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Le cortex cingulaire antérieur : une structure clé dans les conséquences émotionnelles de la douleur neuropathique.

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The ACC is a critical hub for neuropathic pain-induced depression

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Le cortex cingulaire antérieur : une structure clé dans les conséquences émotionnelles de la douleur neuropathique

Résumé

Outre le stress chronique, la douleur chronique représente une cause majeure de dépression. En effet, environ 50% des patients qui souffrent d'une douleur chronique développent des troubles de l'humeur. Les perturbations des structures cérébrales impliquées dans la perception de la douleur pourraient contribuer à cette comorbidité, dont les mécanismes restent pourtant mal compris. Nous avons étudié l'implication du cortex cingulaire antérieur (CCA) dans les conséquences sensorielles et émotionnelles de la douleur neuropathique dans un modèle murin. Nous avons montré qu'une lésion du CCA ou une inhibition des neurones pyramidaux du CCA préviennent l'émergence des désordres émotionnels dans notre modèle. De plus, nos résultats indiquent que ces conséquences émotionnelles coïncident avec une hyperactivité neuronale dans le CCA. En conclusion, nous montrons que le CCA est une structure clé pour la dépression induite par la douleur neuropathique.

Résumé en anglais

Besides chronic stress, chronic pain is one of the prevalent determinants for depression. Indeed, around 50% of chronic pain patients develop mood disorders. Alterations in brain regions implicated in pain processing may also be involved in affective processing, thus potentially be responsible of mood disorders. However, the underlying mechanisms of this comorbidity are not yet elucidated. Here, we studied the role of the anterior cingulate cortex (ACC) in the somatosensory, aversive and anxiodepressive consequences of neuropathic pain. We showed that a permanent lesion or temporal inhibition of ACC pyramidal neurons blocked the development or suppressed the expression of an anxiodepressive phenotype in neuropathic mice. In addition, anxiodepressive-like behavior coincided with ACC hyperactivity. In conclusion we show that the ACC is a critical hub for neuropathic pain-induced depression.

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Bleichner MG, Jansma JM, **Sellmeijer J**, Raemaekers M, Ramsey NF. 2014 Give me a sign: decoding complex coordinated hand movements using high-field fMRI. Brain Topogr 27:248-257.

Oostland M, **Sellmeijer J**, van Hooft JA. 2011 Transient expression of functional serotonin 5-HT3 receptors by glutamatergic granule cells in the early postnatal mouse cerebellum. J Physiol 589:4837-4846.

Présentations

Sellmeijer J, Barthas F, Barrot M, Aertsen A, Veinante P, Yalcin I (Unraveling the role of the Anterior Cingulate Cortex in chronic neuropathic pain induced mood-disorders, NeuroTime Strasbourg 2016)

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Résumé de thèse en français

Ce projet a été financé dans le cadre du programme Erasmus-NeuroTime. Ce programme nécessite la mise en place d'une cotutelle entre 3 laboratoires et conduit à un diplôme reconnu par les 3 universités partenaires : les universités de Strasbourg, Freiburg et Bâle. Les expériences d'optogénétique ont été réalisées grâce aux conseils du Pr Andreas Luthi (Université de Bâle), et le Pr Ad Aertsen (Université de Freiburg) nous a guidés dans les analyses computationnelles des données électrophysiologiques.

Introduction

Les troubles émotionnels, tels que la dépression et l'anxiété, sont fréquemment observés chez des patients souffrant de douleur chronique, ce qui aggrave singulièrement le pronostic global de ces patients (1). Les études chez l'animal permettent de modéliser ces aspects, et notamment l'émergence progressive de troubles apparentés à la dépression et à l'anxiété au cours d'états douloureux chroniques (2-4). Certains auteurs suggèrent que la douleur chronique pourrait représenter une forme de stress chronique à l'origine de l'apparition des troubles émotionnels (5). Cependant, des études récentes montrent que la douleur chronique et le stress chronique ont des effets très différents sur l'axe hypothalamo-hypophyso-surrénalien (HHS). Ainsi, l'augmentation des niveaux de corticostérone et l'altération du rétro-contrôle négatif contrôlant l'activité de l'axe HHS, classiquement observées lors d'un stress chronique, ne sont pas retrouvées dans notre modèle de douleur neuropathique, un modéle de constriction du nerf sciatique (modèle du cuff) chez la souris (3).

Selon une autre hypothèse, il existerait des substrats neurobiologiques communs au traitement des informations nociceptives et à la régulation des états émotionnels. Ainsi, les modifications induites lors de la douleur chronique pourraient perturber les mécanismes de régulation de l'humeur et contribuer à l'émergence de troubles dépressifs. En particulier, le cortex cingulaire antérieur (CCA) jouerait un rôle primordial à l'interface de la douleur et des émotions (6, 7).

Afin de mieux comprendre le rôle du CCA dans l'émergence de désordres affectifs lors d'une douleur neuropathique chronique, nous avons caractérisé, dans notre modèle murin, les réponses somato-sensorielles, aversives et émotionnelles, ainsi que l'activité électrique neuronale de cette structure corticale.

Méthodes

Nous avons utilisé un modèle murin de douleur neuropathique, précédemment mis au point par notre groupe de recherche (8). Celui-ci est induit par la mise en place d'un manchon de polyéthylène (« cuff ») autour de la branche principale du nerf sciatique. Nous avons montré que 5 semaines après l'induction de la neuropathie, les animaux développent des troubles émotionnels apparentés à l'anxiété et la dépression (3). Au cours de ce travail de thèse, nous avons approfondi la caractérisation de ce modèle sur une période de 22 semaines, et étudié (i) l'allodynie mécanique, en utilisant des filaments de Von Frey, (ii) la douleur spontanée, en utilisant un conditionnement de préférence de place (CPP), ainsi que (iii) les comportements de type anxio-dépressifs à travers une batterie de tests (boîte clair-obscur, test d'hyponéophagie, splash test, test de la nage forcée). Afin d'évaluer le rôle du CCA au cours de la douleur neuropathique chronique, nous avons réalisé des lésions de cette structure cérébrale par des injections locales d'acide iboténique. En parallèle, nous avons également examiné les conséquences comportementales d'une stimulation de l'activité neuronale dans le CCA par optogénétique, chez des souris naïves génétiquement modifiées (Thy1-ChR2-YFP). Après avoir ainsi démontré l'implication essentielle du CCA dans la comorbidité entre douleur neuropathique chronique et épisode dépressif majeur, nous avons déterminé, sur une période de 22 semaines, les profils d'activité neuronale dans le CCA en réalisant des enregistrements électrophysiologiques unitaires in vivo.

Résultats

Caractérisation des conséquences à long terme de la douleur neuropathique

Suite à l'induction de la neuropathie l'allodynie mécanique persiste pendant 12 semaines. Elle s'accompagne d'une douleur spontanée qui continue de se manifester même après la rémission spontanée de l'allodynie, c'est à dire au delà de 12 semaines. Cette douleur spontanée régresse à son tour environ 22 semaines après l'induction de la neuropathie. En parallèle, les animaux développent à partir de la 5ème semaine un phénotype anxio-dépressif qui se manifeste jusqu'à la 22ème semaine.

Rôle du CCA sur le phénotype anxio-dépressif induit par la douleur neuropathique

Nos résultats indiquent que la lésion par l'acide iboténique du CCA bloque l'apparition du phénotype anxio-dépressif et de la douleur spontanée chez les animaux neuropathiques, mais

n'affecte pas l'émergence de l'allodynie (9). Nous montrons également, par une approche optogénétique, que l'inhibition du CCA, bloque les conséquences anxiodépressives de la douleur neuropathique et la douleur spontanée. Enfin, chez des souris naïves, en l'absence de douleur neuropathique, l'activation des neurones pyramidaux du CCA est suffisante pour induire des comportements anxio-dépressifs (9). Ni l'inhibition du CCA en condition neuropathique, ni son activation chez des animaux naïfs n'ont d'effet sur le seuil de sensibilité mécanique.

Les conséquences anxio-*dépressives de la douleur neuropathique s'accompagnent d'une* hyperactivité des neurones du CCA

Afin d'explorer le rôle du CCA au cours de la neuropathie, nous avons mesuré l'activité neuronale spontanée à 4 moments clés de la neuropathie.

Stade1 : les animaux expriment une allodynie mécanique mais n'ont pas encore développé de phénotype anxio-dépressif (2-3 semaines après l'induction de la neuropathie).

Stade2 : les comportements anxio-dépressifs sont apparus alors que l'allodynie est toujours présente (6-12 semaines après l'induction de la neuropathie).

Stade3 : les animaux sont en rémission spontanée de l'allodynie mécanique mais présentent toujours des comportements apparentés à l'anxiété et à la dépression (12-18 semaines après l'induction de la neuropathie).

Stade4 : l'allodynie mécanique est absente, et les réponses émotionnelles se sont normalisées (22-26 semaines après l'induction de la neuropathie).

Nos résultats indiquent que l'activité neuronale spontanée du CCA est augmentée chez les animaux neuropathiques aux stades 2 et 3 par rapport aux animaux contrôles. De plus, l'activité en bouffées de potentiels d'action (burst) est également augmentée chez les animaux neuropathiques aux stades 2 et 3. Afin d'identifier le type de neurones impliqués dans cette augmentation d'activité, nous avons réalisé des marquages juxta-cellulaires et des identifications neurochimiques par des marquages par la calbindine, la Cam kinase de type II et la parvalbumine. Les résultats indiquent que ce sont les interneurones, ainsi que les neurones pyramidaux du CCA, qui présentent une augmentation de leur activité électrique aux stades 2 et 3.

Conclusion

Nos résultats confirment que les composantes sensorielles et émotionnelles, ainsi que les conséquences anxio-dépressives, se mettent en place et se développent au cours du temps après l'induction d'une douleur neuropathique. Ils montrent également le rôle crucial du CCA, une structure corticale de la « matrice douleur » à l'interface de la douleur chronique et des régulations émotionnelles. En effet, alors que la lésion du CCA est suffisante pour empêcher l'apparition de conséquences émotionnelles négatives chez les animaux neuropathiques, la stimulation de cette région corticale est suffisante pour induire des troubles comportementaux similaires chez des animaux naïfs. Enfin, nos résultats révèlent une similitude frappante entre la cinétique des troubles anxio-dépressifs et celle des modifications de l'activité électrophysiologique du CCA. Ces modifications, qui correspondent à une augmentation de l'activité unitaire et de l'activité en bouffées de potentiels d'action des neurones pyramidaux et des interneurones, se manifestent uniquement lorsque les troubles thymiques comportementaux sont présents.

Content

Preface

Neuropathic pain is a chronic condition that arises as a consequence of a disease or a lesion affecting the somatosensory nervous system (10). Chronic pain affects approximately 30% of the population, where as neuropathic pain is present in around 8 % of the population (IASP). Neuropathic pain can have dramatic consequences for patients (11). Thirty-four percent of neuropathic pain patients develop mood-disorders (12) which dramatically disrupt patients' quality of life (1).

The most frequently observed mood disorders in chronic pain patients are generalized anxiety and major depressive disorders The Diagnosis and Statistical Manual of Mental Disorders V (DSM-V) criteria for generalized anxiety disorder include the presence of excessive anxiety and worry that lasts at least 6 months and have at least 3 symptoms of the following: edginess or restlessness, tiring easily, impaired concentration, irritability, increased muscle aches or soreness and difficulty sleeping. The DSM-V criteria for major depression disorder include having a depressive episode for at least 2 weeks and at least 5 of the following symptoms: depressed mood, loss of interest or pleasure in almost all activities, a weight loss or gain, sleep disturbances, agitation or motor retardation, fatigue, feelings of worthlessness or guilt, difficulty concentration and thoughts of death and suicide.

This thesis explores the underlying processes of the neuropathic pain and depression comorbidity by using the sciatic nerve cuffing model of neuropathic pain. We characterize the development of the somatosensory and aversive consequences of neuropathic pain as well as anxiodepressive-like behavior. Additionally, we perform electrophysiological single-unit recordings from the ACC, a region implicated in both pain related and mood related processes, and manipulate its activity through optogenetic excitation and inhibition.

In the next introductory chapters we will provide information on pain (I), neuropathic pain (II), the methods of preclinical modeling of neuropathic pain-induced mood disorders (III) and preclinical insights of neuropathic pain-induced mood disorders (IV). We will finish the introduction with a draft of the, as of yet unpublished, review which describes the ACC's connectome, role in mood processing and in pain processing (V). The introduction will be followed by two research papers, one of which is published in Biological Psychiatry (VI-VII), and the general discussion (VIII).

Introduction

I. Pain

The International Association for the Study of Pain (IASP) defines pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. This definition suggests the importance of both sensory and emotional components of pain. In addition, in 1968, Melzack and Casey (13) suggested that the pain experience reflects the interaction of somatosensory, affective and cognitive processes which introduced for the first time the notion of pain matrix. Since, this concept is improved thanks to functional imaging studies and now the pain matrix defines a group of brain regions that take part in the processing of pain information such as the posterior insula, parietal operculum, mid-cingulate, the primary sensory/motor areas, anterior insula, ACC, dorsolateral prefrontal cortex, posterior parietal cortex, perigenual cingulate, orbitofrontal cortex, anterolateral prefrontal cortex and the ventral striatum (14).

According to this new pain matrix concept, pain processing occurs in three steps including the processing of nociceptive information, the translation of nociceptive information to pain experience and, finally, the formation of pain memory. Neurons from spinal laminae I, V and VII convey nociceptive information from the periphery to the posterior, centrolateral, mediodorsal and posterior nuclei of the thalamus (15, 16), forming what is called the spinothalamic system. These regions synapse unto cortical areas responding to noxious stimuli such as posterior insula, medial parietal operculum and mid-cingulate cortex and somatosensory cortices (17-20).

The transition of nociceptive information to the conscious pain experience is mediated by the mid and anterior insulae, the ACC, the prefrontal and posterior parietal areas; with less consistency, the striatum, supplementary motor area, the hippocampus, the cerebellum, and the temporoparietal junction. Mid and anterior insulae are responsible for the posterior to anterior information flux within the insula, which supports the transformation of sensory events into vegetative reactions and associated internal feelings (14, 21). The cognitive section of the ACC, the prefrontal and posterior parietal areas, are thought to be involved in attention, anticipation learning and cognitive control (14).

Lastly, third order networks, constitute by the perigenual cingulate, the orbitofrontal cortex, the temporal pole, and the anterolateral prefrontal areas, are implicated in both pain

memory and the emotional components such as unpleasantness of pain (22, 23). These regions are also involved in the generation of subjective pain experience by observations of other people's suffering, or the pain-relieving effects derived from placebo (24, 25) or strong religious believes (26). Such changes are not elicited by affecting sensory gain but by reevaluation and interpretation of the meaning of a stimulus.

II. Chronic Neuropathic Pain

Pain normally serves to direct behavior towards self-protection and promotes adaptive behavior (27) but when pain becomes chronic, it loses its adaptive and biologic function of signaling injury or disease. Chronic pain affects the lives of 20-60% of the world population (28, 29) and remains difficult to treat (30). Among different types of chronic pain such as inflammatory, musculoskeletal, visceral, in this study, we mainly focused on neuropathic pain. Neuropathic pain is caused by a lesion or disease affecting the somatosensory system (IASP; (31). Depending on whether damages affect the peripheral or central nervous system, neuropathic pain can be further divided into central neuropathic pain and peripheral neuropathic pain (32). For most patients, neuropathic pain has a peripheral origin, arising as a consequence of peripheral nerve injury or as a consequence of a metabolic disease such as diabetes. Nerve injuries and diabetic peripheral neuropathy account for almost two-thirds of the patients. However, neuropathic pain can also result from infectious diseases, as in post-herpetic neuralgia, from exposure to neurotoxic compounds, such as those used for cancer chemotherapy (32, 33). Causes arising from the central nervous system include spinal cord injury, multiple sclerosis, Parkison's disease and stroke related lesions (32, 33). Diagnosis of neuropathic pain is often based on physical examination and questionnaires that assess the characteristics of neuropathic pain (33-35). Symptoms include spontaneous pain, allodynia, hyperalgesia, paresthesia, dysaesthesia and hypoesthesia (32, 33) (Box 1).

According to clinical and preclinical studies (36-38), the physiopathology of neuropathic pain lies in peripheral, at the level of the nerve and dorsal root ganglia, and central mechanisms, at level of spinal cord and brain (36-38). As the central nervous system is the primary topic of this thesis work, we discuss the most studied central nervous system modifications in the next section.

A. Central modifications

One of the consequences of nerve injury is the sensitization of the central nervous system which is associated with the development and maintenance of chronic pain. The nervous system goes through a process called "wind-up", when central sensitization occurs, and gets regulated in a persistent state of high reactivity. This state of reactivity subsequently maintains pain sometimes even after the initial injury has healed. Sensitization of the central nervous system can be caused by a disrupted inhibitory/excitatory balance and plasticity changes in the spinal cord or brain.

Box 1. IASP definitions of neuropathic pain symptoms

Hyperalgesia: increased pain from a stimulus that normally provokes pain

Allodynia: painful response to a normally non-painful stimulus

Paresthesia: abnormal sensation whether spontaneous or evoked

Dysesthesia: unpleasant abnormal sensation, whether spontaneous or evoked

Hypoesthesia: decreased sensitivity to stimulation, excluding special senses

Inhibitory/excitatory imbalance

Disrupted inhibitory/excitatory balance has been observed at both the spinal and supraspinal level in chronic pain conditions (39-45). One of the reasons for this imbalance is the decrease of inhibitory activity. For instance, the loss of inhibition in the dorsal horn by a reduction of GABA-induced inhibitory postsynaptic currents (IPSCs) (39, 40), reduced GABA receptor subunit expression (41), loss of interneurons (42) and calcium-channel subunit upregulation (43) lead to a decrease in firing thresholds contributing to mechanical and cold allodynia (42, 44, 45) as well as hyperalgesia (44) observed in neuropathic pain. At the supraspinal level, clinical studies have shown that glutamate levels were higher, and GABA levels lower in the posterior insula (46) in neuropathic patients. Additionally animal studies have shown disinhibition of ACC layer V pyramidal neurons (47) and disinhibition in the central nucleus of the amygdala due to a decrease of GABAergic inhibition (48) in neuropathic pain animal models.

Spinal and supraspinal plasticity changes in neuropathic pain

In neuropathic pain conditions, both functional (49-52) and morphological (53-55) plasticity changes are observed at the spinal and supraspinal level. A major driving force in initiating change in synaptic strength is the increase of postsynaptic calcium levels. Ionotropic receptors and voltage-gated calcium channels as well as calcium release from intracellular stores can increase calcium influx (56, 57) which in turn induces cellular changes that increase synaptic strength (58). For instance, in the spinal cord calcium influx has been shown to activate extracellular signal-regulated kinase (ERK) which in turn has been shown to increase AMPA and NMDA receptor insertion into the membrane (59). Indeed, dorsal horn AMPA (60) and NMDA receptor (52) expression were found to be increased in an animal models for neuropathic pain (52) and increased NMDA receptor trafficking has also been reported (54).

