription factors which regulate transcription and, furthermore, gene expression. Therefore, an increasing number of studies focus on transcription factors involved in the comorbid relationship of chronic pain and mood disorders. A commonly studied transcription factor, the cyclic AMP

response element binding (CREB) protein (Hoeffler and Habener 1990), has, among others, a modulating role in the comorbidity of pain and depression. Neuropathic pain–induced depressive-like behaviors in mice caused by SNI are associated with a diminished phospo-CREB expression in both the cortex and hippocampus (Li et al. 2017; Zhao et al. 2017). Furthermore, antidepressants reverse the disrupted phospo-CREB expression associated with pain-induced depressive behaviors (Yasuda et al. 2014), and interestingly, even forced exercise in female mice increases CREB gene expression in pups, which in turn acts anxiolytic and antidepressant and increases the tolerability to pain (Motaghinejad et al. 2017). Consequently, activated CREB can further induce the expression of c-Fos, a marker of neuronal activation (Dragunow and Faull 1989; Hoffman et al. 1993), which is also upregulated by comorbid pain and depression in brain regions associated with modulating pain and emotion such as the prefrontal cortex (PFC), ACC, insular cortex, and amygdala (Zhang et al. 2014). For instance, chronic mild stress paired with formalin-induced pain resulted in greater expression of c-Fos-positive cells in the lateral habenula of rats compared to stress and pain alone (Li et al. 2016). In addition, this upregulation was completely reversed by the antidepressant clomipramine. Similarly, c-Fos was upregulated in the ACC of mice displaying neuropathic pain–induced depressive-like behaviors (Barthas et al. 2017) (see Table 2).

Since serotonin is among the most studied targets in the research of both pain and mood disorders, the transcription factors controlling its expression are also of interest for studying the chronic pain–depression comorbidity. One such candidate is the transcription factor Lmx1b, which is crucial in the differentiation of serotonergic neurons in the nervous system. Zhao et al. (2007) showed that Lmx1b knock-out mice display lowered sensitivity to mechanical stimuli and an enhanced inflammatory pain response. In addition, the effect of antidepressant drugs inhibiting serotonin reuptake was abolished in these mice. These results indicate that the transcription factor Lmx1b has a role in modulating both nociception and depressive-like behaviors (see Table 2).

Another group of transcriptional regulators which were recently demonstrated to play an important role in the comorbidity of pain and depression is the nuclear factor-kappaB (NF-kB) family of transcription factors. They regulate the expression of the metabotropic glutamate receptor 2/3 (mGlu2) in the dorsal horn (DH) and dorsal root ganglia (DRG) (Chiechio et al. 2006). It was shown that treatment with L-acetylcarnitine, a drug commonly prescribed for neuropathies associated with diabetes and HIV, activates NF-kB, which in turn upregulates mGlu2 in the DH and DRG of neuropathic rats and successfully alleviates both their nociceptive- and depressive-like symptoms after only 3 days of treatment (Chiechio et al. 2006; Nasca et al. 2013).

The role of neurotransmitters

Monoamines

Monoamine neurotransmitters including serotonin (5-HT), dopamine (DA), and norepinephrine (NE) are among the most studied candidates in both the field of chronic pain and depression (Haase and Brown 2015). Hence, it is imperative to investigate their role in the molecular mechanisms involved in the comorbidity of these two conditions. The following section will examine some of the present literature concerning the role of these monoamines in the comorbidity of pain and mood disorders (see Table 3).

Serotonin

The serotonergic system has been the focus of a substantial number of pre-clinical and clinical studies on the relation between pain and depression. One widely used method to rapidly induce this comorbidity in rodents relies on the intraplantar administration of complete Freund's adjuvant (CFA) which results in sustained inflammatory pain and leads to depression-like behaviors. This procedure has been shown to deplete serotonin levels, as well as those of its precursors involved in its metabolism in the hippocampus of rats (Zhang et al. 2016a). Similarly, infusing a cocktail of inflammatory agents into the dura mater of rats, inducing meningeal nociception (resembling chronic migraine in humans) and anxiodepressive-like behaviors, resulted in decreased levels of 5-HT in the prefrontal cortex in rats (Zhang et al. 2017). Treatment with the tricyclic antidepressant amitriptyline, commonly used to treat mood disorders, neuropathic pain, and migraine in humans, was able to increase the 5-HT levels and restore the pain- and depression-related behavioral responses (Zhang et al. 2017). However, chronic treatment with the selective-serotonin reuptake inhibitor fluoxetine did not affect the mechanical allodynia, but did block the anxiodepressive-like consequences induced by sciatic nerve injury (Barthas et al. 2017). A single dose of ketamine which is sufficient to alleviate both the mechanical allodynia and the associated depression-like behaviors was also shown to increase the serotonin level in the hippocampus of CFA-administered rats (Zhang et al. 2016a).

As rats primarily rely on their sense of smell to interpret the stimuli in their surroundings, the olfactory bulbectomy (OB) is another model that has been commonly used to induce depressive-like behaviors (van Riezen and Leonard 1990). Interestingly, it also causes an increased nociceptive sensitivity displayed by increased response rates in the hot plate (thermal hyperalgesia) and tail-flick tests (Rodríguez-Gaztelumendi et al. 2014), which makes it suitable for studying the relationship between pain and depression. Moreover, this model was shown to exhibit a downregulation of the

Table 3 Monoamines involved in neuropathic pain and concomitant anxiodepressive-like behaviors

	Expression	Species	Region	Models	Pain type	Mood disorder	Reference
Serotonin							
5-HT	Decreased	Rat	PFC	Dura mater IS infusion	Meningeal	AD-L	Zhang et al. (2017)
IDO1	Increased	Rat	HPC	CFA, chronic social stress	Inflammatory	D-L	Kim et al. (2012)
	Increased	Human	Plasma	Disk herniation, radiculitis	Lumbar/cervical	MDD	Kim et al. (2012)
	Increased	Mouse	HPC, spinal cord	CCI	Neuropathic	D-L	Jiang et al. (2018)
SERT density	Decreased	Rat	Spinal cord	OB	Hyperalgesia	AD-L	Rodríguez-Gaztelumendi et al. (2014)
5HTR1A functionality	Decreased	Rat	Spinal cord	OB	Hyperalgesia	AD-L	Rodríguez-Gaztelumendi et al. (2014)
5-HT/TRP ratio	Decreased	Rat	HPC	CFA injection	Inflammatory	AD-L	Zhang et al. (2016a)
Norepinephrine							
TH-positive cells	Increase	Rat	LC	CCI + CMS	Neuropathic	D-L	Bravo et al. (2014)
α2-Adrenoreceptor	Increase	Rat	LC	CCI	Neuropathic	AD-L	Alba-Delgado et al. (2013)
NAT	Increase	Rat	LC	CCI	Neuropathic	AD-L	Alba-Delgado et al. (2013)
NAT and TH+ cells	Decreased	Rat	LC	STZ-induced diabetes	Hyperalgesia	A-L	Alba-Delgado et al. (2016)
Dopamine							
Extracellular DA	Increase	Rat	NAc	SNI	Neuropathic	D-L	Sagheddu et al. (2015)
D2 receptor	Decrease	Rat	NAc	SNI	Neuropathic	D-L	Sagheddu et al. (2015)
DA level	Decrease	Rat	PFC	Dura mater IS infusion	Meningeal	AD-L	Zhang et al. (2017)
DA release	Increase	Rat	NAc	SNL	Neuropathic	AD-L	Kato et al. (2016)

5-HT, serotonin; *5HTR1A*, serotonin 1A receptor; *A-L*, anxiety-like; *AD-L*, anxiodepressive-like; *CCI*, chronic constriction injury; *CFA*, complete Freund's adjuvant; *CMS*, chronic mild stress; *DA*, dopamine; *D-L*, depressive-like; *HPC*, hippocampus; *IDO1*, indoleamine 2,3-dioxygenase 1; *IS*, inflammatory soup; *LC*, locus coeruleus; *MDD*, major depressive disorder; *NAc*, nucleus accumbens; *NAT*, noradrenaline transporter; *OB*, olfactory bulbectomy; *PFC*, prefrontal cortex; *SERT*, serotonin transporter; *SNI*, spared nerve injury; *SNL*, spinal nerve ligation; *STZ*, streptozotocin; *TH*, tyrosine hydroxylase; *TRP*, tryptophan

serotonin transporter and receptor 1A (5-HT1A) functionality in DH of the lumbar spinal cord. The group showed that chronic, but not acute, treatment of OB rats with fluoxetine progressively normalized both the nociceptive responses and depressive-like behaviors, and also restored the transporter density and receptor functionality to normal levels. These findings highlight the role of serotonin signaling at the periphery in the comorbidity of pain and depression.

Besides looking strictly at the serotonin neurotransmitter and its receptor, research has recently focused on studying promising molecular candidates which are involved in its metabolism. It was found that indoleamine 2,3-dioxygenase 1 (IDO1), a rate-limiting enzyme in tryptophan metabolism, is responsible for a decrease of serotonin content in chronic pain–induced mood disorders (Kim et al. 2012). Inhibiting the elevated presence of this precursor of serotonin in the hippocampus of rats and mice attenuated their depressive-like behaviors caused by inflammatory pain and social stress (Kim et al. 2012; Zhang et al. 2016a). A similar increase in the presence of IDO1 was also found in the plasma of human patients who suffered from disk herniation or radiculitis for more than 3 months, which subsequently led to the development of depression (Kim et al. 2012). These results suggest that neurometabolic changes related to serotonin signaling can play a modulatory role in the comorbidity of chronic pain and depression.

Norepinephrine

Alongside serotonin, NE signaling is another prominent target for both studying and treating the comorbid occurrence of pain and mood disorders. Its primary source, the locus coeruleus (LC) nucleus, has been closely associated with nociception and emotion and might play a key role in this comorbidity.

By combining the models of chronic constriction injury (CCI) and chronic mild stress (CMS), it has been found that chronic pain and chronic stress mutually potentiate each other, resulting in more pronounced changes in the noradrenergic transmission compared to the effect of each condition alone (Bravo et al. 2013, 2014). For instance, comorbid neuropathic

pain and stress-induced depressive-like behaviors resulted in an increased expression of tyrosine hydroxylase, the rate-limiting enzyme of NE synthesis, in LC neurons, which also showed a decrease in spontaneous electrophysiological activity. Moreover, prolonged neuropathy induced by CCI also enhanced the expression and sensitivity of alpha 2 adrenoreceptor and increased the expression of the norepinephrine transporter (NET) in the LC of rats (Alba-Delgado et al. 2013). Interestingly, this increase in expression was time-dependent and coincided with the onset of anxiodepressive-like behaviors (28 days post-CCI). These findings point to several modifications related to noradrenergic transmission in the LC in pain and depression comorbidity.

Subsequent research showed that different types of neuropathic pain can employ opposite molecular and neuroplastic mechanisms to result in the development of comparable anxiodepressive-like behaviors. Namely, rats administered with a single systemic injection of streptozotocin (STZ) displayed aspects of type 1 diabetes and showed a gradual increase in nociceptive hypersensitivity. Curiously, although the temporal development of hypersensitivity in these animals was different than in CCI rats, the onset of anxiety-like and depressive-like behaviors was observed at the same time delay (28 days after neuropathy induction). However, in contrast to what is observed in CCI rats, STZ rats showed a decrease in tyrosine hydroxylase and NET levels in the LC, as well as in the overall LC firing activity (Alba-Delgado et al. 2016). This indicates that specific neuroplastic mechanisms take place in different models of pain-depression comorbidity and highlight the importance of studying different models of chronic pain and mood disorders, since it seems that each type induces specific molecular and cellular alterations.

The role of NE in the comorbid relationship between chronic pain and mood disorders has also been demonstrated through clinical treatment of patients with pharmacological agents targeting its synaptic presence. Alongside the commonly used duloxetine, an efficient agent in treating negative symptoms of fibromyalgia, milnacipran, a serotonin norepinephrine reuptake inhibitor (SNRI) with greater affinity for the NE reuptake site (Goldenberg et al. 2010), has been shown to alleviate discomfort, fatigue, and physical dysfunction, while continued treatment gradually improves symptoms of pain and depression. Moreover, besides their antidepressant effect, acute or chronic treatment with norepinephrine reuptake inhibitors (NRIs) such as desipramine and reboxetine has shown robust anti-allodynic and/or anti-hyperalgesic effect in rodents (Leventhal et al. 2007; Yalcin et al. 2009). Also, other compounds which have been shown to have a high selectivity for the NE transporter have provided promising results, such as the conopeptide Xen2174 which produced a long-lasting anti-allodynic response in CCI and SNL rats (Nielsen et al. 2005), or WAY-318068 which, alongside depression, showed efficacy in models of neuropathic, acute, inflammatory, visceral, diabetic, and cancer pain (Whiteside et al. 2010). Hence, although SNRIs may produce more pronounced effects on the pain-depression comorbidity compared to NRIs or SSRIs alone, it is still important to fully elucidate the individual role of each monoamine, in order to better optimize current and new treatment strategies.

Dopamine

Alongside 5-HT and NE, there is compelling evidence that DA is also implicated in the comorbid relationship of pain and depression through its spinal and supraspinal activity involving several brain regions such as the periaqueductal gray (PAG), the thalamus, the basal ganglia, and the limbic system (Hache et al. 2011).

As seen with 5-HT, an infusion of an inflammatory soup in the dura mater of rats, causing a migraine-type of pain-related anxiodepressive-like behaviors, also results in a decrease of DA levels in the PFC. Again, these molecular and phenotypic effects were improved by treatment with the tricyclic antidepressant amitriptyline (Häuser et al. 2008).

Several studies have shown that the neuropathy-induced anxiety and depressive-like behaviors are closely linked to the DA receptor expression, as well as DA release in the rat NAc (Sagheddu et al. 2015; Kato et al. 2016). Specifically, during the early phase of neuropathic pain (2 weeks post-SNI), there is a decrease in DA-2 receptor expression in the NAc of rats (Sagheddu et al. 2015), which is also seen after chronic mild stress (Papp et al. 1994). Accordingly, it has been demonstrated that treatment with commonly used post-surgical pain relief medications such as clonidine or gabapentin during 14 days after SNL increased intra-NAc DA release in rats with neuropathic pain (Xie et al. 2014). Curiously, the same pattern of early phase DA increase in the NAc was also observed after pain relief due to pregabaline treatment or 30% sucrose intake after SNL, suggesting that pain relief might be triggering a reward-like mechanism through DA signaling, which has an effect on the overall mood-related state of the animal (Kato et al. 2016). However, this pattern of DA release after pain relief or sucrose intake was not persistent, and it faded in a later phase of neuropathy (4 weeks after SNL) (Stoy et al. 2012). Interestingly, a hypodopaminergic state is also commonly reported in patients suffering from Parkinson's disease, which is associated with a high incidence of both depression and chronic pain (Conte et al. 2013). These results accentuate the role of altered dopaminergic function in the comorbid development of mood disorders such as depression, and chronic pain, and emphasize the need for further studies regarding the role of DA in these conditions.

Instead of focusing on the individual role of 5-HT, NE, and DA in mediating pain and depression, some studies have looked at the combined action of all three of them in modulating this comorbid relationship. Direct evidence for this comes from a new class of antidepressants, known as the triple reuptake inhibitors. As their name suggest, these compounds simultaneously inhibit the reuptake of 5-HT, NE, and DA, thereby prolonging their presence and activity at postsynaptic levels (Guiard et al. 2009). It was recently shown that commercially available triple reuptake inhibitor drugs such as indatraline, as well as newly synthesized and structurally different ones, such as NS18283, significantly reduce nociceptive hypersensitivity and depressive-like behaviors in a mouse model of chemotherapy-induced neuropathic pain (Hache et al. 2015). Hence, all of these studies, whether tackling the role of monoamines separately or as a group, shed light on the complex nature of the comorbid development of pain and depression.

Glutamate and GABA

Glutamate and gamma-aminobutyric acid (GABA) are the major excitatory and inhibitory neurotransmitters in the nervous system, respectively. The balance between excitation and inhibition guarantees the well-functioning of the brain. Excessive plasma glutamate/glutamine and a significantly lower GABA level were detected in patients with major depression (Northoff and Sibille 2014). Further studies found that drugs targeting glutamate transmission, like the NMDA antagonist ketamine, could alleviate both pain and depressive symptoms in both animals and humans (Ettensohn et al. 2018; Wang et al. 2011), and the AMPA receptor facilitator AMPAkines has similar analgesic and antidepressant effects by acting on the descending inhibitory circuits such as the PAG-rostral ventromedial medulla (RVM) and the NAc-RVM axes (Le et al. 2014) (see Table 4).

Glutamate

A disrupted level of glutamatergic transmission and glutamatergic receptors has been demonstrated in animal models of chronic pain and mood disorder comorbidity (see review of Benson et al. 2015). For instance, enhanced presynaptic glutamate neurotransmission in the ACC, the higher structure for integrating the affective component of painful sensation and mood disorders, was observed in both chronic inflammatory pain (Zhao et al. 2006a, b) and neuropathic pain (Koga et al. 2015). More specifically, Koga and colleagues showed enhanced presynaptic glutamate release probability (Koga et al. 2015; Zhuo 2016), since lower paired-pulse ratio (PPR) was observed in the ACC of mice displaying chronic pain–induced anxiety. Furthermore, they reported that blocking the hyperpolarization-activated cyclic nucleotide–gated (HCN) channels, which are involved in pre-long-term potentiation (LTP), produced analgesic and anxiolytic effects (Koga et al. 2015). In vivo electrophysiological recordings also support the idea that the ACC is hyperactive when animals display chronic pain and depression comorbidity. Interestingly, the temporal inhibition of the glutamatergic neuron of the ACC by optogenetic stimulation blocked the anxiodepressive-like consequences of neuropathic pain (Sellmeijer et al. 2018). In contrast, it has been shown that the decreased activity of pyramidal neurons in the prelimbic cortex plays an essential role in pain-induced affective symptoms (Wang et al. 2015; Kelly et al. 2016) and that an increased GABAergic inhibition is responsible for the reduction of pyramidal activity (Zhang et al. 2015).

Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluRs) are members of the class C family of G protein–coupled receptors, regulating glutamatergic transmission in the CNS. Currently, there are three groups and eight subtypes of mGluRs which have been identified. Group I (mGluR1 and mGluR5) are excitatory G protein–coupled and predominantly expressed post-synaptically, whereas group II (mGluR2/3) and group III (e.g., mGluR4) are inhibitory G protein–coupled and mostly pre-synaptically expressed (see Chiechio 2016 for review). These receptors are involved in many physiological process and diseases like chronic pain (Chiechio 2016) and mood disorders (Zarate et al. 2010). Postmortem studies in major depressive disorder (MDD) and bipolar disorder patients revealed no mGluR (mGluR2/3 and mGluR5) alterations in the ACC (Matosin et al. 2014); however, mGluR2/3 was elevated in the PFC in MDD (Feyissa et al. 2010). mGluR4, expressed in both GABAergic and glutamatergic synapses in the amygdala, modulates sensory and affective components of pain. Indeed, Zussy et al. (2016) showed that the activation of mGluR4, by a photopharmacological approach, reversibly inhibited anxiodepressive consequences of inflammatory pain. Chung et al. (2017) injected [11C] ABP688, an mGluR5-selective radiotracer, via the rat tail vein and used positron-emission tomography to examine the expression level of mGluR5 in different brain regions 16–25 days after spared nerve ligation (SNL). Interestingly, they found bidirectional changes of mGluR5 expression indicating an upregulation in the caudal part of the prelimbic (PrL) cortex and downregulation in the insular cortex and NAc. However, pharmacological blockage of mGluR5 in the PrL was sufficient to ameliorate both tactile hypersensitivity and depressive-like behaviors.

Table 4 Role of the glutamatergic/GABAergic transmission in neuropathic pain and concomitant anxiodepressive-like behaviors

	Expression	Species	Region	Models	Pain type	Mood disorder	Reference
Glutamate and GABA							
Glutamate release	Increased	Mouse	ACC	CFA-injection	Inflammatory	A-L	Zhao et al. 2006a, b; Koga et al. (2015)
mGluR5	Increased	Rat	PrL	SNL	Neuropathic	D-L	Chung et al. (2017)
	Decreased	Rat	IC, NAc	SNL	Neuropathic	D-L	Chung et al. (2017)
GluA1	Increased	Rat	NAc	SNI	Neuropathic	D-L	Goffer et al. (2013); Su et al. (2015)
	Increased	Rat	NAc	CFA injection	Inflammatory	D-L	Su et al. (2015)
	No difference	Rat	NAc	Paw incision	Post-incisional	D-L	Su et al. (2015)
	No difference	Mouse	AMY	Reserpine treatment	Hyperalgesia	D-L	Liu et al. (2014)
GluN2A	No difference	Mouse	AMY	Reserpine treatment	Hyperalgesia	D-L	Liu et al. (2014)
	Increased	Rat	rACC	Formalin injection	Inflammatory	CPA	Li et al. (2009b)
GluN2B	Increased	Rat	rACC	Formalin injection	Inflammatory	CPA	Li et al. (2009b)
	Increased	Mouse	AMY	Reserpine treatment	Hyperalgesia	D-L	Liu et al. (2014)
pGluN1	Decreased	Rat	HPC	CCI	Neuropathic	AD-L	Li et al. (2014)
GluN1	No difference	Rat	rACC	Formalin injection	Inflammatory	CPA	Li et al. (2009b)
GABA	Decreased	Human	Mid-ACC	Osteoarthritis	Joint pain	MDD	Reckziegel et al. (2016)

ACC, anterior cingulate cortex; *AD-L*, anxiodepressive-like; *A-L*, anxiety-like; *AMY*, amygdala; *CCI*, chronic constriction injury; *CFA*, complete Freund's adjuvant; *CPA*, conditioned place avoidance; *D-L*, depressive-like; *GABA*, gamma-aminobutyric acid; *GluA1*, AMPA receptor subunit; *GluN1*, NMDA receptor subunit zeta-1; *GluN2A*, NMDA receptor 2A; *GluN2B*, NMDA receptor 2B; *HPC*, hippocampus; *IC*, insular cortex; *MDD*, major depressive disorder; *mGluR5*, metabotropic glutamate receptor 5; *NAc*, nucleus accumbens; *pGluN1*, phosphorylated GluN1; *PrL*, prelimbic region; *rACC*, rostral anterior cingulate cortex; *SNI*, spared nerve injury; *SNL*, spinal nerve ligation

α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors

AMPA, NMDA, and kainate receptors are ionotropic glutamate receptors, exerting faster effects than metabotropic receptors. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are tetramers composed of four types of subunits: GluA1, GluA2, GluA3, and GluA4. It has been shown that the expression of AMPA is altered in the comorbidity of chronic pain and mood disorders. GluA1 AMPA receptor subunit, for example, was reported increased in the NAc in the SNI rat model (Goffer et al. 2013). Su et al. (2015) confirmed the elevated GluA1 level in NAc of SNI rat model, and more specifically, they compared the GluA1 and GluA2 expression levels in three different models displaying pain-depression comorbidity conditions: post-incisional pain with paw incision (PI), persistent but reversible inflammatory pain (CFA), and chronic neuropathic pain (SNL) models. Their results showed that the GluA1 level in NAc synapses was not altered in the PI model, increased 7 days after CFA injection (but recovered 14 days after), and increased and remained unchanged in SNL model. Both studies (Su et al. 2015; Goffer et al. 2013) point to a unique adaptive mechanism of GluA1 in different types of chronic pain and depression comorbidity. The overexpression of GluA1 subunit exclusively increases the synthesis of the Ca^{2+}-permeable AMPA receptors which are formed by GluA1 homomers, whereas blocking these homomers worsens depressive symptoms (Su et al. 2015).

N-methyl-D-aspartate receptors

N-methyl-D-aspartate (NMDA) receptors are the most studied glutamatergic receptors in the context of depression and chronic pain due to their role in regulating the glutamatergic system and plasticity changes like postsynaptic LTP. Ketamine, a noncompetitive NMDA antagonist, exerts fast antidepressant and analgesic effects (see Zorumski et al. 2016 for review) at a subanesthetic dose. It also blocks the anxiodepressive-like consequences of neuropathic pain in rodents after an administration of a single subanesthetic dose (Wang et al. 2011). In a reserpine-induced pain/depression model, an upregulation of GluN2B, but not GluN2A or AMPA receptor GluA1 expression, was observed in the amygdala of mice (Liu et al. 2014). However, a decreased phosphorylation of the NMDA receptor type 1 (GluN1) in the hippocampus was reported in rats showing anxiety and depressive-like behaviors following CCI (Li et al. 2014). In addition, GluN2A and GluN2B, but not GluN1, were upregulated in the rostral ACC in formalin-injected rats, and selectively blocking these subunits abolished the acquisition of the induced conditioned place avoidance indicating the critical role of the NMDA in pain-related aversion (Li et al. 2009b). Also, it has been recently shown that the neuropathy-induced

Table 5 Role of the endocannabinoids in neuropathic pain and concomitant anxiodepressive-like behaviors

Endocannabinoids							
2-AG	Increased	Human	Plasma	Osteoarthritic pain	Joint pain	MDD	La Porta et al. (2015)
CB1 and CB2 mRNA	Increased	Human	Lymphocytes	Osteoarthritic pain	Joint pain	MDD	La Porta et al. (2015)
AEA	Decrease	Rat	HPC and PFC	WKY (stress-prone strain)	Allodynia	AD-L	Vinod et al. (2012)
CB1 coupling	Decrease	Rat	HPC and PFC	WKY (stress-prone strain)	Allodynia	AD-L	Vinod et al. (2012)
AEA/2-AG	Decrease	Mouse	Brain + GS muscle	CUS + intramuscular NGF	Hyperalgesia	AD-L	Lomazzo et al. (2015)

2-AG, 2-arachidonoylglycerol; *AD-L*, anxiodepressive-like; *AEA*, anandamide; *CB1*, cannabinoid receptor type 1; *CB2*, cannabinoid receptor type 2; *CUS*, chronic unpredictable stress; *GS*, glutamine synthetase; *HPC*, hippocampus; *MDD*, major depressive disorder; *NGF*, nerve growth factor; *PFC*, prefrontal cortex; *WKY*, Wistar Kyoto rat

anxiodepressive-like behaviors could be attributed to an NMDA-contributed hyperactivity of the ACC (Sellmeijer et al. 2018).

Kainate receptors

In addition to AMPA and NMDA receptors, kainate receptors form another group of ionotropic glutamatergic receptors. It has been clearly shown that kainate receptors mediated pre-LTP, increased glutamate release probability measured by paired-pulse facilitation, are implicated in the CFA-induced anxiety-like behaviors (Koga et al. 2015).

GABA

The GABAergic system is studied in both human and pre-clinical animal models, contributing to the overall understanding of GABA level in different brain regions and different pain and mood disorder comorbidity cases. For instance, using proton magnetic resonance spectra optimized for detecting GABA level in patients with osteoarthritis, it has been shown that altered GABA level in mid-ACC is only responsible for pain severity but not affective compound (Reckziegel et al. 2016). In a rat model of chronic inflammatory pain, GABA expression is elevated in mPFC, and administering a $GABA_A$ antagonist could improve mechanical allodynia (Luongo et al. 2013). Another study which focused on the pain-depression comorbidity showed that intra-basolateral amygdala (BLA) administration of muscimol, a $GABA_A$ agonist, decreased formalin-induced pain behavior in CMS rats and increased their sucrose preference, while intra-mPFC administration of muscimol produced no effect, suggesting different roles of the BLA and mPFC mediating pain perception in depressive states and highlighting the brain region–dependent role of GABAergic system (Qi et al. 2013). Similarly, intraperitoneal injections of pregabalin, an alkylated analog of GABA, improved nociceptive, anxiety-like, and anhedonic responses in neuropathic pain induced by PSNL mice model (La Porta et al. 2016), further strengthening the evidence for the role of the GABAergic system in the pain-depression comorbidity.

The endocannabinoid system

There are various accounts from around the world that the plant *Cannabis sativa* has been utilized as medicine for thousands of years (Fitzgibbon et al. 2015). Its main psychoactive compound, delta-9-tetrahydrocannabinol (Δ9-THC), was identified in 1964 (Gaoni and Mechoulam 1964). Successive studies on its activity resulted in the identification of the endocannabinoid system. This system is comprised of the cannabinoid receptors (CB1 and CB2) and their endogenous ligands, with anandamide (AEA) (Devane et al. 1992) and 2-arachidonylglycerol (2-AG) (Mechoulam et al. 1995) being the most investigated ones.

While clinical evidence is still scarce when it comes to the role of the endocannabinoid system in the pain-depression comorbidity, there are several studies suggesting that it is a valid target for future research. For instance, La Porta et al. (2015) found a significant positive correlation between 2-AG plasma levels in osteoarthritic patients with their levels of pain and depression. Moreover, they also report a positive correlation of depression with CB1 mRNA expression in blood lymphocytes and CB2 with pain scores. However, probably the highest number of clinical evidence linking the endocannabinoid system to pain and depression comorbidity comes from the use of cannabis and its natural and synthetic counterparts in patient treatment. Woolridge et al. (2005) report that cannabis intake was associated with decreased muscular and neuropathic pain, alongside anxiety and depression in HIV patients. Similarly, there was a significant pain and depression reduction in fibromyalgia patients after 4 weeks of treatment with nabilone (a Δ9-THC analog), compared to the placebo group (Skrabek et al. 2008).

In spite of a number of studies linking endocannabinoid modulation with pain and depression alone, there is limited pre-clinical evidence associating the co-occurrence of chronic pain and depression to the endocannabinoid system (Fitzgibbon et al. 2015). Nevertheless, Vinod et al. (2012) found that Wistar Kyoto rats, a genetically stress-prone strain displaying increased depressive- and anxiety-like behaviors as well as exacerbated mechanical allodynia, showed decreased

Table 6 Role of the BDNF in neuropathic pain and concomitant anxiodepressive-like behaviors

	Expression	Species	Region	Models	Pain type	Mood disorder	Reference
BDNF	Decreased	Mouse	HPC	SCI	Neuropathic	D-L	Li et al. (2017)
	Decreased	Rat	HPC	CFA-injection	Inflammatory	D-L	Duric and McCarson (2006, 2007)
	Decreased	Mouse	Cortex, HPC	PSNL	Neuropathic	D-L	Brüning et al. (2015)
	Decreased	Rat	ACC, mPFC, HPC	SNI	Neuropathic	D-L	Pan et al. (2018)
	Decreased	Mouse	ACC	SNI	Neuropathic	D-L	Zhao et al. (2017)
	Decreased	Rat	ACC	CCI	Neuropathic	D-L	Ishikawa et al. (2014)
	Decreased	Rat	ACC, RVM	CCI	Neuropathic	D-L	Yasuda et al. (2014)
	Decreased	Mouse	ACC, BLA, CA1/3, RVM	SNL	Neuropathic	D-L	Zhu et al. (2017)
	Decreased	Mouse	mPFC	SNI	Neuropathic	D-L	Guida et al. (2015)
	Decreased	Rat	PFC	SNI	Neuropathic	D-L	Xie et al. (2017)
	Decreased	Mouse	mPFC	CMS	Hyperalgesia	D-L	Liu et al. (2018)
	Increased	Rat	rACC	SNI	Neuropathic	CPA	Zhang et al. (2016b)
	Increased	Human	Colonic biopsies	IBS	Visceral pain	MDD	Yu et al. (2011)
	Increased	Rat	Spinal cord	SNL	Neuropathic	D-L	Wei et al. (2017)
	No difference	Mouse	PFC	PSNL	Neuropathic	A-L	González-Sepúlveda et al. (2016)
	No difference	Human	Serum level	Musculoskeletal pain	Musculoskeletal	MDD	Generaal et al. (2016)

ACC, anterior cingulate cortex; *A-L*, anxiety-like; *BDNF*, brain-derived neurotrophic factor; *BLA*, basolateral amygdala; *CCI*, chronic constriction injury; *CFA*, complete Freund's adjuvant; *CMS*, chronic mild stress; *CPA*, conditional place avoidance; *D-L*, depressive-like; *HPC*, hippocampus; *IBS*, irritable bowel syndrome; *MDD*, major depressive disorder; *mPFC*, medial prefrontal cortex; *PFC*, prefrontal cortex; *PSNL*, partial sciatic nerve ligation; *rACC*, rostral ACC; *RVM*, rostral ventromedial medulla; *SNI*, spared nerve injury; *SNL*, spinal nerve ligation

levels of AEA and increased levels of CB1 coupling in the hippocampus and frontal cortex. Another direct evidence connecting the pain-depression comorbidity with the endocannabinoid system comes from Lomazzo et al. (2015) who utilized the CMS mouse model in combination with intramuscular administration of nerve growth factor to achieve anxiodepressive-like behaviors and widespread mechanical hyperalgesia. They showed that pretreatment of these mice with URB597, an inhibitor of the fatty acid amide hydrolase (FAAH), which is the primary enzyme responsible for the metabolism of AEA, was sufficient to attenuate anxiety-like behaviors in the light-dark test, as well as thermal and widespread mechanical hyperalgesia. Similarly, Hu et al. (2009) utilized the CCI model to investigate the interaction between the CB2 receptor and the comorbid chronic pain and depressive-like behavior. They showed that a systemic administration of GW405833, a CB2 agonist, significantly lowered the CCI-induced depressive-like behaviors and mechanical hyperalgesia, pointing to its potential as an alternative treatment option.

Together, although limited, current evidence suggests a promising role of the endocannabinoid system (see Table 5) in the interactive relationship of chronic pain and depression, and constitutes a valid target for future studies, which could aim at delineating whether the effects observed in the comorbidity follow the same or separate molecular pathway.

Neurotrophic factors

Changes in neurotrophic factors, particularly BDNF, and subsequent alterations in synaptic plasticity and synapse dynamics contribute to the comorbidity of chronic pain and depression. BDNF is the most widely expressed neurotrophin in the peripheral and central nervous system where it regulates neuronal survival and differentiation and critically participates in activity-dependent synaptic plasticity mechanisms (see review of Bramham and Messaoudi 2005). Clinical studies showed increased plasma BDNF levels in patients with fibromyalgia (Haas et al. 2010) and IBS (Yu et al. 2011) displaying severe depressive symptoms. However, pre-clinical studies suggest a region-dependent manner of BDNF regulation in both chronic pain and in depression. Indeed, BDNF transcription and expression is upregulated immediately (24 h) after CFA injection in the DH of rats and remains high even 14 days post-injection (Duric and McCarson 2006, 2007). Consistent with chronic inflammation, the BDNF level in the spinal cord in neuropathic pain models is significantly higher than in the sham group in both mice (Almeida et al. 2015) and rats (M'Dahoma et al. 2015; Zhang et al. 2016b; Wei et al. 2017; Tateiwa et al. 2018). Despite the uniform upregulation of BDNF in the spinal cord, alterations in the hippocampus are time-dependent.

Table 7 Role of neurogenesis in neuropathic pain and concomitant anxiodepressive-like behaviors

	Expression	Species	Region	Models	Pain type	Mood disorder	Reference
Neurogenesis							
BrdU+ cells	Decrease	Rat	DG	CFA, immobilization	Inflammatory	A-L	Duric and McCarson (2006)
DCX+/BrdU+ cells	Decrease	Mouse	HPC	SNI	Neuropathic	A-L	Mutso et al. (2012)
DCX+/BrdU+ cells	Decrease	Mouse	DG	Chronic CFA	Inflammatory	A-L	Zheng et al. (2017)
DCX+/BrdU+ cells	Decrease	Rat	HPC	CCI, immobilization	Neuropathic	A-L	Romero-Grimaldi et al. (2015)

A-L, anxiety-like; *BrdU*, bromodeoxyuridine; *CCI*, chronic constriction injury; *CFA*, complete Freund's adjuvant; *DCX*, doublecortin; *DG*, dentate gyrus; *HPC*, hippocampus; *SNI*, spared nerve injury

The hippocampal transcription and expression level of BDNF decreased right after CFA-injection in rats and remains low (Duric and McCarson 2007). In contrast to the quick drop in the chronic inflammatory model, the reduction of hippocampal BDNF level is slower in the neuropathy cases. For instance, BDNF expression began to downregulate at least 7 days after SCI (Li et al. 2017), SNL (Zhu et al. 2017), PSNL in mice (Brüning et al. 2015), and SNL in rats (Tateiwa et al. 2018), when the depressive-like symptoms emerged. These suggest on the one hand that neuropathic pain has a different mechanism than inflammatory pain, and on the other hand that the opposite regulatory direction of BDNF level in the spinal dorsal horn and hippocampus is due to their different roles in the pain chronification. Respectively, fast and slow regulations also hint at potential interactions between BDNF derived from different sources such as nociceptor neurons, peripheral sensory neurons, and microglia-derived BDNF.

In the forebrain regions like mPFC and ACC, most of the neuropathy studies reported downregulation of BDNF in the SNI model of both rats (Xie et al. 2017; Pan et al. 2018) and mice (Guida et al. 2015; Zhao et al. 2017; Zhu et al. 2017), and CCI rat model (Ishikawa et al. 2014; Yasuda et al. 2014). Moreover, BDNF levels in BLA and RVM were also reported to be downregulated in the SNL mouse model (Zhu et al. 2017) and CCI rat model (Yasuda et al. 2014). There are also some negative results regarding BDNF level changes in neuropathy cases. For instance, contrary to their results in the hippocampus, Tateiwa et al. (2018) reported an unchanged BDNF expression level in other brain regions like the mPFC and ACC, as well as thalamus, cerebellum, and amygdala, 21 days after nerve injury in the SNL model. González-Sepúlveda et al. (2016) also detected no difference in the PFC BDNF level in a PSNL mice model with comorbid anxiety-like behavior. Curiously, one of the common features of the latter studies is the fact that at the time point at which the animals were tested, they did not show any depressive-like behavior; however, according to the Xie et al. (2017), BDNF level changes occur only when there are comorbid pain and depressive-like symptoms observed (see Table 6).

Neurogenesis

Adult neurogenesis is commonly assessed by administering bromodeoxyuridine (BrdU), an analog of thymidine, to detect cells that are actively replicating their DNA, and therefore proliferating (Lehner et al. 2011). Another method involves labeling the microtubule binding protein doublecortin (DCX) which is transiently expressed in proliferating progenitor cells and newly generated neuroblasts (Brown et al. 2003). These techniques are very useful in pre-clinical studies, including those using rodent models to demonstrate a relationship between chronic pain–depression comorbidity and altered neurogenesis in the adult hippocampus. For instance, Duric and McCarson (2006) showed that BrdU-positive cells in the dentate gyrus of rats were significantly reduced after exposure to prolonged inflammation (21 days of CFA) or stress (repeated immobilization for 10 days). Moreover, it was demonstrated that both SNI and CFA models in mice result in a reduction of $DCX^+/BrdU^+$ neuroblasts (Mutso et al. 2012; Zheng et al. 2017). Interestingly, Romero-Grimaldi et al. (2015) found that stress (i.e., chronic immobilization stress) significantly exacerbated the negative effects of CCI and resulted in even greater reduction of proliferation, survival, and differentiation of rat newborn hippocampal cells (see Table 7). Although the research on the relationship of depression and neurogenesis has been recently subjected to serious discrepancies (Hanson et al. 2011), the amount of information available is still exceedingly high in comparison to the very limited information that is available on the potential implications of neurogenesis in chronic pain (Grilli 2017). Therefore, even though the available data suggest that hippocampal neurogenesis is affected and implicated in both chronic pain and mood disorders, its full role is in each of the conditions, and their comorbidity remains to be determined.