Synaptic plasticity changes within cortical areas involved in pain processing has been observed in chronic neuropathic pain (61). For instance, nerve injury induced synaptic remodeling by synaptogenesis, synapse elimination, strengthening of persisting synapses and hyperexcitability have been shown in the somatosensory cortex (53). Besides alteration in synaptic transmission, long term potentiation (LTP) and depression (LTD) are forms of plasticity. LTP and LTD may also be altered in chronic pain conditions. However, alterations in LTP/LTD may be different depending on the brain region. For instance, a reduction in hippocampal LTP

without affecting LTD has been found in neuropathic pain (62, 63) but LTP increase was reported in the ACC (49-51).

An in vivo study showed that digit amputation leads to long-lasting membrane potential depolarization in ACC neurons of adult rats (51). In mice with peripheral nerve ligation, it has been shown that pyramidal neurons in layer II/III of the ACC have an increase of AMPA-receptor-mediated excitatory postsynaptic currents (EPSCs) (49), of membrane AMPA GluA1 receptors (50) and of NMDA GluN2B receptors (51), all of which lead to postsynaptic enhancement. In addition, presynaptic release of transmitters is enhanced in the ACC. Paired-pulse facilitation, a transient form of plasticity and a way to test presynaptic functioning, showed a significant reduction in ACC neurons in neuropathic mice which indicates presynaptic enhancement of excitatory synaptic transmission after nerve injury (49). Increased excitatory transmission has also been observed in other brain regions, such as the amygdala (64), insular cortex, and primary and secondary sensory cortices (65).

Central nervous system activity changes

Cortical activity abnormalities are a common finding in neuropathic pain. However, because different subregions have profoundly different functional roles, there are some discrepancies between studies. However, cortical hyperactivity has been reported in the presence of neuropathic pain most frequently. For instance, an EEG study showed hyperactivity within the theta and beta frequency range originating from the IC, ACC and somatosensory cortices in patients with ongoing neuropathic pain (66). Similarly a PET study reported hyperactivation in ongoing neuropathic pain in the anterior insula, posterior parietal, lateral inferior prefrontal, and the ACC (67). Apart from changes in spontaneous activity, hyperactivity during heat allodynia has also been reported. An fMRI study showed that the bilateral anterio insula, right ventral putamen, orbital frontal cortex, right dorsolateral prefrontal cortex and the ACC are hyperactive during heat allodynia in neuropathic patients(68).

On the other hand, cortical hypoactivity has also been reported. For instance, the ventromedial PFC, an important region for the integration of cognitive and emotionally relevant information (69, 70), has been shown to be hypoactive in neuropathic pain (24, 71, 72). From this region connections trigger inhibition of pain signals via the periaqueductal grey (14). This notion

is supported by the findings that analgesic procedures, such as distraction and motor cortex stimulation, trigger ventromedial PFC activity (73, 74).

In neuropathic pain patients, another common finding is thalamic hypoactivity (14), which is thought to be a compensatory mechanism of functional reorganisation after nerve damage, however, analgesic procedures restore thalamic blood flow without alleviating pain relieve (75-77), and blood flow is not correlated with pain relief (78). Another common finding is an increase of neuronal bursting and electroencephalographic slowing in the thalamus of neuropathic pain patients (14) which is thought to be caused by thalamic cell hyperpolarisation (14).

III. Preclinical modeling of neuropathic pain induced mood disorders

Besides somatosensory alterations, neuropathic pain can also induce mood disorders. For instance, the prevalence rate for major depressive disorder is about 30% in neuropathic pain patients (1, 12). While the comorbidity between neuropathic pain and mood disorders are well established the underlying mechanisms remain unclear. For this purpose, animal studies are needed to model the comorbidity between neuropathic pain and mood disorders.

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 (79-82). While many neuropathic pain models have been developed in rodents (79-82), most of the peripheral neuropathic pain models rely on ligation, compression or partial sections of the sciatic nerve **(Table 1)**. This nerve is of easy access and nociceptive tests can be easily conducted on hind paws. These models rely on three or four loose ligatures around the main branch of the sciatic nerve (chronic constriction injury, CCI) (83), on the tight ligation of the sciatic nerve (partial sciatic nerve ligation, PSL) (84) or of the L5 and L6 spinal nerves (spinal nerve ligation, SNL) (85), on the ligation of the common peroneal nerve (86), or on the implantation of a polyethylene cuff around the main branch of the sciatic nerve (8, 87). The spared nerve injury (SNI) is another frequently used model, for which two of the three terminal branches of the sciatic nerve are tightly ligated before their distal axotomy (88). A shared feature of these models is to induce mechanical allodynia, as well as changes in thermal sensitivity for most of them.

In this study, we used a sciatic nerve cuffing model which is a reliable and stable method to induce neuropathic pain in mice (3). It entails the cuffing of the main branch of the sciatic nerve with a polyethylene tube. This standardized procedure results in neuropathic pain with low inter-individual variability for long lasting mechanical allodynia.

Almost all of the preclinical studies on the anxiodepressive consequences of neuropathic pain were performed on models related to sciatic nerve manipulation, using either nerve compression or section. While, some studies failed to show the relationship between neuropathic pain and anxiodepressive-like behavior in animals (89-93), other studies did report such behavioral phenotypes in neuropathic rodent models (3, 4, 8, 94-98). An explanation for this discrepancy might lie in the timing of the behavioral test after pain induction, since the studies showing negative results were performed only 3 weeks after peripheral nerve surgery. Some

Table 1. Summary of studies on the affective consequences of neuropathic pain (from Yalcin et al., 2014).

Pain Model	Species	Test	Results	References
PSNL	Rat	BT	BB deficits	Andrews et al., 2012
	Mouse	OF, EPM, TST	No effect	Hasnie et al., 2007b
	Mouse	OF	No effect	Kodama et al., 2011
	Mouse	LD, EPM	ALB	Narita et al.,2006a,b
	Mouse	LD, EPM	ALB	Matsuzawa-Yanagida et al., 2008
SNL	Rat	OF, EPM,LD, FST	No effect	Kontinen et al., 1999
	Mouse	OF, EPM, LD, FST	ALB, DLB	Suzuki et al., 2007
CCI	Rat	FST, SP	No effect	Bravo et al., 2012
	Rat	FST, OF	DLB	Zeng et al., 2008
	Rat	EPM	ALB	Roeska et al., 2009
	Rat	FST	DLB	Fukuhara et al.,2012
	Mouse	EZM, FST, OF	no effect	Urban et al., 2011
SNI	Rat	OF, EPM,FST	no ALB, DLB	Gonçalves et al., 2008
	Rat	OF, EPM, FST	ALB, DLB	Leite-Almeida et al., 2009
	Rat	OF, EPM	ALB	Seminowicz et al., 2009
	Rat	FST, SP	DLB	Wang et al., 2011
	Mouse	OF, FST	no ALB, DLB	Norman et al., 2010
	Mouse	EZM, FST, OF	no effect	Urban et al., 2011
	Mouse	DL,NSF	ALB	Mutso et al., 2012
Cuff	Mouse	EPM, MB, SI, TST	ALB	Benbouzid et al., 2008
	Mouse	LD, MB, NSF, Splash, FST	ALB, DLB	Yalcin et al., 2011
SNT	Rat	FST	DLB	Hu et al., 2010
	Rat	BT	BB deficits	Andrews et al., 2012
Antiretroviral	Rat	OF,BT	ALB, BB deficits	Huang et al., 2013

ALB: Anxiety-like behavior, BB: Burrowing behavior, BT:Burying test, CCI: Chronic construction injury,
DLB: Depression-like behavior, EPM: Elevated-plus maze, EZM: Elevated zero maze,
FST: Forced swimming test, LD: Light-dark test, MB: Marble burying, NSF: Novelty-suppressed feeding,
OF: Open field, SI: Social interaction, SNI: Spared nerve injury, SNL: Spinal nerve ligation,
SNT: Spinal nerve transfection, SP: Sucrose preference, PSNL: Partial sciatic nerve ligation,
TST: Tail suspension test.

studies show that anxiodepressive behavior develops over time with anxiety-like behavior developing only after 4 weeks after neuropathic surgery and depressive-like behavior becoming apparent between the 6th and 8th week after neuropathic surgery (3). Other factors such as species, strains of animals, the type of pain surgery and the time of day the tests are performed may also influence the results (99-101).

In order to study the anxiodepressive consequences of neuropathic pain a variety of tests are used. Most of these tests evaluate anxiety-related behavior by measuring exploration behavior, such as the elevated plus maze, the open field or the dark-light exploration test (3, 94, 102). The novelty-suppressed feeding test, measures the conflict between the drive to eat and the fear of venturing into the center of the open field. This test has been used to assess anxiodepressive-like behavior since it has been shown to respond to chronic antidepressant drug treatment (103). Other behavioral tests consist of exposing the animal to stressful situations and measure stress coping, such as the forced swimming and the tail suspension tests (3, 91). Aside from mood, the animal's interest in pleasurable activities such as consuming sucrose solution or engaging in social interactions can also be tested both of which are decreased in neuropathic pain (8, 104). Burrowing, an evolutionarily conserved behavior that likely reflects motivation and general well being, can also be used to test the consequences of neuropathic pain and has been shown to be reduced in neuropathic animals (105). Chronic pain (106, 107) and depressed patients (108) frequently show sleep related issues such as insomnia or hypersomnia. Animal studies have confirmed this by investigating the effect of chronic pain on sleep efficiency, arousals (109), wakefulness and non-rapid eye movement (NREM) sleep (110, 111) showing that wakefulness is increased and sleep is impaired. Neuropathic animals, have been found to show cognitive impairment including; impaired attention (101) but also memory deficits such as altered working memory (62, 112), short term memory (62) and spatial memory (101). For this thesis, we used a battery of tests to evaluate anxiety and depressive-like behavior which will be explained in more detail in chapter VI and VII.

IV. Mechanisms of neuropathic pain-induced mood-disorders: Insights from animal studies

Clinical studies have shown both morphological and functional changes in brain structures including the medial prefrontal cortex (mPFC) (113-115), the ACC, the hippocampus (116, 117), the amygdala (118) and the thalamus (119, 120) in neuropathic pain induced affective and cognitive disorders (**Fig. 1**). As the ACC has been implicated in many mood-disorders and is the main subject of this thesis, we decided to dedicate chapter 3 to this structure.

Neuroanatomical and plasticity changes

The somatosensory, anterior cingulate and the insular cortex mediate and modulate pain processing (14). While the somatosensory cortex plays a role specifically in discriminating the location and intensity of painful stimuli (115), it has no direct implication in affective, motivational and cognitive information of the pain.

The hippocampus plays a major role in learning and memory (121, 122), in addition, it has been shown to be responsible for cognitive consequences of neuropathic pain (123, 124) such as deficits in working memory (62, 101), short-term memory (62) and recognition memory (92). In both neuropathic patients and animals, hippocampus volumes were decreased which was associated with a reduction of neurogenesis in the dentate gyrus (116). Nerve injury has also been shown to reduce presynaptic terminals density at CA3-CA1 synapses (62) and was correlated with impaired contextual fear extinction (116) and memory deficits (62). Additionally, chronic pain-induced hippocampal plasticity changes include the induction of LTP in the dentate gyrus, CA1-CA3 synapses (62, 63).

The central (CeA) and baseloteral amygdala (BLA) play important roles in the affective and cognitive aspects of pain (125-128). For instance, amygdala morphological analyses showed an increase of amygdala volume in animals with spared nerve injury displaying anxiodepressive behavior, which was associated with increased neurogenesis in the CeA and the BLA (94). Additionally, another study showed that CeA neuron excitability was increased and BLA-CeA synapses were potentiated (129). This suggests that NMDA receptors, by facilitating BLA inputs or synaptic signaling between CeA interneurons, help maintain neuropathic pain (130).The GABAergic system (131) also plays a role in facilitating neuropathic pain. GABA-A receptors in the CeA can modify avoidance behavior under the influence of pharmacological manipulation in

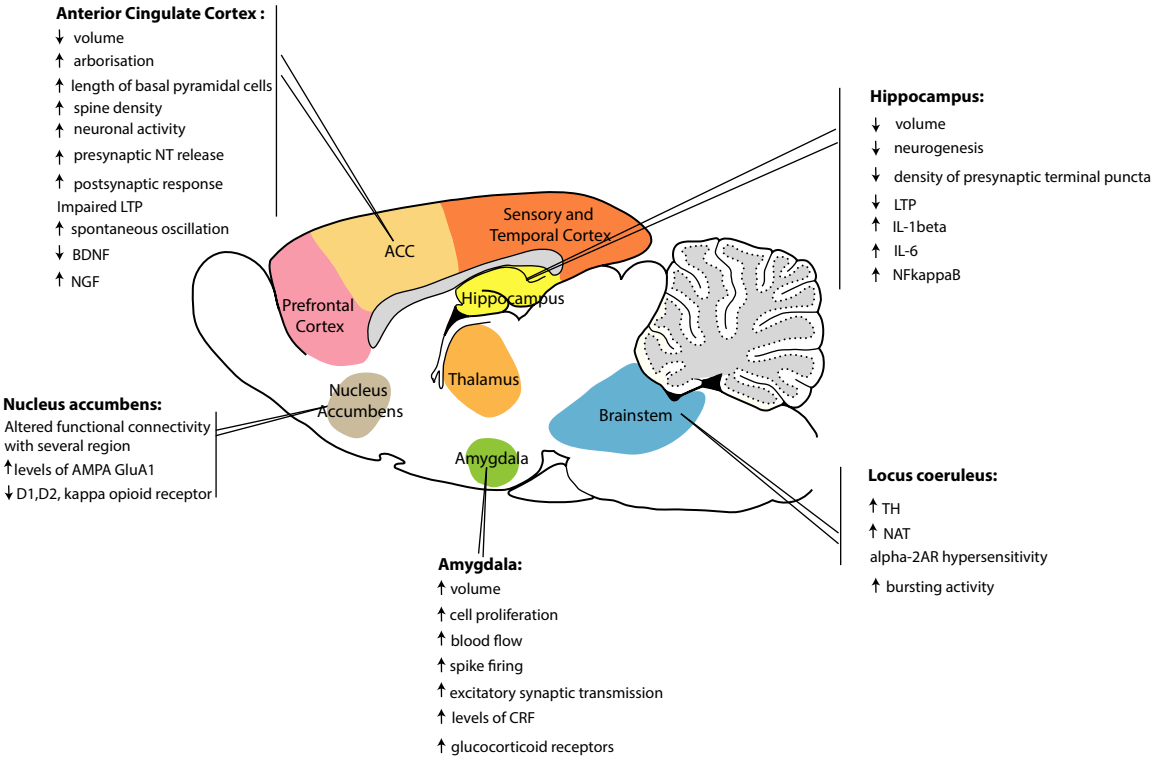


Fig. 1 Summary of functional and morphological alterations in animal with neuropathic pain (From Yalcin et al., 2014.)

ACC: Anterior cingulate cortex, AR: Adrenoceptor, BDNF: Brain derived neurotrophic factor, CRF: corticotropin releasing factor, D: Dopamine, IL: Interleukine, LTP: Long-term potentiation, NAT: noradrenaline transporter, NGF: Nerve growth factor, NFkappaB: Nuclear factor kappa B, NT: Neurotransmitter, TH: Tyrosine hydroxylase.

neuropathic animals which shows the CeA's role in affective processing under the influence of neuropathic pain (131).

The mesolimbic pathway, including the ventral tegmental area (VTA) and the nucleas accumbens (NAc) have been related to depression and its treatment (132, 133). These regions have been found to respond to aversive and nociceptive stimuli (134, 135) and increased functional connectivity between the NAc and the PFC was related to the transitioning of acute pain into chronic pain (136). GluA1 subunits of AMPA glutamate receptors in NAc synapses are increased in rodents with neuropathic pain (137). These changes underlie anxiodepressive-like behavior as the blockage of AMPA receptors increased depression-like behavior in neuropathic animals (137).

Neuroendocrine parameters

Mood disorders are frequently associated with HPA-axis changes (138). However, such alterations do not seem to be prevalent in the context of neuropathic pain. Corticosterone hormone levels are not changed in rats 3 weeks after nerve constriction injury (139, 140). This is further supported by previous results from our team showing no corticosterone level alterations during basal or stress conditions until 8 weeks after peripheral nerve compression (3). It seems that, even though neuropathic pain leads to similar behavioral consequences as chronic stress, sustained neuropathic pain differs from chronic stress concerning the neuroendocrine response.

Another point of interest are thyroxine (T4), free thyroxine (fT4), triiodothyronine(T3) and thyroid stimulating hormone (TSH) levels after nerve ligation. Decreased plasma levels of T4, fT4 but not T3 and TSH have been found in nerve-injured rats that display decreased social dominance behavior towards intruders (141).

Neuroimmune response

Neuroimmune alterations are shown to play a role in the generation of depression (142, 143) and chronic pain (144). The implications of different actors are time-dependent, in which early phases of neuropathic pain are characterized by an increase in microglial staining in the periaqueductal grey and the hypothalamus (145) and astroglial activation in the periaqueductal grey and cingulate cortex is observed during the late phase (146, 147). In the neuropathic pain condition IL-1β, a proinflammatory cytokine involved in the maintenance of neuropathic pain (148), is

upregulated in the brainstem (119), the PFC (119) and the hippocampus (149). In addition, IL-6 levels (150) and Nuclear Factor kappa B expression, a transcription factor involved in the immune response (151) are elevated in the hippocampus. In turn, these changes have been shown to be related to the generation and maintenance of LTP, since LTP can increase the expression of IL-1 β and IL-6 (152, 153) whereas blocking them can impair or prolong LTP maintenance, respectively (152, 153).

Neurotrophic factors

Alterations in neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) are also found to contribute to depression and antidepressant treatment effect (154). BDNF, besides regulating neuronal survival and differentiation, has been found to participate in activity-dependent synaptic plasticity mechanisms (155, 156). BDNF decrease has been shown in the hippocampus of depressed patients (157) while antidepressant treatment activates the BDNF-signaling pathway (138). In the spinal cord, BDNF is found to participate in neuropathic pain pathogenesis by causing disinhibition of pain-transmitting signals (158, 159). In the cingulate cortex, striatum and the hippocampus BDNF levels are decreased whereas nerve growth factor is increased in these regions (150). Supporting these findings is a study that showed that administration of 4-methylcatechol, a BDNF synthesis promoter, suppresses neuropathic pain-induced depression (160). However, since few studies investigate the role of BDNF in neuropathic pain-induced depression, further work is needed to provide conclusive evidence.