Neuroendocrine alterations

Although still not fully elucidated, the role of the hypothalamic-pituitary-adrenal (HPA) axis, one of the

main bodily stress response systems, is likely also playing a role in the relationship of chronic pain and mood disorders. Intriguingly, in both the CCI model and the sciatic nerve cuffing model of neuropathic pain–induced depressive-like behaviors, there was no alteration in the HPA axis (Bomholt et al. 2005; Ulrich-Lai et al. 2006; Kilburn-Watt et al. 2010; Yalcin et al. 2011). On the other hand, it was shown in patients with chronic multi-site musculoskeletal pain who suffer from major depressive disorder that pain induces hypoactive HPA axis function while depression causes a hyperactive function, so it is possible that these two conditions might blunt the individual effect of each other (Generaal et al. 2014). Moreover, Bomholt et al. (2005) observed that in CCI rats, the HPA axis responded normally to novel stressors which are known to activate the HPA axis. Similarly, Ulrich-Lai et al. (2006) reported that the CCI did not affect resting or restraint stress-related HPA activity. However, these studies focused on a specific pain model and, more importantly, only on the presence of corticosteroid and cortisol, the dominant glucocorticoids found in rodents and humans, respectively. Therefore, such approach likely provides incomplete information about the role, if any, of the HPA axis in subjects with pain-depression comorbidity. Hence, it is important to look further into other models and candidate hormones involved in the activation of the HPA axis to determine its participation in the comorbid relationship of pain and depression. For instance, it was shown that female patients who developed depression due to chronic mandibular pain show no difference in cortisol levels compared to healthy controls, but have a decreased dehydroepiandrosterone (DHEA) secretion, which is another highly abundant hormone in the HPA axis, associated with pain intensity, stress, and pain-related depressive states (Jo et al. 2016). Moreover, there is an increase in serum adrenocorticotropic hormone (ACTH) and corticosterone levels in mice submitted to PSNL model, additionally pointing to a role of the HPA axis activation in the pain-depression comorbidity (Brüning et al. 2015). Also, a study by Ji et al. (2007) showed that the function of the corticotrophin releasing hormone receptor CRH1 in the amygdala is implicated in pain-related anxiety-like behavior. Namely, systemic and intra-amygdalar administration of the CRH1 antagonist NBI 27914 was sufficient to reduce the nociceptive and anxiety-like responses in a rat model of arthritic pain. This is in accordance with the results obtained by Ulrich-Lai et al. (2006) which show an increase in the expression of corticotrophin releasing hormone mRNA in the central amygdala of rats after CCI model.

On the other hand, Kilburn-Watt et al. (2010) found a decrease in thyroid hormones in rats which displayed an altered social behavior following a sciatic nerve ligation. Unexpectedly, the decrease was persistent indicating an altered regulation of the hypothalamic-pituitary-thyroid axis. Similarly, other studies also reported that there was an association between thyroid autoimmunity with fibromyalgia and depression (Pop et al. 1998; Ribeiro and Proietti 2004). Interestingly, Pamuk and Cakir (2007) observed that thyroid autoimmunity in fibromyalgia was characteristic of older postmenopausal female patients, suggesting a possible role of estrogen. Indeed, there is cumulative evidence demonstrating the role of the estrogen receptor in various molecular and cellular functions associated with depression and pain processing (Lu and Herndon 2017). This goes in line with a recent finding that endometriosis, an estrogen-dependent inflammatory disorder associated with chronic pelvic pain, anxiety, and depression, triggers differential gene expression in several brain regions (Li et al. 2018). Specifically, mice with endometriosis displayed depressive-like behaviors and nociceptive hypersensitivity, which were accompanied by disrupted expressions of several genes such as Gpr88, Glra3, and Serpina3n in the insula, Chrnb4 and Npas4 in the hippocampus, and Lcn2 and Nptx2 in the amygdala, which are associated with anxiety and chronic pain.

Taken together, the results of these studies (see Table 8) point to a multifaceted role of hormones in the comorbidity of pain and depression and therefore, constitute a justified

Table 8 Role of hormones in neuropathic pain and concomitant anxiodepressive-like behaviors

	Expression	Species	Samples	Models	Pain type	Mood disorder	Reference
Hormones							
CRF1R	Increased	Rat	AMY	Kaolin/carrageenan	Arthritic	AD-L	Ji et al. (2007)
CRF mRNA	Increased	Rat	AMY	CCI	Neuropathic	AD-L	Ulrich-Lai et al. (2006)
Cortisol/DHEA	Increased	Human	Saliva	TMD + BDI-II	Mandibular	MDD	Jo et al. (2016)
ACTH and CORT	Increased	Mouse	Serum	PSNL	Neuropathic	AD-L	Brüning et al. (2015)
Thyroid hormones	Decreased	Rat	Plasma	CCI	Neuropathic	AD-L	Kilburn-Watt et al. (2010)

ACTH, adrenocorticotropic hormone; *AD-L*, anxiodepressive-like; *AMY*, amygdala; *BDI-II*, Beck Depression Inventory-II; *CCI*, chronic constriction injury; *CORT*, corticosteroids; *CRF*, corticotropin-releasing factor; *DHEA*, dehydroepiandrosterone; *PSNL*, partial sciatic nerve ligation; *TMD*, temporomandibular disorder

Table 9 Inflammatory factors and gliosis involved in neuropathic pain and concomitant anxiodepressive-like behaviors

	Expression	Species	Region	Models	Pain type	Mood disorder	Reference
Pro-inflammatory							
IL-1β	Increased	Rat	Liver	SNI	Neuropathic	D-L	Zhou et al. (2015)
	Increased	Mouse	Serum, cortex, HPC	PSNL	Neuropathic	D-L	Brüning et al. (2015)
	Increased	Rat	DH, HPC, PFC, NAc, AMY	SNI	Neuropathic	D-L	Gui et al. (2016)
	Increased	Mouse	HPC	CCI	Neuropathic	D-L	Kim et al. (2012)
	Increased	Rat	AMY	SNI, OB	Neuropathic	D-L	Burke et al. (2013a)
	Increased	Rat	HPC, cortex	Reserpine treatment	Hyperalgesia	D-L	Arora and Chopra (2013)
	Increased	Rat	HPC	SNI, MD	Neuropathic	D-L	Burke et al. (2013b)
	Increased	Mouse	Brain	CFA injection	Inflammatory	D-L	Maciel et al. (2013)
	Increased	Mouse	PFC	SNI	Neuropathic	D-L	Norman et al. (2010a, b)
	Increased	Mouse	PFC	PSNL	Neuropathic	A-L	González-Sepúlveda et al. (2016)
IL-6	Increased	Mouse	HPC	CCI	Neuropathic	D-L	Jiang et al. (2018)
	Increased	Rat	Plasma, HPC	CFA-injection	Inflammatory	D-L	Kim et al. (2012)
	Increased	Human	Plasma	Chronic back pain	Back pain	MDD	Kim et al. (2012)
	Decreased	Rat	AMY	SNI, OB	Neuropathic	D-L	Burke et al. (2013a)
	Increased	Mouse	Serum, cortex, HPC	PSNL	Neuropathic	D-L	Brüning et al. (2015)
TNF-α	Increased	Mouse	HPC	CCI	Neuropathic	D-L	Jiang et al. (2018)
	Increased	Mouse	Sciatic nerve, PFC, HPC	Peripheral nerve crush	Neuropathic	D-L	Nascimento et al. (2015)
	Increased	Rat	HPC, cortex	Reserpine treatment	Hyperalgesia	D-L	Arora and Chopra (2013)
	Increased	Rat	HPC	SNI, MD	Neuropathic	D-L	Burke et al. (2013b)
	Increased	Mouse	Frontal cortex	Reserpine treatment	Hyperalgesia	D-L	Xu et al. (2013)
	Increased	Mouse	Serum, cortex, HPC	PSNL	Neuropathic	D-L	Brüning et al. (2015)
TNF	increased	Mouse	HPC	CCI	Neuropathic	D-L	Dellarole et al. (2014)
Anti-inflammatory							
INF-γ	Increased	Mouse	Serum, cortex, HPC	PSNL	Neuropathic	D-L	Brüning et al. (2015)
IL-10	Decreased	Mouse	Serum, cortex, HPC	PSNL	Neuropathic	D-L	Brüning et al. (2015)
	Increased	Rat	AMY	SNI, OB	Neuropathic	D-L	Burke et al. (2013a)
Astrocytes							
GFAP	Increased	Rat	HPC	SNI, MD	Neuropathic	D-L	Burke et al. (2013b, 2015b)
	Increased	Mouse	PAG	SNI	Neuropathic	D-L	Norman et al. (2010b)
	Increased	Rat	AMY	SNI, OB	Neuropathic	D-L	Burke et al. (2013a)
Microglia							
CD11b	Increased	Rat	AMY	SNI, OB	Neuropathic	D-L	Burke et al. (2013a)
	Increased	Rat	HPC	SNL, OB	Neuropathic	D-L	Burke et al. 2015b
Other mechanisms							
NF-κβ	Increased	Rat	HPC, cortex	Reserpine treatment	Hyperalgesia	D-L	Arora and Chopra (2013)
	Increased	Mouse	HPC, cortex	PSNL	Neuropathic	D-L	Brüning et al. (2015)

A-L, anxiety-like; *AMY*, amygdala; *CCI*, chronic constriction injury; *CFA*, complete Freund's adjuvant; *DH*, dorsal horn; *D-L*, depressive-like; *GFAP*, glial fibrillary acidic protein; *HPC*, hippocampus; *IL-6*, interleukin 6; *IL-10*, interleukin 10; *IL-1β*, interleukin 1beta; *INF-γ*, interferon gamma; *MD*, maternal deprivation; *MDD*, major depressive disorder; *NAc*, nucleus accumbens; *NF-κβ*, nuclear factor-kappa beta; *OB*, olfactory bulbectomy; *PAG*, periaqueductal gray; *PFC*, prefrontal cortex; *PSNL*, partial sciatic nerve ligation; *SNI*, spared nerve injury; *SNL*, spinal nerve ligation; *TNF*, tumor necrosis factor; *TNF-a*, tumor necrosis factor alpha

target for further in-depth studies and even potential new therapeutic options.

Neuroinflammatory factors

Neuroinflammatory alterations play an important role in the pathophysiology of depression (Capuron and Miller 2011; Dantzer et al. 2008) and chronic pain (Clark et al. 2013; Miller et al. 2009) as demonstrated by pre-clinical studies showing a sustained imbalance between pro-inflammatory and anti-inflammatory cytokines and alterations in immune and non-immune cells. During the last 10 years, an increasing number of studies focused on the involvement of these factors in the comorbidity of chronic pain and depression (Burke et al. 2015a). For instance, clinical studies showed that patients with chronic back pain (Kim et al. 2012) and chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) (Hu et al. 2016) with comorbid depression had elevated plasma pro-inflammatory cytokines which initiate, orchestrate, and amplify the inflammatory response of IL-6, IL-1α, IL-1β, and TNF-α. Moreover, in the cerebrospinal fluid of patients with complex regional pain syndrome (CRPS), IL-1β and IL-6, but not TNF-α, were also found to be increased (Alexander et al. 2005).

Pre-clinical studies focusing on different chronic pain models with comorbid anxiety/depression reported upregulation of IL-6 in serum and cerebrospinal fluid in the CP/CPPS syndrome in the hippocampus and cortex, as well as in

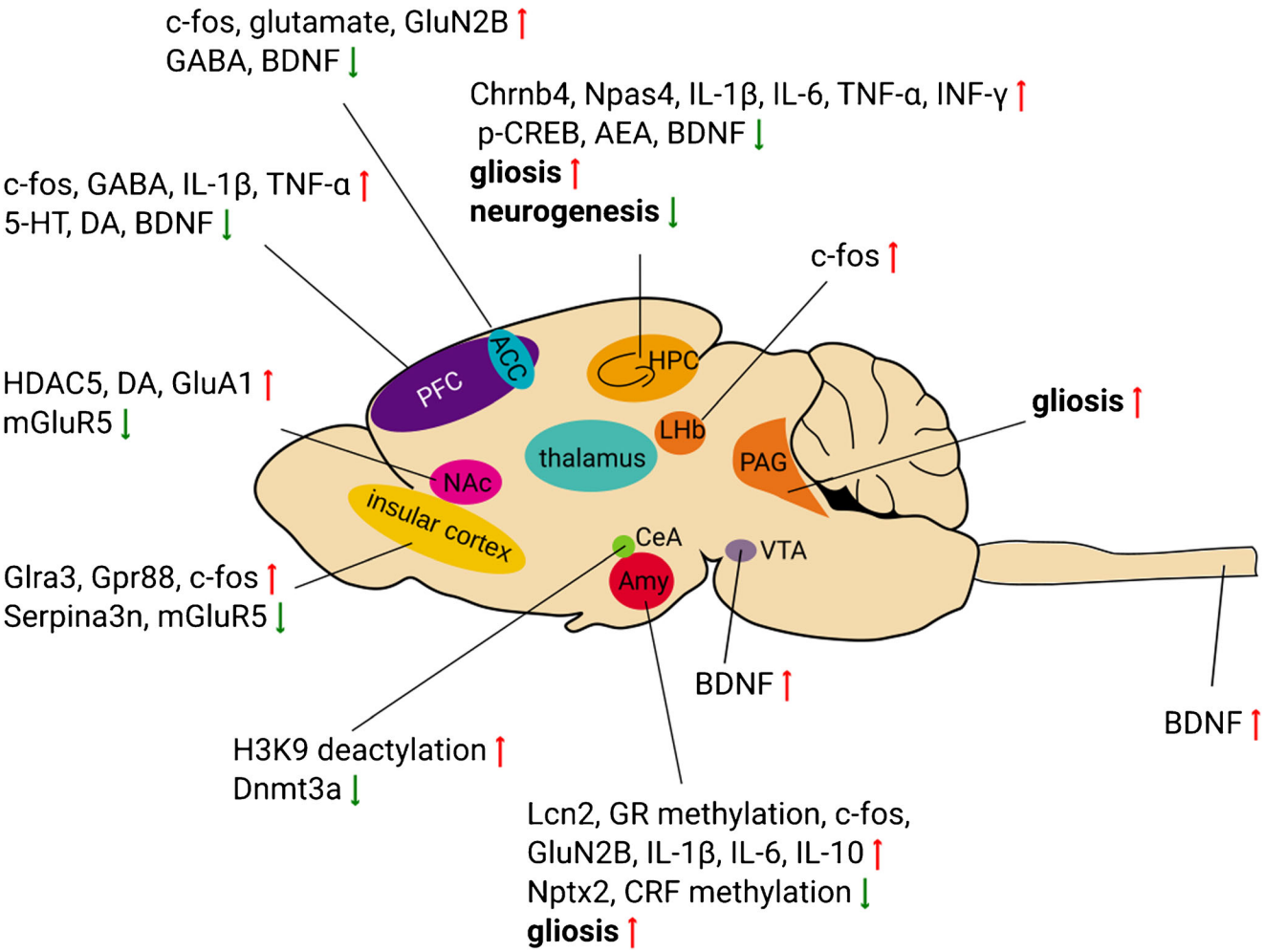

Fig. 1 Summary of molecular alterations involved in pre-clinical rodent models of comorbid chronic pain and anxiodepressive-like behaviors. Red up-arrows: increased level; green down-arrows: decreased level. 5-HT, serotonin; ACC, anterior cingulate cortex; AEA, anandamide; AMY, amygdala; BDNF, brain-derived neurotrophic factor; CeA, central amygdala; Chrnb4, cholinergic receptor nicotinic beta 4 subunit; CRF, corticotropin-releasing factor; DA, dopamine; Dnmt3a, DNA methyltransferase; GABA, gamma-aminobutyric acid; GALR2, galanin-2 receptor; Glra3, glycine receptor alpha 3; GluA1, AMPA receptor subunit; GluN2B, NMDA receptor 2B; Gpr88, G protein–coupled receptor 88; GR, glucocorticoid receptor; HDAC5, histone deacetylase 5; HPC, hippocampus; IL-6, interleukin 6; IL-10, interleukin 10; IL-1β, interleukin 1beta; INF-γ, interferon gamma; Lcn2, lipocalin-2; LHb, lateral habenula; Lmx1b, LIM homeobox transcription factor 1 beta; mGluR5, metabotropic glutamate receptor 5; NAc, nucleus accumbens; NF-κβ, nuclear factor-kappa beta; Npas4, neuronal PAS domain protein 4; Nptx2, neuronal pentraxin-2; PAG, periaqueductal gray; p-CREB, phospo c-AMP-response element binding; PFC, prefrontal cortex; Serpina3n, serpin peptidase inhibitor; TNF-α, tumor necrosis factor alpha; VTA, ventral tegmental area

inflammatory and neuropathic pain models (Kim et al. 2012; Jiang et al. 2018; Brüning et al. 2015). In contrast, the level of IL-6 was downregulated in the amygdala (Burke et al. 2013a). Using diverse neuropathic pain models in rodents, it has also been shown that the IL-1β expression was enhanced in the liver (Zhou et al. 2015), plasma (Brüning et al. 2015; Gui et al. 2016), spinal DH (Gui et al. 2016; Apkarian et al. 2006), PFC (Apkarian et al. 2006; Norman et al. 2010a, b; Arora et al. 2011; Arora and Chopra 2013; Brüning et al. 2015; González-Sepúlveda et al. 2016; Gui et al. 2016), hippocampus (Apkarian et al. 2006; Arora et al. 2011; del Rey et al. 2011; Kim et al. 2012; Arora and Chopra 2013; Burke et al. 2013b; Brüning et al. 2015; Gui et al. 2016), NAc (Gui et al. 2016), amygdala (Burke et al. 2013b; Gui et al. 2016), and brainstem. Another major pro-inflammatory cytokine, TNF-α, was also observed to be increased in the serum (Brüning et al. 2015), the PFC (Nascimento et al. 2015; Brüning et al. 2015), and the hippocampus (Jiang et al. 2018; Nascimento et al. 2015; Burke et al. 2013b; Brüning et al. 2015; Dellarole et al. 2014) in rodent models displaying comorbid neuropathic pain and anxiety/depression. On the other hand, anti-inflammatory cytokines' IL-10 and INF-γ levels were altered in the serum, cortex, hippocampus, and amygdala in neuropathic pain rat models (Brüning et al. 2015; Burke et al. 2013a).

In addition, glial cells like microglia and astrocytes also contribute to the cross talk between immune system and neural system. Microglia activation was observed in the amygdala (Burke et al. 2013a) and hippocampus (Burke et al. 2015b), while astrogliosis was reported in the hippocampus, amygdala, and PAG (Norman et al. 2010b; Burke et al. 2013a, b) of animals displaying anxiodepressive-like behaviors after neuropathic pain (see Table 9).

Conclusion and future directions

As seen from the studies presented in this review (see Fig. 1), the past 10 years have yielded a growing number of studies which tried to understand the molecular mechanisms of the comorbidity of chronic pain and mood disorders. While clinical studies provide genetic data, pre-clinical studies were crucial for divulging cause-effect relations. However, overall, our understanding of the mechanisms underlying the development of chronic pain and mood disorders such as depression remains strikingly limited. Although the recent advances highlight various aspects of brain anatomy and physiology which seem to play an important role in the process, detailed cellular and molecular contributions to the transition to chronic pain–depression comorbidity are not available. One way to get closer to this would be to have a more systemic approach while analyzing animal models, where each model is interpreted as a condition for itself. In fact, different characteristics of a specific model such as species (i.e., mouse, rat), strain, age, sex, type, and duration of pain or stress should all be taken into account when analyzing the molecular and physiological phenotype. It is very certain that different combinations of these characteristics might produce very different, if not opposite, results at the molecular, cellular, and behavioral level. It is thus necessary to make side-by-side comparison of data ranging from molecular to behavioral changes obtained from different animal models. Indeed, a detailed characterization of different models displaying a comorbidity of pain and mood disorders would allow clarifying the temporal development of phenotypic changes at different levels, hence shedding light on the order in which certain molecular, cellular, and behavioral alterations manifest in these conditions. Once this is established and clarified, one of the important questions which needs to be further analyzed is whether the chronic pain and mood/anxiety disorders share similar neural mechanisms or chronic pain modulates neural mechanisms which increase the vulnerability for mood/anxiety disorders. As highlighted in this review, the comorbidity can be explained by shared molecular mechanisms observed in both chronic pain and mood disorders such as polymorphisms of 5-HT transporter and imbalance of inhibitory and excitatory neurotransmission or pro-inflammatory and anti-inflammatory cytokines. However, further clinical and pre-clinical studies are still needed to examine the second hypothesis and to search for indicative biomarkers in order to identify candidates for early diagnosis of developing comorbidity of chronic pain and mood disorders. Finally, forthcoming efforts devoted to exposing the underlying molecular mechanisms of chronic pain and depression should direct to novel therapeutic advancements for prevention and alleviation of the negative consequences of these conditions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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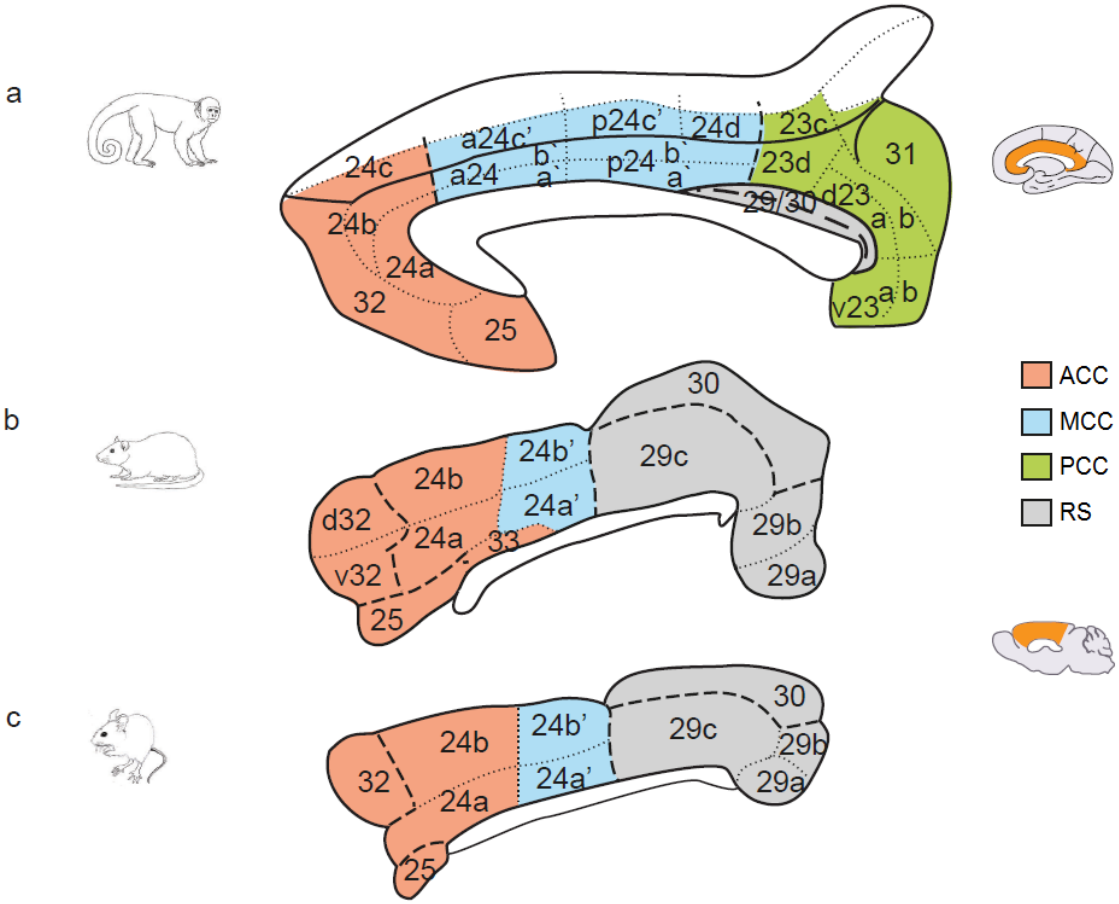
The anterior cingulate cortex: neuroanatomy and function

Location and structure

During the early 20th century, Brodmann (1909) established a numerical nomenclature to define specific cortical locations based on the cytoarchitectonic characteristics of the human cerebral cortex. This was the basis for further detailed anatomical and physiological studies, as well as for modern studies using advanced neuroimaging technology. Another landmark study, which played a crucial role in further decades of structural and functional investigation of the PFC was the macaque cytoarchitectonic map published by Walker (1940). However, an apparent problem which needed to be solved was the lack of comparative studies which would account for neuroanatomical discrepancies between species and provide means of inter-species consistency pertaining to the nomenclature and criteria for regional demarcation. Indeed, overcoming these discrepancies are still among principal challenges in neuroanatomy, but overcoming them leads to greater understanding of neural processes and allows researchers to better model human pathologies in other species such as nonhuman primates and rodents.

The cingulate gyrus has been a prominent research target ever since Broca (1878) described it as a part of the „great limbic lobe“, and the continuous expansion of data pertaining to various aspects of this brain region is yet to cease (Pessoa and Hof 2015). It is a distinguishable tract of fibers spanning from the orbitofrontal cortex, along the dorsal surface of the corpus callosum, and terminating in the isthmus of the cingulate gyrus, anterior to the occipital lobe (Bubb et al. 2018). The current insights about the anatomofunctional properties of the cingulate cortex have been the result of years of multidisciplinary approach in various animal species (Vogt 2005). These integrated, neurobiological assessments led to the current view of the four-region model of the cingulate cortex (Vogt et al. 2003; Vogt et al. 2005; Vogt and Laureys 2005). The four distinct cingulate regions, each one being a cluster of subregions with similar cytoarchitecture, circuitry and functions, are known as: the ACC (s, subgenual; p, pregenual), the midcingulate cortex (MCC; a, anterior; p, posterior), the posterior cingulate cortex (PCC; d, dorsal; v, ventral), and retrosplenial cortex (RSC) (Vogt 2005). The ACC is the most rostral part of the cingulate cortex and it is composed of Brodmann areas 24a, 24b, 25, and 32 in the mouse, while the rat ACC has an additional, area 33, and nonhuman primates and humans have area 24c, which is not found in the rat or mouse (Fig. 2) (Vogt et al. 2005; Vogt and Paxinos 2014). However, although the region of interest in the present work is the mouse ACC, the original data presented in this thesis will concern exclusively the areas 24a and 24b.

The current divisions and their corresponding nomenclature constitute a recent consensus to variations found in previous versions of brain atlases and individual neuroanatomical studies which referred to different subdivisions of the ACC under various names such as prelimbic cortex (currently area 25), infralimbic cortex (currently area 32), cingulate cortex 1 (Cg1; currently area 24b/24b') and 2 (Cg2; currently area 24a/24a') (Heidbreder et al. 2003; Franklin and Paxinos 2007; Paxinos and Watson 2007; Van De Werd et al. 2010). These discrepancies often involved portions of adjacent brain regions to be bundled with the ACC, and the problem of the unspecified rostrocaudal ACC borders was not completely solved until the introduction of the MCC, which was shown to be a distinct, functionally unique cingulate region (Vogt et al. 1995).



d

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 2: Color coded, schematic representation of the organisation and placement of ACC, MCC, PCC, and RS in primates (a), rats (b) and mice (c), and their corresponding nomenclature (d). Unlike primates and mice, rats have ACC area 33, while primates have area 24c. Abbreviations: MCC, midcingulate cortex; PCC, posterior cingulate cortex; RS, retrosplenial cortex. (Adapted from: Vogt and Paxinos 2014)

Cytoarchitecture

The rodent ACC has several defined cortical layers including layer I, II, III, V and VI, however it lacks the granular layer IV containing small neurons, hence the whole structure is referred to as agranular (Fig. 3) (Vogt 2009; Vogt and Paxinos 2014). Layer I is primarily composed of small local interneurons, which receive input from many projecting fibers coming from other central nuclei. The cells in layers II/III are predominantly pyramidal neurons which receive thalamic input and project to deeper layers of the ACC. This input from these layers, alongside thalamic input, is further projected to cortical and subcortical structures via pyramidal cells in layer V (Gabbot et al. 2003; Wang et al. 2004; Zhuo 2007). Layer V, divided into Va and Vb, is characterized by large neurons, with many expressing intermediate nonphosphorylated filaments (Braak 1979; Vogt 2009). Due to lower neuronal density, the layers II/III are thinner compared to layers V/VI which provide the main output of the ACC (Gabbott et al. 2005). In addition, besides layer I, layers II-VI also contain local interneurons which contribute to the intricate functional excitatory and inhibitory connectivity among the different layers (Wu et al. 2009). Further cytoarchitectural analysis of the human ACC revealed that each area displays specific neurotransmitter receptor binding properties (Palomero-Gallagher et al. 2009). For instance, the human subgenual ACC has higher densities of $GABA_A$, $GABA_B$, serotonin $5\text{-}HT_{1A}$, noradrenergic α1 and benzodiazepine receptors, compared to the pregenual ACC (Palomero-Gallagher et al. 2008).

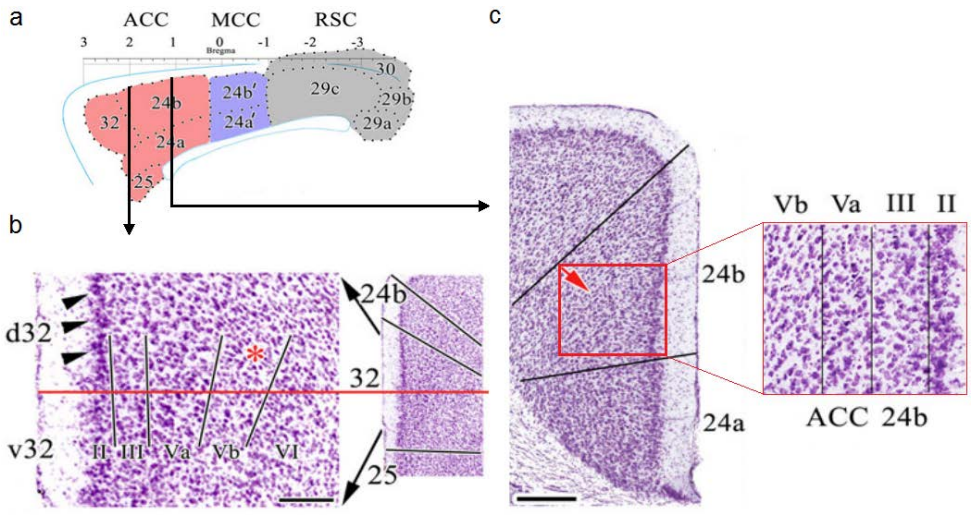


Figure 3: Cytoarchitecture of the mouse ACC. a) A cortical map showing the level of the coronal sections. b) Nissl staining illustrating dorsal and ventral divisions of area 32. The red line represents the margin between the two sub-areas, arrowheads highlight neuron islands in layer II of d32, and the red asterisk note the large neurons in layer Vb. c) Nissl sections of areas 24b and 24a with a magnified image of the different layers in area 24b to emphasize neuronal size differences. The red arrow points to the layer Va which has the largest neurons in this area. (Adapted from: Vogt and Paxinos 2014)

Connectivity

Besides the critical role of proper intralaminar communication of neurons within the ACC (Wu et al. 2009), the various afferent and efferent connections the ACC forms with other brain regions establish the basis for a wide variety of integrative and executive functions spanning from attention, to cognition, to emotional processing, in both healthy and pathological conditions (Stevens et al. 2011). As described earlier in the text, the ACC is composed of several subregions, which have been shown to have a robust communication with each other, as well as with other parts of the cingulate cortex (Gabbot et al. 1997; Fisk and Wyss 1999; Jones et al. 2005; Shibata and Naito 2008; Fillinger et al. 2017, 2018). However, for the sake of convenience, the following sections will regard the ACC as a whole when addressing the main projections from and to important brain areas (Fig. 4), in order speculate on the potential role of those particular connections.

The cerebral cortex is one of the main hubs of both ACC inputs and outputs, as seen by extensive reciprocal connections with the medial prefrontal and orbital cortices, and the secondary motor cortex (Heidbreder and Groenewegen 2003; Hoover and Vertes 2007, 2011; Fillinger et al. 2017, 2018), which might play a role in the integration of sensory input and modulation of executive functions (Hoover and Vertes 2007; Kesner and Churchwell 2011; Shenav et al. 2013). Furthermore, a strong reciprocal connection is also documented with the retrosplenial, parietal associative and the secondary visual cortices (Zingg et al. 2014), suggesting an integrated multimodal exchange and integration of spatial, visual, auditory and somatosensory information (Czajkowski et al. 2014; Torrealba and Valdes 2008).

Interestingly, although afferents from the parahippocampal and hipocampal regions to the ACC have been repeatedly documented (Zingg et al. 2014; Hoover and Vertes 2007; Fillinger et al. 2017), and may have a role in memory encoding of various sensory experiences (Dickie et al. 2011), there is contrasting evidence about the existence of efferents from the ACC to the hippocampus (Rajasethupathy et al. 2015; Fillinger et al. 2018).

Apart from cortical structures, the ACC shows a significant connectivity with non-cortical forebrain regions. For instance, preclinical studies have shown that the claustrum has a dense reciprocal connection with the ACC (Hoover and Vertes 2007; Atlan et al. 2017; Fillinger et al. 2017, 2018), which may play a crucial role in attention, according to recent findings (Mathur et al. 2009; Goll et al. 2015). Another telencephalic structure with strong reciprocal projections with the ACC is the basolateral amygdala (Gabbott et al. 2006; Matyas et al. 2014; Fillinger et al. 2017, 2018), likely involved in processing of emotional information (LeDoux 2000; Likhtik and Paz 2015), including fear conditioning (Erlich et al.

2012; Abiri et al. 2014) and affective aspects of pain (Hasanein et al. 2007; Veinante et al. 2013). An additional region which has reciprocal connections with the ACC, that likely participate in the modulation of emotional responses, is the hypothalamus (Risold et al. 1997; Floyd et al. 2001; Heidbreder and Groenewegen 2003; Gabbott et al. 2005; Fillinger 2017, 2018). Finally, the remaining afferents to the ACC from the basal forebrain are from the corticopetal system (mainly from the diagonal band of Broca, medial septal nucleus and the globus pallidus) (Heidbreder and Groenewegen 2003; Hoover and Vertes 2007; Fillinger et al. 2017), containing both cholinergic (Chandler et al. 2013) and GABAergic projections (Gracia-Llanes et al. 2010; McKenna et al. 2013), potentially involved in attentional processes (Burk and Sarter 2001; Sarter et al. 2001). On the other hand, the main target of ACC efferents to the striatum is the caudate putamen (Deng et al. 2015; Mailly et al. 2013, Fillinger et al. 2018), which might play a role in decision making processes (Friedman et al. 2015) and habitual actions (Yin et al. 2004).

Another prominent source of dense reciprocal projections to and from the ACC are the various nuclei of the thalamus (Hoover and Vertes 2007; Oh et al. 2014; Fillinger et al. 2017, 2018). Specifically, the anterior thalamic nuclei, in combination with hippocampal regions such as the enthorinal and retrosplenial cortices have an important role in spatial learning and navigation (Jankowski et al. 2013; Aggleton and Nelson 2015), as well as task-oriented attention (Wright et al. 2015). Moreover, the communication with the medial and posterior nuclei likely has a role in various different functions including, but not restricted to, working memory, decision making, fear conditioning, visual association (Weible 2013; Matyas et al. 2014; Mitchell 2015; Benarroch 2015), as well as nociceptive information transmission (Yang et al. 2006; Shyu and Vogt 2009).

Neuroanatomical tracing studies suggest that the ACC has long-range reciprocal connections with the brainstem, most notably with the monoaminergic regions such as the dopaminergic centers substantia nigra and the ventral tegmental area, the serotonergic raphe nuclei, and the noradrenergic area locus coeruleus (Gabbott et al. 2005; Chandler et al. 2013; Fillinger et al. 2017, 2018). Given the ACC involvement in processing nociceptive, emotional and affective information, these particular connections and their relation to monoaminergic neurotransmitters, potentially play a crucial modulating role in sensory and mood regulation, including the corresponding pathologies associated with it such as chronic pain and depression (Honda et al. 2007; Mulert et al. 2007).

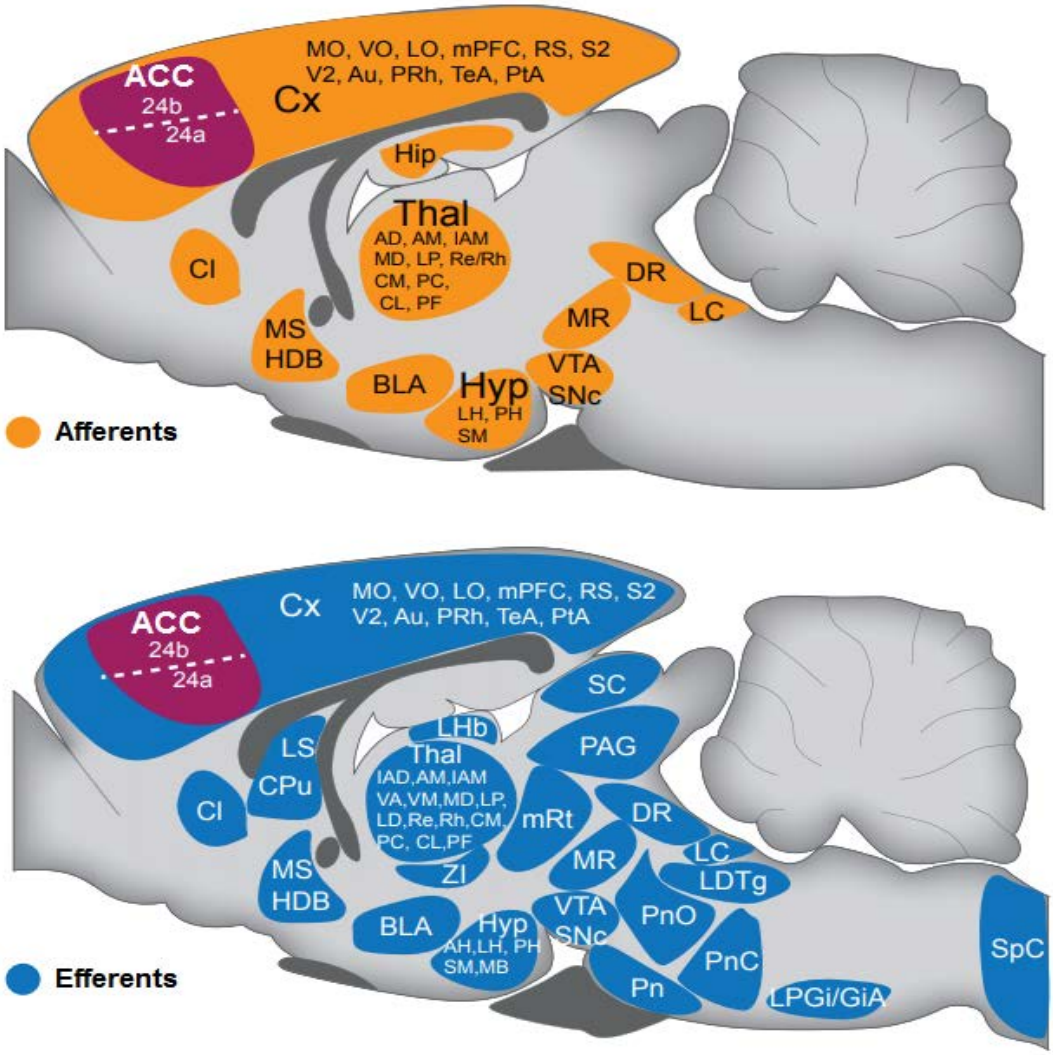


Figure 4: Schematic representation of the afferents/inputs (orange) and efferents/outputs (blue) of the rodent anterior cingulate cortex. Abbreviations: ACC, anterior cingulate cortex; AD, anterodorsal thalamic nucleus; AH, anterior hypothalamic area; AM, anteromedial thalamic nucleus; Au, primary auditory 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 septal 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, substantia 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)

Role in comorbid pain and depression

Pathological states which tend to frequently co-occur, including chronic pain and depression (Bair et al. 2003; Gustorff et al. 2008), should not be researched and managed in isolation in order to infer their individual contribution to the overall condition, but rather addressed and studied as one unique entity. Unfortunately, although there is a relatively substantial amount of both preclinical and clinical studies focusing on the role of the ACC independently in neuropathic pain (Hsieh et al. 1995; Peyron et al. 2004; Tseng et al. 2013; Ning et al. 2013; Tsuda et al. 2017), anxiety (Osuch et al. 2000; Etkin et al. 2011; Kim et al. 2011; Mochcovitch et al. 2014; Zhuo 2016) and depression (Mayberg et al. 2000; Bissiere et al. 2006; Konarski et al. 2007; Yucel et al. 2008; Drevets et al. 2008), the evidence specifically pertaining to the role of the ACC in the comorbidity of these conditions remains scarce.