V. The Anterior Cingulate Cortex

The next chapter contains an early draft of a yet unpublished review that we are about to finalize. This paper was prepared in collaboration with Clementine Fillinger, who wrote the neuroanatomy section and Florent Barthas, who reviewed the ACC's involvement in pain. The sections on the ACC's involvement in emotional processing and mood-disorders as well as the electrophysiological properties of the ACC have been written by me.

A. Multifaceted role of Anterior Cingulate Cortex: Insight from emotional and pain studies.

Over the past 20 years, clinical and preclinical studies depicted the anterior cingulate cortex (ACC) as a site of interest for many neurological and psychiatric conditions. The ACC plays a critical role in emotion, autonomic regulation, pain processing, attention, memory, decision making and visuospatial orientation, as evidenced through imaging, lesion, single-neuron recording and electrical stimulation approaches.

In 1878, Broca described the cingulate cortex as a component of the limbic lobe. Ever since, knowledge of this structure expended greatly, and today it is considered as a heterogeneous structure, composed of subdivisions with different cytoarchitecture, connections and functions. However, whether emotion, cognition and pain processing are segregated according to distinct connectomes or subdivisions within the ACC, is still an ongoing debate (69, 161). For instance, the hypotheses that the dorsal ACC is only involved in cognitive functions while emotional information is processed by the ventral ACC (162), or that the anterior part is an "executive" region whereas the posterior region is "evaluative"(163) are no longer supported by recent studies (162, 163).

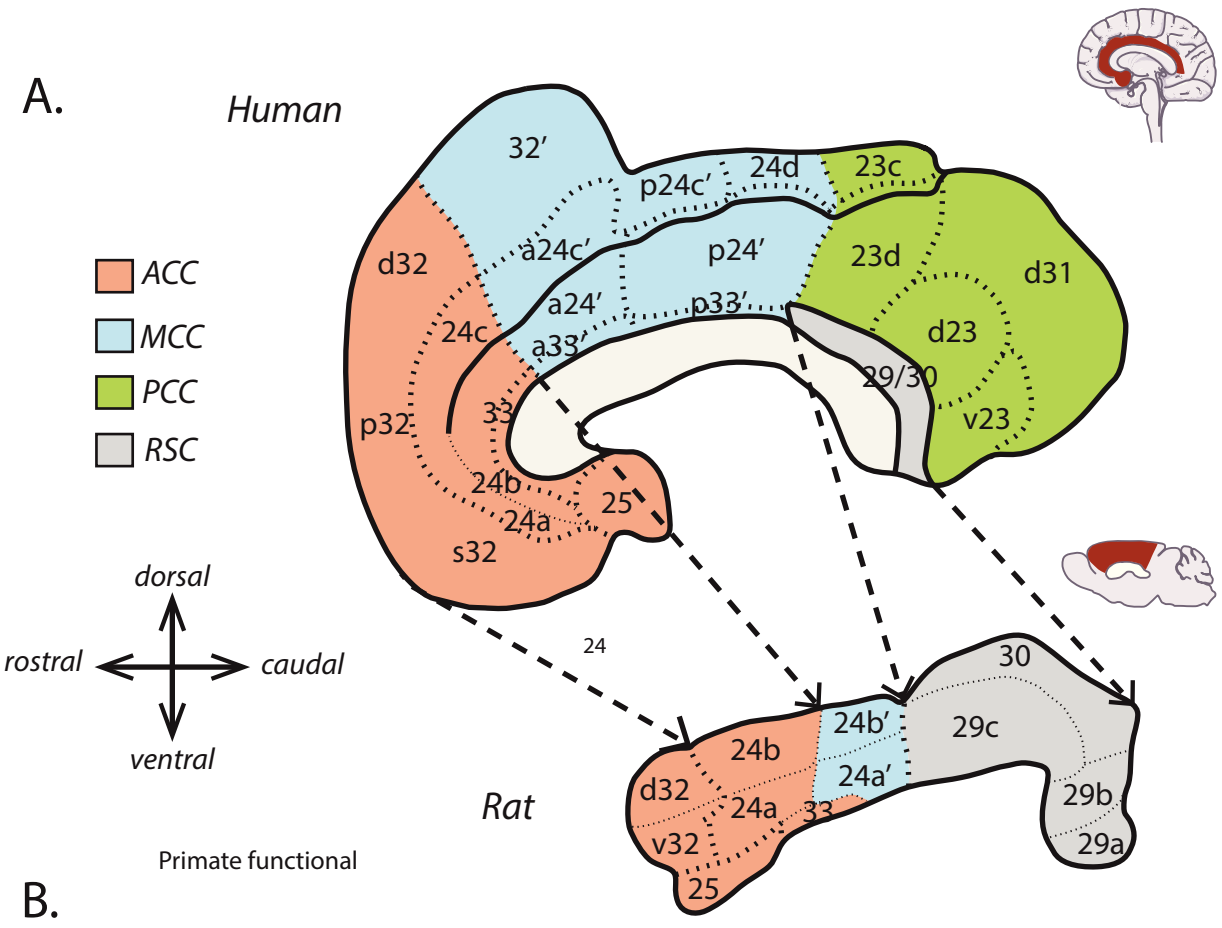
The primary aim of this review is to compile recent information on the impact of the ACC in emotional and pain processing, as well as in their related pathologies, by offering a synthesis of both preclinical and clinical data. We will also discuss the influence of cognitive functions on this processing. While preparing this review, we deemed it necessary to also offer a comparison of the ACC neuroanatomical organization across species, by comparing human, non-human primates and rodents. Indeed, a few contradictory reports concerning the ACC under physiological and pathological conditions may be reconciled by taking the distinct roles of ACC subregions into consideration.

B. The neuroanatomy of the ACC

In primates, the cingulate cortex belongs to the medial wall of the prefrontal cortex, spanning from the supracallosal to cingulate sulcus. Along the anteroposterior axis, four subregions including the anterior (ACC), the middle (MCC) and the posterior (PCC) cingulate cortices as well as the retrosplenial (RS) cortex compose this sulcus (6, 164, 165). Comparison studies based on the cytoarchitecture and the homology with the primate cingulate cortex describe only the ACC, the MCC and the RS in rodents. Interestingly, no region analogous to the primate PCC was found yet (165). The ACC, an agranular structure since it lacks a true layer IV, is composed of areas 24, 25, 32 and 33 both in primate and rodent. The MCC is composed of areas 24', 32' and 33', while the PCC and the RS respectively cover the areas 23, 31 and areas 29, 30. In each species, these areas are divided into additional subdivisions (**Fig. 2A**). Importantly, the rodent's cingulate cortex lacks the sulcal areas found in primates.

This review mainly focuses on the ACC, and more specifically on area 24 which contains both neurons and interneurons distinctly distributed in terms of density over different layer and subdivisions (166). While there are far more glutamatergic (Glu) neurons (around 80%) than GABAergic neurons in all ACC layers except in layer 1, the latter includes heterogeneous subpopulations based on calcium binding proteins (parvalbumin (PV), calbindin or calretinin) and peptides, such as somatostatin (SOM) (167).

Until the 4th edition of the Paxinos and Franklin's "the Mouse Brain in Stereotaxic Coordinates" (2012), different nomenclatures were used to describe the ACC in rodents, making comparisons with primates confusing (**Fig. 2B**). In earlier atlases of the rodent brain (168, 169), areas 24b/24b' and 24a/24a' were respectively labeled cingulate cortex Cg1 (dorsal) and Cg2 (ventral) according to Zilles and Wree (170, 171). Thus, the term "anterior cingulate cortex" has been often employed to refer to Cg1 and Cg2, even if areas 24a' and 24b' actually belong to the MCC and not to the ACC (165). A second source of confusion was due to the concept of medial prefrontal cortex in rodents (mPFC), constituted by the infralimbic cortex (IL), prelimbic cortex (PrL), "anterior cingulate cortex" (Cg or ACC) and of the secondary motor cortex (M2) (172, 173). Since the IL and the PrL actually correspond to the ACC areas 25 and 32 respectively (165), the concept of four structures constituting the mPFC seems inaccurate. To overcome this confusion and to favor inter-species comparisons, the latest revision of the rat (174) and mouse



Primate	Neuroanatomy	25	s32	s24a s24b	p32	p24a p24b 24c		d32	33	(a,p)24a′	(a,p)24b′	(a,p)24c′	24d	(a,p)33′	32′	23,31	29,30
	Functional	subgenual ACC			pregenual ACC					MCC						PCC	RS
Rat	Neuroanatomy	25	v32	24a 24b	v32	24a 24b	~	d32	33*	24a′	24b′	~	~	~	~	~	29,30
	Functional	IL	PrL	Cg2 Cg1	PrL	Cg2 Cg1	~	PrL	33*	Cg2	Cg1	~	~	~	~	~	RS

Figure 2 Organization of the ACC in primates and rodents.
A. Schematic views of cingulate cortex subregions in primates and rodents.
B. Correspondance between cingulate cortex neuroanatomical and functional nomenclatures in primates and rodents.
*not present in mice

(175) brain atlases replaced the terms IL, PrL, Cg1 and Cg2 with their corresponding Brodmanns' area (**Fig.2B**).

C. ACC connections: insights from rodent and non-human primate studies

Inputs

Areas 24a/24b of the ACC receive strong inputs from cortical and thalamic regions, as well as from a few other forebrain structures and from a limited number of brainstem centers (**Fig. 3A**).

At cortical level, besides strong interconnection linking 24b and 24a, the ACC receives moderate to strong inputs from frontal regions, especially from the IL, the PrL and the orbital cortices, in both rodents (176, 177) and monkeys (178, 179). More caudally, the ACC receives a prominent input from the RS, the parietal associative and the secondary visual cortices (173, 176, 177, 180). A slight afferent coming from primary sensory areas in mice (181), rats (173, 177, 180, 182) and monkeys (179, 183) has also been described. Additionally, the hippocampal formation and parahippocampal region moderately participate to ACC inputs in rodents (177, 181, 184) and monkeys (179, 185).

A second major input to the ACC originates from the thalamus, mainly from the anterior nuclei and the lateral parts of the mediodorsal (MD) nucleus, but also from the dorsal, midline and intralaminar nuclei in rodents (173, 177, 182, 186-190) and monkeys (183, 185, 191). An interesting difference is present between rats and monkeys, with the major thalamic projection arising from the anterior thalamic nuclei in rats, especially the anteromedial nucleus, while this projection to areas 24a/24b has been described as minor or almost absent in monkeys (191, 192). Most of the midline and intralaminar nuclei contribute to a moderate projection to the ACC, with a denser projection from the ventral midline nuclei (reuniens and rhomboid) than the dorsal midline nuclei (paraventricular and paratenial), in both rats (177, 182, 186-188, 193) and monkeys (183, 185, 191).

A few additional forebrain regions contribute to ACC afferents. The basolateral nucleus of the amygdala (BLA) and the claustrum (Cl) project heavily to the ACC in rodents and monkeys (173, 177, 181, 190, 192, 194-196). Interestingly, the claustro-cingulate connection is bilateral with an ipsilateral preference in rats, but is exclusively ipsilateral in mice (181, 196, 197). Additionally, the cholinergic corticopetal system, especially the diagonal band of Broca, the medial septum and the nucleus basalis, send efferents to the ACC in rats (173, 177, 198) and monkeys (183, 199).

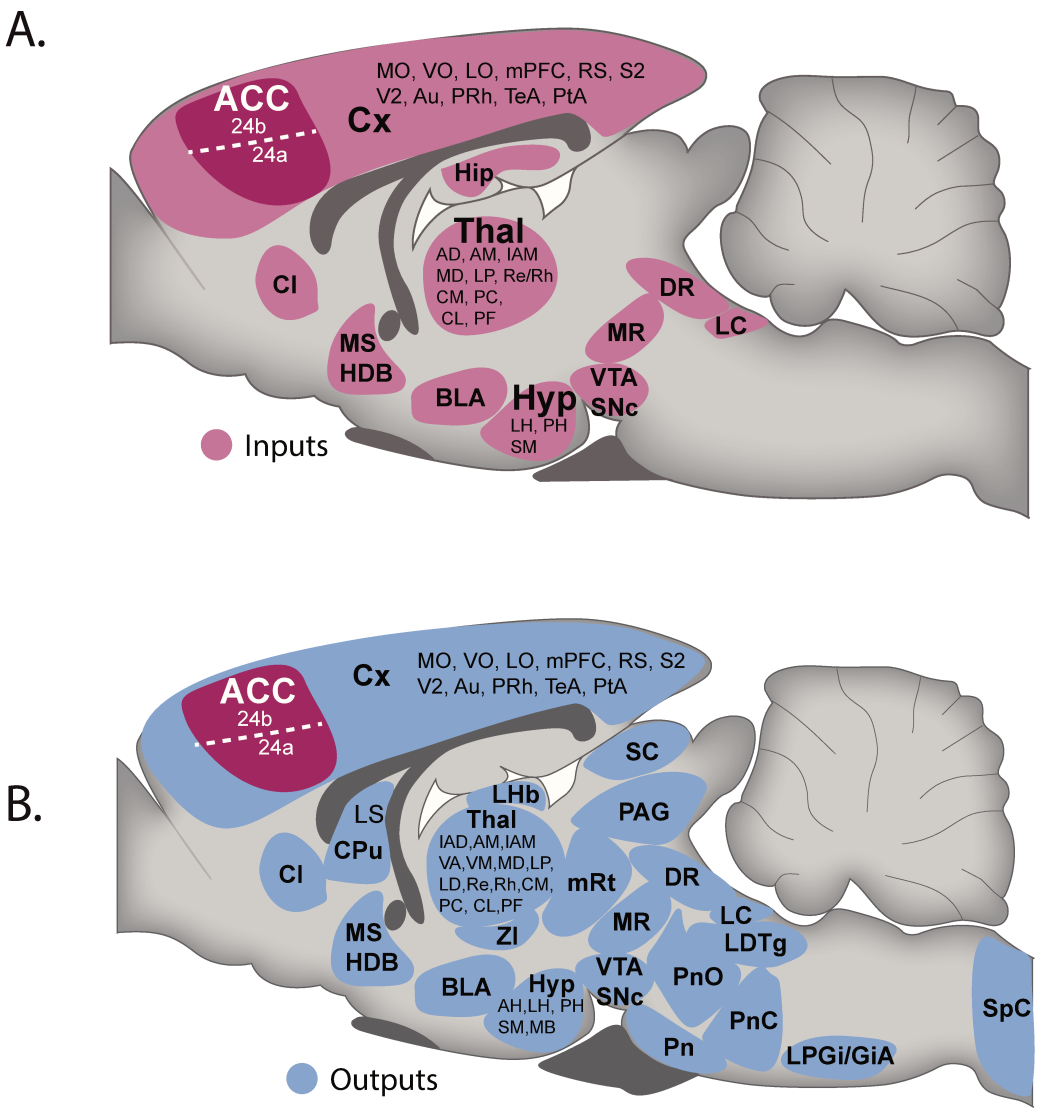


Figure 3 Connectivity of the anterior cingulate cortex in the rat
A. Inputs to the anterior cingulate cortex
B. Outputs from the anterior cingulate cortex

Hypothalamic inputs to the areas 24b/24a appear mostly modest, arising mainly from the lateral hypothalamic area and from the supramammillary nucleus, in rats (173, 177) and monkeys (183, 200).

The ACC receives inputs from brainstem's monoaminergic centers, in both rats and monkeys, arising from the substantia nigra, pars compacta (SNc), the ventral tegmental area (VTA), the median and dorsal raphe nuclei (MnR, DR), and the locus coeruleus (LC) (173, 177, 183, 198, 201, 202). Additional minor inputs have been described from the interpeduncular nucleus, the parabrachial nucleus, the mesopontine reticular formation, the area prerubralis and the periaqueductal gray in rats (173, 177, 198) and monkeys (183, 201, 203).

Outputs

Almost all cortical connections of areas 24b/24a are reciprocal. Indeed, substantial ACC outputs have been described to the orbital, the sensorimotor and the visual cortices, the RS, and in temporal areas such as the insular, piriform and entorhinal cortices in rats (173, 176, 180, 204, 205) (**Fig. 3B**). The hippocampus is a notable exception: it doesn't receive any input from the rat ACC (173, 204). In monkeys, projections to motor and premotor cortices have been described (205-207), as well as to the presubiculum and to CA1 (205, 208).

Similarly, the thalamic nuclei, receiving inputs from the ACC, are generally the same as the ones projecting to it (173, 190, 209). The stronger thalamic outputs of the rat ACC target the anteromedial, the interanteromedial and the lateral part of the MD, and the ventromedial and the reuniens/rhomboid nuclei (190, 209-211). In monkeys, a moderated projection to the pulvinar has also been reported (185, 212-215).

In subcortical regions, efferents from the ACC target the BLA and the Cl in rats (173, 204, 210, 216) and monkeys (185, 195, 208, 215, 217, 218). Contrary to the dominant ipsilateral claustro-ACC projection, the ACC-Cl connection is preferentially controlateral (196). The cholinergic diagonal band of Broca is also a recipient of areas 24b/24a axons, especially its horizontal limb (173, 204). A unidirectional cortico-striatal projection from the ACC targets the dorsal striatum, mainly the medial and intermediate parts of the caudate putamen, but only marginally projects to the ventral striatum in rats (173, 204, 210, 219). The ACC projection to the hypothalamus is mainly directed to the lateral and the posterior hypothalamus (173, 204, 210).

In the brainstem, area 24b/24a axons terminate in monoaminergic centers such as the DR, the VTA, the SNc and the LC in rats (173, 204, 210, 215). In addition, the rat ACC also projects to brainstem centers from where it doesn't receive a major afferent, such as the dorsolateral column of the periaqueductal gray (173, 204, 210, 215, 220) and the superior colliculus (221). Other efferents have been described to the rat pontine nuclei, the midbrain reticular formation and the nucleus of the solitary tract (210, 222), including to a recently defined mesopontine structure: the tail of the VTA (tVTA) also named rostromedial tegmental nucleus (RMTg) (223, 224). Finally, ACC neurons have been found to project to the cervical and the thoracic spinal cord (210, 225, 226). In monkeys, efferents to the brainstem have been identified in similar targets, including the SNc, the VTA, the dorsolateral part of the periaqueductal gray, the midbrain reticular formation, the red nucleus, the DR and the LC (207, 212, 227-229). Regarding the spinal cord, monkeys' cingulospinal projection originates preferentially from the MCC in contrast to the rat ACC (165, 230).