Recent data from our team obtained with a mouse model of neuropathic pain-induced anxiodepressive-like behaviors, suggest that there is an increased firing rate and bursting activity within the ACC of animals displaying depressive-like behaviors (Sellmeijer et al. 2018). In addition, lesioning or temporal optogenetic inhibiton of the ACC prevents the anxiodepressive consequences of chronic pain without affecting the sensory mechanical allodynia (Barthas et al. 2015; Sellmeijer et al. 2018). This is parallel to human ablative surgeries of the ACC which have depression-alleviating results (Shields et al. 2008). Furthermore, neuropathic mice displaying anxiodepressive-like behaviors also show disrupted expression in the ACC, including an increase in the mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1), a key negative regulator of the MAPK pathway, which was reversed by the antidepressant fluoxetine (Barthas et al. 2017). Furthermore, by combining two well-established rat models, chronic constriction injury and chronic mild stress, Bravo et al. (2012) showed that the comorbidity of neuropathic pain and depressive-like behaviors is associated with a diminished neuronal density in the ACC. This is in accordance with previous clinical studies showing that patients suffering from neropathic pain and other chronic pain types display reduced grey matter volume in the ACC (Vartiainen et al. 2009; Rodriguez-Raecke et al. 2009; Ruscheweyh et al. 2011), while patients with MDD show a similar decrease in the subgenual ACC (Drevets et al. 1998; Botteron et al. 2002; Wagner et al. 2008), a subregion activated during negative affective experiences (Shackman et al. 2011).

Interestingly, a recent case report showed that a long-term therapy combining intravenous ketamine and transcranial magnetic stimulation of the ACC for a patient with severe comorbid depression, anxiety and chronic pain substantially reduced all pain- and

depression-related symptoms (Best and Pavel 2017). However, therapeutic success, particularly a positive response to antidepressant medication was shown to be associated with a distinctively different activity pattern in the ACC compared to a negative response (Mulert et al. 2007). Additionally, it has been suggested that subgenual ACC activity normalizes in responders to either an active antidepressant or placebo (Mayberg 2003), while a decreased function and volume of the ACC has been associated with a delayed response to antidepressant treatment (Chen et al. 2007). Hence, given the previous findings that both depression and chronic pain are related to decreased volume of the ACC (Botteron et al. 2002; Rodriguez-Raecke et al. 2009), as well as disrupted ACC activity (Jaworska et al. 2012; Xiao et al. 2019), this might give clues to why comorbid conditions contribute to treatment resistance in patients with depression (Al-Harbi 2012).

Finally, imaging studies suggest that similar neural mechanisms might be at play in depression and pain since negative effects of both physical and emotional distress are mediated by the ACC (Maletic and Raison 2009). Likewise, anticipatory anxiety about pain has also been reported to enhance pain, and the ACC seems to be among the most prominent regions facilitating this interaction (Wise et al. 2007; Wang et al. 2008; Zhuo 2016). Therefore, future research should focus more on studying the common neural substrates employed in co-existing conditions, as well as the role of individual components within those structures, such as the functional and molecular alterations in specific cell populations, which may be associated with a comorbid state.

Neuronal isolation methods

Cellular diversity and cytoarchitectural complexity found in the mammalian brain represent an obstacle to study in-depth the molecular characteristics of individual cell populations. Thus, an important challenge of many clinical and preclinical studies which aim to study specific cell types of interest is the isolation of the desired cell population. Since there is a variety of available techniques, each with its own advantages and disadvantages, choosing the most suitable method depends on several factors, aside from the researcher's aims, such as the availability of instruments, expense, duration, as well as the desired cell quantity and purity (Rahmanian et al. 2017). Although, over the years, an array of different methods have emerged which can separate cells based on different aspects, including size, volume, density, electrophysiological characteristics, light scattering properties, pH, cell contents (Orfao and Ruiz-Arguelles 1996; Almeida et al. 2014), here we will provide a general overview of a few prominent approaches frequently used for isolating neuronal populations of interest.

An affordable and relatively fast and highly reproducible method to separate a heterogeneous cell population is to separate different cells based on size and/or density. This can be achieved by using techniques such as sedimentation (Barkley et al. 1973; Battu et al. 2001), filtration (Unsain et al. 2014) or density gradient centrifugation (Goldenber and De Boni 1983; Graber and Harris 2013). While these techniques prove to be very efficient in isolating high yields of cells, the purity and homogeneity of the collected cells are low in comparison to other available techniques. However, these techniques may serve as an initial step before utilizing further more sophisticated isolation methods on the obtained sample (Jana et al. 2007).

Another category which can be employed to separate neuronal populations is based on the adhesion properties of the cells, when in contact with a solid surface. This was shown to be advantageous when separating glial cells from neurons (Giulian and Baker 1986; Jana et al. 2007), such as Schwann cells (Jirsová et al. 1997) or astrocytes (Goudariaan et al. 2014). Although these techniques are inexpensive and useful when large numbers of viable cells are needed, the limiting factor is that they generally do not provide high purity, especially if the heterogeneous sample contains populations of interest with similar adhesive properties (Almeida et al. 2014).

Furthermore, some methods exploit neuronal physiological or morphological characteristics to obtain the desired cell type of interest. For instance, a specific cell population can be cultured on a medium which selectively allows that target neuron type to

survive and grow, while inhibiting other populations (Needham et al. 1987; Kaech and Banker 2006). Reproducibility and ease of performance make this technique a valuable asset in cell-type specific studies, but it also has some limitations, including high cost of specialized media and contamination risk (Seibenhener and Wooten 2012). Another approach, which relies on the morphological properties of a given neuronal type, is laser capture microdissection, which utilizes a narrow beam laser to cleave cells of interest from brain tissue sections (Pietersen et al. 2011; Morris et al. 2018). This method assures good recovery and cell purity, however it is one of the most expensive tools available and requires extensive training and troubleshooting, hence the efficiency and yield, as well as the viability of the dissected cells, may vary between experiments (Almeida et al. 2014; Chung and Shen 2015; Zhu et al. 2018).

Finally, the most widely used techniques for the specific separation of one or more cell population from a heterogeneous sample belong to the broader category of antibody-mediated isolation methods (Almeida et al. 2014). The most widely used technique in this category is known as fluorescence-activated cell sorting (FACS), a specialized type of flow cytometry that physically separates different cell populations from a heterogeneous mixture of cells, based on the specific light scattering and fluorescent characteristics of those cells (Bonner et al. 1972). This method can employ both the antibody-antigen interaction, by using fluorescent antibodies which can bind to nuclear and non-nuclear epitopes (Guez-Barber et al. 2012; Martin et al. 2017), as well as transgenic animals expressing fluorescent proteins in specific cell types (Lobo et al. 2006). While FACS is a very precise technique due its individual cell analysis, it is less adapted for adult neuron isolation, and also requires expensive equipment and trained staff (Tomlinson et al. 2013). Another method which makes use of antibody-mediated cell labeling, and is considerably faster compared to FACS, is the magnetic-activated cell sorting (MACS). It uses magnetic particles coated with antibodies directed against specific antigens, which can then be separated from the sample with a magnetic field (Rembaum et al. 1982). A recent variation of this technique, which is also utilized in the present study, known as the translating ribosome affinity purification (TRAP) method uses magnetic beads to target translating ribosomes in order to study cell type-specific mRNA profiles of any genetically defined cell type (Heiman et al. 2014).

Overall, regardless of which method is utilized for cell purification, once the desired cell populations have been isolated, they can be used in further molecular analyses such as RNA sequencing to produce transcriptome databases of specific cell types under both normal and pathological circumstances (Zhang et al. 2014; Thomson et al. 2017).

GABAergic cells in pain and depression

Since the late nineteenth century and the pioneering work by Ramon y Cajal using the Golgi stain (Cajal 1899), it is evidenced that the brain is composed of an intricate network of heterogeneous cell populations.

The proper functioning of the mammalian neocortex relies on the coordinated dynamic interaction of two principal cell types: the excitatory and inhibitory neurons (Roberts 1974). Although it has been estimated that inhibitory neurons comprise only around 20% of the total neuronal population in the adult neocortex (Hendry et al. 1987; Sahara et al. 2012), they are found throughout the mammalian brain and their altered function has been long associated with various neurodevelopmental, cognitive and mood disorders including autism, epilepsy, schizophrenia and depression (Gerner and Hare 1981; Petty 1994; Baraban and Tallent 2004; Levitt et al. 2004; Cossart et al. 2005; Woo and Lu 2006). When activated, presynaptic inhibitory neurons release neurotransmitters that then bind to the receptors on the postsynaptic neuron, making it less likely to generate an action potential. The predominant neurotransmitter responsible for the local inhibition of other cortical neurons is the γ-aminobutyric acid (GABA) (Houser et al. 1983; DeFelipe and Fariñas 1992). GABA is synthesized from glutamate by glutamic acid decarboxylases GAD65 and GAD67 which are expressed in most brain regions (Soghomonian and Martin, 1998). Once released, GABA binds two main types of GABAergic receptors in order to dampen neurotransmission by decreasing the firing rate and neurotransmitter release at both the presynaptic and postsynaptic neuron (Watanabe et al. 2002). The fast ionotropic $GABA_A$ receptors, which are ligand-gated chloride (Cl^-) ion channels, are the primary target of GABA and their activation causes a Cl^--mediated hyperpolarization of the cell (Luján et al. 2005; Blednov et al. 2014). On the other hand, the slower metabotropic $GABA_B$ receptor, which are G protein coupled receptors, activate postsynaptic K^+ channels or inhibit presynaptic Ca^{2+} channels in order to prevent the cell from depolarizing and releasing neurotransmitters (Couve et al. 2000).

Alongside other physical and psychiatric pathologies, the fundamental importance of GABA transmission in the etiology and modulation of both depression and pain has been repeatedly demonstrated (Maletic and Raison 2009), and even led to the formulation of the GABAergic hypothesis of MDD (Lloyd et al. 1989; Luscher et al. 2011) and GABA disinhibition hypothesis of pain (Lau and Vaughan 2014).

Generally, depression is associated with a hypofunction of the GABAergic system (Mohler 2012; Pehrson and Sanchez 2015). It has been linked to reduced plasma GABA levels (Kalueff and Nutt 2007), and a decrease in GABAergic cells in the lateral orbital and

the dorsolateral PFC, structures known to play a role in cognition and emotion (Rajkowka et al. 2007). Moreover, reduced GABA levels were reported in the ACC and occipital cortex of patients with treatment-resistant depression, compared to both healthy controls and patients with non-treatment-resistant depression (Price et al. 2009). Interestingly, there is evidence from both preclinical and clinical research suggesting that antidepressant and electroconvulsive therapy can restore GABAergic deficits associated with depression (Gray and Green 1987; Green and Vincent 1987; Sanacora et al. 2002; Esel et al. 2008). However, there is also seemingly conflicting results pertaining to the role of GABA in successful depression treatments. Namely, by using a magnetic resonance spectroscopy to study the brains of recovered depression patients, Hasler et al. (2005) found no difference in the PFC GABA levels of unmedicated subjects with remitted MDD compared to healthy controls. On the other hand, Bhagwagar et al. (2008) reported that GABA levels were, in fact, decreased in the ACC and occipital cortex of recovered depressed subjects compared to healthy controls. These data suggests that further research is required to investigate the role of GABA in the remission and recovery of major depression.

In parallel, GABA is also indispensable in the pain signaling pathways, where it plays important modulatory roles ranging from dorsal horn interneuron communication, to the fine tuning of intricate cerebral networks which process pain signals and regulate the descending pathways (Giordano 2005; Jasmin et al. 2004). In particular, GABA is an important mediator in neuropathic pain (Jasmin et al. 2004) and a loss of GABAergic inhibitory control at the level of the spinal cord is closely associated with neuropathic pain develoment (Zeilhofer 2008; Basbaum et al. 2009). Namely, injury-induced neuropathic pain in rats has been associated with altered functioning of GABAergic interneurons and GABA receptors located in the dorsal horn (Moore et al. 2002; Wang et al. 2007). These neuropathy-related reductions in GABA-mediated inhibitory signaling in the dorsal horn (Moore et al. 2002) and the overall spinal GABAergic disinhibition associated with increased pain (Huang et al. 2012) might be related to apoptosis of GABAergic interneurons observed after peripheral nerve injury in rats (Scholz et al. 2005). However, this has been a matter of debate (Polgár et al. 2005) and thus needs additional clarification. Next, it seems that both $GABA_A$ and $GABA_B$ play a role in the pathophysiology of chronic pain (Goudet et al. 2009). Intrathecal administration of a $GABA_B$ receptor agonist has been shown effective in alleviating nerve ligation-induced neuropathic pain in rats, while administration of $GABA_A$ and $GABA_B$ antagonists was sufficient to induce allodynia and hyperalgesia in naïve (i.e. non-neuropathic) rats (Malan et al. 2002; Yalcin et al. 2011b). This suggests that proper GABA signaling in the spinal cord is required not only for

neuropathic pain processing, but also for discrimination between noxious and innocuous stimuli. In addition, preclinical research also suggests that intrathecal administration of a single dose of GABA during the early period of neuropathic pain development reduces pain symptoms (Eaton et al. 1999a; Stubley et al. 2001). Interestingly, a similiar effect is achieved by a transplantation of neuronal cells bioengeenered to synthesize GABA (Eaton et al. 1999b), GABA progenitors (Jergova et al. 2012) or neocortical precursors of forebrain GABAergic interneurons (Bráz et al. 2012) into the spinal cord of rodents with neuropathic pain.

The majority of neocortical GABAergic interneurons (INs) can be divided into three principal groups, depending on whether they express the Ca^{2+}-binding protein parvalbumin (PV), the neuropeptide somatostatin (SST) or the ionotropic serotonin receptor 3a (5-HT3aR) (Rudy et al. 2011). It has been estimated that PV INs account for 40% of GABAergic cells in the neocortex, while SST and 5-HT3aR INs account for approximately 30% each (Miyoshi et al. 2010; Xu et al. 2010; Lee et al. 2010; Tremblay et al. 2016).

It is important to note that these groups can be further divided into smaller interneuron populations depending on the co-presence of other markers, such as the neuropeptide cholecystokinin (CCK), the neuropeptide Y, and the Ca^{2+}-binding proteins calbindin and calretinin, which can be expressed in one or more of the three groups defined above (Rudy et al. 2011). In addition, 5-HT3aR INs are usually divided into two main subgroups depending on whether or not they express the vasointestinal peptide (VIP). While VIP-positive INs make up 40% (Lee et al. 2010), those that do not express VIP account for 60% of the 5-HT3aR INs and are mostly neurogliaform cells, principally populating the superficial neocortical layer I (Kawaguchi et al. 1997; Ascoli et al. 2008; Tremblay et al. 2016). However, due to the fact that the PV, SST and VIP INs constitute independent, nonoverlapping cell populations (Xu et al. 2010), their distinct morphological, electrophysiological, anatomical connectivity and molecular properties have been extensively studied in recent years (Hangya et al. 2014; Letzkus et al. 2015; Hattori et al. 2017). It has been shown that PV INs primarily form perisomatic connections with pyramidal neurons and with other PV INs throughout cortical layers II-IV (Kubota 2014), while SST INs mostly target distal dendritic regions of postsynaptic excitatory pyramidal cells and other INs, predominantly in the infragranular layers V and VI (Murayama et al. 2009; Xu et al. 2010) (Fig. 5). In contrast, VIP INs preferably synapse with and inhibit SST INs in layer II/III, thus creating a disinhibition of excitatory neurons (Lee at al. 2013; Pfeffer 2014) (Fig. 5). Such distinct organization outlines

the complex, yet distinct inhibitory effects each of these groups have on excitatory and inhibitory neurons throughout the cortex.

Although there is limited evidence directly connecting the activity of GABAergic neuronal subpopulations with specifically the comorbidity of pain and depression, there is preclinical and clinical data pointing to a role of dysfunctional interneuron subgroups in both pain and depression (Chrubasik 1991; Lai et al. 2016; Fee et al. 2017; Fogaça and Duman 2019). For example, MDD is associated with a decrease in *PV* gene expression in the subgenual ACC (Tripp et al. 2011), as well as PV neuron density in a subregion of the orbitofrontal cortex (Rajkowska et al. 2007). Also, PV-depleted mice show reduced nociceptive sensitivity in hot plate test, as well as a reduced social interaction (Wöhr et al. 2015). This is in accordance with data suggesting that selective ablation of PV INs in naive mice induces mechanical allodynia, while their activation results in reduced hypersensitivity in neuropathic mice (Petitjean et al. 2015). Furthermore, it has been demonstrated that chronic social stress and chronic mild stress reduce the number of PV cells in both the medial PFC and hippocampus of rats (Filipović et al. 2013; Czéh et al. 2015, 2018; Todorović et al. 2019).

Similarly, it has been shown that MDD patients have a reduced *SST* gene, mRNA and protein expression in the cerebrospinal fluid, subgenual ACC, dorsolateral PFC and amygdala, and that female subjects show a more pronounced reduction than males (Rubinow et al. 1985; Rajkowska et al. 2007; Sibille et al. 2011; Tripp et al. 2011; Guilloux et al. 2012). In addition, intravenous somatostatin infusions have been shown successful in relieving pain in patients suffering from cluster headaches (Sciuteri et al. 1984), while chronic intrathecal and epidural infusions alleviated pain from cancer patients (Meynadier et al. 1985; Mollenholt et al. 1994). Also, rats exposed to chronic stress display a decreased *Sst* gene expression in the medial PFC and neuron number in the hippocampus (Banasr et al. 2017; Czéh et al. 2015). Curiously, knocking out *Sst* in mice results in anxiodepressive-like behaviors (Lin and Sibille 2015), while activating SST INs in the somatosensory cortex of neuropathic mice prevents the development of mechanical allodynia after spared nerve injury (Cichon et al. 2017).

Likewise, it has been repeatedly shown that 5-HT3R-expressing neurons play an important role in mediating antinociception in the spinal cord (Alhaider et al. 1991; Maricq et al. 1991; Sasaki et al. 2001). Moreover, although the exact mechanism of action has not yet been fully elucidated, it is long known that 5-HT3Rs are potential targets for antidepressant drugs (Gupta et al. 2016). Indeed, preclinical rodent studies have shown that depressive-like behaviors can be reduced by the administration of pharmacological antagonist of 5-HT3Rs

such as tropisteron (Bravo and Maswood 2006), ondasetron (Ramamoorthy et al. 2008) and bemesetron (Kos et al. 2006), while some other, like vortioxetine, have also shown promising results in human MDD treatment (Thase et al. 2016). In addition, it has recently been found that amitriptyline, a tricyclic antidepressant commonly used to treat depression and neuropathic pain, blocks the 5-HT3 receptors, which might be one of its pain- annd depression- relieving mechanisms (Park et al. 2018).

Finally, Cichon et al. (2017) used a mouse model of neuropathic pain to demonstrate that pyramidal neurons in layer V of the somatosensory cortex were hyperactive as a result of reduced activity of PV and SST INs and an increased activity of VIP INs. This highlights the importance of the inhibitory and disinhibitory balance orchestrated by INs, which seems to be not only important in pain (Harding and Salter 2017), but also in other disorders (Zhang et al. 2016), including depression (Fuchs et al. 2017). Thus, further research should be directed at understanding interneuronal cross-talk and its effect on the overall activity of specific brain structures associated with both healthy and pathological states.

Nowadays, it is well established that there are significant differences among the different cell types and their subfamilies in terms of morphology, neurochemical composition, physiology, and other distinguishable features which set apart one group of cells from the other. However, the further we explore and identify new cell-type specific features, the greater the obstacle to understanding the organization and function of these cells in the complex circuits involved in different brain activities under normal and pathological circumstances. The astounding diversity in the physiological and biochemical properties of inhibitory neurons alone (Fritschy and Mohler 1995; DeFelipe 1997; Kawaguchi and Kondo 2002; Markram et al. 2004; Somogyi and Klausberger 2005) illustrates the perplexity in the balance between excitation and inhibition in normal and pathological brain functioning.

Hence, a substantial amount of research has focused on finding different methods for reliable and precise identification and manipulation of specific cell populations. These efforts resulted in the development of a variety of techniques which can help in finding answers pertaining to a wide range of questions, spanning from tissue composition (Siri 1993; Borga 2018), cell type identification and dissociation (Tomlinson et al. 2013) to single cell isolation and analysis (Hu et al. 2016), and even single nuclei analysis (Grindberg et al. 2013; Lake et al. 2016). Among the available tools, genetic targeting is among the most promising and efficient strategies which allows systemic access to specific cell types based on distinct regulatory mechanisms associated with particular cell populations. In combination with novel molecular, optical and electrophysiological technologies, genetic targeting can result in

precise labeling of a cell type of interest which enables further chemical, morphological, physiological and circuit analyses (Luo et al. 2008).

Therefore, although over the years, it has been suggested that more than 20 different types of inhibitory GABAergic neurons exist in the hippocampus and neocortex (Klausberger and Somogyi 2008; Tremblay et al. 2016) it was not until recent extensive transcriptomic analyses, that we got a glance at the full extent of the GABAergic cell diversity (Zeng and Sanes 2017; Tasic et al. 2018; Meyer et al. 2018). On top of the high variety and intervariability in gene expression under normal conditions, pathologies are associated with further genetic, genomic and epigenetic alterations (Smith and Flodman 2018). Due to these variations, alongside environmental circumstances, unique cell-type specific implications are at the heart of pathogenic conditions (Schor and Schor 2001). In addition, genomic profiling of neuronal cell types under normal (Sugino et al. 2006) and pathological conditions might help describe the critical temporal interval in the development of certain diseases.

Therefore, it is clear that further comprehensive genomic analyses of specific cell types in different disorders are required to reach a more extensive understanding of individual pathological conditions and the interaction between them. Such approach has the potential to yield more effective diagnostics and pathology-customized treatment options.

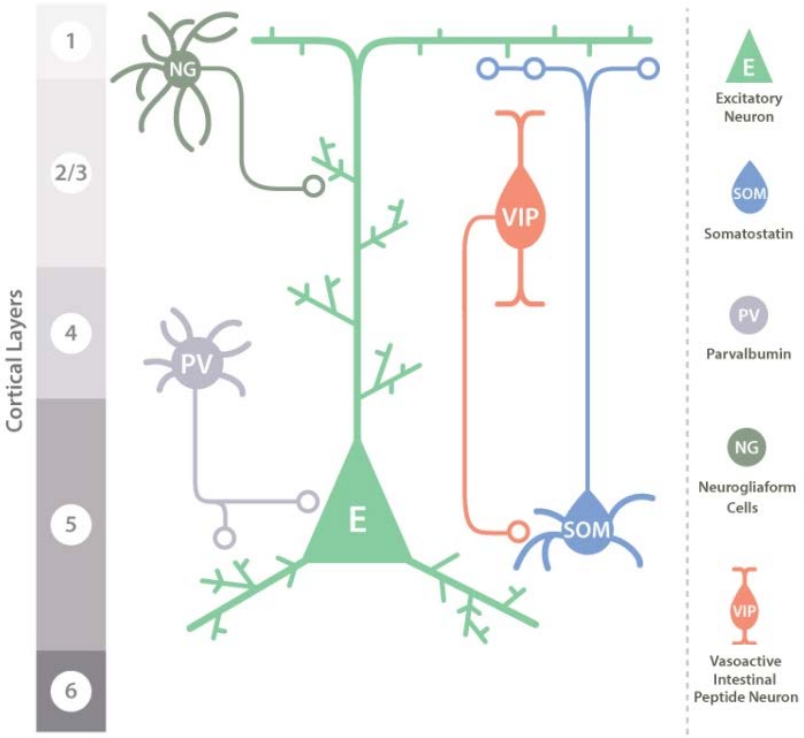


Figure 5: Schematic representation of major subgroups of GABAergic interneurons, their preferred connectivity and predominant location in the neocortical layers (adapted from: https://knowingneurons.com, illustration by: Jooyeun Lee)

Pharmacological treatment of comorbid pain and depression

There is a noteworthy overlap in the pathophysiology of depression and chronic pain pertaining to genetic, neurochemical and functional processes, resulting in comparable phenomenology observed in clinical practice (Narasimhan and Campbell 2010). Hence, it is not surprising that certain medication initially intended for the treatment of one disorder can be effective in treating the symptoms of the other condition.

For instance, it has been shown that the mild analgesic acetaminophen, also known as paracetamol, was successful in reducing distress of social rejection and the corresponding neural response recorded in the ACC and insula (Dewall et al. 2010), further supporting a shared behavioral and neural mechanism between social and physical pain (Eisenberger 2012). Similarly, it was shown that social rejection activated the opioid system in healthy subjects (Hsu et al. 2013). Although opioids, a globally used pain remedy (Kunnumpurath et al. 2018), have long been replaced by antidepressants in the treatment of mood disorders due to their highly addictive properties, there is still evidence showing their efficiency in treating depressive symptoms (Bodkin et al. 1995; Tenore 2008; Ehrich et al. 2015). Along these lines, buprenorphine, usually used at high doses for treating opioid addiction and pain symptoms, is successful in reducing suicidal ideation of depressed patients when administered at very low doses (Yovell et al. 2016). This medication was also shown effective in combination with samidorphan, a μ-opioid receptor antagonist, in decreasing depressive symptoms in patients with treatment-resistant major depression (Fava et al. 2016). Finally, preclinical studies showed that even low doses of morphine can reduce separation/isolation-induced distress vocalizations in juvenile animals (Panksepp et al. 1988; Kalin et al. 1988).

However, since it is generally advised to use non-opioid medication for the treatment of comorbid pain and depression (Dowell et al. 2016), a more common practice is the use of antidepressant medication in the treatment of neuropathic pain in patients with depression. Although antidepressant medication has been used in clinical settings as supplementary treatment for pain, their efficacy depends on the type being used, as well as on the pain condition itself (Watson 1994). Nevertheless, the analgesic properties of tricyclic antidepressants (TCA) and serotonin noradrenaline reuptake inhibitors (SNRI) place these pharmacological agents among the first line treatment options of neuropathic pain (Attal et al. 2010; Finnerup et al. 2010; Finnerup et al. 2015). Indeed, unlike the selective serotonin reuptake inhibitors (SSRIs), whose efficacy in the treatment neuropathic pain is moderate at best (Lee and Chen 2010), TCAs such as amitriptyline, nortriptyline, imipramine, and desipramine, and SNRIs venlafaxine, duloxetine, and milnacipran, have been shown effective

in treating comorbid neuropathic pain and depression (Haanpää et al. 2010; Lee and Chen 2010). One mechanism by which these effects are achieved may be the modulation of descending pain inhibition through serotonergic and noradrenergic projections coming from the brain (Testa et al. 1987; Viisanen and Pertovaara 2010; Wei et al. 2014). Likewise, even though antidepressants and anticonvulsants such as the gabapentinoids, which are initial treatments of choice for neuropathic pain, have different targets, they appear to have converging mechanisms in their mode of action. Indeed, both seem to effect some important pain modulatory processes such as decreasing central sensitization and activating descending noradrenergic inhibitory pathways (Kremer et al. 2016). Also, it has been shown that antidepressants elevate the levels of allopregnanolone (Nechmad et al. 2003; Pinna et al. 2006), a neurosteroid with anxiolytic and analgesic effects (Patte-Mensah et al. 2014). However, antidepressants used for chronic pain treatment such as TCAs amitriptyline (Moore et al. 2015) and imipramine (Tanay et al. 2001), or SNRIs such as duloxetine (Lunn et al. 2009) and venlafaxine (Aiyer et al. 2017), are usually prescribed without precision while the long-term efficacy and safety is still highly limited (Gierthmühlen and Baron 2016). In fact, more than 60% of Europeans who take pain prescription medication report inadequacy of their treatment, while a significant portion stops their treatment due to side effects (Breivik et al. 2006).

Therefore, it is imperative to find new treatment venues for addressing the debilitating conditions of comorbid chronic pain and depression. This could be through finding new compounds effective in ameliorating affective and painful symptoms, such as the plant-derived compound puerarin (Zhao et al. 2017) or patient-optimized interdisciplinary treatment which integrates existing methods ranging from psychological to pharmacological approaches (de Heer et al. 2018). Among promising experimental treatments for comorbid chronic pain and depression is also the noncompetitive N-methyl-d-aspartate (NMDA) antagonist ketamine (Doan et al. 2015). It has a wide range of use in clinical and non-clinical settings, spanning from anesthetic and analgesic benefits in surgical procedures, to recreational purposes, to cancer, postoperative and neuropathic pain management (Li et al. 2011). Interestingly, ketamine has been drawing increasing attention in the recent decades due to its fast, potent and robust antidepressant effects in patients with treatment-resistant major depressive disorder (Berman et al. 2000; Zarate et al. 2006; Murrough et al. 2012). Its versatility also reflects in the multitude of routes by which it can be administered including oral, nasal, rectal, intravenous, intrathecal, subcutaneous, intramuscular, epidural and transdermal (Hocking and Cousins 2003). Although ketamine has shown promising results in alleviating the detrimental

nociceptive and affective consequences of co-existent chronic pain and depression in both preclinical models (Wang et al. 2011; Zhang et al. 2016) and human patients with complex regional pain syndrome (Correll et al. 2004; Schwartzman et al. 2009), our current understanding of the efficacy of ketamine in the treatment of comorbid pain and depression remains scarce. Thus, alongside finding new treatment methods, future research should be directed at further delineating the physicochemical properties of already known compounds such as ketamine, which has already shown efficacy in treating both pain and mood disorders.

Research objectives

Although a substantial amount of literature has been accumulated over the past several decades pertaining to pain and emotion (Keefe et al. 2001), the comorbidity between them is insufficiently described. The high incidence of mood disorders in patients suffering from chronic pain (Bair et al. 2003), notably the high frequency of depression (34%) in neuropathic pain patients (Gustorff et al. 2008) causes this comorbidity to be the rule rather than the exception. Therefore, the present work aims at extending our knowledge and understanding about the molecular alterations associated with the co-existence of chronic neuropathic pain and anxiodepressive symptoms.

For this, our team utilizes a preclinical mouse model of neuropathic pain, employing sciatic nerve constriction, which results in prolonged mechanical allodynia and has been shown to induce anxiodepressive consequences in a time-dependent manner (Yalcin et al. 2011a; Barthas et al. 2015; Barthas et al. 2017). Our work primarily focuses on the ACC, a brain region known to process noxious stimuli, as well as the corresponding unpleasant emotional responses associated with it (Johansen et al. 2001), in order to make behavioral adjustments (Bush et al. 2000). With the help of various genomic, molecular and behavioral techniques, we set out to describe in more depth the role of the ACC in neuropathic pain-induced anxiodepressive like behaviors at the molecular and cellular level.

Hence, based on the published and preliminary data, the current thesis project was built around three general objectives:

1) To study the molecular alterations within the whole ACC using well validated animal models, and focus on a specific molecular target;

2) To study the therapeutic effect of ketamine on behavioral and molecular properties associated with comorbid neuropathic pain and depression;

3) To characterize the functional and molecular role of GABAergic neurons in the ACC in this comorbidity.

The first and third objective utilize an open genomic approach to identify the molecular blueprint of co-existing neuropathy and depressive behavior. While the first objective provides molecular targets at the level of the whole structure, the second one does the same at a cell-type specific level. Together, these approaches will yield specific molecular targets which can be further modified and analyzed in depth to discern their role in the development of the comorbidity, at both the cellular and the whole tissue level. The second objective concerns the effect of systemic ketamine administration on the behavioral and molecular characteristics associated with neuropathic pain-induced depressive-like behaviors.

With the completion of the current projects, we aim to acquire detailed insight about the molecular impact of chronic pain-induced depression on the ACC and the GABAergic neurons within. We intend to generate genomic maps that will shed light on the regional and neuronal type-specific cortical alterations in the given comorbidity. This approach may potentially provide a first step toward preclinical target validation that could ensure establishing a causal link between altered gene expression and the comorbidity of chronic pain and depression. Ultimately, such findings have a potential to lead to more patient-optimized treatment strategies.

Results

General overview

The following section contains three manuscripts with original data, obtained over the course of my master and doctoral education. The published and unpublished work presented in the following section is organized into individual scientific articles, each pertaining to one of the previously defined research objectives.

The first project titled „*Cingulate overexpression of mitogen-activated protein kinase phosphatase-1 as a key factor for depression*“ was started by my co-first author Florent Barthas during his last year of PhD thesis and was taken over by me in 2014 during my second year master internship. It looks at the molecular characteristics in the ACC related to pain and stress-induced depression and identifies a potential candidate for future research and treatment options. The manuscript was completed and published in 2017, in the 82nd volume of the Biological Psychiatry journal, where it was highlighted by an editorial comment (Di Benedetto 2017). Later, this publication was recommended by F1000 Prime and selected by the National center for scientific research (CNRS) Alsace delegation and the French society for the study and treatment of pain (SFETD) as a remarkable event of the year.

The second project presented in this section is titled „*Ketamine induces rapid and sustained antidepressant-like effects in chronic pain induced depression: role of MAPK signaling pathway*“. It extends the findings of the first by looking at the therapeutic effect of ketamine on the nociceptive and anxiodepressive-like behaviors induced by neuropathic pain. The study also focuses on the effect of ketamine on the molecular target identified in the first project. This work has been recently completed and submitted for publication.

The last project titled „*The role of ACC GABAergic cells in neuropathic pain-induced depression*“ represents a more in-depth approach to what has been done in the first project and a step further towards understanding the molecular underpinnings of comorbid chronic pain and depression. Compared to the earlier performed open genomic approach at the whole tissue level, here we employ an open transcriptomic analysis of a defined neuronal population. Additionally, we are interested in studying the relationship between the activation of this cell type and the behavioral phenotype in the aforementioned comorbidity. This project is still ongoing.

Biological Psychiatry

Cingulate Overexpression of Mitogen-Activated Protein Kinase Phosphatase-1 as a Key Factor for Depression

Florent Barthas*, Muris Humo*, Ralf Gilsbach, Elisabeth Waltisperger, Meltem Karatas, Samuel Leman, Lutz Hein, Catherine Belzung, Anne-Laurence Boutillier, Michel Barrot, and Ipek Yalcin

ABSTRACT

BACKGROUND: Depression is frequently associated with chronic pain or chronic stress. Among cortical areas, the anterior cingulate cortex (ACC, areas 24a and 24b) appears to be important for mood disorders and constitutes a neuroanatomical substrate for investigating the underlying molecular mechanisms. The current work aimed at identifying ACC molecular factors subserving depression.

METHODS: Anxiodepressive-like behaviors in C57BL/6J male mice were induced by neuropathic pain, unpredictable chronic mild stress, and optogenetic ACC stimulation and were evaluated using novelty suppressed feeding, splash, and forced swim tests. ACC molecular changes in chronic pain–induced depression were uncovered through whole-genome expression analysis. Further mechanistic insights were provided by chromatin immunoprecipitation, Western blot, and immunostaining. The causal link between molecular changes and depression was studied using knockout, pharmacological antagonism, and local viral-mediated gene knockdown.

RESULTS: Under chronic pain–induced depression, gene expression changes in the ACC highlighted the overexpression of a regulator of the mitogen-activated protein kinase pathway, mitogen-activated protein kinase phosphatase-1 (MKP-1). This upregulation is associated with the presence of transcriptionally active chromatin marks (acetylation) at its proximal promoter region as well as increased cyclic adenosine monophosphate response element–mediated transcriptional activity and phosphorylation of cyclic adenosine monophosphate response element binding protein and activating transcription factor. MKP-1 overexpression is also observed with unpredictable chronic mild stress and repeated ACC optogenetic stimulation and is reversed by fluoxetine. A knockout, an antagonist, or a local silencing of MKP-1 attenuates depressive-like behaviors, pointing to an important role of this phosphatase in depression.

CONCLUSIONS: These data point to ACC MKP-1 as a key factor in the pathophysiology of depression and a potential target for treatment development.

Keywords: Anterior cingulate cortex, Chronic pain, Chronic stress, Depression, MAPK, MKP-1

*equally contributed

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Besides chronic stress (1), chronic pain is also clinically associated with the development of mood disorders (2). Epidemiological studies report a mean prevalence rate of around 50% for major depressive disorder in patients with chronic pain (3). Preclinical research further revealed that the anxiodepressive consequences of chronic pain can be studied in murine models (4,5) and highlighted the time dependency of these affective phenotypes (6). These models now offer a reliable tool to explore original mechanisms leading to depression.

A conceptualized view of depression relates this pathology to specific structural and functional changes in the brain neurocircuitry. Among the candidate regions, the anterior cingulate cortex (ACC) appears to be critical because it is known to display functional and morphological alterations in depressed patients (7) such as decreased connectivity to the amygdala (8), altered glucose metabolism (9), and reduced gray matter volume (10). Preclinical studies also showed functional and morphological alterations in the ACC following chronic stress exposure (11,12). Recently, we reported that the optogenetic activation of pyramidal neurons within the ACC is sufficient to induce anxiety and depressive-like behaviors in naïve animals (13). Furthermore, the lesion of the ACC prevents chronic pain–induced depressive-like behaviors and the aversiveness of spontaneous pain without affecting the sensory mechanical sensitivity (13). Thus, the ACC is a critical hub for mood disorders, including anxiodepressive consequences observed in chronic pain, and for studying their underlying molecular aspects.

In this context, open approaches such as genome-wide studies can be powerful to identify molecular blueprints of

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depression. Here, we first performed a whole microarray analysis within the ACC in both sham and sciatic nerve–injured animals. Based on this genome expression analysis, the current study then focused on one critical regulator of the mitogen-activated protein kinase (MAPK) pathway, MAPK phosphatase-1 (MKP-1), in chronic pain–induced depression. Namely, our microarray results indicate that *Mkp-1* is significantly upregulated in the ACC of sciatic nerve–injured animals. Reports of MKP-1 overexpression in the hippocampus of stressed animals (14,15), as well as in patients with major depressive disorder (14), supported our interest to further investigate MKP-1 in chronic pain and mood disorder comorbidity.

MKP-1, also known as dual specificity phosphatase 1, is the main negative regulator of the MAPK signaling cascade (14,16). Cell culture studies showed that the expression of MKP-1 can be induced by a wide variety of extracellular factors such as growth factors, lipopolysaccharides, heat shock, and hydrogen peroxide (17). Studies focusing on the mechanisms of MKP-1 expression suggest that its transcription can be preceded by chromatin remodeling at the promoter region. External stressors (e.g., arsenite or ultraviolet C) can initiate phosphorylation and acetylation of histone H3 at the *Mkp-1* promoter region (17) and/or activate several transcription factors such as cyclic adenosine monophosphate response element binding protein (CREB), activating transcription factors 1 and 2 (ATF1 and -2), and activator protein 1 (18,19), which leads to the induction of *Mkp-1*.

These data raise questions about whether ACC MKP-1 is a key factor in depression pathophysiology, particularly for chronic pain–induced depression. Thus, using animal models, we studied the link between MKP-1 and anxiodepressive-like behaviors, identified molecular upstream mechanisms leading to its increased expression in the ACC, and tested the therapeutic potential of targeting this phosphatase. We show that neuropathic pain (NP)–induced depressive-like behaviors are associated with an increase in c-Fos expression, an increase in phosphorylated CREB and phosphorylated ATF levels in the ACC, and increased histone H3 lysine 9/lysine 14 (H3K9/K14) acetylation at the promoter regions of *C-fos* and *Mkp-1*. We further demonstrate that ACC MKP-1 overexpression is present in several animal models of depression, suggesting a general link between depression and increased level of ACC MKP-1. We also show that chronic treatment with a classical antidepressant drug, fluoxetine, suppresses the increase in MKP-1 levels within the ACC. Moreover, knocking out, antagonizing, or locally silencing its presence in the ACC attenuates depressive-like behaviors induced by NP, pointing to an essential role of MKP-1.

METHODS AND MATERIALS

Animals

Approximately 350 adult male C57BL/6J mice (Charles River, L'Arbresle, France) were used in all experiments. For the optogenetic experiments, we used Thy1-ChR2-YFP mice (13,20). The cyclic adenosine monophosphate response element (CRE)–related activity was examined by using CRE-LacZ mice (21) (see Supplement). Protocols were approved by the local ethical committee of the University of Strasbourg (No. 2015012909428166) and Comité d'expérimentation animale du Val de Loire (No. 19).

Surgical Procedures

All surgical procedures were done under general anesthesia (ketamine/xylazine, 68/10 mg/kg, intraperitoneally; Centravet, Taden, France). Viral transfection and optogenetic procedures used standard in vivo stereotaxic procedures, and coordinates for the ACC (areas 24a and 24b) were 0.7 mm anterior and 0.3 lateral to the bregma, based on the Mouse Brain Atlas (22).

NP Model and Nociceptive Testing

Chronic NP was induced by placing a polyethylene cuff around the right common sciatic nerve of the animal (23). The control group (sham) underwent the same procedure without cuff implantation. The mechanical sensitivity was scored using von Frey filaments (Bioseb, Vitrolles, France) (see Supplement).