D. ACC electrophysiological properties

Eighty percent of ACC neurons are pyramidal cells which function through glutamate (231). Glutamate receptors such as AMPA, kainate and NMDA receptors are all found throughout the ACC (232, 233). It has been shown that AMPA and kainate receptors are responsible for fast synaptic responses (232, 233) whereas NMDA receptors mediate slow synaptic responses in mice (234, 235). AMPA receptor density is highest in the supragranular layer with local maxima in layers II and III whereas NMDA receptors are present in low quantities with minima in layers V and VI (167). Kainate receptor densities are high in layers I, II, V and VI with low densities in layer III (167).

Twenty percent of ACC neurons are interneurons which can be classified in three major groups that together make up almost 100% of all interneurons; interneurons expressing parvalbumin (PV), somatostatin (STT) and the ionotropic serotonin receptor (5HT3aR) (236). Other calcium binding proteins can be used to identify interneurons as well, such as calretinin and calbindin, but they either colabel with one of the major three groups or comprise only a very small percentage of the total population (236). Parvalbumin neurons are either basket cells, neurons that synapse at the soma and proximal dendrite of target cell, or chandelier cells, neurons that project to axons of pyramidal neurons (236). These fast spiking interneurons are located in

all layers but constitute about 45% of interneurons in layer V and VI and only 25% in layer II and III (236). Small populations of somatostatin neurons are present in layer I but they are more abundant throughout layer II, III, V and VI. These cells can be identified by their bursting activity (236). 5HT3aR neurons primarily populate the superficial layers of the cortex, constituting about 60% of all interneurons in the layers I, II and III. GABA is the major inhibitory neurotransmitter in the ACC. Inhibitory postsynaptic currents are mainly caused by GABAA receptors (237) and are thought to modulate pain-induced avoidance behavior (238). GABAA receptors are most prevalent in layer III, V and VI, and are highest in the latter (167). GABAergic interneurons have different electrical properties in relation to where they are situated in the cortex. For instance, interneurons that fire with a burst-like pattern are mostly found in layer V whereas regular spiking interneurons have been recorded in layer II, III and V (231). Irregular spiking interneurons typically fire an initial burst that is followed by irregularly timed single action potentials. This small fraction of interneurons is located in layer II and III. Many connections from pyramidal cells onto interneurons are excitatory in layers II until V. However, some interneurons receive depressing synapses from pyramidal neurons in layers II and III (231).

Apart from glutamate and GABA receptors, the ACC contains other receptors such as muscarin, serotonin and dopamine receptors. Muscarin receptor densities are highest in layer II and III whereas noradrenaline receptors are present in layer I and III. Serotonin receptors are most prevalent in layer I, II, V en VI and dopamine receptor concentrations are highest in layer I through III.

E. Role of the ACC in the emotional processing

Although many clinical studies showed the ACC's role in emotional processing (69), the exact functional contribution is still unclear as the ACC plays a role in both the generation and regulation of emotions [69, 70]. For instance, activation of the ACC has been documented in the induction of emotions such as envy (239), fear (240), sadness (241, 242), disgust (243) and anger (244). Besides, the ACC participates in affective processing indirectly by modulating functional connectivity with other brain regions, such as the insula and the amygdala (245). The ACC has also been related to the modulation of autonomic responses during meditation (246), emotional coping (247) and in response to rewarding stimuli (248).

Interestingly, different subregions of the ACC respond differently to emotional stimuli depending on the type of experimental tasks used. Imaging studies have shown hypoactivity in dorsal portions of the ACC (162) and hyperactivity in the ventral regions during the processing of emotional conflict (249), while the latter is deactivated during the passive viewing of emotional pictures (250). Together with other cortical regions (ventro- and dorsomedial prefrontal cortex, posterior cingulate cortex, lateral parietal cortex and superior temporal gyrus), the ACC is considered to be part of the default-mode network that is characterized by high neuronal resting-state activity (251). In contrast, some studies have demonstrated that the ACC is deactivated when it is a part of the default-mode network (250, 252). For instance, a study by Northoff and colleagues, in which functional Magnetic Resonance Imaging (fMRI) was combined with resting-state magnetic resonance spectroscopy, indeed showed a negative blood oxygen level-dependent (BOLD) response which was correlated with increased GABAergic and decreased glutamatergic activity within the ACC during the judgment of scenic photographs (252). Another explanation for the distinct roles of the ACC subregions in emotional processing might be related to the ACC's influence on other brain structures such as the amygdala. Given the strong reciprocal connectivity between the ACC and the amygdala, any assessment of the role of the ACC in the processing of emotional information should benefit from the consideration of cinguloamygdala interaction. Indeed, emotional conflict is associated with the activation of the rostral ACC followed by decreased amygdala activation suggesting that the rostral ACC has top down control over amygdala (253).

F. Role of the ACC in fear and fear learning

The ACC has a role in the various components of fear processing such as the perception of threat or of unconditioned stimuli (appraisal), the pairing of unconditioned stimuli and conditioned responses (acquisition and conditioning), the execution of fear response, and the regulation of these processes (extinction). Most of the time these processes have been investigated with classical Pavlovian fear conditioning paradigms, which consist of pairing a neutral conditioned stimulus (CS), such as a tone, with an aversive unconditioned stimulus (US), such as a foot shock.

Human neuroimaging studies pointed out the important role of the dACC throughout the ACC for fear processing such as CS appraisal (254), expression of fear responses (240) and fear

learning processes (255) by showing the activation of the dACC during acquisition of fear conditioning (256, 257) and during the expression of conditioned fear responses (240). In the latter study, both MRI and fMRI showed that the thickness and the activity of the dACC positively correlated with fear conditioned skin conductance while participants underwent a stimulus-shock association (240). Recent studies using instructed fear paradigms, in which subjects are told before the experiment that a given CS might be followed by an US, had shown that the dACC is specifically involved in conscious appraisal but not in the expression of the fear response (254, 258). In animals, fear can also be acquired through observation of others suffering. A preclinical study in which observer mice developed freezing behavior while watching demonstrator mice receiving repetitive foot shocks reported that the inactivation of the ACC by injecting lidocaine or deleting Cav1.2Ca^{+2} channels blocked observational fear learning and reduced pain responses (255).

It has also been shown that trace fear conditioning, in which the US follows by a stimulus free empty interval separating the cessation of the CS from the onset of the US, also engages the ACC (259-262). Furthermore, it has been reported that calcium/calmodulin-dependent protein kinase IV overexpressing mice showing increased ACC synaptic long-term potentiation have increased acquisition of trace fear (260, 263), while mice with ACC synaptic plasticity deficiency, due to either FMR1 knockout or chronic pain, have impaired trace fear conditioning (261, 262). Although the lesioning of the ACC has no impact on delay fear conditioning, it impairs the increased ACC neuronal activity observed in trace fear conditioning (264). Based on in vivo single-unit and local field potential recordings, a recent study implicated ACC pyramidal and non-pyramidal neurons in reinstating movement after freezing behavior during trace fear conditioning (259). Clinical studies have also shown the involvement of the ACC in delay fear conditioning (265).

Learning processes including acquisition, consolidation, storage and retrieval of fear memory are important parts of fear processing. There is an abundance of studies showing the ACC's role in generating and retaining fear memory (266, 267). Interestingly, clinical fear conditioning studies revealed that activation of the rACC occurs after learning of repeated CS-US pairing (265, 268, 269). Not surprisingly the ACC-amygdala interaction plays a role in generating fear memory. A study aiming to investigate top-down control of the rACC subregion over amygdala during pavlovian fear acquisition showed that exitotoxic lesions, temporal inactivation

or activation of the rACC projecting to the basolateral amygdala altered the acquisition of the tone-shock associative learning (270). Similarly, injecting methylphenidate into the ACC or the amygdala facilitates fear memory consolidation in rats, probably by increasing dopamine and norepinephrine in the synaptic clefts (271). Apart from generating fear memory, the ACC is also involved in the consolidation and reconsolidation of recent and remote contextual fear memory (266). Indeed, rats receiving protein synthesis inhibitor anisomycin immediately after consolidation or after re-exposure to the context of the foot shock, showed decreased freezing behavior during the retest. Non human primate studies further showed that the dACC is involved in retaining aversive memory since low frequency stimulation of the structure prevents spontaneous recovery of aversive memory during extinction (272).

Some mechanistic studies showed that fear processing induces plastic changes within the ACC (266, 273). For instance, Descalzi and colleagues demonstrated that memory consolidation within a trace fear learning paradigm is mediated by GluN2B dependent trafficking of CP-AMPARs. Besides that, inhibition of NMDAR-NR2B in the ACC disrupts memory formation and memory consolidation (266).

G. Role of the ACC in mood disorders and other psychiatric disorders

With the advent of neuroimaging techniques, a growing number of studies investigate the brain systems involved in mood disorders and develop neurocircuitry models. Unsurprisingly, the physiological role of the ACC in emotional and cognitive processing is reflected in its role in such models of mood disorders (**Table 2**). Indeed, the ACC is known to display functional and morphological alterations in major depressive disorder (274-278), bipolar disorder (279), obsessive compulsive disorder (280), anxiety (281, 282), post traumatic stress disorder (283-285) and schizophrenia (286-288). In this part, we focus on major depressive disorders, obsessive compulsive disorders (OCD), schizophrenia as well as generalized anxiety disorders and post traumatic stress disorders (PTSD). We describe various functional/anatomical imaging studies and neurochemical findings supporting the role of the ACC in mood disorders, revealing the effect of treatment on the ACC function and evaluating the relationship between the ACC and other limbic structures in mood disorders.

A common finding seems to be that the ACC's size is a predictor for the development of mood disorders. Clinical structural analysis of the ACC revealed that a reduction of its volume is

Table 2. Summary of studies on ACC activity changes in mood-disorders.

Mood-disorder	Structure	Approach	Results	References
MDD	sACC	fMRI, PET, EEG	Activity decrease	Pizzagalli et al., 2004
		PET	Activity increase	Drevets et al., 2002
	vACC	fMRI, PET	Activity increase	Mayberg et al., 1999, Yoshimuro et al., 2010
	dACC	fMRI, PET, SPECT	Activity decrease	Mayberg et al., 1999
PTSD	rACC	fMRI	Activity decrease	Shin et al., 2001, 2005
GAD	rACC	fMRI	Activity decrease	Swartz et al., 2014, Weaton et al., 2014 Fonzo et al., 2016
PD	ACC	fMRI	Activity increase	Pillay et al., 2007
Borderline	vACC	fMRI	Activity increase	Scherpiet et al., 2014, Minzenberg et al., 2007
	dACC	fMRI	Activity decrease	Gruber et al., 2004, Scherpiet et al., 2014 Soloff et al., 2015
	ACC	fMRI	Activity increase	Winter et al., 2015
	ACC	EEG	ERN decrease	de Bruijn et al., 2006
OCD	rACC	fMRI	Activity increase	Fitzgerald et al., 2005, Cavanagh et al., 2010
	dACC	fMRI	Activity decrease	Cavanagh et al., 2010
	ACC	fMRI	Activity increase	Hou et al., 2012, Cheng et al., 2013
Schizophrenia	ACC	fMRI	Activity increase	Mendrek et al., 2005, White et al., 2011
	dACC	fMRI	Activity decrease	Lee et al., 2014
	ACC	EEG, PET	Activity decrease	Gallinat et al., 2002, Kim et al., 2003 Boksman et al., 2005, Fu et al., 2005 Wagner et al., 2013, Lee et al., 2014 Yan et al., 2012a

MDD: Major depression disorder, PTSD: Post-traumatic stress disorder, GAD: General anxiety disorder, PD: Panic disorder, OCD: Obsessive-compulsive disorder
ACC: Anterior cingulate cortex, sACC: subgenual ACC, vACC: ventral ACC, dACC: dorsal ACC, rACC: rostral ACC
fMRI: Functional magnetic resonance imaging, PET: Positron emission tomography, EEG: Electroencephalogram
SPECT: Single photon emission computed tomography
ERN: Error-related negativity

correlated with the number of depression episodes (274). Additionally, less sACC (275-277) and vACC (278) gray matter volume correlated to an increased risk of major depressive disorder. Somewhat contrasting with these findings is a study relating a smaller dACC to having a higher quality of life-questionnaire score (289). Reduction of the ACC volume is also found in various other psychiatric disorders, including post traumatic stress disorder (PTSD) (283-285), borderline personality disorder (290, 291), schizophrenia (286-288) and OCD (280). In anxiety disorder an increase of dACC grey matter volume has been reported (281). In both the PTSD (285) and borderline personality disorder (291, 292), the symptom severity was negatively correlated with the size of the ACC. Apart from grey matter volume changes, ACC white matter integrity can also be affected in mood disorders. White matter reductions have been reported in depression (293, 294), anxiety (295), PTSD (296) and schizophrenia (297). In depressed patients it has been shown that multiple fiber tracks, including that of the ACC, are decreased (294). Therefore, loss of ACC white matter alone might not always explain depression prevalence. Nevertheless, white matter alterations can lead to functional connectivity changes (293, 298, 299) which is hypothesized to underlie mood disorder symptoms. As can be read in the following sections, apart from ACC volume many clinical and preclinical studies relate connectivity and activity abnormalities of the ACC to mood disorders.

1. Role of ACC in Major Depressive Disorder (MDD)

Insight from imaging studies

Volumetric imaging is a commonly used technique when studying depression. A common finding is that the ACC's volume can be used as a predictor for the development of MDD (274-278). Hastings et al. found that this volumetric alteration is gender dependent since depressed males had two times less sACC volume than depressed females (300). Genetic factors have also been implicated in the volume differences. For instance, it has been shown that healthy subjects carrying the allele for the short version of the gene for the promoter region of the serotonin transporter had smaller sACC and impaired coupling between the sACC and amygdala (301). This polymorphism had already been associated with development of depression (302, 303) and anxiety (304). This volumetric alteration could be explained by glial cell (305) or oligodentrite loss (306). Additionally, synaptic dendrite decrease might be involved (307). Although not many animal studies have focused on cell loss in animal depression models, one study showed that the

loss of glial cells in prefrontal areas, including the cingulate areas, is enough to induce depressive-like behavior (308). On the contrary, some studies didn't find any volumetric differences between depressed and non-depressed patients (309-311). This might be explained by a different definition of the subregions of the ACC, heterogeneity of patients, the influence of comorbid diseases, stage of the illness (312) or differences in treatment history (313).

Functional neuroimaging studies using positron emission tomography (PET), single photon emission computed tomography (SPECT), functional magnetic resonance imaging (fMRI) or magnetic resonance spectroscopy (MRS) have identified subregion dependent ACC abnormalities in depression which were, in some cases, even correlated with the severity of depression (314). By measuring glucose metabolism and blood flow during resting state, several studies showed hypoactivity in the dACC (315) while the ventral part, such as the perigenual ACC, was reported to be hyperactive (315, 316) in depressed patients. These different functional responses in distinct subregions of the ACC were correlated with symptom clusters such as sadness/depressed mood which were accompanied by an increase of vACC activity and decreased activity in the dorsal ACC (315). However, results concerning the sACC activity could be contradictory since both increased (317) and decreased activity (311) in depressed patients have been reported. The supposed shift between dorsal and ventral activity in resting state fMRI may correspond to the frequently observed awareness shift between internal and external mental contents in depressed patients (318). Indeed, studies focusing on resting state activity in both healthy and MDD subjects point out that internal mental contents are related with the perigenual ACC and the default-mode network while external mental contents associated with dorsolateral prefrontal cortex and the executive network (318). Several studies showed that these functional alterations within the ACC could be reversed by antidepressant treatment (317, 319-321), TMS (322), ECT (323), DBS (324), cognitive behavioral therapy (325) and psychotherapy (326). Interestingly, some studies have even reported that the activity of the ACC is a predictor for the treatment response including antidepressant medications (327-329), sleep deprivation (330) and cingulotomy (331).

Imaging studies also showed alterations in various neurotransmitter systems previously implicated in depression physiopathology such as decreased glutamatergic activity (332-334), abnormal GABA level (335-337), decreased serotonin synthesis (338), increased serotonin transporter (339), decreased dopamine 2 receptor binding (340) in the ACC of the depressed

patients. Northoff and Sibille (2014), hypothesized that the abnormal balance between dACC and vACC, thus between internal and external mental contents in awareness, in MDD can be associated with the changes in GABA interneurons. Indeed, somatostatin (341) and parvalbumin levels (342), neuropeptides expressed in GABA neurons that regulate the excitatory input or output of pyramidal cells respectively, are low in MDD patients which could translate into an imbalance between pyramidal cell input and output and lead to abnormal resting state activity (318).

Alterations in network connectivity within the ACC might also contribute to the development of mood-disorders. For instance, resting state fMRI studies reported a decrease in local dACC network connectivity among individuals susceptible to the depressogenic effects of early life stress (343). Treatment resulted in symptom improvement and improved information processing (344). Besides cortico-cortical connectivity, Ho and colleagues showed an increase in functional connectivity between the sgACC and the amygdala and a decrease in connectivity between sgACC and fusiform gyrus, precuneus, insula, middle frontal gyrus in MDD patients in a fear facial recognition task (345). The sgACC dysregulation thus might be responsible for the biased processing of negative stimuli and results in an imbalance of functional brain networks (345). In another study, analyzing structural connectivity in patients, showed a decrease in connectivity between the right rACC and the bilateral superior frontal gyrus (346), which was correlated with depression severity.

Insight from lesion studies

Clinical studies showed that anterior cingulotomy, a lesion of the cingulum bundles, can be clinically used as a last resort treatment for patients suffering from treatment resistant MDD. This type of ablative surgery leads to symptom improvements in around 75% of the patients with intractable major depression (347). Anterior cingulotomy is more successful when the lesion is performed more anteriorly and interrupts the connectivity with the posterior part of the ACC (348). Preclinical studies also showed that the lesion of the ACC modulates mood related parameters (349, 350) as well as blocks the chronic pain-induced anxiodepressive-like behaviors (9).

Neuroimmune and neuroendocrine hypothesis

A growing number of studies suggest that inflammatory processes, characterized by increases in proinflammatory cytokine activity, have a significant impact on emotional regulation and mood disorders (351-353). A study with 31 healthy participants showed an association between increased soluble TNF-alpha, markers of inflammation, in oral fluids which was correlated with the hyperactivity of the dACC (351) support the idea that neuroinflammation in MDD could be associated with structural and functional anomalies in various brain regions (352). Another study indicates that inflammation-associated mood deterioration correlates with increased activity within the sACC during performance in an emotional face processing task. In the same study, inflammation-associated mood change was associated with reduced connectivity of sACC to amygdala, medial prefrontal cortex, nucleus accumbens, and superior temporal sulcus, which was correlated with peripheral IL-6 levels (353).