Unpredictable Chronic Mild Stress Model

Mice were subjected to a variety of stressors several times a day/night for 8 weeks, including altered cage and bedding, altered light/dark cycle, cage tilting (45°), and predator smell exposure (24,25). At the end of 8 weeks, the novelty suppressed feeding (NSF) test was performed and the ACC was harvested for protein analyses (see Supplement and Supplemental Table S3).

Anxiodepressive-Related Behaviors

Behavioral phenotyping was performed by using the NSF, splash, and forced swim tests (FST) (see Supplement).

Optogenetic

Independent sets of Thy1-ChR2-YFP mice implanted with optic fibers were tested either 5 minutes and 1 day after a single stimulation or 1 day and 2 weeks after four consecutive (30 min/day) stimulations. Animals were stimulated with a blue light–emitting diode (peak wavelength: 460 nm; intensity: 4–6 mW) (see Supplement).

Viral-Mediated Gene Knockdown

Three weeks after sciatic nerve surgery, NP- or sham-operated animals received either lentiviral vectors expressing a set of *Mkp-1* small interfering RNA (siRNA)/short hairpin RNA/RNA-mediated interference and green fluorescent protein under the cytomegalovirus promoter (Applied Biological Materials, Richmond, BC, Canada) or a scrambled version of the virus, bilaterally (2 μL/site) into the ACC. Four weeks after transfection, behavioral tests were conducted on three groups: 1) sham-operated (control for NP) mice that express siRNA (sham/siRNA), 2) mice displaying NP-induced anxiodepressive-like behaviors administered with scramble virus (NP/control), and 3) mice displaying NP-induced anxiodepressive-like behaviors expressing siRNA (NP/siRNA). In a separate experiment, the impact of local deletion of ACC MKP-1 was assessed in naïve animals. Details are shown in the Supplement.

Pharmacological Agents

The selective serotonin reuptake inhibitor fluoxetine (20 mg/kg/day) mixed with 0.2% saccharine was administered in drinking water for 3 weeks (starting 5 weeks after the sciatic nerve injury), while the control group drank 0.2% saccharine. The MKP antagonist dusp-(*E*)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1*H*-inden-1-one (BCI) dissolved in 2% dimethyl sulfoxide was administered systemically (10 mg/kg, intraperitoneally, twice a day) for 4 days, followed by a behavioral test 1 hour after the last injection. Control animals received 2% dimethyl sulfoxide. All drugs were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France) (see Supplement).

Tissue Harvesting and Analysis

For genomic analysis, chromatin immunoprecipitation (26), and Western blot, all animals were killed by cervical dislocation and the ACC, primary somatosensory cortex, and whole hippocampus were harvested and stored at −80°C. For detailed procedures of the microarray analysis, chromatin immunoprecipitation, Western blot, and immunostaining, see the Supplement.

Statistical Analysis

Results are expressed as mean ± SEM. Statistical analyses were performed with Statistica 7.1 (StatSoft, Tulsa, OK) by using unpaired Student's *t* tests or multifactor analysis of variance with independent or repeated measures and Duncan post hoc analyses. Immunoblotting experiments were analyzed with the nonparametric Kruskal-Wallis test, followed by multiple comparisons with the Wilcoxon or Mann-Whitney *U* test when data did not fit with the rules of parametric analyses. The significance level was set at $p \leq .05$. For detailed information, see Supplemental Table S4.

RESULTS

Mkp-1 Messenger RNA and Protein Levels Increase in the ACC of NP Mice Displaying Depressive-like Behaviors

To characterize molecular changes within the ACC, we conducted a whole-genome expression analysis in NP-induced depressed-like animals. The ACC tissues were collected at 8 weeks after sciatic nerve surgery, which corresponds to the presence of both nociceptive and anxiodepressive-like phenotypes, as illustrated by decreased mechanical thresholds in the von Frey test (Figure 1A; $F_{7,70} = 6.04$, $p \leq .001$; post hoc: weeks 1–7, $p \leq .001$) and increased latency to feed in the NSF test (Figure 1B; $p \leq .01$), respectively. At this time point, no change in food intake or in spontaneous locomotor activity was present in NP mice (Supplemental Figure S1A). The microarray data revealed several changes in gene expression within the ACC (Figure 1C and Supplemental Table S1). A Kyoto Encyclopedia of Genes and Genomes signaling pathway analysis from WebGestalt highlighted a major alteration in the MAPK pathway (Figure 1D; $p = 1.40e\text{-}06$), in particular concerning its negative regulator *Mkp-1*, whose expression was robustly upregulated in the ACC of animals displaying anxiodepressive-like behaviors (1.7-fold) (Figure 1E; $p \leq .01$). This finding was then confirmed by Western blot analysis, showing that the increase was also present at protein level in NP mice within the ACC (Figure 1F; $p \leq .001$; see also Supplemental Figure S1B) but not in the primary somatosensory cortex and whole hippocampus (Supplemental Figure S2). Interestingly, microarray data showed that *Mkp-1* was already upregulated at the messenger RNA (mRNA) level 2 weeks after sciatic nerve surgery in animals displaying nociceptive hypersensitivity but not yet detectable anxiodepressive-like behaviors (Supplemental Figure S3A; $p \leq .001$; see also Supplemental Table S2). However, this upregulation was not significant at the protein level at this 2-week early time point (Supplemental Figure S3B), but it slightly increased at 5 weeks postsurgery (Supplemental Figure S3C; $p = .10$), when animals displayed anxiety-like behavior (6) in the light/dark box (Supplemental Figure S3C; $p \leq .001$).

NP-Induced Depressive-like Behaviors Are Associated With Transcription Factors' Recruitment

To identify potential upstream factors responsible for *Mkp-1* overexpression, we looked at the involvement of relevant transcription factors within the genomic data. Based on transcription factor target analyses from WebGestalt, we focused on CREB (Figure 2A; $p = 2.55e\text{-}08$) and ATF-1 (Figure 2A; $p = 4.76e\text{-}12$) as well as on Fos proteins (Figure 2A; $p = 5.02e\text{-}10$, serum response factor) that displayed significant changes in the microarray data (for *C-fos* and *Fosb*: 1.94- and 1.47-fold increases in NP animals, respectively). CREB and ATF are known to bind CREs at the *Mkp-1* promoter region (27), and functional activator protein 1 binding sites are also present within this promoter region (18).

Western blot analyses confirmed that both phosphorylated forms of CREB (Figure 2B; $p \leq .05$) and ATF-1 (Figure 2B; $p \leq .01$) increased in the ACC of NP animals. To have a functional assessment of CREB/ATF activity, we used CRE-LacZ transgenic reporter mice. By using a line of CRE-LacZ mice displaying very high basal reporter activity in the cortex, we previously reported no alteration in ACC CRE-mediated transcription (6). However, this could have been related to a ceiling effect preventing effective detection of increased activity in this brain region. Thus, here we used another CRE-LacZ line with lower basal reporter activity. In this line, β-galactosidase immunostaining showed a significant increase in the presence of CRE-positive cells in the ACC of NP animals (Figure 2C, D; $F_{16,48} = 3.98$, $p < .001$; post hoc: −0.47 to +1.41 from the bregma; $p \leq .05$) but not in the prelimbic (A32) and infralimbic cortices (Supplemental Figure S4). Because *Fos* expression was upregulated by NP in our microarray analysis (Figure 1C and Supplemental Table S1; $p < .05$), we also confirmed increased c-Fos protein levels in the ACC of NP animals (Figure 2E, F; $F_{14,48} = 5.75$, $p < .001$; post hoc: −0.47 to +1.33 from the bregma; $p \leq .001$). This alteration was also observed in the rostal part of the infralimbic cortex (A25) ($F_{1,5} = 2.47$, $p < .05$; Supplemental Figure S4) but not in the prelimbic cortex.

NP-Induced Depressive-like Behaviors Are Associated With Epigenetic Changes at *Mkp-1* and *C-fos* Promoters in the ACC

As immediate early genes, it is intriguing that *C-fos* and *Mkp-1* are still present at high levels in the ACC 8 weeks after NP induction,

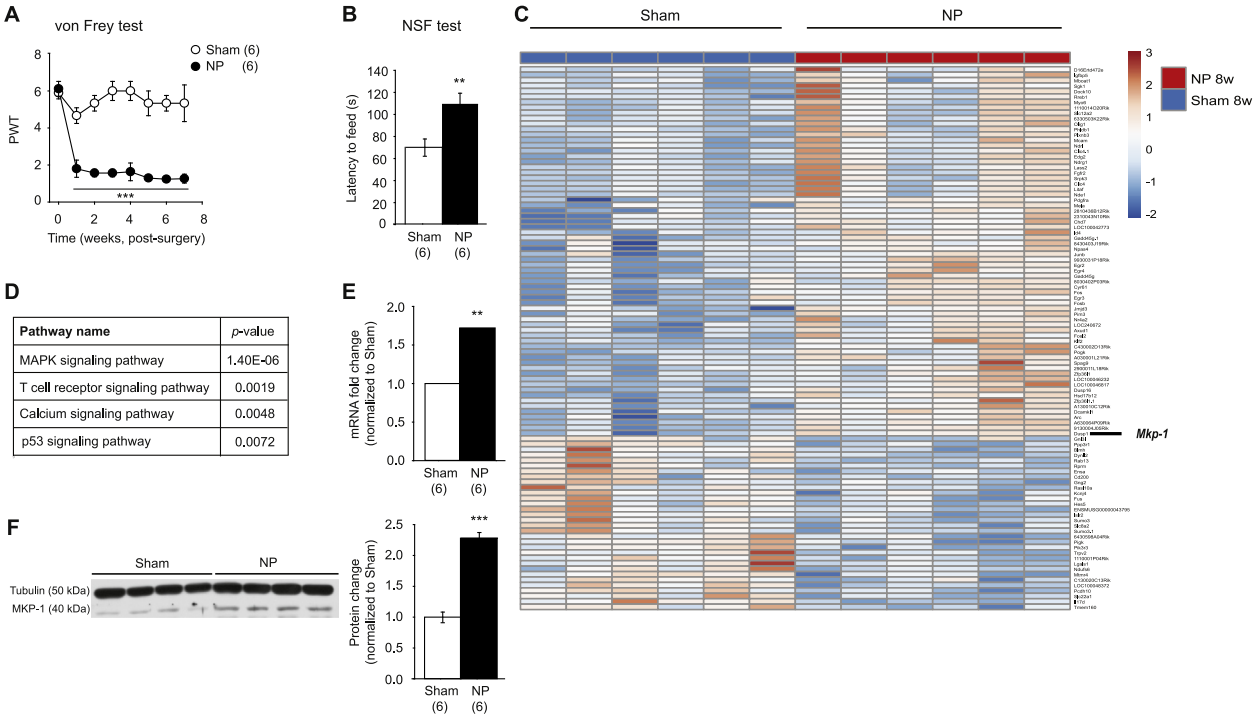

Figure 1. After 8 weeks of sciatic nerve compression, mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) messenger RNA (mRNA) and protein levels increase in the anterior cingulate cortex (ACC) of neuropathic pain (NP) animals displaying depressive-like behaviors. **(A)** von Frey test shows long-lasting ipsilateral allodynia, as evidenced by decreased mechanical thresholds of the right paw of NP animals. **(B)** NP animals display an anxiodepressive-like behavior, as shown by an increased latency to feed compared with their sham-operated littermates in the novelty suppressed feeding (NSF) test 8 weeks after surgery. **(C)** Heatmap representing dysregulated genes in the ACC 8 weeks after the surgery. **(D)** Results of Kyoto Encyclopedia of Genes and Genomes signaling pathway analysis showing that the MAPK pathway was most significantly enriched. **(E)** Microarray result showing an overexpression of *Mkp-1* (1.7-fold) in the ACC of NP animals compared with controls. **(F)** Western blot results illustrating an upregulation of ACC MKP-1 protein levels in animals displaying NP-induced depressive-like behaviors. Data are expressed as mean ± SEM; $^{**}p \leq .01$, $^{***}p \leq .001$. PWT, paw withdrawal thresholds.

and we tested whether specific epigenetic alterations relevant for active gene transcription could be found at their promoters. We used chromatin immunoprecipitation followed by quantitative polymerase chain reaction to look for induction of specific acetylated marks on H3K9/K14ac, a marker of transcriptionally active chromatin found mainly in the proximal promoter/transcription start site (TSS) regions, and histone H3 lysine 27 (H3K27ac), a mark of both active enhancers and TSS regions (28,29). We controlled immunoprecipitation enrichment and specificity for H3K9/K14ac and H3K27ac on *Gapdh* as ubiquitously expressed positive control and *Tsh2b* as negative control (i.e., not expressed in the brain) (Supplemental Figure S5). Both histone marks were enriched on *Mkp-1* and *C-fos* promoter/TSS regions, with H3K9/K14ac (Figure 2G), but not H3K27ac (Figure 2H), showing a significant increased enrichment on these promoters in the NP group ($p \leq .05$). Together, these data show that 8 weeks after the surgery, NP maintains active epigenetic regulations on the *Mkp-1* gene.

MKP-1 Level Increases in Other Models of Depression

We then assessed whether NP-induced alterations in ACC MKP-1 levels could be generalized to other models of depression. Hence, mice were subjected to unpredictable chronic mild stress (UCMS) during 8 weeks (see Supplemental Table S3) (25). Similarly to what was observed in NP animals, mice that underwent the UCMS procedure displayed an increase in both anxiodepressive-like behavior in the NSF test (Figure 3A; $p \leq .001$) and MKP-1 protein level in the ACC (Figure 3B; $p \leq .01$). Moreover, we previously showed that sustained activation of pyramidal neurons of the ACC leads to anxiodepressive-like behavior in naïve Thy1-ChR2-YFP mice (13). By using the same protocol, we determined the influence of ACC activation on MKP-1 levels. Repeated, but not single (Supplemental Figure S6A), optogenetic activation of the ACC of naïve Thy1-ChR2-YFP mice (Figure 3C) induced depressive-like behavior 1 day after the last stimulation, as shown by the increased latency to eat in the NSF test (Figure 3D; $p \leq .01$). More notably, this repeated optogenetic stimulation of the ACC also increased local MKP-1 levels (Figure 3E, F; $p \leq .05$). The anxiodepressive-like behaviors totally disappeared 2 weeks after the last stimulation (Supplemental Figure S6B), while MKP-1 levels still remained significantly high (Supplemental Figure S6C). However, this upregulation was lower than that obtained on day 1 following the last consecutive stimulation (1.54 ± 0.15-fold vs. 2.29 ± 0.63-fold; $p = .06$) (Figure 3E).

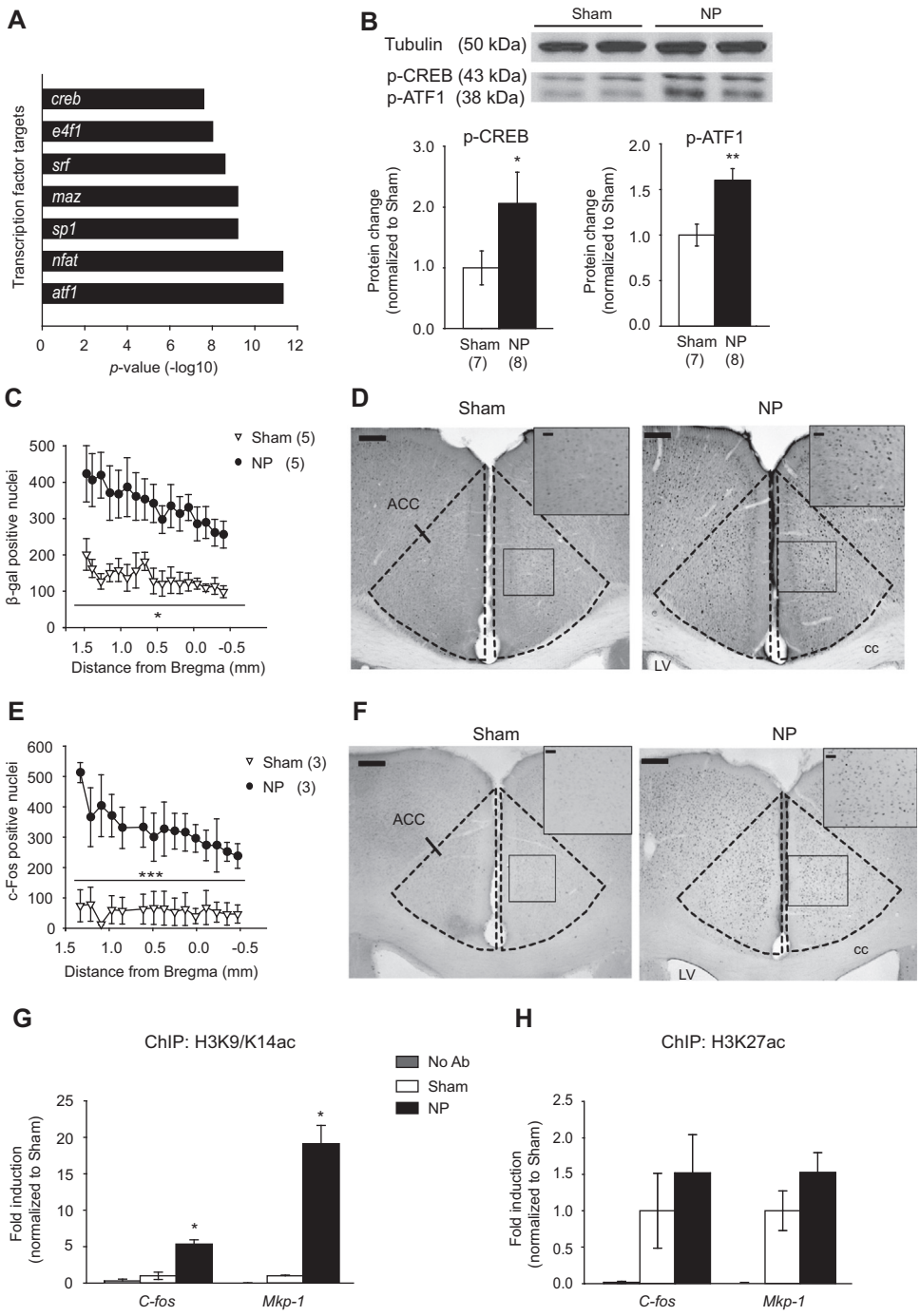

Figure 2. Neuropathic pain (NP)–induced depressed animals display enhanced cyclic adenosine monophosphate (cAMP)–driven transcriptional activity and H3 acetylation at the promoter of *C-fos* and *Mkp-1* in the anterior cingulate cortex (ACC). **(A)** Microarray-based transcription factor target analysis showing the 7 most probable transcription factors regulating the prominently changed genes in the ACC of animals displaying NP-induced depressive-like behaviors. **(B)** Western blot analysis showing an increase in phosphorylated cAMP response element binding protein (p-CREB) and phosphorylated activating transcription factor (p-ATF) in the ACC of the NP group. **(C)** Quantitative representation of β-galactosidase (β-gal)-positive nuclei in the ACC of sham and NP animals at various distances from the bregma, showing a higher presence of CRE positive cells in the ACC of NP animals after 8 weeks of sciatic nerve compression. **(D)** Representative pictures comparing the expression and distribution of β-gal labeling in the ACC of sham and NP CRE-LacZ mice. Large scale bar = 300 μm, inset scale bar = 30 μm. **(E)** Increased c-Fos expression in NP animals compared with sham animals after 8 weeks of sciatic nerve compression. **(F)** Representative pictures comparing the expression and distribution of c-Fos-positive cells in the ACC of sham and NP animals. Large scale bar = 300 μm, inset scale bar = 30 μm. **(G, H)** Quantitative polymerase chain reaction results from chromatin immunoprecipitation (ChIP) experiments performed in the ACC of NP animals compared with sham, demonstrating the presence of histone H3 lysine 27 acetylation (H3K27ac) at the proximal promoter/transcription start site regions of *C-fos* and *Mkp-1* and a significant increase in histone H3 lysine 9/lysine 14 acetylation (H3K9/K14ac) binding on both genes in the NP group. No Ab, no antibody. Data are expressed as mean ± SEM; $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$. cc, corpus callosum; LV, lateral ventricle.

Fluoxetine Decreases ACC MKP-1 Levels

Chronic oral treatment (3 weeks) with the selective serotonin reuptake inhibitor fluoxetine did not affect mechanical hypersensitivity (Figure 4A; $F_{1,44} = 0.65$, $p = .42$), but it blocked the anxiodepressive-like behavior in the NSF test (Figure 4B; $F_{3,42} = 14.10$, $p \leq .001$; post hoc: NP vehicle > sham vehicle, $p \leq .001$; NP fluoxetine < NP vehicle, $p \leq .001$). Interestingly, fluoxetine treatment also significantly decreased the ACC MKP-1 in the NP and sham groups (Figure 4C; $H = 13.93$, $p \leq .01$; post hoc: sham vehicle < NP vehicle, NP vehicle > NP fluoxetine, sham vehicle > sham fluoxetine, $p \leq .01$).

MKP-1-Deficient Mice Are Resistant to NP-Induced Depression

To investigate the causal link between MKP-1 and depression, we obtained *Mkp-1*$^{-/-}$ mice from the laboratory of Andrew

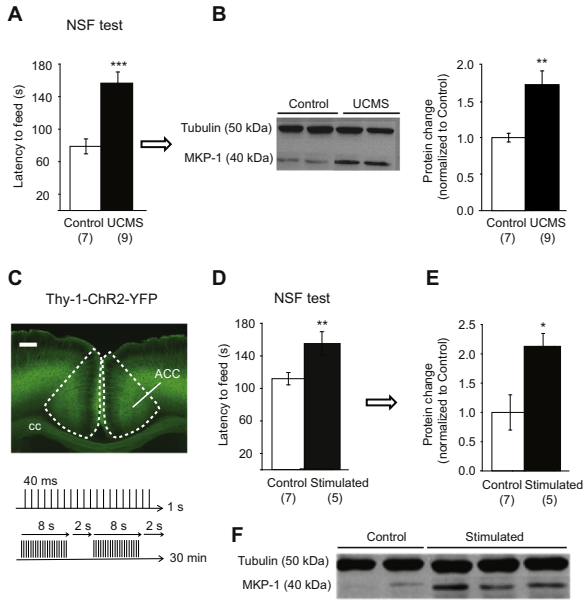

Figure 3. Anterior cingulate cortex (ACC) mitogen-activated protein kinase phosphatase-1 (MKP-1) levels increase in other models of depression. **(A)** Novelty suppressed feeding (NSF) test performed after unpredictable chronic mild stress (UCMS) procedure illustrating an anxiodepressive-like behavior in stressed animals compared with nonstressed animals, as shown by an increased latency to feed. **(B)** Western blot analysis showing increased levels of ACC MKP-1 in stressed animals compared with nonstressed animals. **(C)** Representative picture of the ACC in the Thy-1-ChR2-YFP mice (top) and a scheme of the stimulation protocol used (20 Hz; 4 days; 30 min/day; 8 seconds stimulation; 40-ms pulses; 2 seconds no stimulation) (bottom). Scale bar = 300 μm. **(D)** NSF test at day 5 after ACC stimulation showing that stimulated animals display an anxiodepressive-like behavior, as shown by an increased latency to feed. **(E, F)** Western blot results illustrating an upregulation of ACC MKP-1 in stimulated animals compared with controls. Data are expressed as mean ± SEM; $^{*}p \leq .05$, $^{**}p \leq .01$, $^{***}p \leq .001$. cc, corpus callosum.

Cato (30) and bred them in our animal facilities. We characterized the nociceptive sensitivity and anxiodepressive-like behavior in *Mkp-1*$^{-/-}$ and *Mkp-1*$^{+/+}$ animals before and after NP induction. It is important to note that we did not observe any alteration in the mechanical paw withdrawal thresholds in *Mkp-1*$^{-/-}$ mice compared with *Mkp-1*$^{+/+}$ mice before surgery (Figure 5A; $F_{1,43} = 3.33$, $p > .05$). After surgery, NP animals developed mechanical allodynia regardless of their genetic background (Figure 5B; $F_{3,123} = 50.25$, $p \leq .001$), showing that the loss of MKP-1 expression did not modify the NP somatosensory component. However, by using the NSF and splash tests, we showed that *Mkp-1*$^{-/-}$ mice did not display the anxiodepressive-like behaviors normally induced by NP. Indeed, the loss of MKP-1 suppressed the NP-induced increased latency to feed in the NSF test (Figure 5C; $F_{1,41} = 4.09$, $p \leq .05$, sham < NP in *Mkp-1*$^{+/+}$, sham = NP in *Mkp-1*$^{-/-}$). Similarly, *Mkp-1*$^{-/-}$ NP animals did not display decreased grooming behavior in the splash test (Figure 5D; $F_{1,41} = 12.60$, $p \leq .001$, sham < NP in *Mkp-1*$^{+/+}$, sham = NP *Mkp-1*$^{-/-}$). Interestingly, *Mkp-1* deficiency had no effect per se on behavioral tests in sham *Mkp-1*$^{-/-}$ mice (Figure 5C, D).

BCI, an MKP Antagonist, Reduces NP-Induced Depressive-like Behavior

Beyond constitutive depletion of MKP-1, we assessed whether a reversible pharmacological blockade of this phosphatase could also lead to antidepressant-like effects by testing a systemic subchronic treatment (4 days, intraperitoneally) with MKP-1/6 antagonist BCI (31). Before treatment, NP animals showed an increased latency to feed in the NSF test (Figure 5E; $F_{1,20} = 10.34$, $p \leq .001$; post hoc: NP > sham, $p \leq .001$), thereby confirming the presence of an anxiodepressive-like state. Subsequent BCI treatment increased the grooming time of NP animals in the splash test (Figure 5E; $F_{1,19} = 3.72$, $p \leq .05$; post hoc: NP saline < NP BCI, $p \leq .01$) without affecting their mechanical sensitivity threshold (Figure 5F; $F_{2,23} = 92.2$, $p \leq .001$; post hoc: NP BCI right < sham right, $p \leq .001$; NP saline right < sham right, $p \leq .001$).

Local Suppression of MKP-1 Within the ACC Blocks NP-Induced Depression

While constitutive MKP-1 depletion and pharmacological antagonism brought information for evaluating the link between MKP-1 and depression, they lack neuroanatomical selectivity. Thus, we performed a local viral-mediated manipulation (Figure 5G) of *Mkp-1* to identify its role in the ACC.

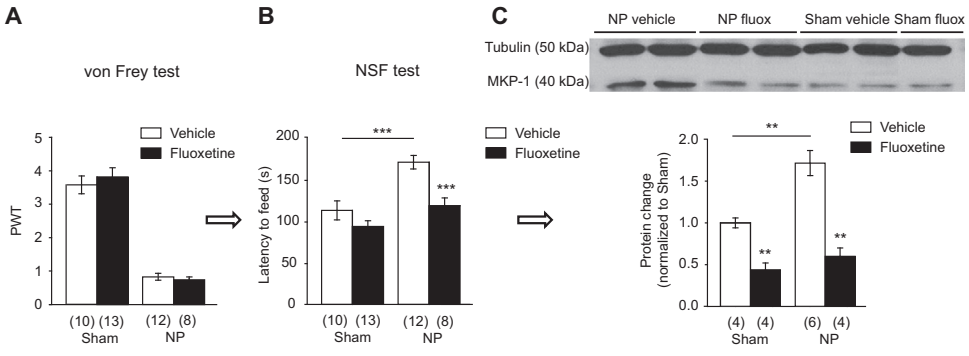

Figure 4. Prolonged fluoxetine treatment blocks anterior cingulate cortex (ACC) mitogen-activated protein kinase phosphatase-1 (MKP-1) upregulation. **(A)** von Frey results for the right hind paw showing that fluoxetine treatment (3 weeks) did not affect the development of allodynia after neuropathy induction. **(B)** Novelty suppressed feeding (NSF) test results illustrating that fluoxetine treatment decreases the anxiodepressive-like behavior in neuropathic pain (NP) animals, as shown by a lowered latency to feed. **(C)** Western blot results demonstrating a decrease in MKP-1 in the ACC of both sham and NP animals after fluoxetine treatment. Data are expressed as mean ± SEM; $^{**}p \leq .01$, $^{***}p < .001$. PWT, paw withdrawal thresholds.

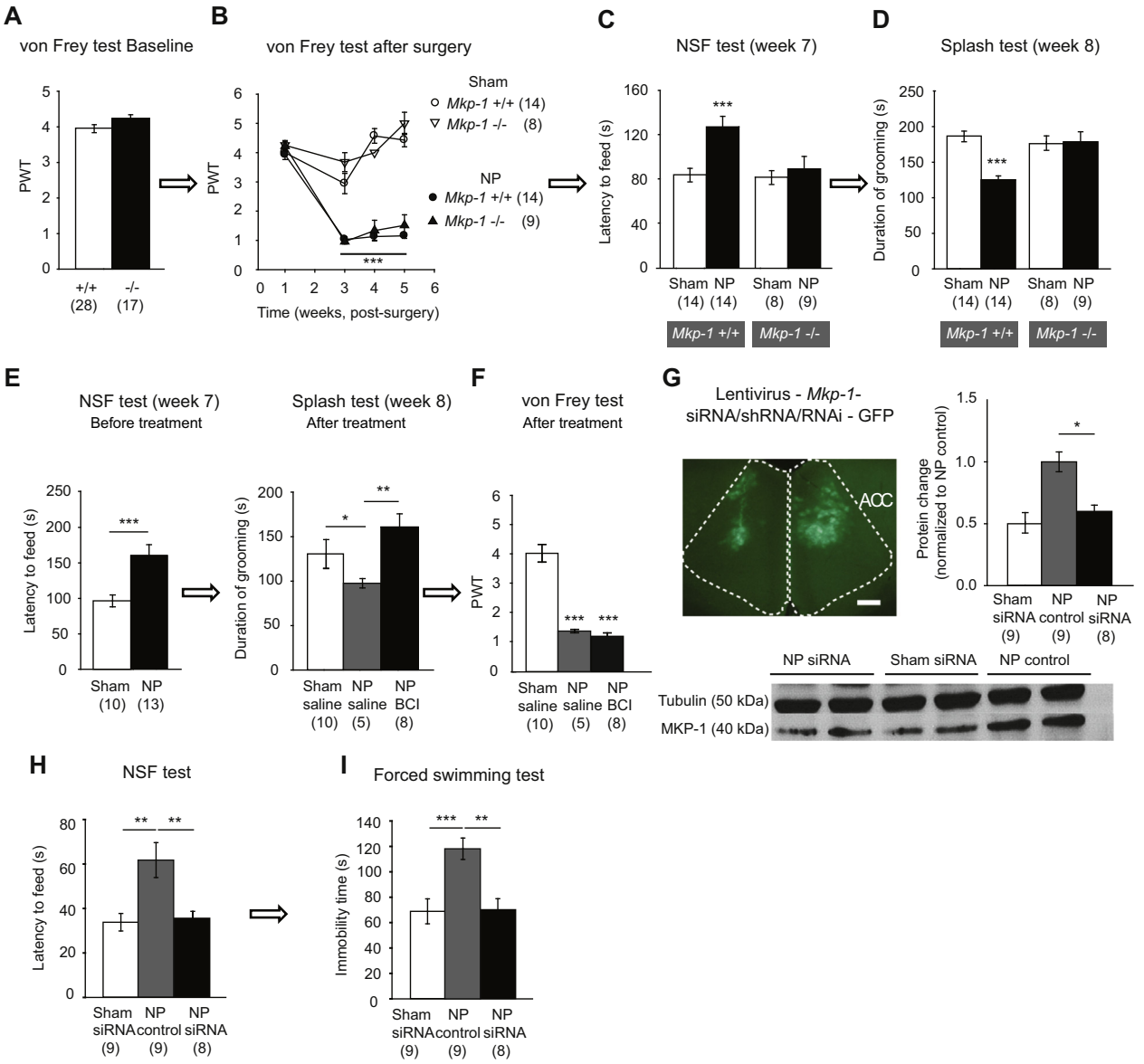

Figure 5. Total, systemic, and local suppression or blockade of mitogen-activated protein kinase phosphatase-1 (MKP-1) prevents and/or blocks neuropathic pain (NP)–induced depression. **(A)** Baseline von Frey results (right paw) illustrating that *Mkp-1*$^{-/-}$ mice show no initial difference in mechanical sensitivity compared with *Mkp-1*$^{+/+}$ animals before surgery. **(B)** von Frey results showing that both *Mkp-1*$^{+/+}$ and *Mkp-1*$^{-/-}$ mice develop mechanical allodynia after cuff surgery. **(C, D)** Behavioral tests demonstrating that there is no difference in the latency to feed in the novelty suppressed feeding (NSF) test or duration of grooming in the splash test between *Mkp-1*$^{-/-}$ sham and NP animals, suggesting an absence of development of depressive-like behaviors 8 weeks after NP induction, contrary to that normally observed in *Mkp-1*$^{+/+}$ cuff mice. **(E)** NSF test before BCI treatment demonstrating that NP animals display anxiodepressive-like behaviors 8 weeks after surgery. Splash test results showing that the NP group treated with BCI spent more time grooming compared with the nontreated group, suggesting a decrease in depressive-like behavior. **(F)** von Frey results demonstrating that BCI treatment did not affect the NP-induced mechanical allodynia in the right hind paw. **(G)** Representative picture of the anterior cingulate cortex (ACC) after bilateral injections of lentiviruses (Lentivirus-*MkP-1*-small interfering RNA (siRNA)/short hairpin RNA (shRNA)/RNA-mediated interference (RNAi)-GFP, 2 μL/site) (top left) and Western blot results showing a decrease in ACC MKP-1 after *Mkp-1* silencing (top right and bottom). Scale bars = 300 μm. **(H)** NSF test illustrating that NP animals with *Mkp-1* silencing have shorter latency to feed compared with NP animals that received the control virus, suggesting a decrease in anxiodepressive-like behavior. **(I)** Forced swim test data showing a decreased immobility time in NP animals with *Mkp-1* silencing compared with NP control animals, suggesting a reduction in depressive-like behavior. Data are expressed as mean ± SEM; $^{**}p \leq .01$, $^{***}p \leq .001$. PWT, paw withdrawal thresholds.

In naïve animals, the local deletion of the MKP-1 within the ACC did not affect behaviors (Supplemental Figure S7). In NP mice, the local *Mkp-1* suppression had a prominent global effect on both the NSF test (Figure 5H; $F_{2,23} = 7.280$, $p \leq .01$) and the FST (Figure 5I; $F_{2,23} = 8.35$, $p \leq .01$). Indeed, this silencing lowered both the latency to feed in the NSF test

(Figure 5H; $p \leq .01$) and the immobility time in the FST (Figure 5I; $p \leq .01$) compared with the scramble-injected NP group. This suggests that silencing ACC *Mkp-1* leads to a decrease in anxiodepressive-like behaviors and further reinforces the causal link between ACC MKP-1 and depression.

DISCUSSION

Here we show that anxiodepressive-like behaviors in mice are associated with an upregulation of ACC MKP-1, which is reversed by fluoxetine. This upregulation is accompanied by increased phosphorylated CREB/ATF-1 levels and Fos expression as well as increased enrichment of H3K9/K14ac at *Mkp-1* promoter in animals displaying depressive-like behaviors. Antidepressant-like effects produced by experimentally preventing, blocking, or decreasing MKP-1 activity within the ACC further reinforce its crucial role in depression.

While depression is the most prevalent lifetime disorder, our limited knowledge of its etiology, alongside the lack of efficient treatment strategies, points to an obvious need for objectively quantifiable abnormalities at the molecular, cellular, or circuit level. Because the ACC is an integration center interconnecting neurons from brain regions implicated in pain and affective-related processing, this study mainly focused on identifying molecular alterations within this structure. Through genomic analyses, we identified *Mkp-1* as one of the most prominently upregulated genes in the ACC, extending previous studies showing increased expression of *Mkp-1* in other brain regions in animal models of depression and in depressed patients (14,15).

By exploring the expression dynamics of MKP-1, we observed that its mRNA, but not the protein, starts being overexpressed after 2 weeks of NP, when mice display mechanical hypersensitivity without anxiodepressive-like behaviors. This delay between gene expression and protein synthesis might involve negative feedback mechanisms controlling MKP-1 degradation (32,33) and/or synthesis (34,35). Furthermore, MKP-1 protein shows a tendency for overexpression at 5 weeks of NP, when anxiety-like behaviors are present, and significant overexpression after 8 weeks, when animals fully display anxiodepressive-like behaviors. Because *Mkp-1* deletion or local downregulation suppresses these behaviors, MKP-1 overexpression appears to be necessary to anxiodepressive phenotypes. However, it might not be sufficient. Indeed, following optogenetic stimulation of the ACC, the increase in MKP-1 levels lasted longer than the anxiodepressive phenotype. Together, these results suggest that other molecular actors (which still remain to be identified) should also be critical and/or that the increase in MKP-1 levels should reach a certain threshold in order to translate into a behavioral outcome.

Various studies (16,36,37) focused on the downstream targets of MKP-1, showing that its main functional role is to modulate MAPK-CREB signaling pathways by inactivating several downstream targets, such as extracellular signal-regulated kinases, c-Jun N-terminal kinases, and P38 (16,36), which further affects the expression of various genes implicated in depression (14,38). However, the molecular events triggering MKP-1 upregulation remain unknown. Previous studies showed that *Mkp-1* mRNA has a short half-life of 1 to 2 hours (17,39); however, our genomic analysis at 8 weeks of NP points out a sustained expression of ACC *Mkp-1*. Increased presence of activated transcription factors thus could be responsible for such prolonged transcription given that microarray results revealed significant modifications in several of these factors, including mRNA coding ATF-1, CREB, and Fos proteins, which are known to target *Mkp-1* (40).

Besides changes in transcription factors, a prolonged expression of relatively short half-life mRNA, such as *Mkp-1* and *C-fos* mRNAs, might also require sustained changes in chromatin structure. Thus, we looked for epigenetic regulation by testing H3ac at the *Mkp-1* and *C-fos* promoters, which has been previously reported to be altered in response to various stimuli (17,41–43). Interestingly, while both H3K9/K14ac and H3K27ac are present at the promoter/TSS region of *C-fos* and *Mkp-1*, only H3K9/K14ac was increased in response to NP. H3K27ac is generally associated with active enhancers (44), whereas H3K9/K14ac, which is more particularly present at the promoter/TSS region of highly transcribed genes (45), is a highly inducible mark that has been shown to be modulated by behavior at the global and locus-specific levels (46,47). The enrichment of H3K27ac and increased NP-induced enrichment of H3K9/K14ac observed at both genes, demonstrating a favorable chromatin conformation for transcription that may be relevant, possibly with other modifications, for the sustained upregulation of MKP-1. Such epigenetic alterations in the ACC could contribute to the emergence of depressive-like behaviors during NP by modulating *Mkp-1* (and other genes) in the long term.

We further demonstrate that ACC MKP-1 is overexpressed not only in NP-induced depression but also in other models such as sustained optogenetic stimulation of the ACC and UCMS, one of the most valid and relevant models of depression (48), suggesting that this pattern is consistent regardless to the cause of depression and could be a common marker. Interestingly, Duric *et al.* (14) did not report alterations of *Mkp-1* in the whole cortex after chronic stress, whereas they demonstrated an increased level of this gene in the dentate gyrus and the cornu ammonis 1 of animals submitted to UCMS and in patients with major depressive disorder. Likewise, we did not observe any change in the primary somatosensory cortex, suggesting that the overexpression is not a general cortical effect but rather may be selective for the ACC. Conversely, we did not observe alteration in MKP-1 protein level when we studied the whole hippocampus, which may reflect that reported changes (14) affect specific subregions of this structure.

To leap from a correlative analysis to a causal analysis for understanding the link between MKP-1 and depression, we combined several approaches. Knocking out and systemic antagonism of MKP-1 blocks the development as well as the maintenance of depressive-like behaviors without affecting the nociceptive hypersensitivity component of NP, suggesting that the presence of MKP-1 at the systemic level may be crucial for depression. Interestingly, local silencing of *Mkp-1* within the ACC is sufficient to suppress depressive-like behaviors after NP. Besides systemic and local suppression of MKP-1, fluoxetine also induces antidepressant-like effects in our model and decreases the ACC MKP-1 levels. The latter is in

line with studies showing a decrease in MKP-1 in the frontal cortex and hippocampus of rats after chronic fluoxetine treatment (49,50). Unlike tricyclic antidepressants that are successful in treating the nociceptive hypersensitivity component, such as mechanical allodynia, observed in NP (51), selective serotonin reuptake inhibitors, and particularly fluoxetine, show limited to no analgesic efficacy in NP (52). Accordingly, the antidepressant-like effect of fluoxetine that we observed was also independent of its impact on the mechanical hypersensitivity.