Besides, suicidal MDD patients had increased expression levels of stress-related candidate genes such as corticotrophin releasing hormone and neuronal NOS-interacting DHHC domain-containing protein with dendritic mRNA (NIDD), which plays a role in NO signaling (354), in their ACC while major depressed non suicide patients only presented decreased NIDD and increased 5-hydroxytryptamine receptor 1A (5-HT1A) expression levels (355) suggesting that depressed patients who committed suicide have different gene expression patterns within the ACC than depressed patients who died of causes other than suicide.

Other animal studies

Several clinical studies suggest that an increased ACC activity may underlie the depression phenotype. This notion is further supported by animal studies. For instance, in the social-defeat-paradigm, the presence of depressive-like behavior is accompanied by an increased cingulate activity as shown by c-Fos staining (356). Reciprocally, pharmacological inactivation of the rat's infralimbic cortex, which can be argued to be equivalent to the primate subgenual ACC, leads to a decrease of the depression-like phenotype (357). However, the detailed influence of cingulate sub-regions requires further exploration, since lesions of the rACC in the rat has been reported to have either no effect on the immobility in the forced swim test (349), or to result in increased immobility (350).

2. Role of the ACC in Anxiety

Anxiety disorders as well as trauma and stress related disorders such as PTSD (DSMV) are also related with the alterations of the activity and connectivity of the ACC.

Insights from imaging studiesIn PTSD, rACC activity is diminished and amygdala activity is increased in response to trauma related words, suggesting that the r/v ACC has a regulating role by inhibiting the amygdala in response to fearful stimuli (358, 359). This ACC activity decrease was found to negatively correlate with avoidance behavior, as measured by the Clinician Administered Posttraumatic Stress Disorder Scale (360). A similar hypothesis has also been proposed for general anxiety disorder (361) as hypoactivation of the prefrontal cortex and the rACC have been reported (362-364) which results in exaggerated limbic activity due to hyperactivation of the amygdala (362, 363). This exaggerated limbic activity makes it difficult for these patients to divert attention away from threat. The relation between ACC and limbic activity is further supported by a case study in which a patient experienced an instant panic attack during brain surgery when the dACC was damaged accidentally during tumor removal (365).

However, some studies relate an increase of ACC activity to disrupted emotional processing in panic disorder patients. One study shows an increase in ACC activity in response to the presentation of happy faces (366). This increase could be due to the increased conflict monitoring as a result of greater attentional demands or a consequence of compensatory mechanisms. This is further supported by a clinical study showing that healthy subjects with high trait-anxiety do show an increase of glutamine and glutamate in the ACC which indeed suggests an increase in activity (367). Additionally, cholecystokinin-tetrapeptide induces anxiety in humans accompanied by an increase of rACC activity (368).

These activity changes could be caused by the connectivity alterations. For instance, early life stress has been shown to induce anxiety in adult life which coincides with decrease in connectivity between the ACC and the amygdala (369). Half of children with an inhibited temperament develop social anxiety disorder, those that do are shown to have more negative connectivity between the rACC and the bilateral amygdala (370) whereas weaker coupling between the ACC and the amygdala has also been reported (295, 371). Patients with fewer symptoms showed greater ACC activity and better functional connectivity between the ACC and the amygdala (370).

Treatment

Interestingly, different treatment strategies improve symptoms by counteracting ACC activity. The administration of anxiolytic drugs, such as benzodiazepines has been found to influence the ACC activity (372), decrease ACC metabolism (373) and reduce anxiety in the anticipation of pain by reducing ACC activity (374). Apart from these benzodiazepines, tiagabine, a GABA reuptake inhibitor which is usually administered together with benzodiazepines, has been shown to increase ACC glucose levels as well as improve generalized anxiety disorder symptoms (375).

Non-pharmacological treatments can also treat anxiety and alter ACC activity. Cognitive behavior therapy (CBT), for instance, has been shown to increase dACC activity in generalized social anxiety disorder patients (376, 377) and decrease limbic activity in generalized anxiety disorder (378) thereby improve symptoms. CBT treatment responders in panic disorder patients with agoraphobia were primarily carriers of the long version of the serotonin transporter gene for 5-HTTLPR, showing that serotonin signaling might be implicated (379). Treatment with antidepressants acting on the serotonergic system, however, had no effect on the exaggerated error-related negativity, a signal attributed to ACC activity, in obsessive compulsive disorder-related anxiety patients (380). Interestingly, oxytocin administration has been shown to improve functional connectivity between the ACC and amygdala in generalized social anxiety disorder patients which shows that oxytocin has a role in enhancing the integration and modulation of social responses (381, 382)

Insights from preclinical studies

A number of animal studies suggest that anxiety-like behavior coincides with ACC hyperactivity. For instance, rats submitted to chronic unpredictable mild stress showed an increase in ACC fos immunohistochemistry and more avoidance behavior (383). Additionally, anxiety can be induced by traumatic events that coincide with firing rate changes in ACC neurons, in which the majority of these neurons show a transient hyperactivity (384). Acute intraperitoneal injection of midazolam, a benzodiazepine, reduces anxious behavior in the elevated plus maze (385). Supporting this notion; inhibition or ablation of the ACC is found to have anxiolytic effects (386). For instance, when the ACC is lesioned animals no longer show an anxiety-like phenotype in a mouse model of neuropathic pain (9). Additionally, administration of GABA receptor agonists into the ACC has been shown to reduce anxiety-like behavior in mice (386). Similarly,

low-frequency stimulation, a stimulation protocol known to induce long-term depression, of the dACC prevents recovery of aversive memories after traumatic events under a tone air-puff paradigm (272).

A range of molecular mechanisms seem to be involved in anxiety. For instance, pain-related anxiety seems to be mediated by expression of extracellular signal regulated-kinase (ERK) in the ACC (387, 388) and inhibiting ERK activation in the ACC after hind paw incision attenuated pain-related anxiety-like behavior (389). In addition, oral treatment with MAPK inhibitor reduced anxiety-like behavior in rats and decreased microglia count and specific NMDA receptors in the ACC after spinal cord injury which might indicate the involvement of LTP (390). This is further supported by a study showing anxiolytic and analgesic effects of LTP blockage in the ACC (391). Postsynaptic LTP requires NMDA in the ACC whereas presynaptic LTP requires kainate receptors (391). Blocking the presynaptic LTP mechanism has anxiolytic and analgesic effects (391). Apart from the glutamatergic system, anxiety might also be mediated by the serotoninergic system. In a mouse model of neuropathic pain by nerve ligation, injection of SSRIs into the ACC reduced anxiety-like behavior (392). Lower serotonin levels in the amygdala were associated with smaller ACC volumes in high trait anxiety marmosets (393). This is similar to the findings from an earlier study in which animals from an unpredictable chronic mild stress model showed an increase in serotonin metabolism in the ACC and a decrease of kynurenic acid, a byproduct of serotonin metabolism, in the amygdala (394).

3. Role of the ACC in borderline personality disorders

Borderline disorder personality (BPD) is a serious mental illness marked by unstable moods, behavior and relationships. Patients have problems with regulating emotions and thoughts, show impulsive and reckless behavior and frequently endure unstable relationships with other people (NIMH).

Insights from clinical studies

In BPD both cognitive and emotional processing are altered. Volumetric studies have shown that both ACC and amygdala grey matter volume is decreased in BPD patients (395, 396). Furthermore, the dACC shows decreased activity during the anticipation and the perception of non-emotional stimuli in BPD patients as compared to healthy controls (397, 398). This is

further reflected by the finding that error-related negativity amplitudes, a signal generated by the ACC after erroneous responses, were decreased in BPD (399). This reduced action monitoring might be the reason why BPD patients do not learn from their errors in comparison to healthy individuals, which might be caused by a reduction of functional connectivity between the ACC and other regions involved in cognitive processes. For instance, in a task in which participants had to attribute mental states to someone else, it was found that functional connectivity between the ACC and regions such as the left superior temporal lobe, the inferior parietal lobes and the right mid-cingulate cortex were decreased (400).

An abundance of studies indicate that BPD patients suffer from increased emotionality. For instance, in the anticipation of negative stimuli BPD patients show an increase of pregenual activity (398) whereas increased ventral activity during anger processing has also been reported (401). dACC activity seem to altered as well; in a study showing dACC involvement in BPD, emotional experiences of exclusion were studied using a virtual ball tossing game in which the participant was eventually excluded from participating (402). BPD patients felt more excluded than healthy controls during the non exclusion condition which was correlated with increased dACC activity (402). A number of studies show that the functional connectivity between the amygdala and the vCC is increased while processing emotional information (403), this then leads to amygdala hyperactivity (404) which disrupts cognitive functioning.

Emotional processes also interfere with cognitive processes such as attention, working memory and episodic memory. For instance, BPD patients previously exposed to emotional imagery before partaking in a cognitive stroop task perform worse than when they are not previously exposed to such imagery. In both conditions, however, they show an increase of ACC activity (405). Working memory is affected as well in these patients. In a recent study it was shown that emotional distractions lead to a stronger positive connectivity between the dACC and areas involved in attention and salience detection such as the left posterior cingulate, insula, and frontoparietal regions in BPD patients (406). Additionally, episodic memory can be affected by emotional distractions. Another study showed that during an episodic memory task using emotional pictures, the amygdala became hyperactive and the ACC hypoactive in BPD patients (407). These findings indicate that borderline patients pay more attention to emotional and social information while performing cognitive tasks than healthy control.

Treatment

Borderline personality disorder is usually treated by talk therapy such as CBT. Due to the complexity of this mental illness, medications generally target specific symptoms such as depression, impulsivity and anxiety (408). To our knowledge no studies have been done on effects of pharmacological treatment on cingulate functionality. CBT however, has been found to decrease ACC activity during the viewing of emotional stimuli (409). This reduction of ACC activity might underlie symptom improvement.

4. Role of the ACC in obsessive compulsive disorder

Insights from clinical studies

Obsessive-compulsive disorder (OCD) is characterized by unreasonable thoughts and fears (obsessions) that lead patients to do repetitive behaviors (compulsions). Several studies suggest ACC dysfunction in patients with OCD. For instance, imaging studies demonstrate increase resting state activity (410, 411) and increase ACC grey matter volume (412). Hyperactivity of the ACC in patients with OCD has been shown to increase with symptom provocation and to normalize with treatment. Electrophysiological evidence linked this region to error-related processing as it generates activity during the detection of errors, which is shown to be affected in OCD patients (413). For instance, the severity of OCD symptoms has been correlated with an increased rACC error-related processing (413).

Functional connectivity studies further showed that connectivity between the ACC and the orbital frontal cortex (OFC) is decreased in OCD patients (410, 414). As OFC neurons evaluate current choices and the ACC encodes the prediction of the choices and evaluates prediction errors, this might be part of the neuronal basis for the impaired error detection phenotype in OCD patients (410, 414). Additionally, decreased connectivity between these regions after sad mood might reflect why patients experience more OCD symptoms during negative emotional states (415).

Moreover, it has been shown that the rACC is more active in OCD patients during response competition tasks while the dACC is deactivated during reinforced learning tasks (416). The increase of rACC activity was related with the difficulties in conflict processing and dACC deactivation during reinforced learning tasks could underlie the patient's inability to learn from their futile repetitive behavior (416).

Treatment

Accordingly, a chronic anterior capsular electrostimulation decreased both the subgenual ACCactivity and the OCD rating scores (417). High ACC metabolic rate has been found to predict clinical response to risperidone, a serotonin and dopamine antagonist (418). On the other hand, responders to antidepressant drug treatment exhibit significantly lower rACC activity after treatment (419) while the CBT responders displayincrease dACC activity. (420).

5. Role of the ACC in schizophrenia

Schizophrenia is a chronic and severe mental disorder that affects how a person thinks, feels, and behaves (NIH). The Symptoms of schizophrenia falls into three categories: positive, negative and cognitive symptoms. Positive symptoms are psychotic behavior that under normal circumstances are not experienced by healthy individuals such as; delusions, disordered thought and hallucinations (DSMV). Negative symptoms are commonly expressed as lack of emotion, poverty of speech and the inability to experience pleasure or the lack of motivation (DSMV). Finally, cognitive symptoms include memory and attention deficits (DSMV).

Most studies relate alterations in ACC connectivity to negative and cognitive symptoms in schizophrenia. Concerning ACC activity in schizophrenia, both increased (421, 422) and decreased activity (423-427) have been reported. Nevertheless, the predominant part of these studies report decreased activity during cognitive tasks (423-427).

Insights from clinical studies

In a study investigating emotional salience in schizophrenia, patients were asked to rate their emotional response to emotionally laden pictures. When both positive and negative scenes were presented at the same time, decreased dACC activity was observed which might explain deficits in the processing of emotional information (428). Indeed, decreased ACC activity might be the cause of negative and cognitive symptoms in schizophreniasince an abundance of evidence report cortical thinning and ACC activity decrease in the presence of negative symptoms in schizophrenia (429). Additionally, ACC activity decrease has been correlated with cognitive task performance, providing a neuronal basis for the cognitive deficit in schizophrenia patients (430). For instance, functional connectivity decrease between the ACC and the mediodorsal thalamus

has been reported and may disrupt cognitive processing (423). Interestingly, an increase in dorsolateral prefrontal cortex and the mediodorsal thalamus connectivity might function as a compensatory mechanism (423). Aside from interregional connectivity changes, hemispheric asymmetries are also reported in schizophrenia (430). Study by Yan et al. showed decreased positive connectivity with the bilateral putamen together with increased negative connectivity with the posterior cingulate cortex (430).

Functional connectivity decrease has been related to aggression in schizophrenia also. In a study using the Urgency Premeditation, Perseverance and Sensation-Seeking scale, cortical thickness in areas including the rACC was found to correlate with urgency scores (431) in which the subconstruct of 'urgency' is thought to play an important role in aggression in schizophrenia patients. In addition, resting-state fMRI showed that reduced functional connectivity of the rACC correlated with urgency scores (431). These findings are supported by another study showing that violent men with antisocial personality disorder or schizophrenia show a significant ACC volume decrease (432).

Some molecular mechanisms have been proposed for understanding the schizophrenia physiopathology concerning the ACC. Molecular findings indicate that α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-Methyl-D-aspartate (NMDA) signaling might be responsible for the disrupted ACC activity in schizophrenia. For instance, transmembrane ionotropic glutamate receptor regulatory protein (TARP) dysregulation is hypothesized to result in decreased AMPA receptor activity (433). Neurogranin, which is associated with NMDA receptor signaling (434), and N-acetyl aspartate (NAA) concentration, whose release is increased due to NMDA receptor activation, are both reduced in schizophrenia patients (435). This disturbed glutamate signaling might be the cause for the decreased ACC activity observed in schizophrenia patients.

Treatment

Acute tryptophan and tyrosine/phenylalanine depletion was found to significantly improve attention in schizophrenia patients, which could indicate that reduced dopamine and serotonin activity in the striatum, ACC and PFC may play a role in attention (436). This is further supported by a study showing that flupentixol and quetiapine, serotonin and dopamine antagonists, improve prefrontal function especially in patients with weak initial ACC function

(437). Modafinil, a serotonin antagonist, increased ACC activity during working memory tasks which was correlated with cognitive performance in schizophrenia patients (438). Additionally, cognitive therapy improves activity in attention and working memory networks including dorsolateral prefrontal cortex, ACC and frontopolar cortex (439). Functional connectivity between the striatum and ACC can be improved by risperidone or aripiprazole, serotonin and dopamine antagonist, treatment (440) and reduced ACC blood flow in schizophrenia patients (441) can be restored by clozapine treatment in schizophrenia patients (442).

H. The role of the ACC in pain

Physical pain represents a necessary alarm signal for body integrity and survival. According to the literature, it is composed by at least two distinct dimensions: the sensory-discriminative component, encompassing qualities such as the localization and intensity of pain, and the affective-motivational component, characterized by the unpleasantness or negative aspect of pain. Imaging studies highlighted the implication of many brain regions during physiological and pathological pain processing, including the ACC (443). Indeed, a growing number of clinical and preclinical studies explored its critical role in sensory-discriminative and affective-motivational, as well as in the cognitive and attentional aspects of pain.

1. Insights from clinical studies

With the first cingulotomy by Ballantine et al. (1967) in the 60's, the ACC became an important target for patients' suffering from intractable pain resistant to classical treatments (444). Manipulating this structure allows a decrease in the perception of the unpleasantness of pain without inducing any change in the sensoridiscriminative aspect of the noxious stimulus (444). These days, ablation of the ACC remains a possible treatment for pain relief (445), even when less invasive procedures targeting the ACC such as deep brain stimulation (446) or repetitive transcranial magnetic stimulation (447) are favored.

The role of the ACC in physiological conditions

The ACC can be activated by different type of noxious stimulation. For instance, it has been demonstrated in healthy subjects that mechanical (448-452), electrical (453, 454), thermal (455-462), incisional (463) and visceral (464-469) noxious stimuli activates different subregions of the

ACC. Indeed, some report activation of the contralateral (470) or the bilateral ACC (471), others of the midcingulate and of the perigenual part of the ACC (472) or both of the anterior, ventral and posterior part of the ACC (462). In fact, the subregional patterns of activation partly change according to the type of stimulus. Indeed, the ventral pACC is abundantly activated following an electrical stimulus, while a more dorsal part of the pACC is activated after thermal stimulation. Besides skin stimulation, several studies have been performed with direct nerve (473) and gastrointestinal tract (474) stimulation. Concerning nerve stimulation, only painful stimulations induce ACC activation (473, 475).

The role of the ACC in the affective component of pain

Pain is a multidimensional experience involving both somatosensory and emotional components. As a part of the limbic system, the ACC has been associated with the affective/emotional component of pain. In the late 90's, a study using hypnosis and live imaging in humans confirmed this role of the ACC (476). Without changing the perceived intensity (i.e. the somatosensory component) of a nociceptive stimulus, hypnotic suggestions to healthy subjects were made in order to vary the unpleasantness (i.e. the affective motivational component) of the stimulation. It appears that the variation of unpleasantness was correlated with the variation of regional cerebral blood flow (rCBF) in the ACC, but not in other structures (476). Using a PET correlation analysis, another study confirmed the role of the pACC in the encoding of pain unpleasantness induced by a hot painful stimulus (477). Interestingly, the pACC was the only region where the activation was correlated with an increase of the reported pain unpleasantness.

Pain relief can be considered as rewarding (478), and another argument for the involvement of the ACC in the affective/motivational aspects of pain is the activation of the ACC both in human and in rats in response to the offset of an noxious heat stimulus (479).

The role of the ACC in the cognitive component of pain

Cognition is a major aspect of the pain experience. For example, pain involves different cognitive features such as expectation, anticipation, attention, appraisal, empathy, learning and memory (480) which are necessary for the defensive behavior in response to actual or potential pain (481). Intense pain demands personal attention and, as a consequence, diminishes the cognitive functions to process other internal and external information. When pain becomes chronic, the

focus towards the pain becomes more prominent (482) and can impair cognitive functions, work and social interactions of chronic pain patients. The involvement of the ACC in different cognitive parameters is detailed in the following sections.

Expectation – Anticipation of pain

Pain expectation and anticipation are related but different phenomena. In theory, pain expectation is linked to an uncertain painful event whereas pain anticipation is associated with a later, but certain painful event. In other words, does the brain react in the same way when the painful event is uncertain (expectation) or certain (anticipation) beforehand to the stimulus? Trying to anticipate an unpredictable and unfamiliar painful stimulus has been shown to activate the right ACC while anticipating a learned painful stimulus decreased the activity in this same region (483), showing that expectation and anticipation do not necessarily recruit the same pathway. Then, do these cognitive processes modify brain activity related to painful stimuli? A study, in which subjects were submitted to either a simple non painful stimulus or non painful stimulus alternated with a painful stimulus reported that the activity of the ACC increased in the latter condition after the non painful stimulus (484). Another interesting question is whether the intensity of pain and the activation of pain-related regions can be influenced by the degree of expected pain. When expected pain is manipulated, expectations of decreased pain reduce both the subjective experience of pain and the activation of regions involved in pain processing such as the ACC and the insular cortex (485).

Attention – Distraction induced by a pain state

It has been stated that pain modifies attention (481). In the case of acute pain, this directs attention to the source of pain, aimed to stop or avoid it. Chronic pain patients can develop hypervigilance towards pain (486), which may change social interaction, the ability to work and the overall quality of life (487). To evaluate the interaction between pain and attention, studies monitoring brain activity during attention demanding tasks and painful stimuli have been developed (488). It has been shown that during high demanding attentional tasks, pain ratings following thermal noxious stimuli diminish, which was correlated with an increase in rACC activity and a decrease in the activity of the cognitive mACC (medial) (488). The decrease in

ACC activity is confirmed in another study using CO_2 laser pulses as noxious stimuli and MEG as a brain activity monitoring technique (489).

2. Insights from preclinical studies

In this section, we review preclinical studies and discuss them from macroscale (imaging and behavioral studies) to microscale level (electrophysiology and molecular studies).

The role of the ACC in the pain processing

Insights from lesioning studies

Although some studies showed that lesions of the ACC decreased the sensory component of thermal (490) or inflammatory pain (491), others reported no effects of lesions on inflammatory response such as licking and biting of the inflamed paw (492) and on mechanical allodynia (493). A recent study further showed that the excitotoxic lesion of the ACC did not affect mechanical allodynia observed in neuropathic pain induced by peripheral nerve injury. In the same study, authors however showed that the lesion of the posterior insular cortex blocks the maintenance but not the development of mechanical allodynia (493). These discrepancies may be explained by the nature of the painful stimulus that was used (cold vs nerve injury), by the precise localization (rostral vs medial, dorsal vs ventral) of the lesion or by the extent (unilateral, bilateral, rostro-caudal) of the lesion.

Insights from ex vivo electrophysiology

In the context of a neuropathic pain model, it has been shown that the spontaneous membrane-potential oscillations and action potential firing of pyramidal neurons of the layers II/III of the ACC are higher in neuropathic rats (494). In another study, the nerve injury enhanced the probability of presynaptic glutamate release and postsynaptic glutamate AMPA receptor-mediated responses (49). In chronic inflammatory pain, by using in vitro patch-clamp recordings, the group of Zhuo reported an enhancement in neurotransmitter release probability in the ACC synapses mediated, at least in part, by calmodulin-stimulated adenylyl cyclase AC1, AC8 (261) and TNF-alpha (495). Using a bee venom persistent pain model, Gong et al. observed an increase in the frequency and the amplitude of spontaneous excitatory post-synaptic currents (sEPSCs)

and a decrease in the spontaneous inhibitory post-synaptic currents (sIPSCs) in ACC slices compared to the controls (496).

Insights from in vivo electrophysiology

Several teams have highlighted an increase of ACC activity in the context of pain processing. Electrical stimulation of supraspinal pain centers such as the medial thalamus induces the activation of the ipsilateral cingulate cortex (497) confirming the ACC as a target of the medial thalamic pain pathway. In anesthetized rats, spontaneous activity of the pyramidal neurons of layers II/III is characterized according to their firing pattern: regular spiking, intermediate and intrinsic bursting. Acute noxious but not non noxious mechanical stimulation of the hindpaw evoked spike responses in all three types of neurons (498). In freely moving rats, noxious laser stimulation to the hindpaw induced neuronal activation in the ACC (499). Altogether, these studies show that the ACC neurons could be activated by different kinds of peripheral nociceptive stimuli (mechanical, thermal) in both anesthetized and freely moving animals.

Insights from imaging studies

Imaging studies in animals have shown an activation of the ACC during noxious stimulation in anesthetized (500) or awake animals (501), whereas innocuous stimulation failed to activate the ACC (502). For instance, electrical or chemical stimulation of the forepaw induced bilateral activation of the ACC which was decreased by pre-treatment with morphine (503). While most of the imaging studies have measured ACC activity following peripheral stimulation in naïve animals (500), there are some studies that focus on chronic pain conditions such as neuropathic pain. Using autoradiographic techniques to monitor changes in rCBF in a model of chronic constriction injury (83), an activation of the ACC can be observed 10 days (504), 2 weeks (505), 8 weeks or 12 weeks (506) after the induction of neuropathy. Structural MRI studies showed a decrease of ACC volume (507) in the spared nerve injury model (88) and functional MRI studies confirmed the activation of the ACC 3 weeks after the induction of the neuropathy (508).

Insights from molecular studies

Molecular manipulations of the ACC modify the expression of several pain behaviors. For instance, inhibiting protein kinase M zeta (PKMζ), an atypical isoform of protein kinase C

thought to be involved in long term potentiation (LTP), resulted in a reduction of mechanical allodynia in neuropathic animals (509). AC1 and AC8, two major adenylate cyclases in the brain which link NMDA receptor activation to the cAMP intracellular signaling pathway are overexpressed in the ACC (510). Furthermore, it has been shown in the same study that double AC1 and AC8 KO mice responses to acute noxious stimulation in the hot plate, in tail flick and in the paw mechanical pressure test are similar to those observed in wild type mice. However, following paw injection of formalin, nociceptive behavior (licking and biting the formalin injected paw), and mechanical allodynia were reduced in double KO AC1/AC8 mice. Mechanical allodynia secondary to partial nerve ligation (a neuropathic pain model) was also reduced in the same animals (510). Moreover, overexpression of the NMDA receptor subunit NR2B in the ACC of mice enhanced nociceptive behavioral response (licking and biting the formalin injected paw) and mechanical allodynia to paw injection of formalin although response to acute nociceptive stimulation (hot plate and cold plate tests, tail flick test) was unaltered (511). Moreover, local injection of NR2B antagonist within the ACC in the model of formalin-induced inflammation reduced nociceptive responses (licking and biting the formalin injected paw) (512).

The role of the ACC in the aversive-motivational component of pain

Pain has emotional dimensions which, for a long time, were not evaluated in animal research due to difficulties to assess and quantify the emotional state of animals. Since the 2000's, several paradigms have addressed this question.

Some articles reported a role for the ACC in the aversive aspects of evoked mechanical allodynia in neuropathic pain models by using the place escape/avoidance paradigm (493). This test measures the escape behavior of animals towards evoked pain. Interestingly, this avoidance behavior was abolished after bilateral lesion of the ACC while the paw withdrawal response, reflecting mechanical sensitivity, remained unchanged. Therefore, the ACC seems to mediate the aversive component of evoked pain without affecting its somatosensory aspect in a model of neuropathic pain.

A study published in 2001 associated formalin injection into the hind-paw, which induces inflammatory pain, with a place-conditioning paradigm. In this test, the rats developed aversion towards the formalin paired context. Interestingly, ACC lesions prior to this place-conditioning test prevented the avoidance for the formalin-paired compartment without having any effects on

nociceptive behaviors such as paw lifting, licking and flinching (492). These results suggest that the ACC mediates the aversive-motivational component but not the sensory component of inflammatory ongoing pain.

Conditioned place preference experiments have been developed recently to study the spontaneous pain in a rat model of neuropathic pain (513). The conditioning stimulus is the relieving effects of a non-rewarding analgesic drug such as clonidine. The animal shows a preference for the analgesic drug-paired context, in absence of any evoked painful stimuli, showing that the drug relieves the spontaneous pain state. The lesion of the ACC blocked the preference for the analgesic-paired compartment, indicating that the ACC is involved in the aversive component of spontaneous pain (514). These results were generalized in other models of pain. Indeed, the ACC is also implicated in the affective component of visceral pain (515) and of cephalic pain (516).

Discrepancies between studies

We already showed some discrepancies between the effects of ACC lesions on the response in inflammatory and neuropathic pain conditions, with most studies showing no effects of the ACC lesions (492, 493, 513) on behavior. However, the molecular studies show involvement of the ACC in response to nociceptive stimuli in chronic inflammatory and neuropathic but not in response to acute nociceptive stimulation. The observed differential involvement of the ACC in pain responses depend probably on the type of pain model used (acute model of pain or tonic inflammatory model or chronic neuropathic model) and the precise localization of the manipulations in the ACC.

VI. Thesis objectives

This thesis' main objective was to study the role of the ACC in the comorbidity of neuropathic pain and mood-disorders. Our team previously showed the development of anxiety and depressive-like behavior after the induction of neuropathy in mice. Here, we sought to understand the neurobiological basis underlying this co-occurrence.

This work was organized around two objectives:

- Determine the role of the anterior cingulate in the sensory and affective components of pain as well as in emotional consequences.

We hypothesize that the anterior cingulate cortex is involved in the affective component as well as the anxiodepressive consequences of neuropathic pain. By using a lesional approach, we compared the role of the ACC with the posterior insular cortex, a cortical region implicated in pain processing, in mechanical hypersensitivity, aversiveness of spontaneous pain and anxiodepressive consequences of neuropathic pain. This work was completed by activation of the ACC in naive animals through optogenetic stimulation to further characterize the role of the ACC in mood.

- Determine the physiological alterations of the ACC over time in the neuropathic pain condition

Based on both clinical and preclinical literature, we hypothesize that chronic pain and mood disorder comorbidity are accompanied by ACC hyperactivity. For this, we performed various behavioral tests to evaluate the presence of various aspects of neuropathic pain, and anxiodepressive-like behavior within a 6 month time-course. In addition, we studied the electrophysiological alterations of the ACC accompanied by these various symptoms in vivo, and correlated them to different stages of the pathology. In order to explore a causal link between ACC hyperactivity and the affective aspects of neuropathic pain, we completed this work by studying the impact of the optogenetic inhibition of the ACC in neuropathic animals. The next two chapters detail the results for each of these two goals.

Results

I. The Anterior Cingulate Cortex is a Criticical Hub for Pain-induced Depression

Florent Barthas, **Jim Sellmeijer**, Sylvain Hugel, Michel Barrot, Ipek Yalcin

In this paper, I participated in designing and performing the optogenetic experiments which was, until then, novel at the laboratory. I also contributed to ex vivo electrophysiological and immunohistochemical validation of the technique.

Archival Report

Biological Psychiatry

The Anterior Cingulate Cortex Is a Critical Hub for Pain-Induced Depression

Florent Barthas, Jim Sellmeijer, Sylvain Hugel, Elisabeth Waltisperger, Michel Barrot, and Ipek Yalcin

ABSTRACT

BACKGROUND: Besides chronic stress, chronic pain is a prevalent determinant for depression. Changes induced in specific brain regions by sustained pain may alter the processing of affective information, thus resulting in anxiodepressive disorders. Here, we compared the role of the anterior cingulate cortex (ACC) and the posterior insular cortex in the anxiodepressive, sensory, and affective aspects of chronic pain.

METHODS: Neuropathic pain was induced by cuffing the right sciatic nerve of C57BL/6J mice. Lesions were performed by local injection of ibotenic acid and chronic activation of the ACC by optogenetic stimulation. Anxiodepressive-related behaviors were evaluated through the novelty suppressed feeding, marble burying, splash, and forced swimming tests. Mechanical thresholds were determined using von Frey filaments, and the relief of spontaneous pain was determined by using place conditioning.

RESULTS: The ACC lesion prevented the anxiodepressive consequences of chronic pain without affecting the sensory mechanical allodynia. Conversely, the tonic or spontaneous pain and the anxiodepressive consequences of pain remained present after posterior insular cortex lesion, even though the mechanical allodynia was suppressed. Furthermore, optogenetic stimulation of the ACC was sufficient to induce anxiety and depressive-like behaviors in naïve animals.

CONCLUSIONS: Our results show that, at cortical level, the sensory component of chronic pain remains functionally segregated from its affective and anxiodepressive components. Spontaneous tonic pain and evoked allodynia can be experimentally dissociated. Furthermore, the ACC appears as a critical hub for mood disorders, including for the anxiodepressive consequences of chronic pain, and thus constitutes an important target for divulging the underlying mechanism.

Keywords: Anterior cingulate cortex, Anxiety, Behavior, Depression, Insular cortex, Neuropathic pain, Optogenetics

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Depression, the most common mental disorder, is a disabling and long-lasting medical condition, estimated to be the foremost contributor to the worldwide burden of disease by 2030 (1). Among several precipitating factors, chronic pain is a prevalent determinant for depression. Indeed, a mean prevalence rate of around 50% for major depressive disorder is reported in patients with chronic pain (2). The existence of pain-induced affective disorders is further supported by preclinical studies showing that chronic pain models can induce anxiety-like and/or depression-like behaviors in animals in a time-dependent manner (3–5). While it could be suggested that chronic pain may be a chronic inescapable stress (6), preclinical and clinical studies have shown that sustained neuropathic pain strongly differs from a simple stress regarding neuroendocrine hypothalamic-pituitary-adrenal (HPA) alterations, even if it induces similar behavioral consequences. Indeed, neuropathic pain does not modify the basal or stress-induced levels of corticosterone or the HPA axis negative feedback (4,7), while this is the case in the several models of stress-induced depression (8,9). Another hypothesis for pain-induced depression could be based on a shared neuroanatomical substrate, proposing that specific brain regions processing pain are also involved in mood-related processing and that the alterations induced in these regions by chronic pain may alter the processing of affective information, thus resulting in mental disorders. Among the candidates, the anterior cingulate cortex (ACC) and the insular cortex (IC) appear to be critical in the networks involved in both pain and mood (10–12).

The ACC is a relay that interconnects neurons from the frontal cortex, the thalamus, and the amygdala, integrating cognitive, emotional, and autonomic functions (10,11). Clinical imaging studies have shown the recruitment of the ACC in pain processing (13), and preclinical studies have more precisely associated the activation of the ACC neurons with pain-like aversive behavior, while the inhibition of these neurons blocks such behavior (14). The IC is another cortical area of interest since both human and animal studies have shown its recruitment in acute and chronic pain (15–17). The complexity of IC connectivity and the variability of pain-related

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activity between different IC subregions suggest that this cortical area may play a multifaceted role in pain processing. For example, some studies have reported a preferential pain activation of the posterior IC (pIC) (18), whereas others have also described it in the mid insula (19) or in the operculoinsular area (15,20). The activation of the IC has been implicated in both antinociceptive and pronociceptive processes (19), while its role in the aversive component of pain is still unclear.

Despite the lack of direct evidence, the ACC and the IC could also play a role in the anxiodepressive consequences of chronic pain. Indeed, both cortices are known to display functional and morphological alterations in depressive states (21–23), such as the observation of decreased connectivity (24), altered glucose metabolism (25), and reduced volume (26) in the ACC of depressed patients and also altered basal neuronal resting state activity in the IC (22). Studies showing the alleviation of depressive symptoms in treatment-resistant patients by ablative surgery (27) or deep brain stimulation (28) of the ACC further support the implication of this region in major depression. However, these data come from the psychiatric field and the involvement of these regions in the affective consequences of chronic pain has not yet been studied.

Although clinical and preclinical studies strongly suggest a role of the ACC and the IC in pain processing, respective functions of these cortical areas in the anxiodepressive consequences as well as in the sensory and affective components of chronic pain remain unknown. Using a lesion approach in a murine model of neuropathic pain, we demonstrate that the ACC is critical in the anxiodepressive consequences of chronic pain, while conversely the pIC is only critical in mechanical allodynia. The repeated stimulation of the ACC by an optogenetic approach induces anxiodepressive behaviors in naïve animals, which further reinforces the essential role of this cortical region in mood disorders.

METHODS AND MATERIALS

Animals

The lesion experiments were conducted in adult male C57BL/6J mice (Charles River, L'Arbresle, France). Genetically modified mice expressing channelrhodopsin-2 and yellow fluorescent protein (Thy1-ChR2-YFP) in a subset of pyramidal neurons were used (29) for optogenetic studies (Supplement 1).

Surgical Procedures

Chronic neuropathic pain was induced by placing a cuff around the right common sciatic nerve (4). Bilateral excitotoxic lesions of the ACC and the pIC by local injection of ibotenic acid were performed under stereotaxic surgery (see Supplement 1).

Optogenetic Procedures

Animals were anesthetized (ketamine 17 mg/mL, xylazine 2.5 mg/mL, intraperitoneal 4 mL/kg) before being placed in a stereotaxic frame (David Kopf Instruments, Tujunga, California). Single glass fiber cannulas, 1.7 mm long with a diameter of 220 μm (MFC_220/250-0.66_1.7 mm_RM3_FLT, Doric Lenses, Quebec, Canada) were implanted in the left ACC. Coordinates derived from the Franklin and Paxinos atlas (30) were set to .7 mm anterior and .3 mm lateral to the Bregma. The cannula was lowered until 1.5 mm of optic fiber was inserted into the brain, covering the whole vertical span of the ACC.

After 3 to 7 days of recovery period, the ACC was stimulated with a blue light emitting diode (LED) with a peak wavelength of 463 nm (LEDFRJ-B_FC, Doric Lenses). From the LED, the light traveled through the fiber optic patch cable (MFP_240/250/2000-0.63_0.75m_FC_CM3) to the implant cannula. Light pulses were generated through a universal serial bus connected transistor-transistor logic pulse generator (OPTG_4, Doric Lenses) connected to a LED driver (LEDRV_2CH v.2, Doric Lenses). Transistor-transistor logic pulses were generated by open source software developed by Doric Lenses (USBTTL V1.9). Optical power was measured at the fiber tip using a photodetector (UNO, Gentec, Quebec, Canada).

Optogenetic stimulation took place on 4 consecutive days for 30 minutes. Stimulated animals received repetitive stimulation sequences of 10 seconds consisting of 8 seconds at 20 Hz with 40 milliseconds pulses and 2 seconds without stimulation (31). Light intensity was measured before implantation and was set between 4 mW and 5 mW. Control animals underwent the same implant procedures but the light was turned off during stimulation time.