In conclusion, our results indicate that the upregulation of ACC MKP-1 is necessary for the expression of depressive symptoms induced by chronic pain, chronic stress, and optogenetic activation of the ACC. From a drug discovery perspective, dual specificity phosphatase family members are promising drug targets given that several MAPK inhibitors are already in different states of development for inflammatory disease and cancer (53). Our results further provide a preclinical target validation for potential treatment of depression given that systemic pharmacological blockade and local suppression of the MKP-1 display antidepressant-like effects.

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The authors report no biomedical financial interests or potential conflicts of interest.

ARTICLE INFORMATION

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Cingulate Overexpression of Mitogen-Activated Protein Kinase Phosphatase-1 as Key Factor for Depression

Supplementary Information

Supplemental Methods and Materials

Animals

Adult male C57BL/6J (approximately 350) mice (Charles River, L'Arbresle, France) were used in all the experiments. For the optogenetic experiments, we used Thy1-ChR2-YFP (B6.Cg-Tg (Thy1-COP4/EYFP) 18Gfng/J) animals (1, 2). The cAMP response element (CRE)-related activity was examined by using CRE-LacZ mice containing six tandem CRE sequences controlling the expression of beta-galactosidase (β-gal) upstream of a minimal RSV promoter (3). We presently limited experiments to one sex because the models and tests we used were set-up and well characterized, in our hands, in male mice and we also aimed to limit the total number of animals used in this study. All procedures were performed in accordance with the local animal care and use committee as well as with European Community Council Directive (EU 2010/63). The Strasbourg Chronobiotron (UMS3415) animal facilities and the Tours animal facilities are registered for animal experimentation (Agreements A67-2018-38). Protocols were approved by the local ethical committee of the University of Strasbourg (#2015012909428166) and Comité d'expérimentation animale du Val de Loire (N°19).

Experiments started with 8 to 12 weeks-old mice, group-housed five per cage and kept under a 12-h light/dark cycle with food and water available *ad libitum*. For optogenetic studies and UCMS, mice were individually housed.

Neuropathic pain (NP) model and nociceptive testing

Chronic NP was induced by placing a 2 mm section of split PE-20 polyethylene cuff around the right common sciatic nerve of the animal (4). Before surgery, mice were assigned to experimental groups so that mechanical nociceptive thresholds were balanced. The control group (sham) underwent the same procedure without cuff implantation. The NP procedure has been shown to result in anxiodepressive-like behaviors in a time-dependent manner (5). The mechanical sensitivity was scored by placing the animals in plastic boxes on an elevated mesh platform and applying von Frey filaments (Bioseb, Chaville, France) of various values (0.4-8 g), in an ascending manner, to the plantar surface of each hind paw. Three responses, as displayed by withdrawal, shaking or licking the paw, out of five applications of each filament were considered a positive response for the given filament.

Unpredictable chronic mild stress model (UCMS)

In this procedure, mice were subjected to a variety of random low-intensity socio-environmental stressors several times a day/night for 8 weeks (6). The stressors included: altered cage and bedding (wet bedding, frequent change of sawdust, substituting bedding with 21°C water, change of homecage), altered light/dark cycle, cage tilting (45°) and predator smell exposure (addition of rat droppings to the cage). See Supplementary Table S3 showing an example of one of the week of whole UCMS procedure. The order of stressors was altered every week (7). UCMS-exposed mice were maintained under standard laboratory conditions, but were isolated in smaller individual cages (24 × 11 × 12 cm), while non-stressed control mice were group-housed in normal cages (42 × 28 × 18 cm) with plastic tunnels and shelter. Body weight was assessed weekly. At the end of 8 weeks, NSF test was performed in order to compare the behavioral outcomes of the UCMS model with the chronic pain- and optogenetic

ACC activation-induced anxiodepressive-like behavior. The ACC was harvested immediately after the test.

Anxiodepressive-related behaviors

All behavioral experiments were conducted during the dark cycle, under red light. The forced swimming test was always considered as terminal.

Novelty suppressed feeding (NSF) test

After being food deprived for 24 h, mice were placed in a 40 x 40 x 30 cm plastic arena with the floor covered with 2 cm of sawdust which contained a food pellet in the center. The latency to start eating the pellet was recorded within a 5 min period. This test induces a conflict between the drive to eat the pellet and the fear of venturing towards the center of the box. In one experiment (Supplementary Figure S1A), food intake was also controlled by returning the animal to the home cage immediately after the test and measuring the amount of food consumed over a period of 5 minutes as described in (8). For the optogenetic studies, animals were tested with the NSF test 1 day after the last stimulation.

Splash test

A 10% sucrose solution was sprayed onto the dorsal coat of the animal and the duration of grooming behavior was recorded over 5 min (8). Decreased grooming time suggests a loss of motivational behavior, parallel to some symptoms of depression observed in humans, such as apathetic behaviors.

Forced swimming test (FST)

Mice were individually lowered into a glass cylinder (height 17.5 cm, diameter 12.5 cm) containing 12 cm of water (23-25°C). The test lasted 6 min, however since usually little or no immobility is present during the first 2 min, the duration of immobility was recorded only

during the last 4 min of the test (9). “Immobility” was defined as a state in which the animal displays no active movement or only small movements to balance their bodies and keep their heads above the water, mirroring hopelessness seen in human subjects.

Dark-light test

The apparatus consisted of light and dark boxes (18 x 18 x 14.5 cm each) connected by a dark tunnel (8.5 x 7 x 6 cm). The lit compartment was brightly illuminated (1500 lux). 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 (5).

Locomotor activity

Eight weeks after NP induction, locomotor activity was monitored for both Sham and NP mice. Mice were individually placed in activity cages surrounded with photocell beams. The number of beam breaks was recorded over 2 hours. The results were presented for the first 5 min, the first hour and the 2 hours of recording.

Optogenetics

The Thy1-ChR2-YFP mice were anesthetized before being placed in the stereotaxic frame. Single glass cannulas (1.7 mm, Doric lenses, Quebec, Canada) were lowered 1.5 mm from the skull into the left ACC near the venous sinuses (0.7 mm anterior and 0.3 lateral to the bregma) and fixated onto the skull with Paladur denture cement. After recovery (3-7 days), the animals were stimulated with a blue light emitting diode with a peak wavelength of 460 nm and intensity between 4-6 mW. Such approach is sufficient to induce ACC activation in both hemispheres, as seen by local c-Fos expression (2). Previous functional validation using *ex vivo* electrophysiological recordings confirmed that the optogenetic stimulation reliably

enables to trigger action potential firing of the ACC pyramidal neurons (2). In this study, we used either single or repetitive (4 consecutive days for 30 min) ACC activation with repetitive stimulation sequences of 10 s consisting of 8 s at 20 Hz with 40 ms pulses and 2 s without stimulation. The same protocol was used for the control group, except that the light was kept off during stimulation time. We then determined the effect of a single or repetitive stimulation on behaviors in independent sets of animals. In a separate experiment, we also evaluated long term impact of a repetitive stimulation (2 weeks after the last stimulation). For the single stimulation, NSF test performed 5 minutes and splash test 1 day after the stimulation. For the repetitive stimulation protocol, the stimulation took place during 4 consecutive days for 30 min and tests were performed 1 day and/or two weeks after the last stimulation. Immediately after behavioral tests, animals were killed and their ACC harvested.

Viral-mediated gene knock-down

For silencing *Mkp-1*, lentiviral vectors expressing a set of *Mkp-1* siRNA/shRNA/RNAi and a green fluorescent protein under the cytomegalovirus promoter (piLenti-siRNA-GFP, Applied Biological Materials Inc., a vector for *mkp-1* knock-down) were delivered by using a Hamilton syringe. Control animals underwent the same procedure and received a scrambled version of the same lentiviral vector (Applied Biological Materials Inc).

Three weeks after sciatic nerve surgery, NP or sham-operated animals received either piLenti-siRNA-GFP or a scrambled version of the virus bilaterally (2 µl/site) into the ACC. Four weeks after transfection, behavioral tests were conducted on three groups: i) sham-operated (control of NP) mice that express siRNA (SHAM/siRNA; $n = 9$), ii) mice displaying NP-induced depressive-like behaviors administered with scramble virus (NP/control; $n = 9$), and iii) mice displaying NP-induced depressive-like behaviors expressing siRNA (NP/siRNA; $n = 8$). Either the fresh or perfused ACC tissues were collected at the end of behavioral

experiments in order to verify the transfection efficiency. In a separate set of experiment, we studied the impact of local deletion of the ACC MKP-1 on behavioral phenotype in naive animals.

Tissue harvesting and processing

Animals were anesthetized with pentobarbital (54.7 mg/ml; i.p., 4 ml/kg, Centravet, Taden, France) and perfused with 100 ml 0.1M phosphate buffer (PB) for 2 min, followed by 500 ml of 4% paraformaldehyde fixative (PFA) for 18 min, except for CRE-LacZ mice which were perfused with PFA for 5 min only. Brains were harvested immediately and stored in PFA at 4°C for 12h, before being embedded in 4% agarose blocks, and cut using a vibrating microtome (Leica, Rueil-Malmaison, France). The brain was cut into 40 µm serial sections, stored in phosphate buffered saline (PBS) at 4°C.

For microarray analysis and protein blotting, the ACC, primary somatosensory cortex (S1) and whole hippocampus were freshly dissected from animals killed by cervical dislocation and tissues were stored at -80°C.

Pharmacological agents

The selective serotonin reuptake inhibitor (SSRI), fluoxetine (20 mg/kg/day) (10) mixed with 0.2% saccharine was administered in drinking water for 3 weeks (started 5 weeks after the sciatic nerve injury), while the control group drank 0.2% saccharine. 20 mg/kg/day corresponds to 200 µg/ml per day (mice weight approximately 30 grams, they consume approximately 3 ml fluoxetine solution per day). For a 100 ml bottle, we thus added 20 mg fluoxetine hydrochloride.

The MKP antagonist dusp-(E)-2-benzylidene-3-(cyclohexylamino)-2, 3-dihydro-1H-inden-1-one (BCI) has been identified by Molina et al., 2009 (11) as an inhibitor of MKP-3

and MKP-1 using zebrafish chemical screen. They determined the IC50 values for MKP-3 and MKP-1 inhibition from six independent experiments which were 12.3 ± 4.0 µM and 11.5 ± 2.8 µM, respectively. They further showed that BCI also inhibited human MKP-1, whose catalytic activity, like MKP-3, is induced by substrate binding. Concerning the dose, since there is no study using BCI at systemic level in rodents, we based our procedure on IC50 values and on local injection in zebrafish (12) where the authors administered 50 µl of 10 µM intrathoracic.

In this study, the same batch of animals including Sham and NP groups were behaviorally tested before and after BCI administration. We first used NSF test to confirm that NP animals develop anxiodepressive-like behaviors 8 weeks after the surgery as expected. According to our previous experiences, we avoid repeating the NSF test as this paradigm is based on the novelty. Indeed, we had previously observed significant improvement for latency to feed in any given condition when the animals were resubmitted to this test. It was thus necessary to use 2 different behavioral outputs. We chose the splash test as it provides reliable and robust phenotype in the NP model.

Immunohistochemistry

Sections were washed in PBS 3 times (10 min) and incubated in 3% H_2O_2/50% ethanol solution for 15 min. After additional 3 washes in PBS (10 min), sections were pre-incubated for 45 min (PBS; 0.2% Triton X-100; 5% goat serum) before being incubated overnight at room temperature in PBS (0.2% Triton X-100; 1% goat serum) and a chicken anti-β-gal primary antibody (1:2000; #9361, Abcam) or a rabbit anti-c-Fos (1:10000; Santa Cruz Biotechnology, E1008). The following day, sections were washed 3 times with PBS (10 min) and incubated with a biotinylated either goat anti-chicken (1:400 in PBS with 0.2% Triton X-100, 1% donkey serum) or donkey anti-rabbit secondary antibody (1:300 in PBS containing

Triton X-100, 1% donkey serum) for 1 h 30 min. Next, after washing in PBS (3 x 10 min), sections were incubated with PBS containing the avidin-biotin-peroxidase complex (ABC kit; 0.2% A and 0.2% B; Vector Laboratories, Burlingame, CA) for 1 h 30 min. Following a wash in 0.05 M Tris-HCl buffer, sections were incubated in 3,3'diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 in Tris-HCl for approximately 4 min and washed again. After mounting, sections were dehydrated in graded alcohols, cleared in RotiHistol (Carl Roth, Karlsruhe, Germany) and coverslipped with Eukitt. Coronal brain slices (40 μm) were used to count β-gal or c-Fos positive nuclei bilaterally, taken anteroposterior levels into account (+1.41 to 0.47 from bregma for ACC, +1.91 to 1.77 for prelimbic and infralimbic areas). We analyzed a section every 120 μm for the targeted regions, considering the full structure for the ACC, and a 250 x 250 μm area per section for the prelimbic and infralimbic cortices in order to minimize the risk of data contamination from adjacent regions. Data were expressed per whole bilateral structure and images were acquired using a microscope (Eclipse E600, Nikon Instruments, Kingston, UK) equipped with a Neurolucida software. Images for siRNA transfection were acquired on a Leica SP5 II confocal microscope.

For siRNA experiments, half of the experimental groups were perfused for immunohistochemistry. Sections were washed in PBS (3 × 10 min), incubated 10 min in a PBS-Tween 20 0.2% and 5% donkey serum. Sections were then incubated overnight at room temperature in PBS with Tween 20 and the primary antibody (1:1000, rabbit antieGFP). Sections were washed in PBS (3 × 10 min), incubated with Alexa 488 fluorophore-labeled secondary antibody (#1719656; 1:2000, Life Technology) for 1 h 30 min, and washed in PBS (3 × 10 min) before being mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). After visual control of the injection site, the ACC for the other half of the experimental groups was freshly dissected for Western blot analysis.

Western Blot

For protein extraction, tissues were lysed in 150 µl of lysis buffer (30 mM Tris pH 7.5, 100 mM NaCl, 10% glycerol, 1% NP40, 15% EDTA) and mechanically dissociated with a plastic stirring rod. The lysate was centrifuged (10000 rpm/4°C/10 min) and 100 µl of the supernatant was collected. 5 µl of the samples was used to determine the protein concentration with the Quick Start Bradford protein assay (Bio-Rad, Munich, Germany), followed by spectrophotometry (Mitrhras LB940, Berthold Technologies). The concentration of each sample was adjusted to 1 µg/µl with lysis and Laemmli buffers. For SDS-PAGE gel electrophoresis, 15 µL of the denatured proteins were loaded and separated on 8% polyacrylamide gels, which were then electroblotted onto polyvinylidene fluoride membrane (Millipore). The membrane was incubated with primary antibody (anti-MKP-1 #1199, 1:2000; Santa Cruz Biotechnology or ab 108342 lot GR43400-13, 1:150000; Abcam) at 4°C under agitation overnight. After washing with TBST, secondary antibody (goat anti-rabbit; 1:15000 or 1:30000, respectively) was added and incubated for 1 h. The membranes were developed using the enhanced chemiluminescence (ECL) detection system (Amersham) using Hyperfilm substrates (Amersham Biosciences). The relative protein expression was determined using the densitometry tool of Adobe Photoshop CS3 software.

RNA extraction

Total RNA was extracted from ACC tissues with the Qiagen RNeasy Mini Kit (Hilden, Germany). Around 20 mg of ACC tissue was disrupted and homogenized with the Kinematica Polytron 1600E in 1.2 ml QIAzol Lysis Reagent for 45 s and left at room temperature for 5 min. Next, 240 µl of chloroform was added and thoroughly mixed before centrifugation for 15 min at 12000 rpm at 4°C. The aqueous phase (600 µl) was transferred to a new collection tube and mechanically resuspended with 600 µl of 70% ethanol. The mix was transferred into an

RNeasy Mini spin column in a 2 ml collection tube and centrifuged at 10000 rpm for 15 s. Next, 350 µl of RW1 buffer was added and centrifuged at 10000 rpm for 15 s, before adding 10 µl of DNAse and 70 µl of RDD buffer. The mix was left at room temperature for 15 min and 350 µl of RW1 buffer was added and centrifuged at 10000 rpm for 15 s. The column was transferred to a new 2ml collection tube and washed with 500 µl of RPE buffer before being centrifuged at 10000 rpm for 15 s. Next 80% ethanol was added and centrifuged for 2 min at 10000 rpm. Finally, the column was dry centrifuged at 10000 rpm for 5 min and transferred to a new 1.5 ml collection tube to which 14 µl of RNase-free water was added. At last, the RNA was eluted by centrifugation for 1 min at 10000 rpm.

Microarray gene expression analysis

The Illumina® TotalPrep™ RNA Amplification Kit (Life) was used to in-vitro transcribe biotinyaled copy RNA. Gene expression was analysed with Illumina Mouse WG-6 v2.0 Expression BeadChips. All steps were carried out according to the manufacturer's protocol. Data were analyzed using the Gene expression analysis module of GenomeStudio V2011.1 (Illumina) by applying quantile normalization. The Diff Score is a transformation of the *P*-value that provides directionality to the *P*-value based on the difference between the average signal in the reference group vs. the comparison group. The formula is: DiffScore = 10*sgn(µcond-µref)*log10p; for a *p*-value of 0.05, DiffScore = ± 13; for a *p*-value of 0.01, DiffScore = ± 22; for a *p*-value of 0.001, DiffScore = ± 33. Heatmaps were generated using ClustVis (13). Data have been uploaded to the Gene Expression Omnibus (NCBI) database under accession GSE92718.

Chromatin immunoprecipitation (ChIP)

The ACCs (3 animals pooled for one sample) were mechanically dissociated and fixated in PBS containing 1% formaldehyde. The "cross-linking" was stopped with glycine (0.125 M). The samples were resuspended in nuclei lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-Cl pH 8.1, 1 mM sodium butyrate) and sonicated with a Diagenode Bioruptor (25 cycles of 30 s on/30 s off on high power). Sonicated samples containing DNA/protein complexes were diluted 10 times with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl pH 8.1, 167 mM NaCl) and 50 µL of each supernatant was saved to serve as "total input chromatin". The remaining part was incubated overnight at 4°C with 2–5 µg of primary antibody against acetyl-histone H3K27 (#ab4729, Abcam) or against acetyl-histone H3K9K14 (#06-599, Merck Millipore), while no antibody was used for the negative control. This was followed by incubation with protein A Dynabeads (#10001D, Life Technologies) for 2 h and a sequence of washing steps (low salt, high salt, LiCl, and TE buffers). The elution buffer (1% SDS, 0.1 M $NaHCO_3$) was added to elute the complexes from the beads, followed by reverse-crosslinking over night at 65°C. DNA was purified with RNAse (30 min, 37°C) and proteinase K (2 h, 45°C), followed by phenol/chloroform extraction and ethanol precipitation.

Quantitative polymerase chain reaction (qPCR)

The purified DNA was resuspended in DNAse free water and analyzed by qPCR (CFX96, Bio-Rad; 98°C for 3 min, and 98°C for 15 s, 63°C for 45 s for 50 cycles, then 98°C for 10 s; melt curve, 65 to 98°C; increment, 0.5°C, 5 s) with SYBR-Green-based reagents (BioRad). The relative quantities of immunoprecipitated DNA fragments were compared with a standard curve generated by input DNA. IP enrichment and specificity were checked with control genes either expressed ubiquitously (*Gapdh*) or not expressed in the ACC (*TsH2B*)

(Supplementary Figure S5). The following primers were used for qPCR: *Mkp-1*, F_tcagcggggagtttttgtg and R_ctgtgagtgaccctcaaagtgg; (14) *C-fos*, F_ctgaggcgcctactactcca and R_gaaacccgagaacatcatgg; *Gapdh-86*, F_ccactccccttcccagttt and R_ccgcatcttcttgtgcagt; *TsH2B-96*, F_tgcacttgtttcagcacctt and R_ggtcaccaagacccagaaaa.

Supplementary Table S1. Microarray-derived expression levels of significantly dysregulated genes 8 weeks after sciatic nerve injury. The Diff Score is a transformation of the p-value that provides directionality to the p-value based on the difference between the average signal in the reference group vs. the comparison group. The formula is: DiffScore = 10*sgn(μcond-μref)*log10p; For a p-value of 0.05, DiffScore = ± 13; For a p-value of 0.01, DiffScore = ± 22; For a p-value of 0.001, DiffScore = ± 33. These data are publicly available on GEO.

Probe ID	Symbol	fold (cuff/sham)	Diff score
ILMN_2623983	*Egr2*	2.151134	25.43628
ILMN_2750515	*Fos*	1.944119	25.34301
ILMN_2622983	*Dusp1*	1.722762	28.99455
ILMN_2597827	*Arc*	1.692703	23.40138
ILMN_1230397	*A630064P09Rik*	1.604632	38.52967
ILMN_1215713	*Egr4*	1.579094	23.65104
ILMN_1251414	*Npas4*	1.570782	14.16639
ILMN_1220034	*Junb*	1.532342	16.48909
ILMN_2794645	*Cyr61*	1.487082	19.26342
ILMN_2778279	*Fosb*	1.471494	19.6849
ILMN_2604029	*Klf2*	1.429929	25.12996
ILMN_2665490	*Litaf*	1.421849	14.37777
ILMN_2636403	*Axud1*	1.421225	23.80956
ILMN_1252481	*Fosl2*	1.420379	30.89858
ILMN_2484278	*9930031P18Rik*	1.408275	34.44762
ILMN_1250195	*Ndrg1*	1.398072	17.73933
ILMN_2491182	*A130010C12Rik*	1.388131	28.5413
ILMN_2493030	*2310043N10Rik*	1.382175	19.19125
ILMN_2497395	*9130004J05Rik*	1.360177	41.96212
ILMN_2571616	*C430002D13Rik*	1.345294	29.2088
ILMN_2595732	*LOC100046232*	1.33299	21.26001
ILMN_1255511	*Rreb1*	1.314741	16.45536
ILMN_2761109	*Clic4*	1.314361	17.22352
ILMN_1217458	*8430403J19Rik*	1.311905	15.85766
ILMN_1259689	*Dcamkl1*	1.310838	30.87304
ILMN_1234796	*Hsd17b12*	1.310265	28.22738
ILMN_2749063	*Dock10*	1.296374	13.03217
ILMN_1225071	*Fgfr2*	1.296051	15.18099
ILMN_2893993	*Lass2*	1.291665	13.81431
ILMN_2998976	*Edg2*	1.288145	19.75181
ILMN_2643423	*LOC100042773*	1.286278	25.043
ILMN_2903945	*Gadd45g*	1.282244	16.13248
ILMN_1213954	*Sgk1*	1.280731	16.8066
ILMN_1247853	*Zfp36l1*	1.273829	21.53874
ILMN_2646166	*Ndrl*	1.272271	17.96134
ILMN_1230546	*Clic4*	1.271297	16.06288

Probe ID	Symbol	fold (cuff/sham)	Diff score
ILMN_2700059	*Slc12a2*	1.270331	13.11338
ILMN_2780424	*Zfp36l1*	1.257683	25.22087
ILMN_3095624	*Jmjd3*	1.256677	23.26166
ILMN_1260073	*D16Ertd472e*	1.252439	13.74546
ILMN_1239398	*LOC100046817*	1.250313	23.87175
ILMN_2717667	*Pim3*	1.24932	32.14536
ILMN_1225029	*Mboat1*	1.24898	14.72014
ILMN_2760105	*Olig1*	1.246317	14.67719
ILMN_1254547	*Nr4a2*	1.242276	16.90362
ILMN_2678714	*Id4*	1.239599	21.30532
ILMN_1254358	*Igfbp5*	1.239225	20.12765
ILMN_2656854	*Myo6*	1.239201	14.2908
ILMN_1233987	*1110014O20Rik*	1.238253	14.2697
ILMN_2555264	*A030001L21Rik*	1.231348	29.57129
ILMN_3006575	*6330503K22Rik*	1.230999	16.66642
ILMN_2744890	*Gadd45g*	1.229202	15.72554
ILMN_2759012	*Plxnb3*	1.22325	20.71529
ILMN_1227494	*Egr3*	1.221462	16.58524
ILMN_2505047	*Pogk*	1.22108	17.84131
ILMN_2439044	*Nde1*	1.220349	14.61858
ILMN_2531737	*LOC240672*	1.21965	18.01757
ILMN_2585137	*8030402P03Rik*	1.218561	13.40303
ILMN_1226904	*2900011L18Rik*	1.218514	15.19334
ILMN_2884126	*Phldb1*	1.218296	13.73285
ILMN_3112011	*Dusp16*	1.211043	24.61516
ILMN_1255287	*Mela*	1.209343	26.62116
ILMN_2684515	*Srpk3*	1.204282	14.02832
ILMN_2955919	*Mcam*	1.204088	14.99178
ILMN_1235932	*Pdgfra*	1.203742	16.19095
ILMN_1215908	*Chd7*	1.20316	20.79864
ILMN_2750850	*Spag9*	1.201715	19.31693
ILMN_2506162	*2810436B12Rik*	1.200858	18.79312
ILMN_1238480	*Kcnj4*	0.8333142	-15.45115
ILMN_1247071	*Slc22a1*	0.8332675	-23.30318
ILMN_2647793	*Rab13*	0.8331625	-22.30456
ILMN_2953411	*Ppp3r1*	0.8327924	-28.3036
ILMN_1248658	*1110001P04Rik*	0.8315538	-22.27083
ILMN_2621130	*6430598A04Rik*	0.8309487	-15.00347
ILMN_2837779	*Trpv2*	0.8283483	-17.91199
ILMN_1214255	*Cd200*	0.827318	-19.02263
ILMN_2914295	*Tmem160*	0.8268025	-25.80542
ILMN_1227024	*LOC100048372*	0.8256795	-31.9827
ILMN_2619107	*Lgals1*	0.8192174	-13.65513

Probe ID	**Symbol**	**fold (cuff/sham)**	**Diff score**
ILMN_2491756	*C130020C13Rik*	0.8191088	-18.64958
ILMN_2624153	*Hes5*	0.8189178	-18.0659
ILMN_3120797	*Pigk*	0.8173604	-24.43718
ILMN_2627995	*Mtmr4*	0.8173051	-22.49778
ILMN_2715429	*Slc8a2*	0.8156307	-18.41099
ILMN_1233115	*Ndufs6*	0.8143693	-13.20841
ILMN_2674979	*Fus*	0.8112372	-13.41019
ILMN_3143604	*Gng2*	0.8108346	-14.64544
ILMN_1259400	*ENSMUSG00000043795*	0.8107359	-16.38556
ILMN_2763294	*Ensa*	0.809377	-13.72145
ILMN_2646878	*Blmh*	0.8052573	-25.86331
ILMN_1228833	*Pcdh10*	0.7951223	-26.79246
ILMN_2719702	*Rasl10a*	0.7947692	-13.33951
ILMN_2792502	*Il17d*	0.786956	-31.9591
ILMN_1226157	*Pik3r3*	0.7788444	-24.24179
ILMN_2776230	*Sumo3*	0.7689706	-15.45785
ILMN_2776231	*Sumo3*	0.7565039	-16.14939
ILMN_2609052	*Dynll2*	0.7545545	-25.05101
ILMN_2643212	*Rprm*	0.7538992	-19.32226
ILMN_2944610	*Islr2*	0.7147037	-27.07377
ILMN_2628122	*Gnl3l*	0.5415065	-15.47511

Supplementary Table S2. Microarray-derived expression levels of significantly dysregulated genes 2 weeks after sciatic nerve injury. The Diff Score is a transformation of the p-value that provides directionality to the p-value based on the difference between the average signal in the reference group vs. the comparison group. The formula is: DiffScore = 10*sgn(μcond-μref)*log10p; For a p-value of 0.05, DiffScore = ± 13; For a p-value of 0.01, DiffScore = ± 22; For a p-value of 0.001, DiffScore = ± 33. These data are publicly available on GEO.

Probe ID	Symbol	fold (cuff/sham)	Diff score
ILMN_2623983	*Egr2*	2.884263	54.1489
ILMN_2622983	*Dusp1*	2.101475	30.458
ILMN_1220034	*Junb*	1.976938	33.27056
ILMN_2750515	*Fos*	1.968218	35.92749
ILMN_2597827	*Arc*	1.956073	28.63409
ILMN_2636403	*Axud1*	1.773838	34.33604
ILMN_1215713	*Egr4*	1.674954	35.17773
ILMN_1230397	*A630064P09Rik*	1.5571	33.79842
ILMN_2778279	*Fosb*	1.505233	19.39018
ILMN_2903945	*Gadd45g*	1.470593	26.47491
ILMN_2491182	*A130010C12Rik*	1.419416	23.96638
ILMN_2744890	*Gadd45g*	1.383928	21.01858
ILMN_2813484	*Per1*	1.366199	14.54538
ILMN_2484278	*9930031P18Rik*	1.363501	25.75892
ILMN_2794645	*Cyr61*	1.328553	15.21315
ILMN_2619330	*Plk3*	1.306549	15.75533
ILMN_1218037	*Tmie*	1.303445	14.32105
ILMN_2604029	*Klf2*	1.301454	16.04022
ILMN_2900653	*Gadd45b*	1.294986	27.38207
ILMN_1234796	*Hsd17b12*	1.294562	22.76217
ILMN_2596396	*Grasp*	1.291017	42.94044
ILMN_1260036	*AW120700*	1.289904	17.66716
ILMN_1243921	*5330431K02Rik*	1.286933	18.12642
ILMN_2701321	*Dusp6*	1.280657	23.49825
ILMN_1240598	*Samd14*	1.278993	16.10585
ILMN_2634083	*Cdkn1a*	1.277543	22.82927
ILMN_2789692	*Wnt7b*	1.269918	13.22498
ILMN_2932662	*Rasl11a*	1.266342	25.1568
ILMN_2777175	*2310004N11Rik*	1.265659	20.15939
ILMN_1239398	*LOC100046817*	1.264578	19.4126
ILMN_2656031	*Grasp*	1.261559	22.58492
ILMN_1259689	*Dcamkl1*	1.260055	39.33811
ILMN_2925711	*Dusp6*	1.250648	28.24568
ILMN_2927565	*Pkp2*	1.248443	18.33701
ILMN_2666980	*Bdnf*	1.244049	16.84228
ILMN_2736478	*Doc2b*	1.242431	13.72908

Probe ID	Symbol	fold (cuff/sham)	Diff score
ILMN_1214228	*Bdnf*	1.240936	15.90103
ILMN_1226904	*2900011L18Rik*	1.240399	22.1435
ILMN_1216953	*Prmt2*	1.236314	15.69452
ILMN_2733185	*Cdc42ep3*	1.231942	14.82857
ILMN_2713285	*Fhl1*	1.230988	14.14655
ILMN_2700292	*H13*	1.227593	22.98152
ILMN_2622354	*Arfl4*	1.226161	18.32549
ILMN_1248791	*Sntb2*	1.223965	31.87044
ILMN_2755936	*Rasl11a*	1.223551	22.01701
ILMN_1225192	*Nfkbid*	1.221613	42.93407
ILMN_1217734	*Pak6*	1.221284	27.31698
ILMN_1227494	*Egr3*	1.219947	20.48035
ILMN_2710253	*Cyr61*	1.219919	15.86413
ILMN_2742152	*Gadd45a*	1.219503	14.76482
ILMN_2769330	*Cd6*	1.218885	13.38414
ILMN_1215689	*A130062D16Rik*	1.218307	26.98904
ILMN_1252481	*Fosl2*	1.218286	15.9244
ILMN_1250011	*Tob1*	1.218094	19.31383
ILMN_2705119	*LOC100047353*	1.216285	17.92424
ILMN_1228267	*Otud1*	1.215947	23.52409
ILMN_2526204	*LOC381256*	1.214983	13.5768
ILMN_2885277	*Nnmt*	1.213722	15.92181
ILMN_1227605	*Adcyap1*	1.211078	13.38002
ILMN_2561029	*9930033H14Rik*	1.209087	18.83563
ILMN_1234565	*P4ha1*	1.208978	28.25821
ILMN_2429484	*4933439C10Rik*	1.202947	20.51163
ILMN_2576984	*C230095J06Rik*	0.8330899	-21.19187
ILMN_2984219	*BC048546*	0.8327226	-13.65731
ILMN_2652989	*BC016608*	0.8322424	-13.24153
ILMN_2481763	*4930425H11Rik*	0.829174	-16.22371
ILMN_2993661	*Trim59*	0.8287371	-13.22312
ILMN_2695098	*Esrrg*	0.8285379	-16.1526
ILMN_1215810	*Mcts1*	0.8282366	-13.51298
ILMN_2838727	*Trhde*	0.8277799	-15.30907
ILMN_1238511	*4930402H24Rik*	0.8273045	-20.07526
ILMN_2571414	*Ndr3*	0.8209289	-15.27389
ILMN_2951446	*Ypel5*	0.8183398	-19.93944
ILMN_2527779	*LOC381400*	0.8177646	-22.06687
ILMN_1236868	*Idb2*	0.8157483	-14.31931
ILMN_2574997	*A630024K09Rik*	0.8152267	-22.54935
ILMN_3023610	*Sgpp2*	0.8140745	-13.23612
ILMN_1242658	*C130066B05Rik*	0.8110857	-19.674
ILMN_1255126	*C030014C12Rik*	0.8078758	-15.94013

Probe ID	**Symbol**	**fold (cuff/sham)**	**Diff score**
ILMN_1242437	*C130090G16Rik*	0.8003336	-16.19595
ILMN_1235327	*Rbbp4*	0.8002388	-19.92503
ILMN_2534207	*LOC380706*	0.7975193	-14.08823
ILMN_3074259	*Lamp2*	0.7951882	-19.0439
ILMN_2419660	*mtDNA_ND4L*	0.7767119	-13.77478
ILMN_1233537	*Gucy1a3*	0.7756321	-28.6486
ILMN_1259747	*Il33*	0.7643566	-13.19717
ILMN_1213286	*Ccl21a*	0.7386547	-13.58464
ILMN_2746783	*Fa2h*	0.6622255	-13.32465

Supplementary Table S3. Example of stressors schedule for a week period.

Day	Time	Evaluation	Stress Type
Monday	9 a.m.		Social stress
Monday	10-12 a.m.		Cage Tilting
Monday	2-2:30 p.m.		Restraint stress
Monday	2:30 p.m.		Predator sounds
Tuesday	10 a.m.	Weight	
Tuesday	10:30 a.m.	Coat State	
Tuesday	11 a.m.		Sawdust change
Tuesday	1-3 p.m.		Rat sawdust
Tuesday	2-4 p.m.		Social stress
Wednesday	9:30-11:30 a.m.		Damp sawdust
Wednesday	12 a.m.-2 p.m.		Light
Wednesday	2 p.m.-4 p.m.		Dark
Wednesday	4 p.m.-6 p.m.		Light
Thursday	9-9:30 a.m.		Restraint stress
Thursday	10:30 a.m.		Sawdust change
Thursday	11:00 a.m.		Sawdust change
Thursday	1-1:30 p.m.		Bath
Thursday	3 p.m.		Social stress
Friday	9:30-11:30 a.m.		Without sawdust
Friday	10-11 a.m.		Cage Tilting
Friday	12 a.m.-2 p.m.		Cage Tilting
Friday	3-4 p.m.		Social stress
Saturday	9-11 a.m.		Light
Saturday	11 a.m.-1 p.m.		Dark
Saturday	1-3 p.m		Light
Saturday	3-7 p.m.		Dark
Saturday	7-9 p.m.		Light
Saturday	9 p.m.-1 a.m.		Dark
Saturday	1-3 a.m.		Light
Saturday	3-9 a.m.		Dark
Sunday	9-11 a.m.		Light
Sunday	11 a.m.-1 p.m.		Dark
Sunday	1-3 p.m		Light
Sunday	3-7 p.m.		Dark
Sunday	7-9 p.m.		Light
Sunday	9 p.m.-1 a.m.		Dark
Sunday	1-3 a.m.		Light
Sunday	3-9 a.m.		Dark

Supplementary Table S4. Detailed statistical analysis.