Electrophysiological Recordings

Patch-clamp recordings of the ACC pyramidal neurons were performed using acute slices prepared from 9- to 12-week-old Thy1-ChR2-YFP mice, the ACC being illuminated with the same system used for the in vivo experiments (Supplement 1).

Pain- and Anxiodepressive-Related Behaviors

The mechanical threshold of hindpaw withdrawal was determined using von Frey filaments (Bioseb, Chaville, France) (4), while the spontaneous pain was evaluated using conditioned place preference in response to the intrathecal administration of the analgesic α2-adrenoceptor agonist clonidine (10 μg) (32) (Supplement 1).

For lesion studies, the novelty suppressed feeding (NSF), splash, and forced swimming tests (FST) were conducted 6, 7, and 8 weeks after the peripheral nerve injury, respectively. For the optogenetic studies, animals were tested with the NSF test 1 day after the last stimulation. Splash test and marble burying test were performed the fourth and the fifth days after the final stimulation (Supplement 1).

Immunohistochemistry, Analysis, and Illustrations

For the lesion study, after the behavioral testing, the animals were perfused and NeuN immunostaining was performed. Lesions were indicated by neuronal cell loss localized bilaterally and extended from 1.18 to .14 mm from the bregma for the ACC lesion and from .38 to −1.22 mm from the bregma for the pIC lesion. Concerning the optogenetic study, after the completion of the behavioral tests, the animals were stimulated once with the same procedure as described before and

perfused 90 minutes later. c-Fos immunohistochemistry then allowed to control for both the implant location and the activation of the ACC by the optogenetic procedure. Blind verification and lesion three-dimensional reconstructions were done using a Nikon Eclipse 80i microscope with the Neurolucida 8.0 software (MicrobrightField, Williston, Vermont). Pictures were taken with a Nikon E80i microscope. Adobe Photoshop CS5 (Adobe, San Jose, California) was used to adjust contrast, brightness, and sharpness.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analyses were performed using multifactor analysis of variance (ANOVA) with independent or repeated measures. In case of significant effect following ANOVA, multiple group comparisons were performed with Duncan post hoc analysis. Significance level was set at $p < .05$. All the analyses were performed with STATISTICA 7.1 (Statsoft, Tulsa, Oklahoma).

The full description and details of the experiments are available in Supplement 1.

RESULTS

Excitotoxic Lesions of the ACC or the pIC

To concurrently analyze the role of the ACC and the pIC in the consequences of chronic pain, we used a murine model of chronic neuropathic pain (4) and performed localized excitotoxic lesions of these cortices with ibotenic acid. NeuN immunostaining allowed visualization of the extent of the lesions at the end of the experiments (Figure 1A–C, and see three-dimensional image in Supplement 2). To control whether the behavioral phenotypes were independent from possible activity deficits, the spontaneous locomotor activity was evaluated. We observed no difference between control and neuropathic animals and no influence of the lesion on spontaneous activity (Figure 1D) or on body weight (Figure 1E).

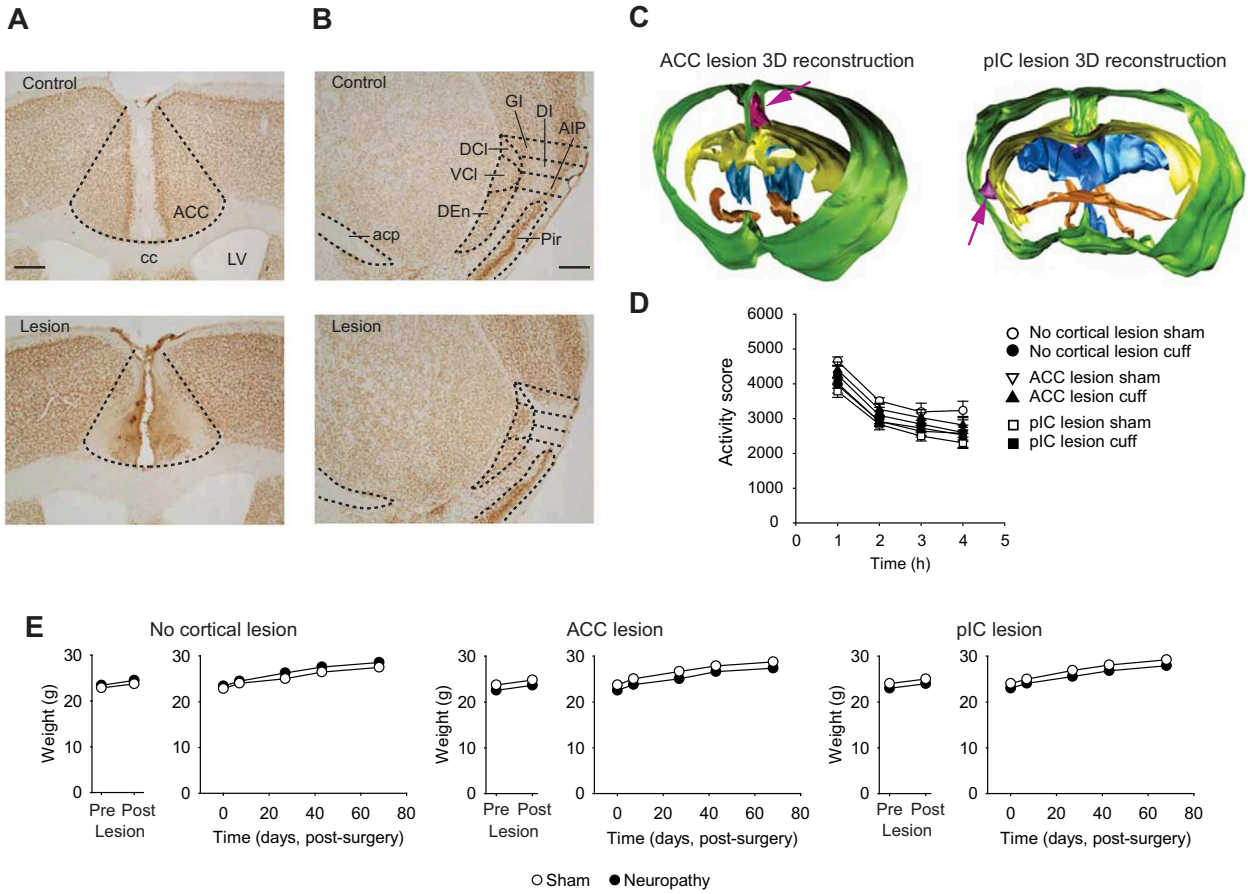

Figure 1. Illustrations of the anterior cingulate cortex (ACC) and the posterior insular cortex (pIC) lesions and their effects on locomotor activity and body weight. **(A)** Representative examples of NeuN immunohistochemistry of the ACC lesion or nonlesioned control sections at the same level. **(B)** Representative examples of NeuN immunohistochemistry of the pIC lesion or nonlesioned control section at the same level; scale bar = 300 μm. **(C)** Three-dimensional (3-D) reconstructions of representative ACC and pIC lesions; borders of the brain are colored in green, corpus callosum and external capsule in yellow, anterior commissure and fornix in orange, lateral and third ventricles in blue, and the lesions of the ACC and the pIC in magenta. **(D)** The lesion of the ACC and the pIC did not affect the general locomotor activity (n = 10–16 animals per group). **(E)** Neither the lesion of the ACC nor the pIC has an effect on the weight gain (n = 10–16 animals per group). Data are expressed as mean ± SEM. acp, anterior comissure, posterior part; AIP, agranular insular cortex, posterior part; cc, corpus callosum; DCI, dorsal part of claustrum; DEn, dorsal endopiriform claustrum; DI, dysgranular insular cortex; GI, granular insular cortex; LV, lateral ventricle; Pir, piriform cortex; VCI, ventral part of claustrum.

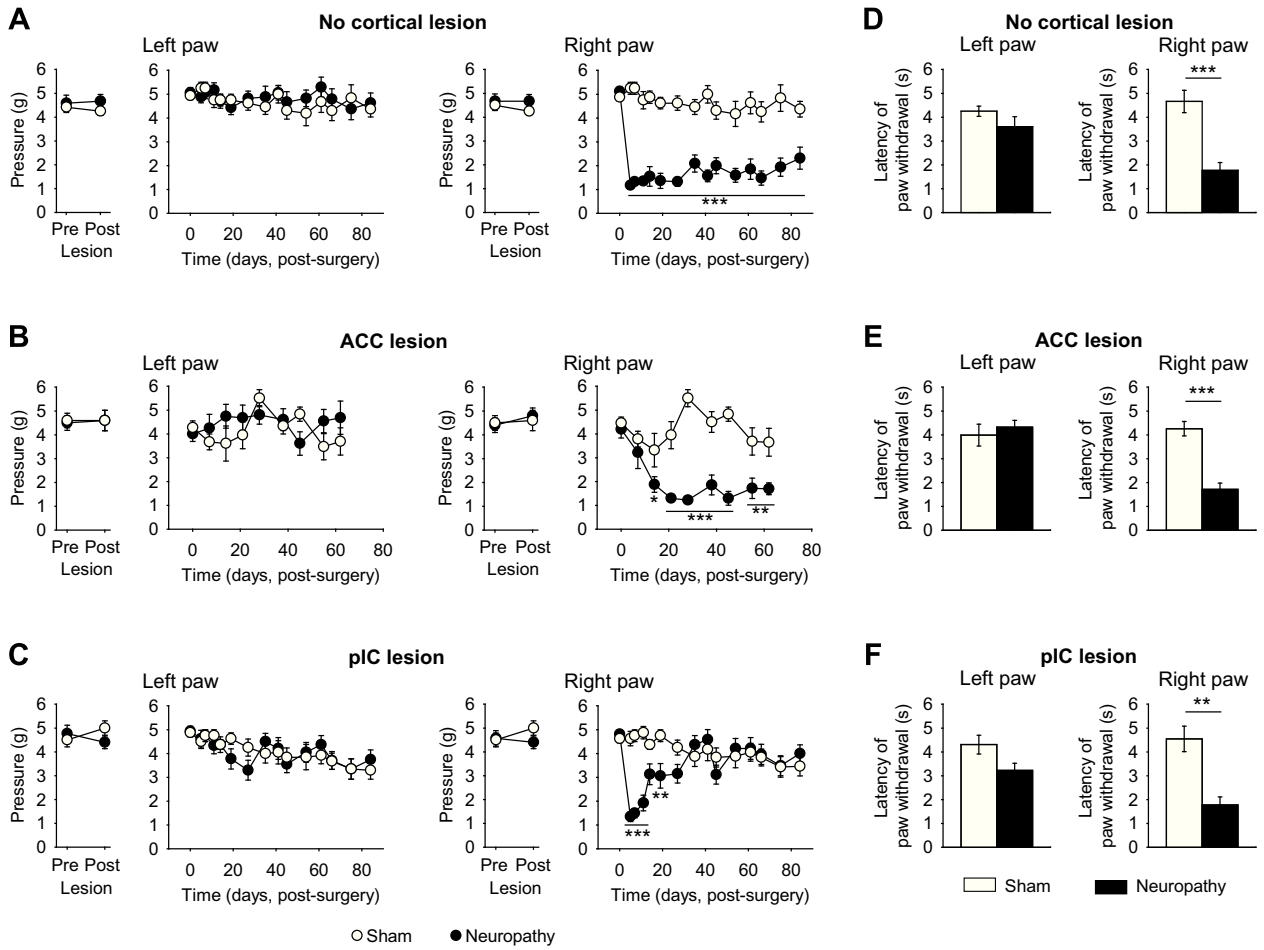

Figure 2. Influence of the anterior cingulate cortex (ACC) and the posterior insular cortex (pIC) on the somatosensory components of neuropathic pain. Neuropathic animals display a unilateral increase of mechanical **(A)** and thermal **(D)** sensitivity in the right hindpaw. The lesion of the ACC has no effect on these behaviors **(B, E)**. The lesion of the pIC prevents the maintenance of the long-term mechanical hypersensitivity **(C)** without affecting short-term thermal sensitivity **(F)**. Data are expressed as mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$ sham versus neuropathy; n = 10–17 animals per group for von Frey test, n = 5–6 animals per group for the radiant heat test.

The pIC but not the ACC Is Necessary for the Somatosensory Component of Chronic Pain

In the neuropathic pain model, we observed decreased mechanical sensitivity thresholds, referred to as mechanical allodynia, by using von Frey filaments ($F_{14,434}$ = 7.41, $p < .001$, Figure 2A). Current preclinical data suggest different influences of the ACC and the IC (14,16) in neuropathic mechanical allodynia. In accordance with this prediction, we found that while the lesion of the ACC did not affect mechanical allodynia ($F_{8,160}$ = 4.79, $p < .001$, Figure 2B; $F_{4,160}$ = 20.617, $p < .001$ Figure S2 in Supplement 1), the lesion of the pIC completely suppressed its long-term development ($F_{14,420}$ = 9.25, $p < .001$; Figure 2C). More precisely, the early postsurgical allodynia remained present in the pIC lesioned mice during the first 2 weeks after sciatic nerve surgery, whereas the long-term allodynia reflecting the chronicity of this symptom was abolished. The pIC is thus a core substrate of long-term allodynia in chronic neuropathic pain. Human studies also support these results, showing that IC subdivisions are activated by mechanical allodynia in neuropathic pain (33). Clinical studies showed that the IC lesion may modify nociceptive sensitivity in humans, but these studies remain difficult to interpret due to the interindividual variability in the extent of the lesion (15,34). Interestingly, our results show that neither the lesion of the ACC nor the lesion of the pIC altered the mechanical thresholds per se in control animals (Figure 2B,C). This suggests that neuropathic mechanical allodynia is integrated by the pIC, being selectively revealed under chronic pain conditions.

Thermal hyperalgesia is another sensory symptom that may be present in neuropathic pain patients. We evaluated the role of the ACC and the pIC by using the radiant heat paw-withdrawal test. Similar to mechanical allodynia, neither the ACC nor the pIC lesion modified the thermal sensitivity in control animals or the hypersensitivity observed during the early phase of the neuropathy (12 days postsurgery) (Figure 2D–F, lesion × surgery $F_{2,27}$ = .09, p = .91). In

addition, the lesion of the targeted cortical areas had no effect on the thermal sensitivity in the later phase of the neuropathy (46 days postsurgery) when thermal hyperalgesia was no longer present in the neuropathic animals (data not shown).

The ACC but not the pIC Is a Core for the Anxiodepressive Consequences of Chronic Pain

The above data revealed a distinct role of the ACC and the pIC in the sensory components of neuropathic pain. It was then of interest to determine the implication of these cortices in the anxiodepressive consequences of neuropathic pain. We observed the anxiodepressive-like behaviors accompanying chronic pain through the increased latency to first bite in the novelty suppressed feeding test (two-way ANOVA; lesion × surgery $F_{2,62} = 5.70$, $p < .01$; no lesion sham < cuff, $p < .002$; Figure 3A), the decreased grooming duration in the splash test ($F_{2,81} = 3.796$, $p < .02$; no lesion sham > cuff, $p < .001$; Figure 3B), and the prolonged immobility in the forced swimming test ($F_{2,78} = 10.24$, $p < .001$; no lesion sham < cuff, $p < .001$; Figure 3C). In the NSF test, which relies on both anxiety- and depression-like aspects, the lesion of the ACC ($p = .65$) but not the pIC ($p < .001$; Figure 3A) suppressed the chronic pain-induced delayed latency to feed. Similarly, depressive-like behaviors observed in neuropathic animals were prevented by the lesion of the ACC ($p = .55$) but not the pIC in the splash test ($p < .01$; Figure 3B) and the FST (for the ACC $p = .22$, for the pIC $p < .001$; Figure 3C). The lesion of either the ACC or the pIC had no effect per se on the behavioral tests in control animals (for NSF, ACC $p = .91$, pIC $p = .71$; for splash test, ACC $p = .16$, pIC $p = .37$; for FST, ACC $p = .95$, pIC $p = .72$; Figure 3A–C). These data, to the best of our knowledge, are the first evidence showing that the ACC is a hub for the anxiodepressive consequences observed in chronic pain.

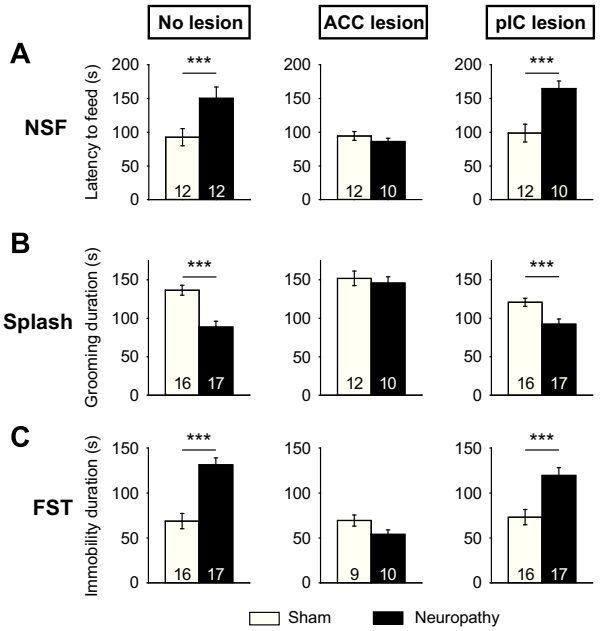

Figure 3. Influence of the anterior cingulate cortex (ACC) and the posterior insular cortex (pIC) on the anxiodepressive consequences of neuropathic pain. An increased latency to feed in the novelty suppressed feeding (NSF) test **(A)**, a decrease in the grooming behavior in the splash test **(B)**, and an increase in the immobility duration in the forced swimming test (FST) **(C)** are observed in neuropathic mice compared with control mice. While these effects are prevented by the ACC lesion **(A–C)**, the lesion of the pIC has no effect on the anxiodepressive behaviors observed in neuropathic mice **(A–C)**. Data are expressed as mean ± SEM. ***$p < .001$ sham versus neuropathy. The number of animals per group is indicated on the bar graphs.

The Optogenetic Stimulation of the ACC Induces Anxiodepressive-like Behavior

Since the ablation of the ACC blocked the anxiodepressive-like behaviors induced by chronic pain, we wondered whether an activation of this structure could induce depressive-like behavior. We thus performed sustained optogenetic stimulation of the pyramidal neurons of the ACC using naïve Thy1-ChR2-YFP mice (Figure 4A). The functional validation of ChR2-YFP expression using ex vivo electrophysiological recordings confirmed that the optogenetic stimulation reliably enables trigger action potential firing of the ACC pyramidal neurons (Figure 4C,D). In vivo, we also observed that the ACC optogenetic stimulation led to a robust induction of c-Fos compared with control mice ($F_{1,9} = 16.3$, $p < .01$; Figure 4B). Behavioral results showed that the repeated activation of the ACC induces anxiodepressive behavior (Figure 4F) in naïve animals without affecting mechanical threshold ($F_{1,10} = .2$ $p = .66$; Figure 4E). Indeed, the stimulated animals displayed an increased latency to first bite in the NSF test ($F_{1,16} = 28.88$, $p < .001$; Figure 4F) and a decrease in overall grooming time ($F_{1,15} = 13.01$, $p < .01$; Figure 4F) and buried more marbles in the marble burying test ($F_{1,13} = 7.45$ $p < .05$; Figure 4F). These data confirm the major role of the ACC in mood disorders.