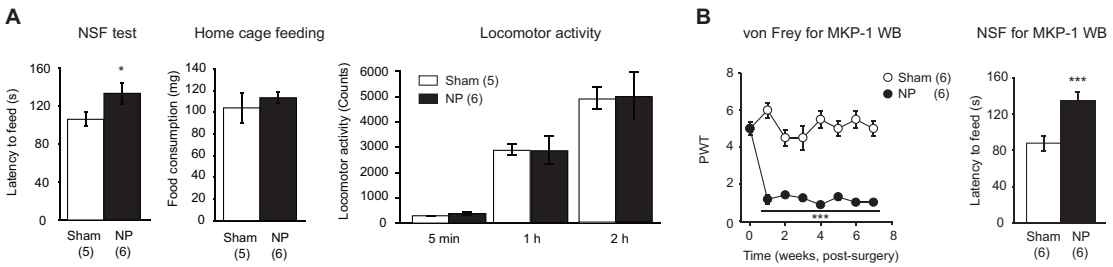
Experiment	Assessment	Treatment	Test	Groups (*n*)	Analysis	Statistics	Post-hoc	Figure
Neuropathic model	Mechanical (paw withdrawal) threshold	NP surgery	Von Frey	Sham right paw (6) NP right paw (6)	Time X Surgery ANOVA Repeated Measurement	$F_{(7, 70)} = 6.04$, $p \leq 0.001$	NP < Sham, $p \leq 0.001$ (1-8 weeks post-surgery)	**1a**
Neuropathic model	Anxio-depressive behavior	NP surgery	NSF (8 weeks after)	Sham (6) NP (6)	Student t-test	$t(10) = 3.05$, $p \leq 0.01$		**1b**
Neuropathic model	Gene expression in the ACC	NP surgery	DNA Microarray (8 weeks after)	Sham (6) NP (6)	KEGG signaling pathway analysis	Pathways : MAPK, $p = 1.4^{e}$-06 ; T-cell, $p = 0.0019$; Calcium, $p = 0.0048$; p53, $p = 0.0072$		**1d**
Neuropathic model	ACC *mkp-1* gene expression	NP surgery	DNA Microarray (8 weeks after)	Sham (6) NP (6)	Student t-test	$p \leq 0.01$		**1e**
Neuropathic model	ACC MKP-1 protein abundance	NP surgery	Western Blot (8 weeks after)	Sham (6) NP (6)	Mann-Whitney U	$p \leq 0.001$		**1f**
Neuropathic model	Gene expression	NP surgery	DNA Microarray (8 weeks after)	Sham (6) NP (6)	Transcription factor targets analysis	Transcription factors: *atf1*, $p = 4.76e\text{-}12$; *nfat*, $p = 4.92e\text{-}12$; *srf*, $p = 2.57e\text{-}09$; *sp1*, $p = 6.41e\text{-}10$; *maz*, $p = 6.41e\text{-}10$; *e4f1*, $p = 9.73e\text{-}09$; *creb*, $p = 2.55e\text{-}08$		**2a**
Upstream transcription factors of MKP-1	ACC p-CREB protein abundance	NP surgery	Western blot (8 weeks after)	Sham (7) NP (8)	Mann-Whitney U	$p \leq 0.05$		**2b**
Upstream transcription factors of MKP-1	ACC p-ATF1 protein abundance	NP surgery	Western blot (8 weeks after)	Sham (7) NP (8)	Mann-Whitney U	$p \leq 0.01$		**2b**
Upstream transcription factors of MKP-1	CRE activity in the ACC	NP surgery	Immunohistochemistry (8 weeks after)	Sham (5) NP (5)	Distance X Surgery ANOVA Repeated Measurement	$F_{(6, 42)} = 14.10$, $p \leq 0.01$	Distance from bregma (mm): -0.05 – 1.37, $p \leq 0.05$	**2c**
Upstream transcription factors of MKP-1	c-Fos activity in the ACC	NP surgery	Immunohistochemistry (8 weeks after)	Sham (3) NP (3)	Distance X Surgery ANOVA Repeated Measurement	$F_{(14, 48)} = 5.75$, $p \leq 0.01$	Distance from bregma (mm): -0.47 – 1..33, $p \leq 0.001$	**2e**
Epigenetic modifications	H3K9K14ac at *c-fos* promoter	NP surgery	ChIP + qPCR (8 weeks after)	No antibody (6) Sham (6) NP (6)	One way ANOVA	$F_{(2, 3)} = 34.38$, $p \leq 0.01$	NP > Sham, $p \leq 0.05$	**2g**
Epigenetic modifications	H3K9K14ac at *mkp-1* promoter	NP surgery	ChIP + qPCR (8 weeks after)	No antibody (6) Sham (6) NP (6)	One way ANOVA	$F_{(2, 3)} = 55.32$, $p \leq 0.01$	NP > Sham, $p \leq 0.05$	**2g**
Epigenetic modifications	H3K27ac at *c-fos* promoter	NP surgery	ChIP + qPCR (8 weeks after)	No antibody (6) Sham (6) NP (6)	One way ANOVA	$F_{(2, 3)} = 3.22$, $p > 0.05$	NP > Sham, $p > 0.05$	**2h**

Experiment	Assessment	Treatment	Test	Groups (*n*)	Analysis	Statistics	Post-hoc	Figure
Epigenetic modifications	H3K27ac at *mkp-1* promoter	NP surgery	ChIP + qPCR (8 weeks after)	No antibody (6) Sham (6) NP (6)	One way ANOVA	$F_{(2, 3)}$ = 12.01, $p \leq 0.05$	NP > Sham, $p > 0.05$	**2h**
MKP-1 in depression models	Anxio-depressive behavior	UCMS	NSF	Non-stressed (7) Stressed (9)	Student t-test	$p \leq 0.001$		**3a**
MKP-1 in depression models	MKP-1 Protein abundance	UCMS	Western blot	Non-stressed (7) Stressed (9)	Mann-Whitney U	$p \leq 0.01$		**3b**
MKP-1 in other models	Anxio-depressive behavior	Optogenetic ACC stimulation	NSF	Non-stimulated (7) Stimulated (5)	Student t-test	$p \leq 0.01$		**3d**
MKP-1 in other models	ACC MKP-1 Protein abundance	Optogenetic ACC stimulation	Western blot	Non-stimulated (7) Stimulated (5)	Mann-Whitney U	$p \leq 0.05$		**3e,f**
Antidepressants and MKP-1	Mechanical (paw withdrawal) threshold before treatment	Fluoxetine	Von Frey	Sham saline (10) Sham fluoxetine (13) NP saline (12) NP fluoxetine (8)	Surgery X Treatment ANOVA	$F_{(1, 45)} = 0.18$, $p = 0.66$		**4a**
Antidepressants and MKP-1	Anxio-depressive behavior	Fluoxetine	NSF	Sham saline (10) Sham fluoxetine (13) NP saline (12) NP fluoxetine (8)	Surgery X Treatment ANOVA	$F_{(3, 42)}$ = 14.10, $p \leq 0.001$	NP vehicle > NP fluox, $p \leq 0.001$ NP vehicle > Sham vehicle, $p \leq 0.001$	**4b**
Antidepressants and MKP-1	ACC MKP-1 protein abundance	Fluoxetine	Western blot	Sham saline (4) Sham fluoxetine (4) NP saline (6) NP fluoxetine (4)	Mann-Whitney U Kruskal-Wallis	H = 13.93 $p \leq 0.05$	NP vehicle > Sham vehicle, $p \leq 0.01$ NP vehicle > NP fluox, $p \leq 0.01$ Sham vehicle > Sham fluox, $p \leq 0.01$	**4c**
MKP-1 KO	Mechanical (paw withdrawal) threshold	Baseline threshold	Von Frey	WT right paw (28) KO right paw (17)	One way (genotype) ANOVA	$F_{(1,43)} = 3.33$, $p = 0.075$		**5a**
MKP-1 KO	Mechanical (paw withdrawal) threshold	NP surgery	Von Frey	WT right paw Sham (14) WT right paw NP (14) KO right paw Sham (8) KO right paw NP (9)	Two way (genotype X surgery) ANOVA	$F_{(3, 123)}$ = 50.25, $p \leq 0.001$	WT Sham > WT NP, $p \leq 0.001$ KO Sham > KO NP, $p \leq 0.001$ (2-4 weeks post-surgery)	**5b**
MKP-1 KO	Anxio-depressive behavior	NP surgery	NSF (7 weeks after)	WT Sham (14) WT NP (14) KO Sham (8) KO NP (9)	Two way (genotype X surgery) ANOVA	$F_{(1. 41)} = 4.09$, $p \leq 0.05$	WT Sham < WT NP, $p \leq 0.001$	**5c**

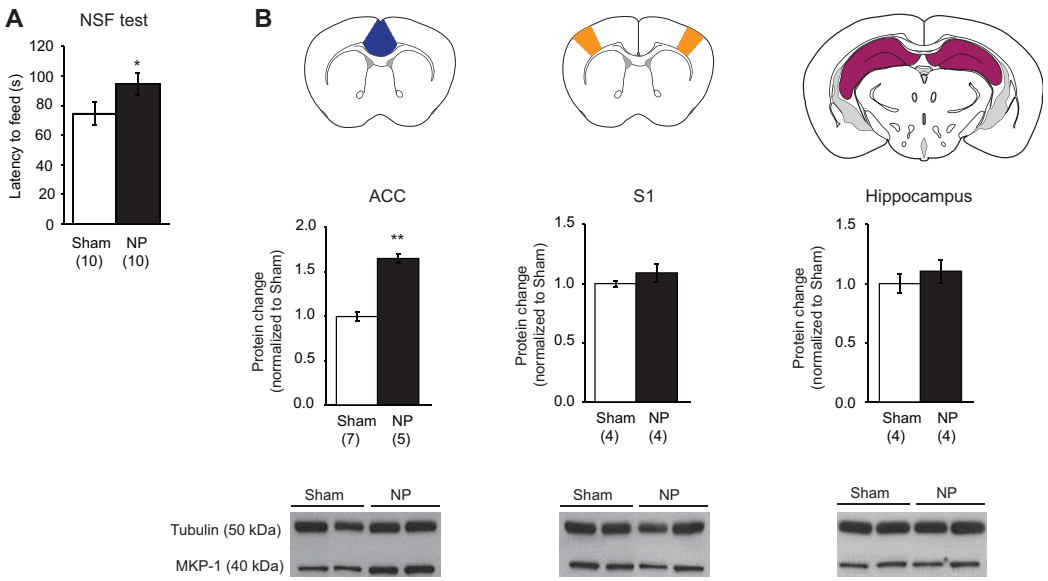
Experiment	Assessment	Treatment	Test	Groups (*n*)	Analysis	Statistics	Post-hoc	Figure
MKP-1 KO	Depressive-like behavior	NP surgery	Splash test (8 weeks after)	WT Sham (14) WT NP (14) KO Sham (8) KO NP (9)	Two way (genotype X surgery) ANOVA	$F_{(1, 41)} = 12.6$, $p \leq 0.001$	WT Sham > WT NP, $p \leq 0.001$	**5d**
MKP-1 systemic antagonist	Anxio-depressive behavior	NP surgery	NSF (before BCI treatment)	Sham (10) NP (13)	One way (surgery) ANOVA	$F_{(1,20)} = 10.34$, $p \leq 0.001$	NP saline > Sham saline, $p \leq 0.001$	**5e**
MKP-1 systemic antagonist	Depressive-like behavior	BCI	Splash test	Sham saline (10) NP BCI (8) NP saline (5)	Two way (surgery X treatment) ANOVA	$F_{(2, 19)} = 3.72$, $p \leq 0.05$	Sham saline > NP saline, $p \leq 0.05$ NP BCI > NP Saline, $p \leq 0.01$	**5e**
MKP-1 systemic antagonist	Mechanical (paw withdrawal) threshold	Mechanical sensitivity	Von Frey (after BCI)	Sham saline right paw (10) NP BCI right paw (8) NP saline right paw (5)	Two way (surgery X treatment) ANOVA	$F_{(2,23)} = 92.2$, $p \leq 0.001$	NP BCI < Sham saline, $p \leq 0.001$; NP saline < Sham saline, $p \leq 0.001$	**5f**
ACC MKP-1 silencing	ACC MKP-1 Protein abundance	siRNA	Western blot	Sham siRNA (6) NP siRNA (4) NP control (4)	Kruskal-Wallis Mann-Whitney U	$H_{(2)} = 6.16$ $p \leq 0.05$	NP siRNA < NP control, $p \leq 0.05$	**5g**
ACC MKP-1 silencing	Anxio-depressive behavior	siRNA	NSF	Sham siRNA (9) NP siRNA (9) NP control (8)	Two way (surgery X treatment) ANOVA	$F_{(2, 23)} = 7.28$, $p \leq 0.01$	NP siRNA < NP control, $p \leq 0.01$	**5h**
ACC MKP-1 silencing	Depressive-like behavior	siRNA	FST	Sham siRNA (9) NP siRNA (9) NP control (8)	Two way (surgery X treatment) ANOVA	$F_{(2,23)} = 8.35$, $p \leq 0.01$	NP siRNA < NP control, $p \leq 0.01$	**5i**
Neuropathic model	Mechanical (paw withdrawal) threshold	NP surgery	Von Frey	Sham right paw (6) NP right paw (6)	Two way (surgery X treatment) ANOVA	$F_{(7,70)} = 9.577$, $p \leq 0.001$	NP < Sham, $p \leq 0.001$ (1-8 weeks post-surgery)	**Suppl. S1b**
Neuropathic model	Anxio-depressive behavior	NP surgery	NSF (8 weeks after)	Sham (6) NP (6)	Student t-test	$t(10) = 3.61$, $p \leq 0.001$		**Suppl. S1b**
NP control experiment	Anxio-depressive behavior	NP surgery	NSF (8 weeks after)	Sham (5) NP (6)	Student t-test	$t(9) = 1.88$, $p \leq 0.05$		**Suppl. S1a**
NP control experiments	Home cage feeding	NP surgery	Home cage feeding assessment after NSF (8 weeks after)	Sham (5) NP (6)	Student t-test	$t(9) = 0.56$, $p > 0.05$		**Suppl. S1a**
NP control experiments	Locomotor activity after NP surgery	NP surgery	Open field (8 weeks after)	Sham (5) NP (6)	Student t-test	5 mins : $t(9) = 1.02$, $p > 0.05$; 1 hour : $t(9) = 0.03$, $p > 0.05$; 2 hours : $t(9) = 0.09$, $p > 0.05$		**Supp1a S1a**
MKP-1 in other brain regions	Anxio-depressive behavior	NP surgery	NSF	Sham (10) NP (10)	Student t-test	$t(18) = 1.88$, $p \leq 0.05$		**Suppl. S2**

Experiment	Assessment	Treatment	Test	Groups (*n*)	Analysis	Statistics	Post-hoc	Figure
MKP-1 in other brain regions	ACC MKP-1 protein abundance in the ACC	NP surgery	Western Blot (8 weeks after)	Sham (7) NP (5)	Mann-Whitney U	$p \leq 0.01$		**Suppl. S2**
MKP-1 in other brain regions	Somatosensory cortex MKP-1 protein abundance	NP surgery	Western Blot (8 weeks after)	Sham (4) NP (4)	Mann-Whitney U	$p > 0.05$		**Suppl. S2**
MKP-1 in other brain regions	Hippocampus MKP-1 protein abundance	NP surgery	Western Blot (8 weeks after)	Sham (4) NP (4)	Mann-Whitney U	$p > 0.05$		**Suppl. S2**
Neuropathic model	ACC *mkp-1* gene expression	NP surgery	DNA Microarray (2 weeks after)	Sham (6) NP (6)	Student t-test	$p \leq 0.001$		**Suppl. S3a**
MKP-1 in ACC	ACC MKP-1 protein abundance in the ACC	NP surgery	Western Blot (2 weeks after)	Sham (4) NP (5)	Mann-Whitney U	$p > 0.05$		**Suppl. S3b**
MKP-1 expression before depression	ACC MKP-1 protein abundance in the ACC	NP surgery	Western Blot (5 weeks after)	Sham (5) NP (4)	Mann-Whitney U	$p > 0.05$		**Suppl. S3c**
MKP-1 in ACC	Anxiety-like behavior	NP surgery	Dark-light test (5 weeks after)	Sham (5) NP (5)	Student t-test	$t(8) = 3.80$, $p \leq 0.01$		**Suppl. S3c**
Upstream transcription factors of MKP-1	c-Fos activity in the prelimbic region	NP surgery	c-Fos Immunohistochemistry (8 weeks after)	Sham (3) NP (4)	One way (surgery) repeated measurement ANOVA	$F_{(1, 5)} = 3.42$, $p > 0.05$		**Suppl. S4**
Upstream transcription factors of MKP-1	c-Fos activity the infralimbic region	NP surgery	c-Fos Immunohistochemistry (8 weeks after)	Sham (3) NP (4)	One way (surgery) repeated measurement ANOVA	$F_{(1, 5)} = 7.7$, $p<0.05$ Posthoc:0.05		**Suppl. S4**
Upstream transcription factors of MKP-1	CRE activity in the prelimbic region	NP surgery	β-gal Immunohistochemistry (8 weeks after)	Sham (6) NP (6)	One way (surgery) repeated measurement ANOVA	$F_{(1, 10)} = 2.47$, $p > 0.05$		**Suppl. S4**
Upstream transcription factors of MKP-1	CRE activity in the infralimbic region	NP surgery	β-gal Immunohistochemistry (8 weeks after)	Sham (6) NP (6)	One way (surgery) repeated measurement ANOVA	$F_{(1, 10)} = 1.83$, $p > 0.05$		**Suppl. S4**
Single optogenetic stimulation effect	Depressive-like behavior	Optogenetic ACC stimulation	NSF (5 min after stimulation)	Non-stimulated (4) Stimulated (4)	Student t-test	$t(6) = 0.4$, $p > 0.05$		**Suppl. S6a**
Single optogenetic stimulation effect	Depressive-like behavior	Optogenetic ACC stimulation	Splash test (1 day after stimulation)	Non-stimulated (4) Stimulated (4)	Student t-test	$t(6) = 0.23$, $p > 0.05$		**Suppl. S6a**

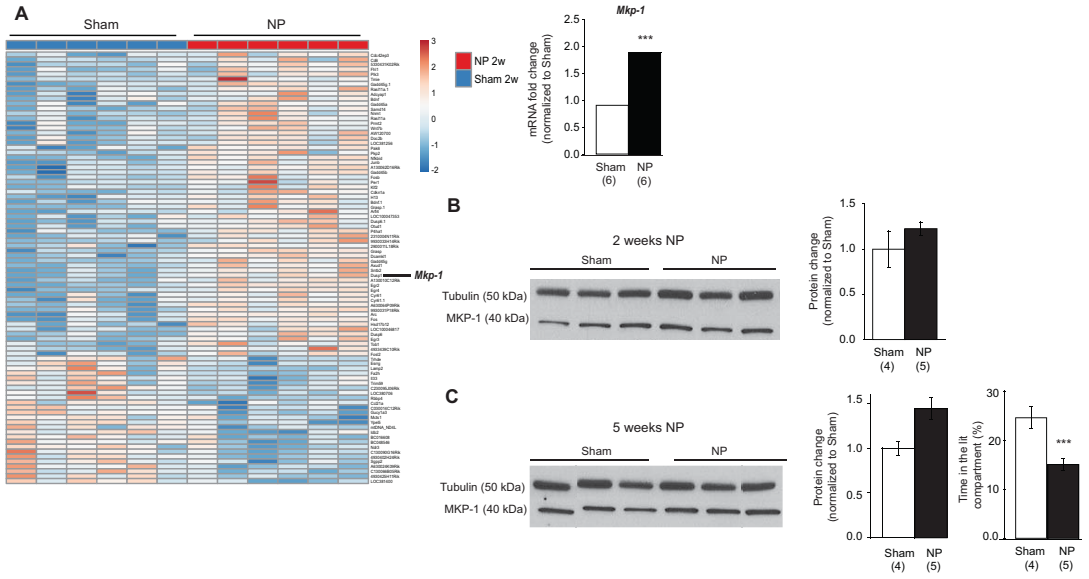
Experiment	**Assessment**	**Treatment**	**Test**	**Groups (*n*)**	**Analysis**	**Statistics**	**Post-hoc**	**Figure**
Long term effects of optogenetic stimulation	Depressive-like behavior	Optogenetic ACC stimulation	Splash (1 day after stimulation)	Non-stimulated (4) Stimulated (5)	Student t-test	$t(7) = 2.19, p \leq 0.05$		**Suppl. S6b**
Long term effects of optogenetic stimulation	Depressive-like behavior	Optogenetic ACC stimulation	Splash (2weeks after stimulation)	Non-stimulated (4) Stimulated (5)	Student t-test	$t(7) = 0.03, p > 0.05$		**Suppl. S6b**
Long term effects of optogenetic stimulation	ACC MKP-1 protein abundance	Optogenetic ACC stimulation	Western Blot (2 weeks after)	Non-stimulated (4) Stimulated (5)	Mann-Whitney U	$p \leq 0.05$		**Suppl. S6b**
ACC MKP-1 silencing control	Anxio-depressive behavior	siRNA	NSF	WT control (5) WT siRNA (5)	Student t-test	$t(8) = 0.88, p > 0.05$		**Suppl. S7**
ACC MKP-1 silencing control	Depressive-like behavior	siRNA	FST	WT siRNA (5) WT control (5)	Student t-test	$t(8) = 0.36, p > 0.05$		**Suppl. S7**



Supplementary Figure S1. Behavioral data. (**A**) Neuropathic animals displaying increased latency to feed in the novelty suppressed feeding (NSF) test did not show any change in food consumption and in spontaneous locomotor activity. (**B**) Behavioral data for mice used for Western blot analyses (as reported in Figure 1F). von Frey results for the right paw, showing that NP animals displayed lower mechanical thresholds compared to sham animals. NSF results showing that NP animals displayed a higher latency to feed compared to sham animals, suggesting a presence of NP-induced anxiodepressive-like behaviors. Data are expressed as mean ± s.e.m.; *$p \leq 0.05$, ***$p \leq 0.001$.



Supplementary Figure S2. MKP-1 protein levels increased in the anterior cingulate cortex (ACC), but not in the primary somatosensory cortex (S1) or in the whole hippocampus, of neuropathic pain (NP) animals displaying depressive-like behaviors. (**A**) Behavioral data showing that the NP animals displayed increased latency to feed after 8 weeks of sciatic nerve injury in the novelty suppressed feeding (NSF) test. (**B**) Western blot data demonstrated increased level of *Mkp-1* in the ACC but not in the somatosensory cortex I or in the whole hippocampus. Data are expressed as mean ± s.e.m.; *$p \leq 0.05$;**$p \leq 0.01$.



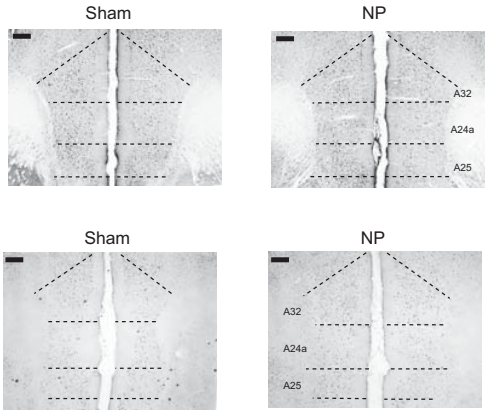
Supplementary Figure S3. 2 weeks after the sciatic nerve injury, *Mkp-1* mRNA but not protein levels increase in the anterior cingulate cortex (ACC) of neuropathic pain (NP) animals displaying increased mechanical hypersensitivity without anxiodepressive-like behaviors. (**A**) Heatmap representing dysregulated genes in the ACC 2 weeks after the surgery. Microarray result showing an overexpression of *Mkp-1* in the ACC of NP animals compared to controls. (**B**) Western blot results illustrating no change in ACC MKP-1 protein levels in animals at 2 weeks post-surgery. (**C**) Western blot results illustrating slight increase in ACC MKP-1 protein levels in animals at 5 weeks post-surgery, the time point in which NP animals showed anxiety-like behaviors as demonstrated here with decreased time spent in the lit compartment in the dark-light test. Data are expressed as mean ± SEM; *** $p \leq 0.001$.

A β-gal positive nuclei

Distance	Treatment	Prelimbic	Infralimbic
1.95	Sham (6)	19.33 ± 3.42	18.70 ± 2.56
	NP (6)	30.34 ± 4.42	27.90 ± 4.73
1.77	Sham (6)	21.86 ± 4.19	22.13 ± 5.52
	NP (6)	25.25 ± 2.82	27.58 ± 3.79

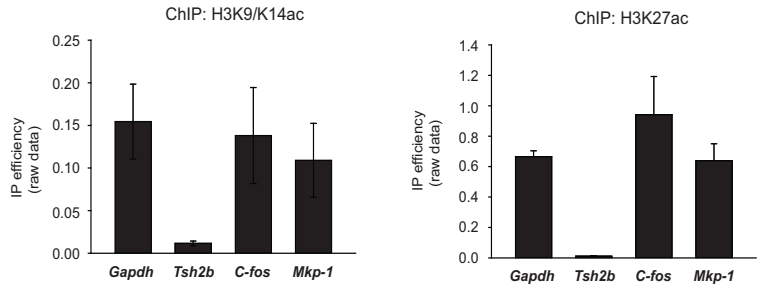
B c-Fos positive nuclei

Distance	Treatment	Prelimbic	Infralimbic
1.95	Sham (3)	23.33 ± 4.33	28.16 ± 3.16
	NP (4)	26.12 ± 4.37	49.00 ± 0.50*
1.77	Sham (3)	16.50 ± 4.33	27.33 ± 12.27
	NP (4)	21.75 ± 1.19	37.47 ± 4.74



Supplementary Figure S4. NP-induced depressed animals displayed altered c-Fos expression but not cAMP driven transcriptional activity in the rostral infralimbic (A25) area. (**A**) Quantitative analysis of β-gal positive nuclei in the prelimbic (A32) and infralimbic (A25) cortex of Sham and NP animals at various distances from the Bregma, showing no difference between sham and NP animals for the presence of CRE-positive cells after 8 weeks of sciatic nerve injury. Representative pictures comparing the expression and distribution of β-gal labeling in sham and NP CRE-LacZ mice. (**B**) Increased c-Fos expression in NP animals compared to sham animals in the rostral infralimbic cortex after 8 weeks of sciatic nerve injury. Representative pictures comparing the expression and distribution of c-Fos positive cells of sham and NP animals. Scale bars 300 μm. Data are expressed as mean ± SEM; *$p \leq 0.05$.

A



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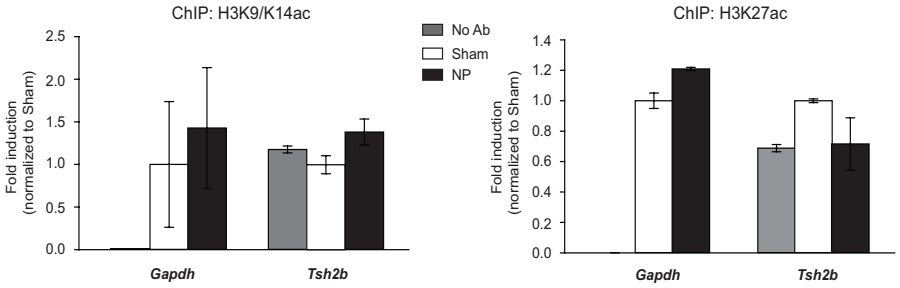
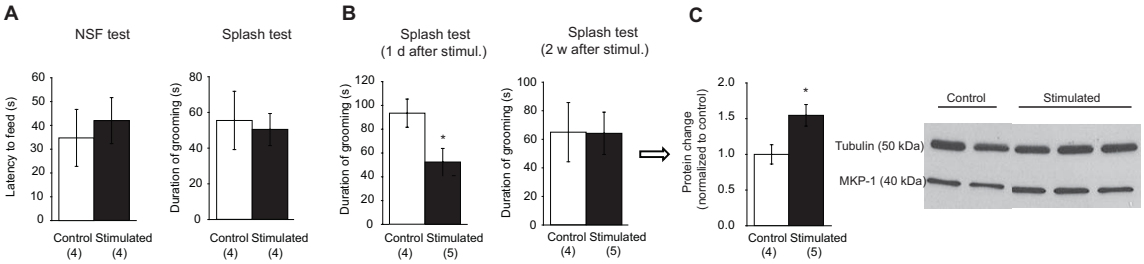
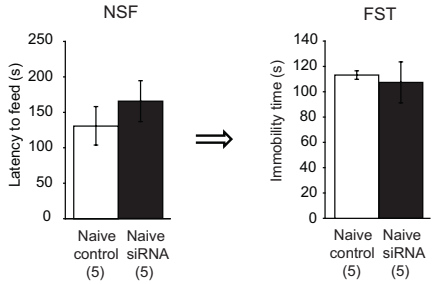


Supplementary Figure S5. Controls for chromatin immunoprecipitation (ChIP) data (related to Figure 2G and 2H). (**A**) IP enrichment results for H3K9/K14ac and H3K27ac, showing the significant enrichment on *Gapdh*, a control gene expressed ubiquitously, and the absence of enrichment on *Tsh2b*, which is not expressed in the cortex. (**B**) IP enrichment results showing that there was no difference between NP and sham animals for fold induction of the control genes. Data are expressed as mean ± s.e.m..



Supplementary Figure S6. Impact of different optogenetic stimulation paradigms on anxiodepressive-like behavior and MKP-1 protein level. (**A**) NSF performed 5 minutes and splash test performed 1 day (d) after a single stimulation did not induce any significant anxiodepressive-like behaviors. (**B**) The anxiodepressive-like behaviors observed one day after 4 consecutive stimulation days disappeared after 2 weeks (w) in the splash test while (**C**) MKP-1 levels still remained significantly high. Data are expressed as mean ± SEM; * $p \leq 0.05$.



Supplementary Figure S7. Impact of local deletion of *Mkp-1* on behavior in naive animals. *Mkp-1* silencing within the ACC did not modify mood related behaviors in naive animals in the NSF and FST tests.

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Ketamine induces rapid and sustained antidepressant-like effects in chronic pain induced depression: role of MAPK signaling pathway (Article)

Intrigued by the results obtained in our first objective, particularly by the potential involvement of the MAP kinase pathway in the antidepressant activity of the SSRI fluoxetine, we decided to perform a side project which would examine whether a similar molecular pattern is associated with the activity of another pharmacological agent shown to improve depressive symptoms. The evident candidate for us was ketamine, due to its previously mentioned, ever increasing popularity as a rapid-acting, long-lasting antidepressant. Hence, we were interested to study its antidepressant-like and anti-allodynic properties in our mouse model of sustained neuropathic pain, as well as to determine whether it also exerts its therapeutic activity through modulating the MAP kinase pathway. The following manuscript, describing the completed experiments pertaining to this objective has been submitted for publication.

Title Page

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

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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 individuals, which urges the development of treatment strategies targeting this comorbidity. Ketamine is a noncompetitive antagonist of the N-methyl-D-aspartate receptor shown 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 explored the behavioral and molecular effects of a single systemic administration of ketamine (15 mg/kg, i.p.) on mechanical hypersensitivity and anxiodepressive-like consequences of chronic neuropathic pain. We show that ketamine transiently alleviated mechanical hypersensitivity (lasting < 24h), while reducing depressive-like behaviors over a longer period of time, present 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 neuropathy-induced anxiodepressive-like behaviors. These findings provide insights into the behavioral and molecular changes associated with single ketamine administration in the comorbidity of chronic pain and depression.

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

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.

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 abundant evidence accumulated over the past several decades about the antidepressive and antinociceptive activity of ketamine, including the molecular and cellular changes that accompany its administration (Cohen et al., 2018; Sleigh et al., 2014; Zanos and Gould, 2018a, b), ketamine's therapeutic potential has not yet been fully elucidated.

We have recently shown that neuropathy-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 associated with processing both pain-related and affective stimuli (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 AMP (adenosine monophosphate) 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 neuropathy-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 pathway.

Specifically, the present study evaluated the effect of systemic ketamine administration on the mechanical hypersensitivity and emotional consequences of neuropathic pain as well as its effect on MAPK pathway. Our main results show that a single intraperitoneal (i.p.) injection of ketamine (15 mg/kg) results in rapid reduction of depressivelike behaviors lasting several days, while only transiently lowering mechanical hypersensitivity. This ketamine-mediated phenotype was accompanied by a decrease in the MKP-1 and 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

Ninety adult male C57BL/6J 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 12h 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 three independent cohorts of animals, wherein two were used for behavioral testing and one was 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 (10mg/kg) (Centravet, Taden, France) intraperitoneally (i.p.) before neuropathy was induced by unilateral implantation of a 2mm 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 presence of mechanical allodynia 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 pressure points.

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 post neuropathy induction. All the tests were performed during the animals' active phase (darkcycle), under red light and as previously described (Yalcin et al., 2011).

2.4.1. Novelty-suppressed feeding test

Food deprived (24 h) mice were placed in the corner of an open plastic box (40 cm x 40 cm x 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 minutes 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 minutes. The test measures the animal's motivation for self-hygiene, and a lack of it indicates loss of motivation, 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 minutes. 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 minutes 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 1h 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 and 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 $p \leq 0.05$. For detailed information see Supplementary table S1.

3. Results

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

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; $F_{(4, 100)} = 6.86$, $p \leq 0.001$). 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', $p \leq 0.001$). This rapid but transitory allodynia relief was not observed in neither sham nor neuropathic animals administered with saline (Fig. 1D and D').

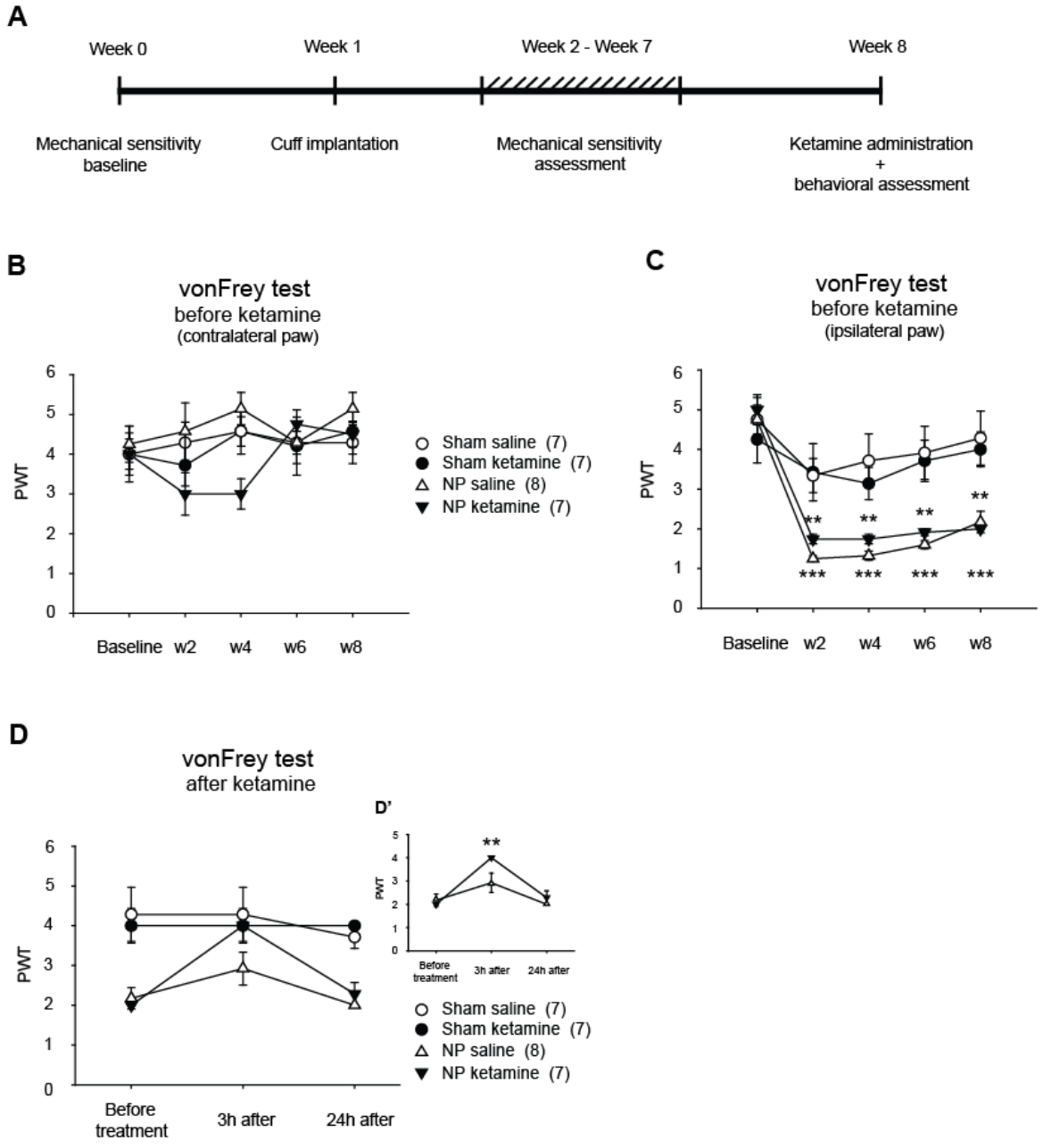

Fig. 1. Antinociceptive effects of single systemic ketamine administration. A) Timeline of surgical and behavioral procedures. B) Pre-ketamine treatment mechanical sensitivity showing no difference in the post-surgery threshold of the contralateral paw of sham and neuropathic (NP) mice as assessed by 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 3h (D'), but not 24h, after administration.

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

Compared to the control animals which received a sham surgery, cuff implantation around the sciatic nerve resulted in anxiodepressive behaviors 8 weeks after the surgery, as displayed by an increased latency to feed in the NSF test (Fig. 2B; $p \leq 0.05$), a decreased grooming duration in the Splash test (Fig. 2C; $p \leq 0.05$) and an increased immobility in the FST (Fig. 2D; $p \leq 0.001$). While the anti-allodynic effect was transient, a single injection of a subanesthetic dose of ketamine was sufficient to reduce neuropathy-induced anxiodepressive-like behaviors for a prolonged period of time (Fig. 2B-E). Specifically, the neuropathic animals showed a decrease in anxiety and depression-related behaviors 1h after ketamine administration in the NSF test (Fig. 2B; $p \leq 0.01$), 24h after in the Splash test (Fig. 2C; $p \leq 0.05$) and 72h after in the FST (Fig. 2D; $p \leq 0.05$). However, 2 weeks after single ketamine injection, depressive like behaviors were the same in both the treated and nontreated neuropathic group (Fig. 2E; $p > 0.05$).

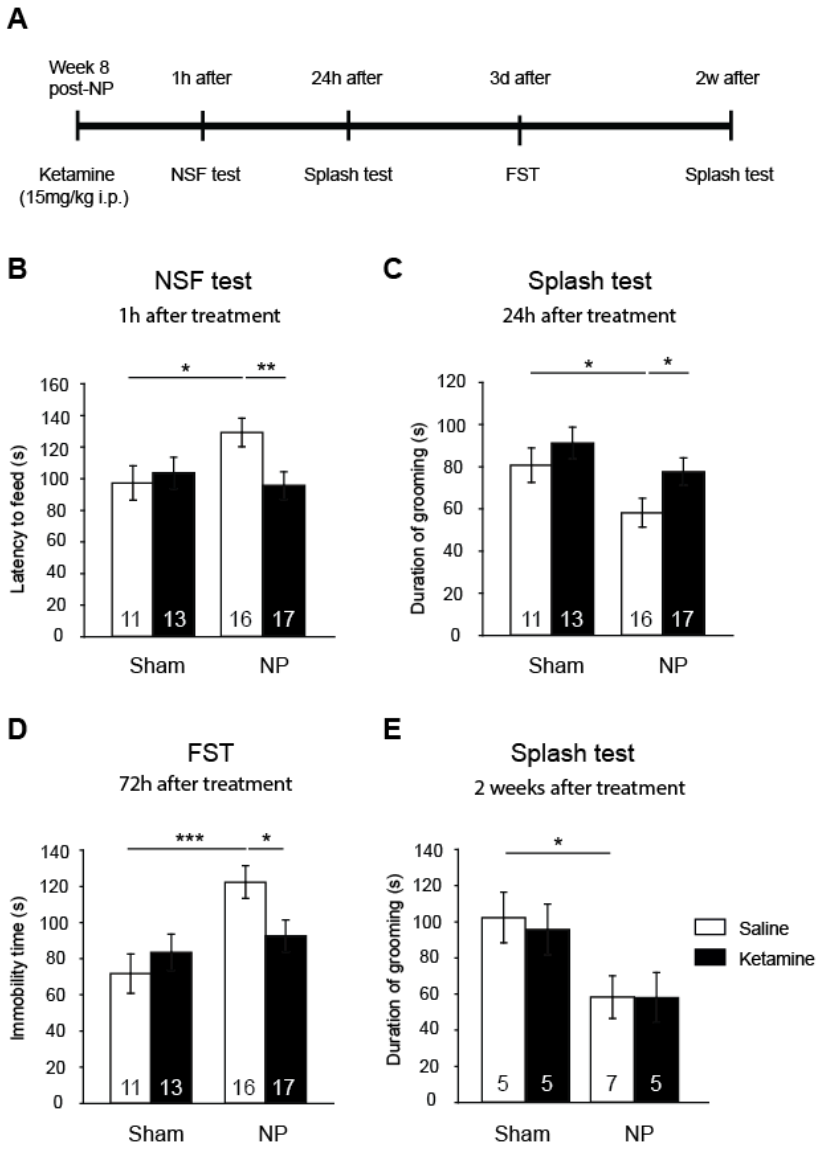

Fig. 2. Antidepressant-like effect of acute systemic ketamine administration. A) Timeline of surgical and behavioral procedures. B) NSF test showing a decrease in the latency to feed of NP mice 1h after ketamine injection. C) Acute ketamine treatment resulted in an increased grooming duration of NP animals in the splash test 24h later. D) Ketamine-treated NP mice showed a decrease in immobility time during FST 72h 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.

3.3. Single ketamine administration restores the disrupted MAPK signaling pathway

The last batch of animals was used to perform molecular analyses on the ACC tissues. Western blot analysis showed that MKP-1 protein level was increased in the ACC of animals displaying neuropathic pain-induced anxiodepressive-like behaviors (Fig. 3A; $F_{(1, 19)} = 4.08$, $p \leq 0.05$; post hoc: sham saline < NP saline, $p \leq 0.01$). However, this increase was diminished 30 minutes after single ketamine administration (Fig. 3A; $p \leq 0.01$). Similarly, neuropathic animals displayed a decreased p-ERK protein level in the ACC (Fig. 3B; $F_{(1, 20)} = 0.42$, $p \leq 0.05$; post hoc: sham saline > NP saline, $p = 0.09$) which was restored 30 minutes after single ketamine administration (Fig. 3B; NP saline < NP ketamine, $p \leq 0.05$).

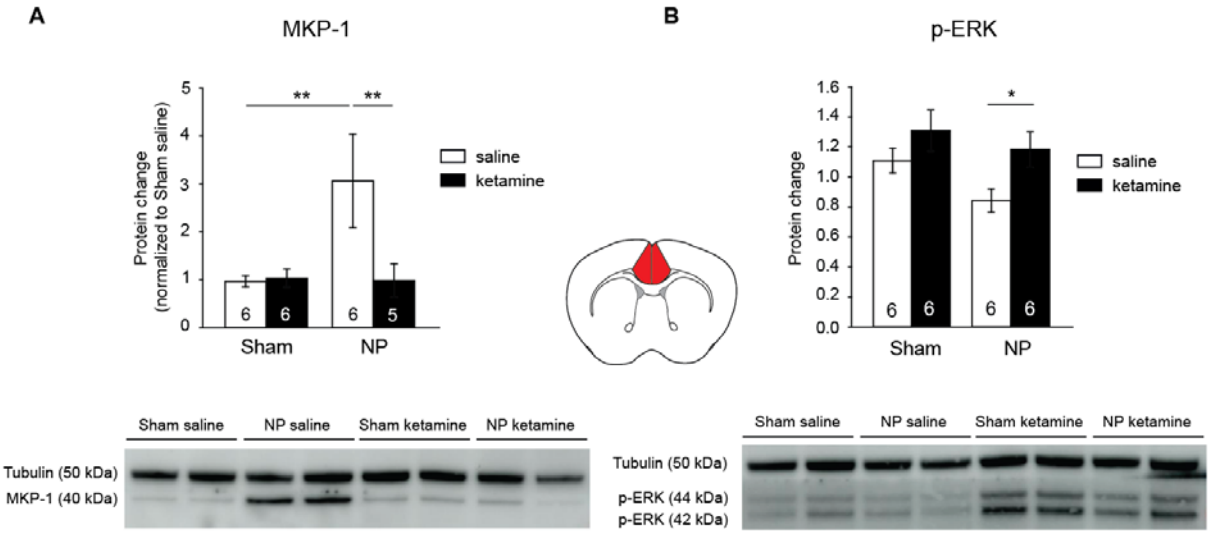

Fig. 3. The effect of ketamine on the MAPK pathway in the ACC. A) Western blot analysis showing an increase in MKP-1 protein level in the ACC of NP mice before treatment, and a decrease 30 minutes after acute systemic ketamine treatment. B) Western blot results showing decreased phosphorylated ERK in the ACC of NP mice, which is restored 30 minutes after ketamine administration. Sample sizes are presented in brackets next to experimental groups.

4. Discussion

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

4.1. Ketamine's pain-relieving properties

Current first-line treatments for neuropathic pain include tricyclic antidepressants, serotonin noradrenaline reuptake inhibitors and anticonvulsants such as gabapentin and

pregabaline (Gilron et al., 2015). 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 3h 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; Schwartzman et al., 2009; 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). When ketamine was administered during the early postsurgical period, starting at the neuropathic pain surgery, there was a progressive recovery of mechanical allodynia which did not fully reappear until 2 months after the cessation of the treatment. On the other hand, starting ketamine administration with a 25-day delay after neuropathy induction produced significantly less robust pain relief, where animals only partially recovered 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). However, the exact mechanisms underlying ketamine's effects on nociception remain poorly understood. One possibility might involve the desensitization of NMDA receptors in the spinal cord which leads to a prolonged pain relief (Sigtermans et al., 2009). As the desensitization in NMDA receptors is relatively slow compared to other glutamatergic receptors such as AMPA and kainate receptors (Traynelis et al., 2010), an extended ketamine treatment might be required

to initiate adaptations that lead to long-term decrease of dorsal horn excitability, and thereby sustained nociception.

Although it has been shown that 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), little is known about the effect of ketamine on the MAPK pathway in neuropathic pain. However, recent evidences suggest 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 spinal astrocyte JNK activation in rats after spinal nerve ligation (Mei et al., 2011). Moreover, besides reducing proinflammatory cytokines in the spinal cord of neuropathic rats, Kwon et al. (2014) demonstrated that ketamine also diminished the increased expression of p38 and phospho-p38 in these animals. Finally, ketamine infusions successfully decreased upregulated ERK in the spinal cord of rats which underwent spinal cord injury (Choi et al., 2009). These studies suggest that ketamine's efficacy in treating neuropathic pain may be related to its suppressive effects on the disrupted expression of MAPKs. Notably however, the effects of ketamine on MAPKs function are both time and dose-dependent. While sedative anesthetic doses acutely reduce MAPK phosphorylation, subanesthetic (i.e. antidepressant) doses produce opposite effects (Kohtala et al., 2019; Kohtala et al., 2016).

4.2. Ketamine's antidepressant properties

The present study showed that a single injection of ketamine at a sub-anesthetic dose is sufficient to relieve neuropathic pain-induced anxiodepressive-like behaviors in mice 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 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 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). With this in mind, neuroimaging studies have demonstrated a relationship between the activity of limbic brain areas such as the ACC and amygdala, and ketamine's antidepressant efficiency. 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. Besides cortex' activity (Ahnaou et al., 2017; Liu et al., 2015; Niesters et al., 2012), ketamine also blocks neuronal bursting in the lateral habenula of rats with depressive-like behaviors (Yang et al., 2018) reinforcing the ideas that ketamine might act through restoring the altered neuronal activity in different brain regions.

Hence, the transient antinociceptive and prolonged antidepressant effect of acute ketamine administration observed in the current study illustrates 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 depression-relieving 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).

4.3. Molecular mechanisms underlying ketamine´s antidepressant effects

In the present study, we showed that an acutely administered ketamine (15 mg/kg) 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, while it had no effect on the expression of MKP-1 in the ACC of non-neuropathic control mice. In addition, ketamine restored the decreased p-ERK in the ACC of mice with comorbid neuropathic pain and anxiodepressive-like behaviors.

Accordingly, postmortem studies have demonstrated that *MKP-1* is overexpressed in the hippocampus of depressed patients (Duric et al., 2010), while ERK1/2 mRNA and protein

levels are decreased (Dwivedi et al., 2001). A similar pattern of expression, where MKP-1 was increased and p-ERK was decreased, was also found in the hippocampus of mice displaying depressive-like behaviors due to morphine-withdrawal, and these behaviors, alongside the altered expressions, were prevented by intrahippocampal infusions of an MKP-1 inhibitor (Jia et al., 2013). This is not surprising given that a substantial body of recent preclinical studies have reported a downregulation of ERK in the prefrontal cortex associated with depressive-like behaviors following exposure to various stressors, including chronic forced swimming (Qi et al., 2008; Qi et al., 2006), social defeat (Wang et al., 2018), chronic restraint (Oh et al., 2018; Wang et al., 2016) and chronic unpredictable stress (First et al., 2011; Li et al., 2017).

Ketamine has been shown to alter the expression of *Mkp-1* in brain regions like the striatum or hippocampus (Ficek et al., 2016) and also normalize its expression associated with susceptibility to depression (Bagot et al., 2017). Additionally, previous evidence suggests that a single dose of subanesthetic ketamine recruits the MAPK signaling cascade (Kohtala et al., 2019; Li et al., 2010) and ameliorates chronic stress-induced deficits in spine number and function (Li et al., 2011), which is a common characteristic in depression (Banasr et al., 2011). Moreover, Réus et al. (2014) found that acute blockade of MAPK signaling is sufficient to induce depressive-like behaviors and, moreover, 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).

While these 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 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.

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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).

Supplementary table: Statistical analysis data

Experiment	Assessment	Treatment	Test	Groups (*n*)	Analysis	Statistics	Post-hoc	Fig
Neuropathic model	Mechanical (contralateral paw withdrawal) threshold	0-8w post-NP; before ketamine	Von Frey	Sham saline (7) Sham ketamine (7) NP saline (8) NP ketamine (7)	ANOVA	Surgery vs Treatment vs Time: $F_{(4, 100)} = 1.34$, $p > 0.05$		**1B**
Neuropathic model	Mechanical (ipsilateral paw withdrawal) threshold	0-8w post-NP; before ketamine	Von Frey	Sham saline (7) Sham ketamine (7) NP saline (8) NP ketamine (7)	ANOVA	Surgery vs Treatment vs Time: $F_{(4, 100)} = 0.20$, $p > 0.05$ Time vs Treatment: $F_{(4, 100)} = 0.26$, $p > 0.05$ Surgery vs Time: $F_{(4, 100)} = 6.86$, $p \leq 0.001$	Duncan: NP saline < Sham saline, $p \leq 0.001$ (2-8 weeks post-surgery) NP ketamine < Sham ketamine, $p \leq 0.01$ (2-8 weeks post-surgery)	**1C**
Neuropathic model	Mechanical (ipsilateral paw withdrawal) threshold	8w post-NP; 0-24h post-ketamine	Von Frey	Sham saline (7) Sham ketamine (7) NP saline (8) NP ketamine (7)	ANOVA	Surgery vs Treatment vs Time: $F_{(2, 50)} = 1.00$, $p > 0.05$ Time x Treatment: $F_{(2, 50)} = 0.78$, $p > 0.05$ Surgery vs Time: $F_{(2, 50)} = 3.59$, $p \leq 0.05$		**1D**
Neuropathic model	Mechanical (ipsilateral paw withdrawal) threshold	8w post-NP; 0-24h post-ketamine	Von Frey	NP saline (8) NP ketamine (7)	ANOVA	Treatment vs Time: $F_{(2, 26)} = 2.97$, $p > 0.05$	Duncan: Ketamine: 3h > 24h, $p \leq 0.001$ Ketamine: 3h > 24h, $p \leq 0.001$ Ketamine 3h > saline 3h, $p \leq 0.01$	**1D'**
Neuropathic model + ketamine (15mg/kg)	Anxiodepressive-like behavior	8w post-NP; 1h post-ketamine	NSF	Sham saline (11) Sham ketamine (13) NP saline (16) NP ketamine (16)	ANOVA	Treatment vs Time: $F_{(1, 53)} = 4.33$, $p \leq 0.05$	Duncan: Sham saline < NP saline, $p \leq 0.05$ NP saline > NP ketamine, $p \leq 0.01$	**2B**
Neuropathic model + ketamine (15mg/kg)	Depressive-like behavior	8w post-NP; 24h post-ketamine	Splash test	Sham saline (11) Sham ketamine (13) NP saline (16) NP ketamine (17)	ANOVA	Treatment vs Time: $F_{(1, 53)} = 0.37$, $p > 0.05$ Treatment: $F_{(1, 53)} = 4.24$, $p \leq 0.05$	Duncan: Sham saline > NP saline, $p \leq 0.05$ NP saline < NP ketamine, $p \leq 0.05$	**2C**
Neuropathic model + ketamine (15mg/kg)	Depressive-like behavior	8w post-NP; 72h post-ketamine	FST	Sham saline (11) Sham ketamine (13) NP saline (16) NP ketamine (17)	ANOVA	Treatment vs Time: $F_{(1, 53)} = 4.55$, $p \leq 0.05$	Duncan: Sham saline < NP saline, $p \leq 0.001$ NP saline > NP ketamine, $p \leq 0.05$	**2D**
Neuropathic model + ketamine (15mg/kg)	Depressive-like behavior	8w post-NP; 2w post-ketamine	Splash test	Sham saline (5) Sham ketamine (5) NP saline (7) NP ketamine (5)	ANOVA	Treatment vs Time: $F_{(1, 18)} = 0.06$, $p > 0.05$ Surgery: $F_{(1, 18)} = 9.30$, $p \leq 0.01$	Duncan: Sham saline > NP saline, $p \leq 0.05$	**2E**
Neuropathic model + ketamine (15mg/kg)	MKP-1 protein abundance in ACC	8w post-NP; 30min post-ketamine	Western blot	Sham saline (6) Sham ketamine (6) NP saline (6) NP ketamine (5)	ANOVA	Treatment vs Time: $F_{(1, 19)} = 4.08$, $p \leq 0.05$	Duncan: Sham saline < NP saline, $p \leq 0.01$ NP saline > NP ketamine, $p \leq 0.01$	**3A**
Neuropathic model + ketamine (15mg/kg)	p-ERK protein abundance in ACC	8w post-NP; 30min post-ketamine	Western blot	Sham saline (6) Sham ketamine (6) NP saline (6) NP ketamine (6)	ANOVA	Treatment vs Time: $F_{(1, 20)} = 0.42$, $p \leq 0.05$ Treatment: $F_{(1, 20)} = 0.53$, $p \leq 0.05$	Duncan: Sham saline > NP saline, $p \leq 0.09$ NP saline < NP ketamine, $p \leq 0.05$	**3B**

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The role of ACC GABAergic cells in neuropathic pain-induced depression (Article)

The following manuscript contains data pertaining to the third objective presented in the current thesis, focusing on the functional and genomic characteristics of GABAergic neurons in the ACC associated with neuropathic and depressive states. The data we aim to obtain upon the completion of the current project will serve as an extension and continuation of the results obtained in the first objective. Specifically, while the first objective concerned the genomic characterization of the whole ACC, which served to identify specific key factors important in the etiology of neuropathic pain-induced anxiodepressive-like behaviors, the current objective tackles this matter from a cell-type specific perspective. This project is still ongoing.

Title Page

The role of ACC GABAergic cells in neuropathic pain-induced depression

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Abstract

Chronic pain frequently results in the development of mood disorders such as major depressive disorder. The increasing socio-economic burden of comorbid chronic pain and depression urges for more in-depth understanding of this pathology, which has a potential to yield more efficient treatment strategies. Still, the individual role of defined neuronal types within the brain in the development and persistence of comorbid pain and depression remains largely unexplored. Here, by using a mouse model of peripheral neuropathic pain-inducing anxiodepressive-like behaviors, in combination with optogenetic stimulation and viral translating ribosome affinity purification (vTRAP), we explored the functional and molecular characteristics of GABAergic cells in the anterior cingulate cortex (ACC) in comorbid chronic pain and depression. We demonstrated that optogenetic activation of Dlx5/6-expresssing GABAergic neurons in the ACC induces an antidepressant-like effect in naive mice at 10 and 7.5, but not 5 Hz frequency. However, stimulation at 5 Hz is sufficient to block anxiodepressive-like behaviors resulting from peripheral neuropathic pain. Furthermore, we showed that the vTRAP approach is a useful method for isolating cell type-specific mRNA from GABAergic neurons in the ACC of adult mice, which can further be used to study the genomic expression of this cell type in the pain-depression comorbidity. Once completed, the present study will shed light on the functional and molecular properties of GABAergic cells within the ACC in comorbid chronic pain and depression.

Keywords: Depression, Neuropathic pain, Anterior cingulate cortex, GABAergic neurons, Optogenetics, vTRAP

INTRODUCTION

Chronic pain and depression are debilitating pathologies with a growing prevalence around the world (Bair et al. 2003; Rayner et al. 2016). Their comorbidity leads to poorer quality of life, recovery prognoses and treatment success than either condition by itself (Gallagher and Verma 1999; Arnow et al. 2006; Kirsh 2010) resulting in a continuously growing socioeconomic burden (Attal et al. 2011; Dominick et al. 2012). It has been estimated that mood disorders such as depression affect more than 30% of patients suffering from neuropathic pain (Gustorff et al. 2008), a chronic pain condition caused by a disease or lesion of the somatosensory system (Loeser and Treede 2008). While clinical research and advanced neuroimaging studies are an indispensable asset in understanding the anatomofunctional properties of human comorbid chronic pain and depression (Maletic and Raison 2009, Malfliet et al. 2017; Yoshino et al. 2017), the existence of preclinical animal models (Yalcin et al. 2014a; Humo et al. 2019), provides an additional approach which allows to tackle specific cellular and molecular characteristics of this issue in a more in-depth way.

One way to study the underlying mechanisms of comorbid pain and depression is to focus on a neuroanatomical substrate involved in processing information related to both conditions. Based on extensive clinical and preclinical data, one such region, showing both morphological and physiological alterations in the presence of pain and mood disorders is the anterior cingulate cortex (ACC) (Drevets et al. 1997; Drevets et al. 1998; Johansen et al. 2001; Mayberg et al. 2005; Seminowicz et al. 2009; Barthas et al. 2015).

Our team recently showed that optogenetic activation of pyramidal neurons in the ACC of naive mice induces depressive-like behaviors (Barthas et al. 2015), while inhibition of the same cell population alleviates aversive and anxiodepressive-like behaviors (Sellmeijer et al. 2018) in a mouse model of peripheral neuropathic pain (Yalcin et al. 2014b). In addition, we showed that whole tissue genomic analysis of the ACC from mice displaying comorbid neuropathy and depressive-like behaviors can be advantageous in identifying molecular candidates, such as the mitogen activated protein kinase (MAPK) phosphatase-1 (MKP-1), which seem to have an important modulatory role in this comorbidity (Barthas et al. 2017).

In order to go a step further, the current study aimed to investigate the functional and molecular properties of γ-aminobutyric acid (GABA)ergic neurons within the ACC in comorbid pain and depression. This will be achieved by optogenetic activation and genomic analysis of cells expressing *Dlx5/6*, homeobox genes associated with development and migration of forebrain GABAergic neurons (Wang et al. 2010; Wang et al. 2011).

While GABAergic neuron dysfunction has been associated with both neuropathic pain (Jasmin et al. 2004; Zeilhofer 2008; Basbaum et al. 2009) and depression (Rajkowska et al. 2007; Mohler et al. 2012; Pehrson and Sanchez 2015), there is limited evidence describing the role of this neuronal population in the comorbidity of these two conditions. Here, we showed that optogenetic activation of GABAergic Dlx5/6 neurons in the ACC of naive animals produces an antidepressant-like effect at 10 and 7.5, but not 5 Hz frequency. On the other hand, 5 Hz frequency was sufficient to block neuropathic pain-induced depressive-like behaviors in mice. Furthermore, we demonstrated that the viral translating ribosome affinity purification (vTRAP) technique is a useful tool for extracting GABAergic neurons from the ACC of adult mice, and can be used for further genomic profiling of defined cell populations.

METHODS

Animals

Sixty four adult male Dlx5/6-cre (C57BL/6J background) mice (Chronobiotron, Strasbourg, France) were used in the study. All mice were 8-12 weeks old at the start of the experiments, housed 2-4 per cage and kept under a 12h light/dark cycle with food/water availability *ad libitum*. Protocols were approved by the University of Strasbourg ethics committee (#2015012909428166) and performed according to animal care and use guidelines of the European Community Council Directive (EU 2010/63).

Neuropathic pain model and mechanical sensitivity assessment

A solution of zoletil (50mg/kg) and xylazine (10mg/kg) (Centravet, Taden, France) was administered intraperitoneally (i.p.) to anaesthetize the animals before neuropathy was induced. This was achieved by surgical implantation of a 2 mm polyethylene tube (cuff) around the main branch of the right sciatic nerve (Yalcin et al. 2014). Unlike neuropathic mice (NP), control (sham) mice underwent the same procedure without cuff implantation. Mechanical allodynia was assessed with the von Frey test before surgery (baseline) and after 2, 4 and 8 weeks during the postoperative period. Each session started with an individual habituation (10 min) in transparent, bottomless plastic boxes located on a mesh platform. Then, plastic filaments of different diameters (pressure: 0.4 - 8.0g; Bioseb, Chaville, France) were applied to the plantar surface of each hind paw in ascending order. A response for a given pressure was considered valid when 3 out of 5 applications caused a withdrawal of the stimulated hind paw. The withdrawal threshold was set after 2 consecutive pressures yielded a positive response.

Behavioral tests

Screening for anxiodepressive-like behaviors was done during the 8th week after cuff implantation. Testing took place under red light, during the animals' active phase (dark-cycle), as previously described (Yalcin et al. 2011).

Novelty-suppressed feeding test

After a 24h-long period of food deprivation, mice were gently lowered into the right corner of a plastic box (40 x 40 x 30cm) whose floor was covered with 2cm of sawdust and which contained a food pellet in the center. Within the next 5 minutes, the latency to approach and start eating the pellet was recorded. During this test, the animal's motivation to venture into an anxiogenic open space and eat food in a novel environment is assessed.

Splash test

During this test each animal was sprayed with a 20% sucrose solution onto the dorsal coat. Next, the total grooming duration was recorded for each animal over the course of 5 minutes. This test measures depressive-like behavior by assessing the animals' motivation for self-hygiene.

Marble burying test

The animals were individually placed in a plexiglass cage (37 x 23 x 18 cm) with 5cm of clean sawdust. Thirty five glass marbles (1cm diameter) were place on top of the sawdust and evenly spaced 2 cm away from the walls, along every side of the cage. Following 30 minutes of undisturbed activity, the mice were removed and the number of non-buried marbles was counted. A marble was considered buried if at least two thirds of its surface was covered by sawdust. This test measures the animals' anxiety level (Nicolas et al. 2006).

Real-time place preference

Mice were tested in a 45 × 20 × 15 cm plastic box separated by a partition into two equal chambers, one having plain grey walls and the other having vertical white stripes on the grey walls. The animals were habituated to the setting on the day prior to testing. With an attached fiber optic patch cord, they were allowed to freely move between the two compartments and their preference for each compartment was recorded over 10 min without photostimulation. The following day, after establishing that there was no preference for a single compartment, the animals received photostimulation in only one, randomly assigned, compartment. The stimulation stopped every time they entered the non-paired side. The entire procedure was recorded with a camera and scored by a person blind to the procedural details.

Viral mediated expression of channelrhodopsin 2 (ChR2) in Dlx5/6 neurons

Independent sets of Dlx5/6-Cre mice used in optogenetic stimulation experiments received viral vectors carrying Cre-dependent ChR2 (AAV5-EF1a-DIO-hCHR2(H134R)-WPRE-hGH; Addgene, Teddington, UK), bilaterally (0.5 µl/side) into the ACC (0.7 mm anterior and 0.3 mm lateral to bregma). Control animals received a viral vector without ChR2 expression. For the behavioral characterization of GABAergic neurons in naive animals, the mice received the virus injection 3 weeks before optogenetic cannula implantation, and 4 weeks before behavioral experiments were performed. Same protocol was also used when the role of GABAergic neurons was assessed in neuropathic animals. However, these mice received the neuropathic surgery 5 weeks prior to virus administration. Finally, all animals used in the TRAP experiments received a viral vector carrying a Cre-dependent ribosomal protein L10 tagged with a green fluorescent protein (GFP) (AAV5-FLEX-EGFPL10a; IGBMC, Strasbourg, France), bilaterally (0.5 µl/side) in the ACC.

Optogenetic stimulation of Dlx5/6 neurons

Three weeks after ChR2 virus transfection, Dlx5/6 mice anesthetized, and single glass optogenetic fibers (1.7 mm; 8-9 mW intensity; Doric lenses, Quebec, Canada) were unilaterally implanted into the left ACC (0.7 mm anterior and 0.3 mm lateral to bregma). The upper metal extension of the cannula was fixated onto the skull with Paladur denture cement. After 3-7 days of recovery, the animals were stimulated during behavioral testing with blue light (460 nm wavelength). The stimulation protocol lasted from start to finish of each behavioral test and consisted of repetitive 20 ms light pulses, at 5, 7.5 and 10 Hz frequency.

Translating ribosome affinity purification (TRAP)

Four weeks after viral transfection, the TRAP procedure was performed as previously described (Heiman et al. 2014). First, the animals were killed by cervical dislocation and the bilateral ACC was dissected in cold Dissection buffer [1X HBSS (Invitrogen), 10 mM HEPES-KOH (pH 7.3) (Affymetrix), 35 mM glucose, 4 mM $NaHCO_3$, 100 µg/ml cycloheximide (Sigma)]. Next, after homogenization in Lysis buffer [10 mM HEPES-KOH (pH 7.3), 150 mM KCl, 5 mM MgCl2, 0.5 mM DTT, 100 µg/ml cycloheximide, RNasin (Promega, Madison, WI), SUPERas-In (Applied Biosystems), Complete-EDTA-free protease inhibitors (Roche)], the polysomes were separated by two centrifugation steps using 10% NP-40 and 300 mM DHPC. Here, a portion (250 µl) of the supernatant containing the EGFP-tagged polysomes was separated from each sample to serve as the whole-tissue "input" for that given sample. The

remaining part (800 µl) was incubated with 200 µl of the affinity matrix [Streptavidin MyOne T1 Dynabeads (Invitrogen), Biotinylated Protein L (Fisher Scientific), GFP antibodies Htz-GFP-19F7 and Htz-GFP-19C8 (50 µg each) (Memorial Sloan-Kettering Monoclonal Antibody Facility)] during 16-18h on a tube rotator at 4°C.

RNA extraction and quantitative polymerase chain reaction (qPCR)

After incubation with the affinity matrix, the mRNA was separated and purified from the ribosome-bead complex with the Absolutely RNA Nanoprep kit (Agilent, Santa Clara, CA). The same procedure was used for both the whole tissue extracts (Inputs) and the immunoprecipitated transcripts (IPs). At last, the isolated RNA was resuspended in 20 µl of RNAse-free water, and a fraction of each sample was used to analyze the quantity and quality of the purified RNA on an Agilent 2100 Bioanalyzer. Further analyses, including qRT-PCR and RNA-seq used only samples with RNA integrity values above 7. For the preliminary enrichment experiments, the extracted RNA was reverse transcribed into cDNA with M-MLV Reverse Transcriptase reagents (Invitrogen, 28025-013). The obtained cDNA was then analyzed by real-time PCR (Applied Biosystems 7300 RT-PCR System; 50°C for 2 min, 95°C for 10 min, 95°C for 15s and 60°C for 1 min for 40 cycles, then dissociation stage 95°C for 15 s, 60°C for 30 s, 95°C for 15 s) with the SYBR-Green mix (Applied Biosystems, A25742). The relative quantities of each immunoprecipitated sample were compared to input cDNA. Enrichment was analyzed with housekeeping genes, expressed ubiquitously, such as glyceraldehyde 3-phosphate dehydrogenase (***GAPDH***; F_aacgacccttcattgac, R_tccacgacatactcagcac) and beta actine (***Actb***; F_cctccctggagaagagctatg, R_ttacggatgtcaacgtcacac), and genes whose expression was expected or not expected in a given sample, including green fluorescent protein (***GFP***; F_agtgcttcagccgctacc, R_gaagatggtgcgctcctg) (Jackson laboratory, #007612), distal-less homeobox 5 gene (***Dlx5***; F_tctctaggactgacgcaaaca, R_gttacacgccatagggtcgc) (Schüle et al. 2007), distal-less homeobox 6 gene (***Dlx6***; F_ggggacgacacagatcaacaa, R_tccccttccgttgaacctg), glutamic acid decarboxylase 65 (***Gad65***; F_cattgataagtgtttggagctagca, R_gtgcgcaaactaggaggtacaa) (Trifonov et al. 2014), vesicular glutamate transporter 1 (***vGlut1***; F_ctcagcccgcctactttgaa, R_ggtcatgacaaggtgaggca), calcium/calmodulin-dependent protein kinase type II alpha (***CamKIIα***; F_tgggtttggctcttgtatgga, R_aagaaaacagtgcagacaggagatc) (Shin et al. 2013), glial fibrillary acidic protein (***GFAP***; F_acagcggccctgagagagat, R_ctcctctgtctcttgcatgttactg) (Masocha 2015).

Statistical analyses

All presented data are expressed as mean ± SEM. Data analysis was performed with STATISTICA 7.1 (Statsoft, Tulsa, Oklahoma) by using unpaired Student t-tests or multifactor analysis of variance (ANOVA), with independent or repeated measures, and Duncan post-hoc analyses. Values of $p \leq 0.05$ were considered statistically significant. Detailed information is summarized in *Supplemental Table*.

RESULTS

Activating ACC Dlx5/6 neurons has a frequency-dependent antidepressant-like effect

To establish whether optogenetic stimulation at 10 Hz of Dlx5/6 neurons in the ACC is aversive to naive mice, we implemented the real-time place preference protocol. It was shown that both the animals which received the control virus and those who received ChR2 spend equal amounts of time in the light-paired and the no light-paired compartment (Fig. 1a). Next, von Frey results demonstrated that 10 Hz stimulation of GABAergic ACC cells does not affect mechanical sensitivity (Fig. 1b). In contrast, behavioral tests indicated that optogenetic activation of ACC Dlx5/6 neurons seems to produce an antidepressant-like effect, as displayed by a decreased latency to feed in the NSF test (Fig. 1c; $p \leq 0.05$), an increased grooming duration in the splash test (Fig. 1d; $p \leq 0.05$) and a decreased number of buried marbles in the marble burying test (Fig. 1e; $p \leq 0.001$) in stimulated animals, compared to the control group. Interestingly, while this antidepressant-like effect was also achieved at 7.5 Hz stimulation frequency (Fig. 1f; $p \leq 0.05$), it was no longer present when the frequency was decreased to 5 Hz (Fig. 1g).

Activation of ACC Dlx5/6 neurons blocks neuropathy-induced depressive-like behaviors

Cuff implantation around the right sciatic nerve induced mechanical allodynia in the ipsilateral paw of NP mice as seen by a lowered mechanical sensitivity threshold in the von Frey test (Fig. 2a; $F_{(3, 72)} = 16.99$, $p \leq 0.05$; post-hoc: Sham EYFP > NP EYFP, $p \leq 0.001$, w2-8; Sham ChR2 > NP ChR2, $p \leq 0.05$, w2-8). This effect was not present in sham-operated animals (Fig 2a, b) or the contralateral paw of NP animals (Fig. 2b). Moreover, optogenetic activation of ACC Dlx5/6 neurons at 5 Hz frequency did not have any effect on the mechanical sensitivity and the presence of allodynia in neuropathic animals (Fig. 2c). On the other hand, in contrast to naive animals, optogenetic stimulation of GABAergic neurons at 5 Hz reduced anxiodepressive-like behaviors in neuropathic mice, as seen by an increased grooming duration in the splash test (Fig. 2d; $F_{(1, 24)} = 4.55$, $p \leq 0.05$; post-hoc: Sham EYFP > NP EYFP, $p \leq 0.05$; NP EYFP < NP ChR2, $p \leq 0.05$) and a decreased latency to feed in the NSF test (Fig. 2e; $F_{(1, 20)} = 3.96$, $p \leq$

0.05; post hoc: Sham EYFP < NP EYFP, $p \leq 0.05$; NP EYFP > NP ChR2, $p \leq 0.05$). However, this decrease was only evident during optogenetic activation of ACC Dlx5/6 neurons, and was no longer present 24h after the stimulation (Fig. 2f).

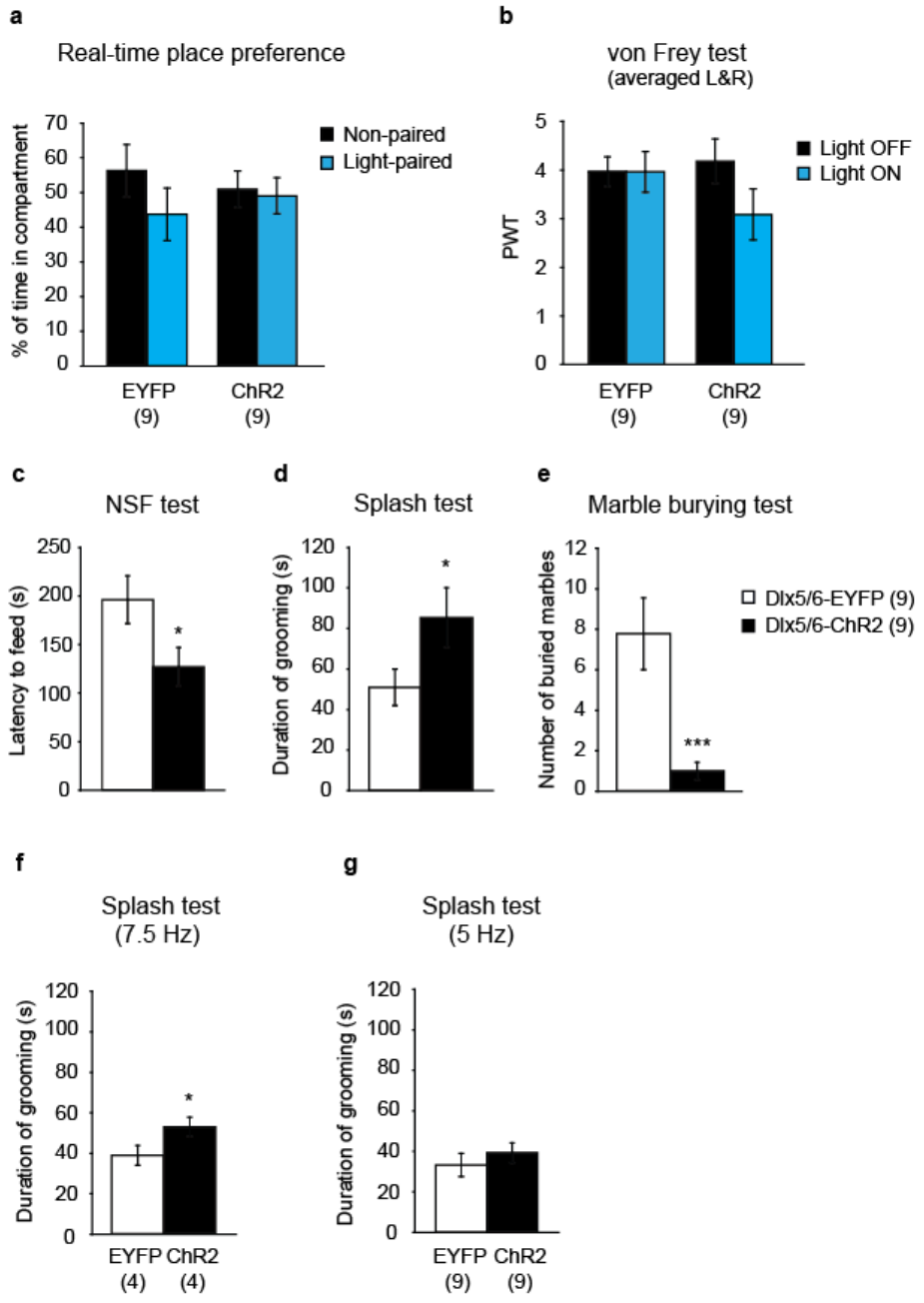

Figure 1: Optogenetic activation of GABAergic neurons in the ACC of naive mice. Real-time conditioned place preference showed that the stimulation of GABAergic neurons at 10 Hz frequency was not aversive since the animals did not display a preference for the non-stimulated compartment (a), nor did it alter the mechanical sensitivity of naive mice in the von Frey test (b). However, this stimulation protocol decreased anxiodepressive-like behaviors in naive mice in the NSF test (c), Splash test (d) and Marble burying test (e). While the antidepressant-like effect was also achieved with stimulation at 7.5 Hz (f), this effect was lost when the frequency was set at 5 Hz (g).

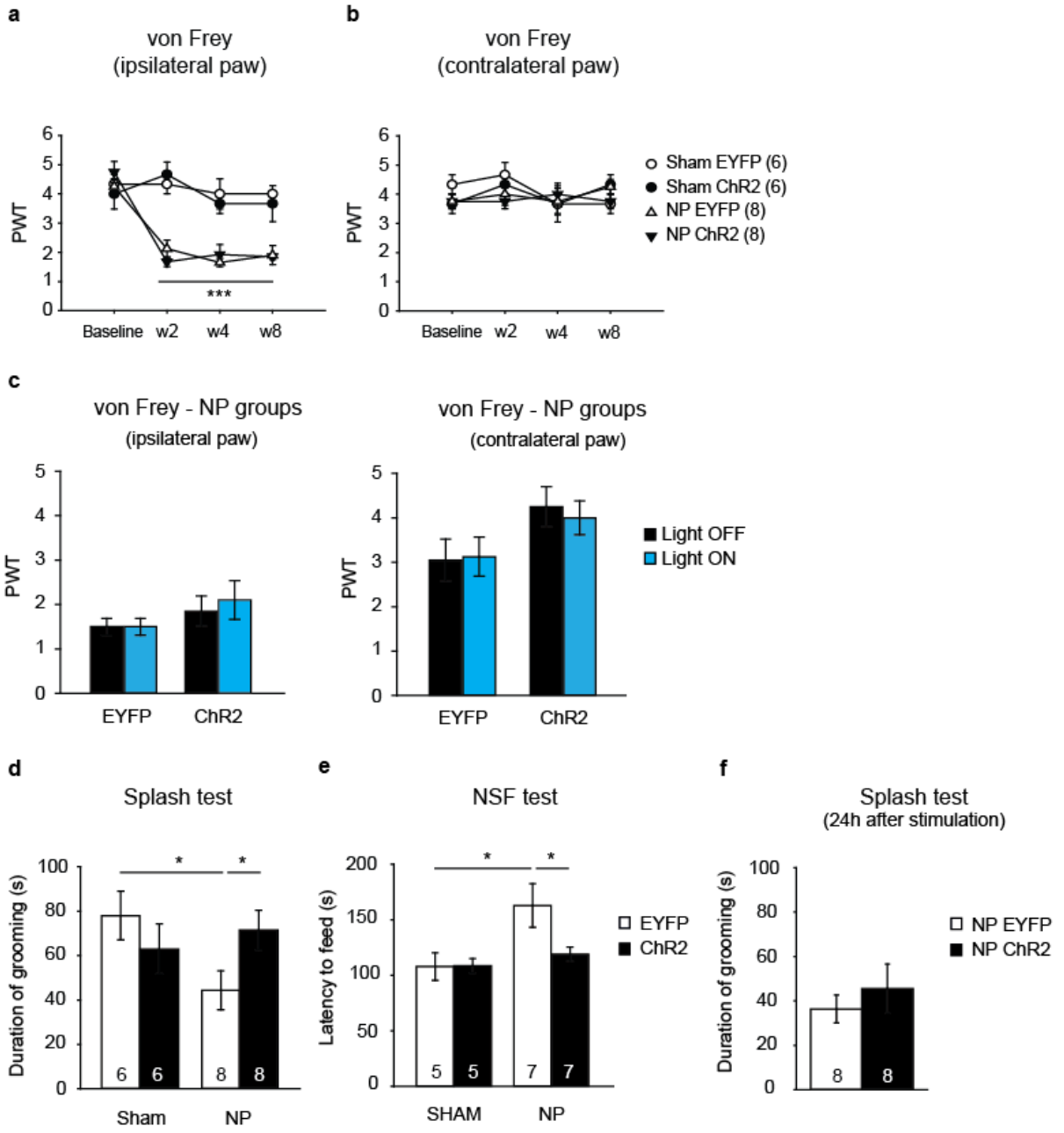

Figure 2: Optogenetic activation of GABAergic neurons in the ACC of neuropathic (NP) and sham-operated mice. Cuff implantation caused a decrease in the mechanical sensitivity threshold of the ipsilateral (a), but not the contralateral paw (b). Stimulation at 5 Hz did not affect mechanical sensitivity of neuropathic animals in the von Frey test (c), however it decreased anxiety and depressive-like behaviors in NP mice, but not sham mice, in both the Splash test (d) and the NSF test (e). A Splash test 24h after the stimulation showed that the antidepressant-like behavioral effect was not lasting beyond the stimulation period (f).

Using vTRAP for genomic profiling of GABAergic cells in the ACC

Fluorescent microscopy confirmed that stereotactic injection of AAV5-FLEX-EGFPL10a into the ACC of Dlx5/6-Cre mice results in a robust GFP transfection, spreading throughout areas 24a and 24b (Fig. 3a). As a first step, we validated the feasibility of the TRAP approach. Indeed,

following TRAP, the purified RNA from both the IPs and whole-tissue inputs were reverse transcribed into cDNA and used in qPCR to screen for the expression of GABAergic cell type-specific genes. Compared to the inputs, the IPs showed an enrichment of GABAergic markers, including *Dlx5*, *Dlx6*, *Gad65*, as well as the viral induced *GFP*, while there was a depletion of excitatory neurons' markers such as *vGlut1* and *CamKIIα*, and also the glial cell marker *GFAP* (Fig. 3b). Once we validated the vTRAP approach, it was applied to a cohort of control and mice displaying mechanical allodynia (Fig 3c; $F_{(3, 60)} = 4.27$, $p \leq 0.01$; NP ipsilateral < Sham ipsilateral, $p \leq 0.001$, w2-8) and neuropathic pain-induced anxiodepressive-like behaviors (Fig. 3d, e; $p \leq 0.01$) and the RNA from IPs and inputs were extracted in order to be analyzed by RNA sequencing. This part of the project is still ongoing.

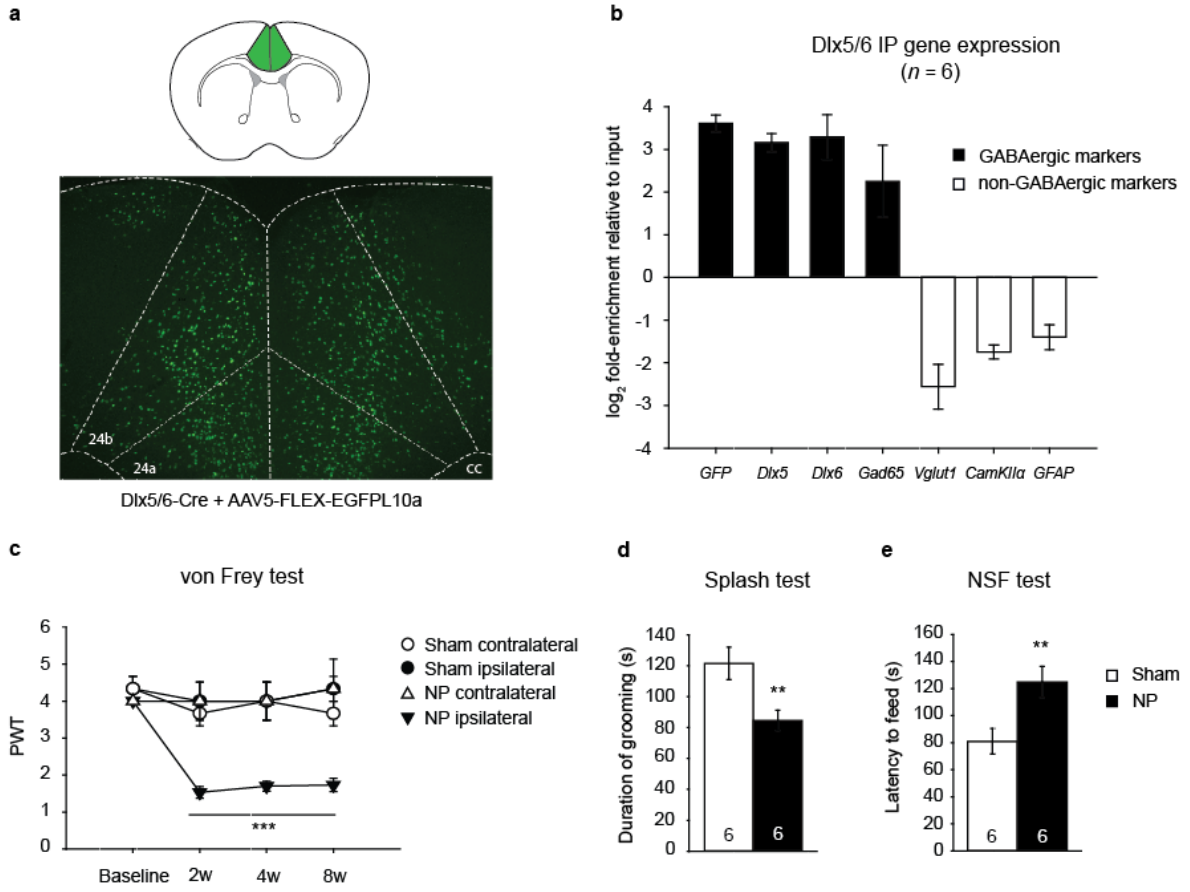

Figure 3: Cell type-specific analyses of GABAergic neurons. Naive Dlx5/6-Cre mice transfected with AAV5-FLEX-EGFPL10 displayed a robust transfection in the ACC (a). After the TRAP procedure, qPCR results showed an enrichment of GABAergic-specific genes in the immunoprecipitate compared to the whole-tissue input: an upregulation of cortical GABAergic markers (Dlx5, Dlx6, Gad65), and downregulation of astrocytic (GFAP) and glutamatergic (vGlut1 and CamKIIα) (b). After these technical validation experiments, a batch of animals displaying mechanical allodynia (c) and anxiodepressive-like behaviors (d, e) was prepared for RNA sequencing.

DISCUSSION

The present study shows that optogenetic activation of GABAergic Dlx5/6 neurons in the ACC of naive mice at 10 Hz and 7.5 Hz, but not at 5Hz, produces an antidepressant-like effect. Next, by using a mouse model of chronic pain, we demonstrated that optogenetic stimulation of GABAergic Dlx5/6 neurons in the ACC at 5 Hz blocks anxiodepressive-like behaviors induced by neuropathic pain, without affecting mechanical sensitivity. Finally, we showed that vTRAP in combination with a Dlx5/6-Cre mouse line is a suitable technique for analyzing the genomic expression in ACC GABAergic neurons in the comorbidity of chronic pain and anxiodepressive-like behaviors.

Optogenetic dissection of the functional role of ACC GABA cells in pain and depression

Due to their capacity to selectively activate or inhibit specific neural circuits in a precise spatio-temporal fashion, optogenetic tools have been shown useful in examining the functional role and connectivity of brain regions whose functioning has been closely associated with mood disorders, affective states and depression (Lammel et al. 2014). Moreover, as different neuronal cell types have different roles in cognition and behavior, optogenetic targeting directly correlates activation or inhibition of defined populations with specific behavioral outcomes, which can be beneficial in studying both depression and chronic pain-related pathologies (Albert et al. 2014; Xie et al. 2018).

However, very few studies used optogenetic tools to investigate the cell-specific behavioral contribution in the comorbid pain and depression paradigm (Cai et al. 2018; Sellmeijer et al. 2018) and this is the first study to investigate the behavioral effect during optogenetic activation of ACC GABAergic neurons in comorbid pain and depressive-behaviors. We show that neuropathic pain-induced depressive-like behaviors are attenuated by activation of ACC GABA neurons, which is in accordance with previous findings showing that PV-expressing GABAergic neurons in the medial prefrontal cortex (mPFC) receive less excitatory input in animals susceptible to stress and the selective suppression of these interneurons promotes helplessness (Perova et al 2015). However, it is important to note the region and circuit-specific behavioral effect of GABAergic cell activation. Indeed, stimulation of GABAergic neurons in the ventral tegmental area, where they provide input to dopaminergic cells, leads to aversive behaviors and disrupted reward consumption (Tan et al. 2012; van Zessen et al. 2012). Similarly, optogenetic inhibition of this neuronal population in the dorsal raphe nucleus, where they synapse onto serotonergic neurons, results in suppressed acquisition of social avoidance (Challis et al. 2013).

In contrast to our results that showed no effect of optogenetic stimulation of ACC GABAergic neurons on the mechanical allodynia induced by peripheral neuropathy, Zhang and colleagues (2015) demonstrated that optogenetic inhibition or activation of GABAergic neurons in the prelimbic cortex decreased and increased pain responses, respectively, in freely moving mice with neuropathic pain due to spared nerve injury. On the other hand, Gu et al. (2015) showed that optogenetic activation of inhibitory neurons in the ACC of mice leads to a reduced pain response. However, compared to the present study, the region of interest in the study of Zhang et al. (2015) was more anterior (1.8 to 2.4mm anterior to Bregma) and showed hypoactivity due to neuropathic pain, while our procedure targeted the ACC (0.7 mm anterior to Bregma) which has been shown to be hyperactive in neuropathic pain conditions (Sellmeijer et al. 2018). In addition, the type of pain studied by Gu and colleagues (2015) was formalin-induced inflammatory pain, compared to the long-lasting peripheral neuropathy model implemented in our study. This might have a role in the difference between the obtained results since inflammatory and neuropathic pain show a different temporal window of molecular and morphological changes during the post-injury period in the mPFC and ACC, respectively (Metz et al. 2009; Wu et al. 2005). The apparent discrepancies in these findings might also be accounted for by the specific connectivity and function of ACC sub-regions, which are differently involved in processing affective and cognitive pain-related stimuli (Rainville et al. 1997; Hofbauer et al. 2001; Davey et al. 2012; Zhang et al. 2013). Finally, different chronic pain conditions result in distinct brain activity patterns which are different from patterns observed in acute nociceptive pain states, since persistent pain recruits brain regions predominantly involved in processing emotion and self-evaluation (Baliki et al. 2006; Apkarian 2011).

Therefore, these data highlight the importance of addressing unique functional units such as specific cell types individually, in the context of different brain regions in specific conditions, in order to achieve a more profound understanding of their role under both normal and pathological circumstances.

Genomic analysis of GABAergic ACC cells in comorbid neuropathic pain and depression

The present study utilized vTRAP on the ACC of transgenic Dlx5/6-Cre mice displaying neuropathic pain-induced depressive-like behaviors in order to perform a region and condition-specific genomic analysis of GABAegic neurons. The obtained purified RNAs from both the whole tissue RNA and the immunoprecipitated transcripts have been sent to RNA sequencing. This part of the study will yield cell-type specific transcriptomes of control and neuropathic

animals, which will be analyzed to obtain individual genomic candidates for further investigation. By additional manipulation of these specific targets with techniques such as viral-mediated silencing or overexpression, gene knock-out, and pharmacological manipulation, we hope to validate potential molecular leads crucial in understanding and treating pain and mood disorder comorbidities.

Genome-wide studies that looked at brains from human patients with major depression using oligonucleotide microarrays showed an altered expression of genes related to GABAergic neurons at the level of the PFC (Klepman et al. 2009) and the ACC (Sequeira et al. 2007), as well as other cortical (Choudary et al. 2005) and subcortical (Sequeira et al. 2009) structures, known to be implicated in the neurobiology of depression. This method of whole-genome expression profiling allowed earlier identification of MKP-1 as an important factor in the hippocampus of depressed humans (Duric et al. 2010) and in the ACC of mice displaying neuropathic pain induced depressive-like behaviors (Barthas et al. 2017).

However, since microarrays are a hybridization-based method, they rely on existing information about the genome sequence and have a high background risk due to cross-hybridization (Okoniewski et al. 2006; Royce et al. 2007). On the other hand, RNA sequencing can identify all transcripts produced by a given gene, and as it is not limited by pre-existing knowledge, it is useful in detecting novel transcripts, including non-coding RNAs (Trapnell et al. 2012).

Some recent studies have used RNA sequencing to study gene expression patterns in brain regions associated with comorbid neuropathic pain and depression. For instance, Alvarado et al. (2013) showed that long term neuropathic pain due to spared nerve injury (SNI) triggers persistent changes in gene expression in the mouse PFC related to various cellular functions including neurite outgrowth, vesicular release, neuronal excitability and plasticity. Descalzi and colleagues (2017) went even further by using RNA sequencing and pathway analysis to compare gene expression in the nucleus accumbens, the mPFC, and the periaqueductal gray between the SNI and chronic unpredictable stress mouse models. They demonstrated that although neuropathic pain and anxiodepressive-like behaviors are associated with unique transcriptome profiles, there were similar changes and adaptations in the expression of a high number of genes pertaining to common signaling pathways and biological functions.

Although still very limited, current evidence suggest that chronic pain and depression have a both unique and common molecular underpinnings, and that future studies should focus more on smaller functional units such as cell types, subtypes and even single cells when studying complex pathologies such as pain and mood disorder comorbidities. Hence, upon

completion, the present study will be the first to closely investigate the role of specifically ACC GABAergic neurons in animals displaying comorbid neuropathic pain and depressive-behaviors. The results will generate genomic maps with neuronal type specific cortical alterations in control and pathological conditions. The findings may potentially serve as a first step toward preclinical target validation at the molecular level and eventually lead to more personalized treatment options.

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Supplemental table: Statistical analysis data

Experiment	Assessment	Treatment	Test	Groups (*n*)	Analysis	Statistics	Post-hoc	Fig
Optogenetic activation of ACC Dlx5/6 neurons in naïve mice	Aversion to stimulation	AAV5-DIO-ChR2 or AAV5-DIO-EYFP + blue light (460 nm) at 10 Hz	Real-time place preference	Dlx5/6 EYFP (9) Dlx5/6 ChR2 (9)	ANOVA	$F_{(1, 32)} = 0.67$, $p > 0.05$		**1a**
Optogenetic activation of ACC Dlx5/6 neurons in naïve mice	Mechanical sensitivity threshold	AAV5-DIO-ChR2 or AAV5-DIO-EYFP + blue light (460 nm) at 10 Hz	von Frey	Dlx5/6 EYFP (9) Dlx5/6 ChR2 (9)	ANOVA	$F_{(1, 32)} = 1.61$, $p > 0.05$		**1b**
Optogenetic activation of ACC Dlx5/6 neurons in naïve mice	Anxiodepressive-like behavior	AAV5-DIO (ChR2 or EYFP) + blue light (460 nm) at 10 Hz	NSF	Dlx5/6 EYFP (9) Dlx5/6 ChR2 (9)	t-test	$t(16) = 2.18$, $p \leq 0.05$		**1c**
Optogenetic activation of ACC Dlx5/6 neurons in naïve mice	Depressive-like behavior	AAV5-DIO (ChR2 or EYFP) + blue light (460 nm) at 10 Hz	Splash	Dlx5/6 EYFP (9) Dlx5/6 ChR2 (9)	t-test	$t(16) = -2.00$, $p \leq 0.05$		**1d**
Optogenetic activation of ACC Dlx5/6 neurons in naïve mice	Anxiety-like behavior	AAV5-DIO (ChR2 or EYFP) + blue light (460 nm) at 10 Hz	Marble burying	Dlx5/6 EYFP (9) Dlx5/6 ChR2 (9)	t-test	$t(16) = 3.72$, $p \leq 0.001$		**1e**
Optogenetic activation of ACC Dlx5/6 neurons in naïve mice	Depressive-like behavior	AAV5-DIO (ChR2 or EYFP) + blue light (460 nm) at 7.5 Hz	Splash	Dlx5/6 EYFP (4) Dlx5/6 ChR2 (4)	t-test	$t(6) = -2.046$, $p \leq 0.05$		**1f**
Neuropathic model + optogenetic activation of ACC Dlx5/6 neurons	Depressive-like behavior (8w post-NP)	AAV5-DIO (ChR2 or EYFP) + blue light (460 nm) at 5 Hz	Splash	Dlx5/6 EYFP (9) Dlx5/6 ChR2 (9)	t-test	$t(16) = -0.80$, $p > 0.05$		**1g**
Neuropathic model + optogenetic activation of ACC Dlx5/6 neurons	Mechanical threshold (0-8w post-NP) Ipisilateral paw	AAV5-DIO (ChR2 or EYFP) + blue light (460 nm) at 5 Hz	von Frey	Sham EYFP (6) Sham ChR2 (6) NP EYFP (8) NP ChR2 (8)	ANOVA	Time x Surgery $F_{(3, 72)} = 16.99$, $p \leq 0.05$	Sham EYFP > NP EYFP, $p \leq 0.001$ (w2-8) Sham ChR2 > NP ChR2, $p \leq 0.05$ (w2-8)	**2a**
Neuropathic model + optogenetic activation of ACC Dlx5/6 neurons	Mechanical threshold (0-8w post-NP) Contralateral paw	AAV5-DIO (ChR2 or EYFP) + blue light (460 nm) at 5 Hz	von Frey	Sham EYFP (6) Sham ChR2 (6) NP EYFP (8) NP ChR2 (8)	ANOVA	$F_{(3, 72)} = 1.37$, $p > 0.05$		**2b**

Neuropathic model + optogenetic activation of ACC Dlx5/6 neurons	Mechanical threshold (8w post-NP)	AAV5-DIO (ChR2 or EYFP) + blue light (460 nm) at 5 Hz	von Frey	Sham EYFP (6) Sham ChR2 (6) NP EYFP (8) NP ChR2 (8)	ANOVA	Ipsilateral $F_{(1, 14)} = 0.91$, $p > 0.05$ Contralateral $F_{(2, 14)} = 2.55$, $p > 0.05$		**2c**
Neuropathic model + optogenetic activation of ACC Dlx5/6 neurons	Depressive-like behavior (8w post-NP)	AAV5-DIO (ChR2 or EYFP) + blue light (460 nm) at 5 Hz	Splash	Sham EYFP (6) Sham ChR2 (6) NP EYFP (8) NP ChR2 (8)	ANOVA	$F_{(1, 24)} = 4.55$, $p \leq 0.05$	Sham EYFP > NP EYFP, $p \leq 0.05$ NP EYFP < NP ChR2, $p \leq 0.05$	**2d**
Neuropathic model + optogenetic activation of ACC Dlx5/6 neurons	Anxiodepressive-like behavior (8w post-NP)	AAV5-DIO (ChR2 or EYFP) + blue light (460 nm) at 5 Hz	NSF	Sham EYFP (5) Sham ChR2 (5) NP EYFP (7) NP ChR2 (7)	ANOVA	$F_{(1, 20)} = 3.96$, $p \leq 0.05$	Sham EYFP < NP EYFP, $p \leq 0.05$ NP EYFP > NP ChR2, $p \leq 0.05$	**2e**
Neuropathic model + optogenetic activation of ACC Dlx5/6 neurons	Depressive-like behavior (8w post-NP)	AAV5-DIO (ChR2 or EYFP) + blue light (460 nm) at 5 Hz	Splash	NP EYFP (8) NP ChR2 (8)	t-test	$t(14) = -0.72$, $p > 0.01$		**2f**
TRAP technical validation	Gene expression	AAV5-FLEX-EGFPL10a (ACC) + TRAP + RNA extraction	qPCR	Dlx5/6 + vTRAP (6)	IP/Input	***Log*$_2$*(ΔΔCt):*** ***GFP*** = 3.61 ± 0.20; ***Dlx5*** = 3.16 ± 0.22; ***Dlx6*** = 3.29 ± 0.52; ***Gad65*** = 2.25 ± 0.84; ***vGlut1*** = -2.56 ± 0.53; ***CamKIIα*** = -1.75 ± 0.16; ***GFAP*** = -1.40 ± 0.29		**3b**
Neuropathic model + vTRAP in ACC	Mechanical sensitivity threshold (8w post-NP)	AAV5-FLEX-EGFPL10a (ACC) + TRAP + RNA extraction	von Frey	Dlx5/6 Sham (6) Dlx5/6 NP (6)	ANOVA	Time x Surgery x Paw: $F_{(3, 60)} = 4.27$, $p \leq 0.01$	NP ipsil. < Sham ipsil., $p \leq 0.001$ (w2-8)	**3c**
Neuropathic model + vTRAP in ACC	Depressive-like behavior (8w post-NP)	AAV5-FLEX-EGFPL10a (ACC) + TRAP + RNA extraction	Splash	Dlx5/6 Sham (6) Dlx5/6 NP (6)	t-test	$t(10) = 2.97$, $p \leq 0.01$		**3d**
Neuropathic model + vTRAP in ACC	Anxiodepressive-like behavior (8w post-NP)	AAV5-FLEX-EGFPL10a (ACC) + TRAP + RNA extraction	NSF	Dlx5/6 Sham (6) Dlx5/6 NP (6)	t-test	$t(10) = -2.94$, $p \leq 0.01$		**3e**

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General discussion

Depression is the most frequent lifetime disorder (Kessler et al. 2005), characterized by a prolonged loss of pleasure and interest in all, or almost all, daily activities (APA 2013; WHO 2018) and estimated to become the foremost contributor to the worldwide disease burden by 2030 (WHO 2008). Alongside stress, chronic pain is a prominent risk factors responsible for the emergence of depression (Attal et al. 2011), as seen by the high prevalence of MDD among patients suffering from chronic pain and vice versa (Bair et al. 2003; Williams et al. 2003; Lee et al. 2009; Agüera-Ortiz et al. 2011). Our limited knowledge of the etiology of this debilitating comorbidity points to a need for objectively quantifiable abnormalities at the molecular, cellular or circuit level. This approach has the potential to yield novel therapeutic targets that could decrease the burden of current inefficient treatment strategies, which are accompanied by a wide range of adverse effects (Gorman et al. 2002; Breivik et al. 2006; Gierthmühlen and Baron 2016).

Therefore, our team was among the first to establish a preclinical murine model to investigate the neurobiological mechanisms implicated in this important clinical issue (Yalcin et al. 2011a; Barthas et al. 2015). This allowed us to characterize the anxiodepressive consequences of neuropathic pain and focus in more depth on a brain region implicated in both neuropathic pain and mood disorders, the anterior cingulate cortex (Peyron et al. 2004; Drevets et al. 2008; Zhuo 2016). Consequently, based on the published, unpublished and preliminary data, the present thesis project gathered information about the molecular, cellular and functional characteristics of the ACC, related to the pathophysiology and treatment of comorbid neuropathic pain and depression.

The following section will individually discuss the findings pertaining to each objective of my thesis and speculate on the possible perspectives which might be implemented that could broaden our understanding about the present matter and bring us closer to finding effective treatment remedies.

Double-edged sword: The many roles of MKP-1

Open approaches such as genome-wide studies can be powerful to identify molecular blueprints of depression. For the first objective, we thus performed microarray analysis within the ACC in both control and depressive-like behaving animals following neuropathic pain induction. Based on this genome expression analysis, we focused on the negative regulator of the MAPK pathway, the dual-specificity phosphatase MKP-1, whose expression is robustly increased in the ACC of neuropathic animals displaying anxiodepressive-like behaviors. We generalized our results by using other models of depression such as chronic mild stress or

ACC optogenetic stimulation, which suggested a general link between depression and increased level of ACC MKP-1. We also showed that the sustained upregulation of the MKP-1 could be associated with increased c-Fos expression, p-CREB and p-ATF levels, as well as increased H3K9/14 ac at the promoter regions of *Mkp-1* in the ACC. In order to leap from correlative to causal analyses we combined several approaches to manipulate MKP-1 and showed that knocking out, antagonizing or locally silencing its presence in the ACC attenuates depressive-like behaviors, while a classically used antidepressant drug (fluoxetine) suppresses the increased MKP-1 levels within the ACC.

Although our study did not show the effects of overexpression of MKP-1 in the ACC due to technical problems, it has been already demonstrated that viral upregulation of *Mkp-1* in the hippocampus of unstressed rats results in depressive-like behaviors (Duric et al. 2010). Interestingly, over the past several decades many studies have suggested that overexpression of MKP-1 is also associated with various types of cancer due to its inhibition of JNK-induced apoptosis (Loda et al. 1996; Magi-Galluzzi et al. 1997; Bang et al. 1998; Wang et al. 2003; Vicent et al. 2004; Moncho-Amor et al. 2011; Shen et al. 2016), as well as chemotherapy resistance (Wang et al. 2006; Chattopadhyay et al. 20006; Huang et al. 2011; Liu et al. 2014; Shen et al. 2016).

However, even though these and similar results, related to depression and cancer, paint a grim picture of the role of MKP-1 activity, it is important to note that this phosphatase has an indispensable role in promoting proper functioning of various biological processes. For instance, it is known that MKP-1 is an important regulatory factor in processes such as the innate immune response (Chi et al. 2006; Wang et al. 2007), T-cell activity (Zhang et al. 2009), suppression of endotoxic shock (Hammer et al. 2006; Zhao et al. 2006) and the management of metabolic homeostasis (Wu et al. 2006). Moreover, it promotes cell survival in a wide variety of stress conditions (Keyse and Emslie 1992; Wu et al. 2005), including apoptosis induced by hydrogen peroxide (Zhou et al. 2006) and UV radiation (Staples et al. 2010). In addition, it has been demonstrated that MKP-1 is critical in promoting axon branching induced by the brain-derived neurotrophic factor (BDNF), which is lost in *Mkp1* knock-out mice (Jeanneteau et al. 2010). Similarly, Collins et al. (2013) showed that MKP-1 promotes the growth of neural processes in dopaminergic neurons and protects them from neurotoxic effects. Nevertheless, it has been suggested that whereas transient MKP-1 expression has neuroprotective properties in the developing cortex, prolonged induction might result in adverse effects on the axonal growth during neuronal development (Jeanneteau and Deinhardt 2011).

Therefore, while MKP-1 targeting has tremendous potential in treating various pathologies (Doddareddy et al. 2012), it is important that future studies address not only the spatial and directional (upregulated vs. downregulated) expression of MKP-1, but also its temporal regulatory nature, including the developmental stage and duration of expression, in both healthy and diseased subjects.

Breaking it down: ketamine's beneficial metabolites

Our work related to the second objective showed that ketamine administration in mice displaying a comorbid neuropathic pain and depressive-like phenotype has both transient and prolonged behavioral effects in alleviating mechanical allodynia and anxiodepressive-like behaviors, respectively. Furthermore, this was accompanied by a normalization of the downregulated MKP-1 and upregulated p-ERK in the ACC of neuropathic mice, suggesting that ketamine might exert its effect, in part, through altering the MAPK pathway.

These results contribute to our limited understanding about the activity of ketamine specifically in comorbid neuropathic pain and depression. Nonetheless, while the increased body of evidence pertaining to ketamine activity, generated over the past several decades, has greatly increased our understanding, it also produced wide variation regarding efficacy in different conditions, dose range, as well as treatment frequency and duration (Cohen et al. 2018). For instance, similar to what is observed in chronic pain patients, there is also evidence suggesting that several weekly subanesthetic infusions of ketamine, spread over two weeks, can extend ketamine's antidepressant effect in treatment-resistant MDD patients (Murrough et al. 2013; Singh et al. 2016). However, among the principal limitations of prolonged ketamine use in clinical treatments are the dissociative side effects and the high risk of abuse (Oye et al. 1992; Krystal et al. 1994; Mathisen et al. 1995; Cohen et al. 2018). In addition, prolonged use of ketamine may induce tissue damage to internal organs (Niesters et al. 2014). Hence, many recent studies have focused on deciphering the activity of specific ketamine metabolites, in order to try harvesting the benefits, while leaving out the addictive and psychomimetic consequences.

This is possible because ketamine is a racemic mixture composed of two optical enantiomers, (S)- and (R)-ketamine (Chaki et al. 2017). It is primarily metabolized in the liver via the cytochrome p450-dependent enzymatic transformations, which results in a wide range of metabolites (Bergman 1999; Zanos and Gould, 2018a) that are potential candidates for future treatment strategies of pain and depression.

Evidence suggest that S-ketamine is four times more potent than (R)-ketamine in reducing pain perception in human patients experiencing neuropathic orofacial pain (Mathisen et al. 1995), as well as in those diagnosed with Complex Regional Pain Syndrome Type 1 (Sigtermans et al. 2009). There is also evidence from rodent studies suggesting that the antinociceptive properties of ketamine are the result of S-ketamine activity (Holtman et al. 2009). Interestingly, the availability of different modes of administration can also be an important asset in using ketamine metabolites in the treatment of pain. Specifically, oral and intranasal S-ketamine administration have been demonstrated as helpful routes in treating chronic or traumatic pain, where intravenous injections are disadvantageous or nonviable (Fanta et al. 2015; Johansson et al. 2013). However, S-ketamine produces more pronounced side effects such as illusions and compared to the less pain-relieving R-ketamine (Oye et al. 1992; Mathisen et al. 1995).

Interestingly, recent research has shown that R-ketamine and its metabolites, in contrast to what is observed in pain, are more successful in alleviating depressive-like behaviors in mouse models of depression (Zhang et al. 2014; Yang et al. 2015; Zanos et al. 2016; Fukumoto et al. 2017). In particular, compared to S-ketamine, R-ketamine showed a more pronounced and longer lasting antidepressant activity in both the learned helplessness and the chronic social defeat mouse models (Yang et al. 2015). Similarly, R-ketamine was also proven to be more efficient than S-ketamine in alleviating depressive-like behaviors in juvenile mice treated with dexamethasone (Zhang et al. 2014). Moreover, Zanos et al. (2016) demonstrated that a single administration of the R-ketamine metabolite, (2R,6R)-hydroxynorketamine, efficiently decreased immobility time in the FST, reduced corticosterone-induced anhedonia and reversed social avoidance resulting from social defeat stress. Finally, R-ketamine's antidepressant superiority was also characterized by the absence of physiochemical side effects associated with racemic ketamine and S-ketamine administration, making it a safer and more efficient antidepressant agent compared to the latter two (Yang et al. 2015; Zanos et al. 2016).

Since R-ketamine is reported to have a two to three times lower affinity for the NMDA receptor (Mion et al. 2013), it has been suggested that the R-ketamine exerts its antidepressant activity through activating AMPA receptors (Zanos et al. 2016; Zanos and Gould 2018b). Accordingly, pretreatment with an AMPA receptor antagonist attenuates the sustained antidepressant effect of both R-ketamine and S-ketamine in rodent models of depressive-like behaviors (Yang et al. 2015; Fukumoto et al. 2017). Although not surprising, given that the antidepressant effects of ketamine and other glutamate receptor antagonists have already been

attributed to AMPA receptor stimulation in previous rodent studies (Karasawa et al. 2005; Koike and Chaki, 2014), the exact pathway of this interaction still remains to be elucidated.

Overall, even though ketamine has been used in clinical settings for half a century and the past decades have yielded a plethora of studies characterizing its activity, we are yet to decipher its full potential in order to harvest its benefits. Its fast-acting, reproducible effects make it a promising research target in the field of psychiatric disorders, which is in urgent need of treatment improvements. Namely, while currently available treatment options require several weeks to exert an effect (Machado-Vieira et al. 2010), it is even more discouraging that 30% of patients suffering from depression do not respond to prescribed antidepressant treatments, and those who do still have a high risk of relapse (Pacher et al. 2001). Therefore, a first step might be to establish a general consensus, categorizing existing knowledge about ketamine's activity related to specific conditions, doses and administration timing, which would already allow constructing an optimal structural framework for developing individualized care plans. Finally, further research pertaining to the molecular and cellular mechanisms behind the antinociceptive and antidepressant actions of racemic ketamine and its metabolites will be invaluable for finding new therapeutic targets and developing more effective and safe treatment strategies.

Looking closer: classification of GABAergic neuronal subtypes

A challenge in neuroscience stems from the fact that brain structures often include a variety of neuronal cell types, and attention is slowly shifting towards studying the functional implications of neuronal heterogeneity in psychiatric disorders (Oldham et al. 2008). Hence, for the third general objective of our study, we are interested in characterizing the molecular traits of the GABAergic neuronal population within the ACC, which will allow us to identify new mechanisms that remain unforeseeable at the level of the whole tissue.

So far, we showed that optogenetic stimulation of ACC neurons expressing the *Dlx5* and *Dlx6* genes, markers of GABAergic cells (Stühmer et al., 2000; Batista-Brito et al, 2008; Wang et al. 2010; Taniguchi 2011), leads to an antidepressant-like effect in naive mice at 10 Hz, and a decrease of depressive-like behaviors in neuropathic mice at 5 Hz stimulation. In addition, we showed that vTRAP can be successfully used to isolate GABAergic neurons from the ACC of mice, which we utilized to purify cell type-specific RNA from mice displaying neuropathic pain-induced depressive-like behaviors that will be further analyzed with RNA sequencing. With the completion of this project, we will acquire new knowledge about the molecular impact of chronic pain-induced depression on cortical GABA neurons

and, for the first time, generate a genomic map that will shed light on the neuronal type-specific cortical alterations. It may provide a first step toward preclinical target validation that could ensure establishing a causal link between altered gene expression and depression and lead to more personalized treatment options. Upon completion, there are several additional steps which might be implemented to validate our approach and further build up on the obtained results. These might include replication of the obtained results using different cell labeling and isolation techniques, or expanding the findings by investigating other brain regions involved in comorbid pain and depression, as well as different neuronal types such as glutamatergic or glial cells.

Nevertheless, it is worth noting that, before validating the current procedure, our aim was to use FACS for cell isolation. Our preliminary experiments involved testing several techniques for labeling and purifying our cell population of interest. The trials included assessments of different viral vectors for transfecting both inhibitory (AAV-Dlx-mCherry) and excitatory (AAV-CamKII-EYFP) neurons in different brain regions. Next, other transgenic mouse lines expressing fluorescent proteins in both GABAergic (GAD65-GFP) and glutamatergic (Thy1-GFP) were used to compare the extent and efficacy of neuronal marker expressions. Moreover, we tried several approaches to dissociate intact adult neurons from several brain regions, including using proteolytic enzymes (i.e. papain) and different density gradients made of silica-based mediums (i.e. Percoll). Finally, additional protocol adjustments, aiming at increasing cell viability, pertained to the assessment of different cell sorting and collection mediums (PBS, Trizol, lysis buffers from several RNA extraction kits, etc.). However, even with all of these refinements, we were not able to obtain the desired outcomes. The encountered problems ranged from low cell count, to inadequate RNA integrity, to low enrichment of target genes in the isolated cell samples. Nevertheless, even though we had more success with the vTRAP approach, it would still be beneficial to replicate the final results with other cell isolation methods such as FACS or laser capture microdissection, in order to rule out potential technique-related perturbations in gene expression patterns (Beliakova-Bethell et al. 2014; Richardson et al. 2015).

Furthermore, another valuable source of information would be to analyze and compare the genomic expression in GABAergic neuron sub-populations associated with specific pathologies such as comorbid pain and depression. This might provide valuable data, since even within subgroups of functionally defined neuronal populations, such as somatostatin (SST) and parvalbumin (PV) GABAergic interneurons, both functional similarities and differences exist depending on the brain region and behavioral output. Namely, Kvitsani and

colleagues (2013) showed that a PV and a subtype of SST neurons form dissociated behavioral correlates in the ACC of mice during a simple foraging task, wherein the SST cells selectively responded at reward approach, while the PV responded at reward leaving. These results suggest that PV and SST neurons uniquely influenced decisive behavioral outputs during foraging (approaching vs. leaving), which is a critical function associated to the ACC (Quilodran et al. 2008; Kolling et al. 2012). On the other hand, it has been shown that PV-positive basket neurons in the motor cortex are predominantly recruited during motor initiation and execution (Isomura et al. 2009), while optogenetic activation of SST neurons of central amygdala results in a change of defensive behavior such as running and avoidance, into a passive response such as freezing and lick suppression (Yu et al. 2016). In addition, the function of these two GABAergic neuron subpopulations can also be differently affected through distinct molecular pathways orchestrated by other neuronal populations found in the network. For instance, pyramidal neurons can regulate the inhibitory input of SST and PV interneurons independently, through unique molecular pathways (Horn and Nicoll 2018). Specifically, Horn and Nicoll (2018) demonstrated that neuronal firing regulates synapses formed by PV interneurons onto hippocampal pyramidal cells, while synapses from SST interneurons are regulated by NMDA receptors. In addition, astrocytes can detect and augment the synaptic inhibition of SST, but not PV interneurons, onto pyramidal cells (Matos et al. 2018). These results highlight the intricate behavioral contribution of different GABAergic neuron subtypes, including unique region, circuit and function-dependent mechanisms that apply to each individual subgroup. Therefore, due to the complexity arising from densely interconnected networks of functionally and molecularly heterogeneous neuronal population, the role of GABAergic cells needs to be further investigated in the context of local networks (Carandini 2012) and cell population-specific molecular profiles (Paul et al. 2017).

Currently, efforts are being made to create anatomically comprehensive human transcriptional maps of different cell types across different brain areas and cortical layers, which have provide an insight into the highly variable molecular profiles of different neuronal types depending on their location (Belgard et al. 2011; Bernard et al. 2012; Hawrylycz et al. 2012). Interestingly, to achieve an even higher resolution and identify previously undetectable changes in specific cell types, Nagy et al. (2018) utilized single-cell (single-nuclei) transcriptome analysis on cells derived from post-mortem dorsolateral PFC tissue of MDD patients. They showed that different classes of brain cells such as excitatory, inhibitory and

glial cells exhibit distinct subtype-specific gene expression patterns, which allowed them find convergent evidence implicating dysregulated synaptic plasticity in the etiology of MDD.

These data do not only provide the means for more reliable comparisons between humans and other species which is of crucial importance in pathophysiological research, but also uncover new avenues for finding novel candidates with therapeutic potential.

Perspectives

Our results give a glimpse about the complex molecular interplay at the level of the ACC in the comorbidity of chronic pain and depression. We provide preclinical evidence about the genomic changes both at the level of the ACC and GABAergic neurons within, and show that manipulation of specific candidate genes such as Mkp-1 can influence the manifestation of depressive-like behaviors in a mouse model of neuropathic pain. Furthermore, collectively, we show that region-specific optogenetic manipulation and treatment with conventional and non-conventional therapeutic treatments such as fluoxetine and ketamine, are all associated with alterations in the MAPK pathway in the ACC, which might be related to the observed changes in the post-treatment behavioral phenotype.

Aside from the already proposed investigation of ketamine metabolites and subtypes of GABAergic neurons in the pathophysiology of co-existing pain and depression, there are additional studies which would further illuminate the molecular complexity of this comorbid relationship. First of all, replicating the current results by utilizing other models of chronic pain, such as visceral pain (e.g. endometriosis or irritable bowel syndrome) or somatic pain (e.g. arthritis or headache) could help identify similarities and differences at the molecular and cellular level which lead to mood disorders. Similarly, taking a similar approach, but focusing on other brain regions implicated in this comorbidity such as the amygdala or habenula, and the communication between them, can yield a different perspective of large scale network involvement, which could then be mimicked by in silico simulations.

Another largely overlooked factor in many preclinical and clinical studies is the high incidence of multiple comorbid pain conditions (Davis et al. 2011). For example, a co-existence of neuropathic pain and nociceptive/inflammatory pain is not uncommon, and based on the World Mental Health Survey, around 40% of patients report having more than one painful condition (Gureje et al. 2008). This co-occurrence can be due to a variety of causes including metabolic disease, cancer, chemotherapy/radiotherapy, traumatic injury, traumatic injury etc. (Pagé et al. 2018). However, due to the apparent ethical caveats related to inducing several types of pain in animal models, this would be best studied in clinical settings, also due

to the fact that different types of pain such as neuropathic and inflammatory pain result in comparable alterations in mobility and physical/social activities (Sheahan et al. 2017), which is not necessarily the case in humans (Yezierski and Hansson 2018). Tackling this issue with clinical neuroimaging techniques might bring us closer to finding specific pain-type neurophysiological signatures, and the interaction of comorbid pain states (Wager et al. 2013; López-Solà et al. 2017).

Nowadays, with the increasing availability of technological advancements, there are various possibilities for advancing our current understanding about the present matter, which may also lead to the overall improvement of our current therapeutic strategies. For instance, by using optogenetic tools, it was shown that ketamine activates the projections from the ventral hippocampus to the medial PFC, since optical inhibition of this pathway abolishes its antidepressant effect (Carreno et al. 2016). In this manner, optogenetic manipulation can be used to dissect the impact of activating and/or inhibiting cell-type specific reciprocal projections of the ACC with other brain regions, known to be implicated in pain and depression. Furthermore, by using a transgenic mouse line (c-fos-TetTag) (Reijmers et al. 2007), Tonegawa and colleagues were able to transfect dentate gyrus neurons activated during fear with ChR2, which enabled them to optically reactivate them in a different context and elicit a fear response (Liu et al. 2012; Ramirez et al. 2013). Interestingly, this approach also proved successful in decreasing stress-induced anxiodepressive-like behaviors in mice by reactivating hippocampal cells that were previously active during a positive experience (Ramirez et al. 2015). Hence, a similar approach could be useful to not only pinpoint ACC-related networks implicated in the development of comorbid chronic pain and depression, but also to identify and activate existing pro-resilient circuits that could help to ameliorate maladaptive behaviors.

All in all, by implementing and combining different technologies and tackling the same problem from different angles, alongside rigorous validation and replication of obtained results, we will be closer to understanding the intricate workings of the mammalian nervous system, which can be used to our advantage in treating various pathologies associated with its function.

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UNIVERSITÉ DE STRASBOURG

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Douleur neuropathique et dépression: les modifications moléculaires dans le cortex cingulaire antérieur

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Résumé

Les troubles d'humeur sont fréquemment associées à une douleur chronique et le cortex cingulaire antérieur (CCA) est une région importante dans cette relation. Notre objectif est d'étudier les bases moléculaires de cette comorbidité, tant au niveau de la globalité du CCA (tissus entier) que dans les différents types cellulaires. Dans un modèle murin de douleur chronique présentant des conséquences anxiodépressives et dans plusieurs modèles de dépression, nous avons mis en évidence une surexpression du régulateur négatif de la voie de la protéine kinase activée par des agents mitogènes (MAPK), la MAPK Phosphatase-1 (MKP-1). La diminution de son expression dans le CCA atténue les comportements de type dépressif, ce qui montre que MKP-1 est un facteur clé de la physiopathologie de la dépression. Nous avons également démontré que l'administration aiguë du kétamine normalise la voie MAPK perturbée, tout en produisant un effet analgésique transitoire et un effet antidépresseur prolongé. Enfin, pour étudier la contribution individuelle de différentes populations de cellules dans le développement de la dépression, nous avons isolé les neurones GABAergiques du CCA pour étudier leur expression génomique afin d'établir une liste de gènes candidats plus spécifiques.

Mots-clés: Dépression, Douleur chronique, CCA, MKP-1, Kétamine, Neurones GABAergiques

Abstract

Mood disorders are frequently comorbid with chronic pain and the anterior cingulate cortex (ACC) appears to be an important region in this relationship. We aimed to investigate the molecular basis of this comorbidity, at both the whole structure and the cell type specific level. A genomic analysis of the ACC in a mouse model displaying chronic pain-induced anxiodepressive consequences evidenced an overexpression of the Mitogen Activated Protein Kinase (MAPK) Phosphatase 1 (MKP-1). An upregulated ACC MKP-1 was also observed in other models of depression, while decreasing its expression attenuates depressive-like behaviors, showing that MKP-1 is a key factor in the pathophysiology of depression. This was further validated by showing that acute ketamine administration normalizes the disrupted MAPK pathway, alongside producing a transient analgesic and a prolonged antidepressant effect. Finally, to address the role of different cell populations in this comorbidity, we have isolated GABAergic neurons from animals showing depressive-like behaviors, which will be used for genomic analysis in order to reveal important cell-type specific candidate genes.

Keywords: Depression, Chronic pain, ACC, MKP-1, Ketamine, GABAergic neurons