Spontaneous Pain Remains Present in the Absence of Allodynia After pIC Lesion

Clinically, the presence of spontaneous pain is often more debilitating than the alteration of evoked nociceptive responses. For a long time, this parameter had remained elusive in animal research. Recently, it has been shown that such spontaneous pain can be unmasked in animal models of neuropathic pain (32) by relieving the tonic-aversive state in chronic pain thanks to nonrewarding analgesic drugs. The cerebral network mediating the aversive component of pain includes the ACC (14,35), but its connections with somatosensory pain pathways are poorly described, while its relations with anxiodepressive behaviors remain unknown. We thus compared the role of the ACC and the pIC in the spontaneous pain component of neuropathic pain 10 weeks after the induction of peripheral nerve injury, using the conditioned place preference (CPP) paradigm. Our results showed that the analgesic effect of clonidine was not affected by the ACC or the pIC lesions (lesion × surgery × treatment, $F_{2,24} = .38$, $p = .68$; Figure 5A). Following spinal clonidine administration (10 μg) at the level of the lumbar spinal cord, we observed a CPP in neuropathic animals (surgery × treatment, $F_{1,42} = 6.7$, $p < .05$; Figure 5B), resulting from pain relief. This effect of clonidine was not

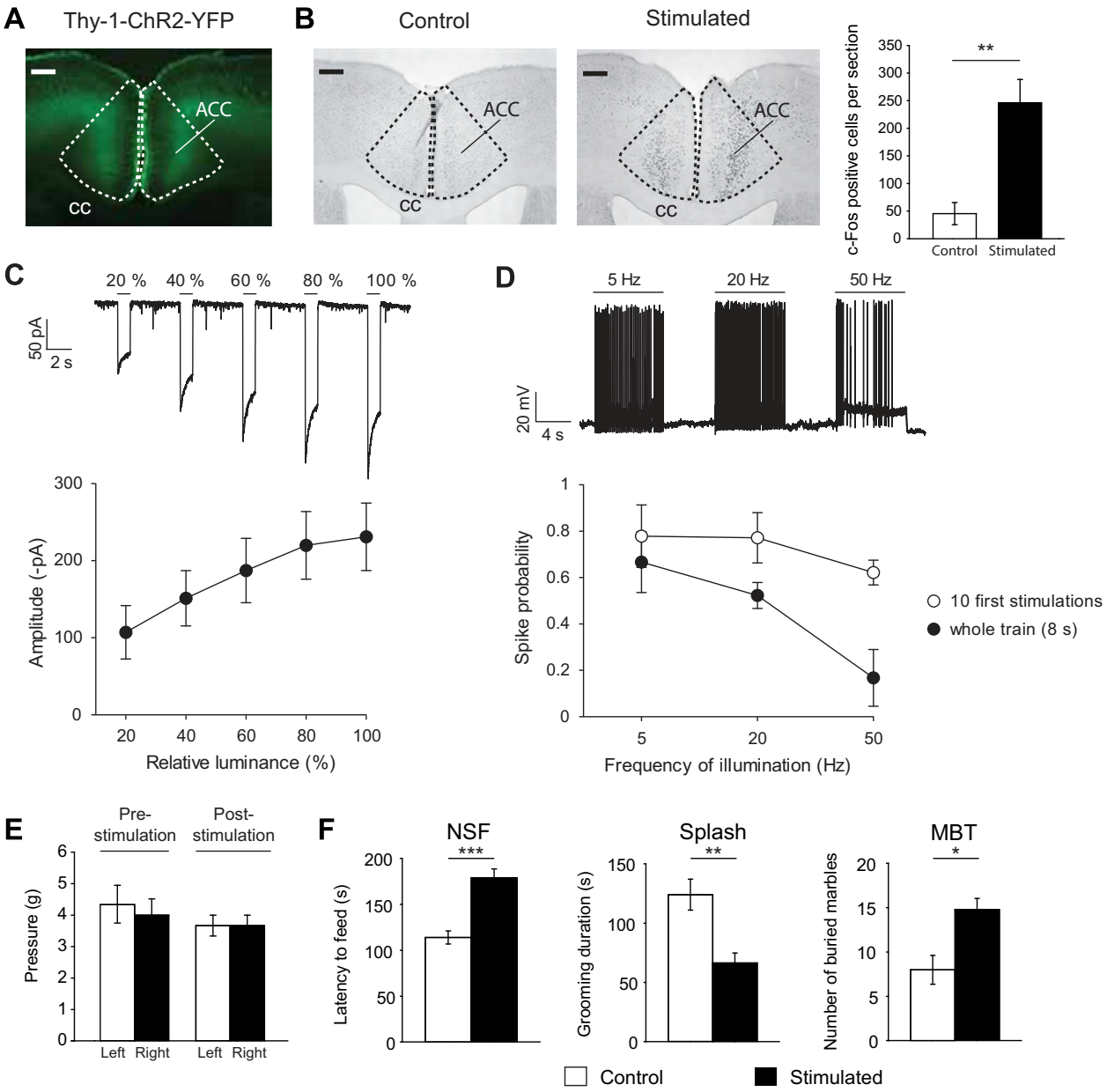

Figure 4. Influence of the optogenetic stimulation of the anterior cingulate cortex (ACC) on anxiodepressive-like behaviors. **(A)** Representative picture of the ACC in the Thy-1-ChR2-YFP mice. **(B)** Stimulation of the ACC induces local c-Fos expression. **(C)** Luminance-response curve of the light-evoked currents recorded in the voltage-clamp mode in the ACC pyramidal neurons of Thy-1-ChR2-YFP mice (top: representative trace; bottom: $n = 6$). The maximal luminance corresponds to 5.5 mW. **(D)** In the current-clamp mode, repetitive illumination at maximal intensity reliably triggers the firing of ACC pyramidal neurons (top: representative trace; bottom: $n = 6$). The probability of an illumination to trigger a spike is reduced at high frequency. **(E)** Repeated stimulation of the ACC had no effect on mechanical sensitivity. **(F)** Repeated activation of the ACC increased the latency to feed in the novelty suppressed feeding (NSF) test, decreased the grooming time in the splash test, and increased the number of buried marbles in the marble burying test (MBT). Data are expressed as mean ± SEM. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ control versus stimulated. For c-Fos expression, $n = 5$–6 per group. For the voltage and current-clamp experiments, $n = 6$ per condition. For the von Frey test, $n = 6$ per condition. For the NSF, $n = 9$ per condition; for the splash test, $n = 8$–9 per condition; for the MBT, $n = 5$–9 per condition. Scale bars 300 μm. cc, corpus callosum.

present in sham mice, supporting the idea that this drug selectively unmasked the tonic-aversive state in chronic pain. Pain relief CPP was still present after pIC lesion (surgery × treatment, $F_{1,44} = 4.3$, $p < .05$; Figure 5D), showing that spontaneous pain arising from injured nerve fibers was still present even though mechanical allodynia was suppressed in these animals (Figure 2C). The lesion of the ACC (surgery × treatment, $F_{1,46} = .42$, $p = .52$; Figure 5C) blocked the pain relief CPP, in agreement with a previous report in rats (35).

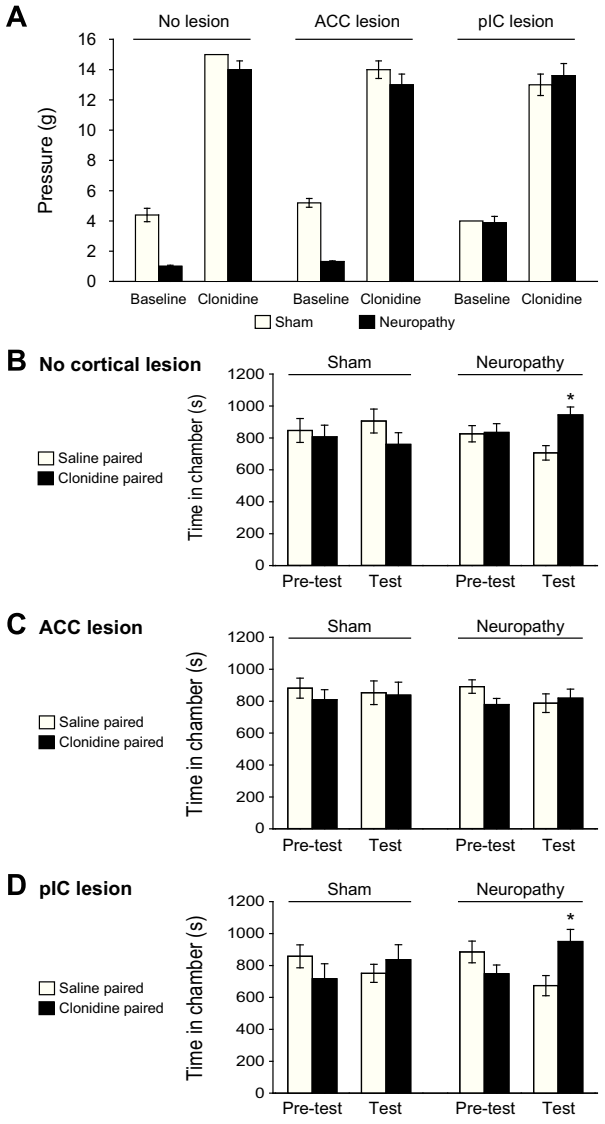

Figure 5. Influence of the anterior cingulate cortex (ACC) or the posterior insular cortex (pIC) on conditioning to spontaneous pain relief. **(A)** Intrathecal injection of clonidine (10 μg) induced analgesia in each experimental group. **(B)** Intrathecal clonidine (10 μg) increased the time spent in the paired chamber, with a corresponding decrease in the saline-paired chamber, in neuropathic but not sham-operated mice. The lesion of the ACC **(B)** but not the pIC **(C)** blocked the clonidine-induced conditioned place preference in neuropathic animals. Data are expressed as mean ± SEM. *$p < .05$ saline paired versus clonidine paired. For the assessment of analgesic effects of clonidine, $n = 5$ per group. For conditioned place preference experiments, $n = 10$–14 per group.

DISCUSSION

Our findings show that the sensory component of chronic pain is functionally dissociated from its affective and anxiodepressive components and that the ACC is a critical brain region for the latter. Indeed, the presence of the ACC is necessary for the anxiodepressive consequences of chronic pain, while the pIC is important only for the somatosensory component. Our optogenetic study further supports the role of the ACC in mood disorders by showing that repeated sessions of ACC stimulation induces anxiodepressive behavior.

Besides chronic stress, chronic pain is another risk factor for depression. However, it differs from chronic stress, as neuropathic pain does not induce alterations of the HPA axis (4,7). In the present study, we showed that the ACC is a critical brain region for the neuropathic pain-induced depression. The ACC has been implicated in the pathophysiology of depression since imaging studies have shown hypoactivity in the dorsal portions of the ACC, hyperactivity in its ventral regions (36), and a reduced volume of the ACC (26) in depressed patients. This notion is further supported by preclinical studies showing that the social-defeat paradigm is accompanied by increased cingulate activity (37). Preclinical studies showed that the ACC is also affected by chronic pain. Indeed, chronic pain can induce functional alterations, such as decreased long-term depression (38) or triggered synaptic potentiation, implicating both presynaptic enhancement of glutamate release and postsynaptic potentiation of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor-mediated responses in the ACC (39). However, the functional role of the ACC in the consequences of chronic pain remains still unexplored. In this context, our results provide a causal link between the ACC and the development of anxiodepressive symptoms following chronic pain.

By using the optogenetic approach, we showed that repeated stimulation of pyramidal neurons in the ACC provokes anxiodepressive-like behavior. This finding may contrast with a previous report of medial prefrontal cortex stimulation that induced antidepressant-like effect (40); however, this study was conducted in the prelimbic and infralimbic regions of the medial prefrontal cortex, rather than in the ACC, with behavioral experiments performed during the optogenetic stimulation itself. In the present study, we stimulated the ACC only, and tests were performed after the completion of the repeated stimulation. Our results also showed that the anxiodepressive-like effect persists several days after the end of the stimulation, suggesting the implication of possible neuroplastic mechanisms. As the ACC is also the core of the aversive component of pain (14,35) (present study), it is possible that the anxiodepressive behaviors observed in neuropathic animals are triggered by the affective component of pain rather than the somatosensory component. Indeed, the affective response to acute pain has an adaptive function, favoring associative learning to avoid re-exposure to harmful stimuli or situations. However, with chronic pain, such sustained emotional response may participate to disadaptation of the affective systems. This hypothesis is supported by our results showing that the lesion of the ACC also blocked the relief of spontaneous pain in neuropathic animals. In this study, the CPP paradigm is used to unmask the tonic aversive state due to nonevoked ongoing pain. In this regard, a nonrewarding analgesic drug (i.e., intrathecal clonidine) induces a place preference when it suppresses the spontaneous pain in the paired compartment, thus revealing that the animal was in a spontaneous (unprovoked) pain state in the other compartment (32). The lesion of the ACC prevents this preference for the intrathecal clonidine-paired side, which could be due to the loss of the

aversive aspect of spontaneous pain (35), to a reduced analgesic action of clonidine, a reduced rewarding effect of pain relief, or to an impaired conditioning. Here, we show that clonidine antiallodynic action remains intact after the ACC lesion (Figure 2C). Moreover, previous studies showed that the lesion of the ACC did not prevent cocaine-induced place preference (35) or κ-opioid receptor agonist-induced place aversion (14), suggesting that this part of the prefrontal cortex may not be critical for conditioning per se. These data may thus be supportive of a role of the ACC in the aversive aspect of spontaneous pain (35), but further investigation will be required to reach conclusion.

The lesion of the pIC had no effect on the anxiodepressive consequences and on the spontaneous pain component of neuropathic pain. However, a clinical study suggests a correlation between the activation of insula and the unpleasantness of tonic pain stimulus (41), and a recent preclinical study showed increased cerebral blood flow in the anterior part of insular cortex during anxiety-like behaviors (42). These observations differ in many variables from present work, such as the type of stimulus (acute versus chronic) or the targeted insular cortex subregion (anterior versus posterior). Indeed, it has been suggested that the posterior division of the insula is involved in somatosensory processing, while the anterior insula codes higher level cognition/emotion related sensory modalities (43,44). Imaging (45) and lesion (16) studies showed that besides the somatosensory cortex II, the insula—especially its posterior part—is one of the main brain regions involved in mechanical allodynia. In addition to the direct projection from the spinothalamic pathway, it also receives information from the somatosensory cortex and it projects to the striatal complex. Our results showing the pIC ablation blocked the long-term development of mechanical allodynia further reinforce the essential role of the pIC in this sensory symptom. While present and previous studies (14,35,46) report that the lesion of the ACC has no effect on nociceptive thresholds or on allodynia, it should be acknowledged that an acute manipulation of this brain area has been reported to induce a transitory decrease in mechanical allodynia at early time points after nerve injury (38).

The nociceptive information is transmitted to the brain by various parallel ascending pathways, such as the spinopontine, the spinomesencephalic, and the spinothalamic pathways (47). These pathways are not fully independent, as they polysynaptically terminate in cortical regions that can be interconnected. They thus participate in higher integration of nociceptive inputs creating the complex sensory and emotional experience constituting pain. In such context of brain circuitry, however, our results support the idea that at cortical level, the sensory component of chronic pain remains functionally dissociated from its affective and anxiodepressive components. This functional segregation that is observed between the ACC and the pIC may rely on differences in respective connectomes. Indeed, even though the ACC and the IC share several common inputs and outputs, such as the paracentral, the intermediodorsal, and the central lateral nuclei of the thalamus (48), these cortical areas also have distinct afferents and efferents. For example, the central medial thalamus projects only to the ACC, but not to the IC, and the ACC sends projections to the basolateral, but not to the central amygdala, while the pIC preferentially innervates the central amygdala (49–51). A network level of analysis would thus be important to further understand this functional segregation.

In control animals, the lesion of either the ACC or the pIC had no detectable effect. This observation is in agreement with previous findings showing a lack of effect of the ACC lesion on the anxiodepressive-like behavior in naïve rats (52). However, it has been reported that more rostral lesions of the ACC can diminish per se the immobility time in the FST (53), which should warrant more detailed studies of the relation between ACC subregions and anxiodepressive behaviors.

In conclusion, this study supports the idea that the ACC and the pIC are integrally involved in pain processing, but our findings go beyond this general assertion by providing direct side-by-side evidence for the cortical dissociation between pain components. Importantly, it reveals the critical role of the ACC, but not the pIC, in encoding anxiodepressive consequences of chronic pain. Our optogenetic study further reinforces the essential role of the ACC in mood disorders. The ACC may thus constitute a primary target for unveiling the precise cellular and molecular bases of the changes occurring in chronic pain-induced depression.

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The Anterior Cingulate Cortex is a Critical Hub for Pain-Induced Depression

Supplemental Information

Supplemental Methods & Materials

Animals

All the Thy1-ChR2-YFP mice were produced onsite from breeders provided by Jackson Laboratory. Experiments started with 8 to12 week-old mice, group-housed five per cage and kept under a 12-hour light/dark cycle with food and water available ad libitum. For optogenetic studies only, mice were separated after the cannula implantation to avoid possible damage to the implant. Animal facilities are registered for animal experimentation (Agreement C67-482-1). The protocols were approved by the local ethical committee of the University of Strasbourg (CREMEAS, n°AL-04).

Excitotoxic Lesion

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (63 mg/kg, 0.035 ml) and installed in a stereotaxic frame (Kopf, Tujunga, CA). Either ibotenic acid (56.7 mM, Biotrend, Köln, Germany) or PBS (1X, Euromedex, Souffelweyersheim, France) was bilaterally injected into the anterior cingulate cortex (ACC) (0.3 µl; anteroposterior: +0.7 mm from bregma, lateral: ±0.3 mm, dorsoventral: -1.7 mm from the skull) or to the pIC (0.2 µl; anteroposterior: +0.2 mm, lateral: ±3.8 mm, dorsoventral: -4 mm) using a 5 µl Hamilton syringe (0.1 µl/minute). The needle remained in place for a further 5 minutes before removal. Following surgery, animals were left undisturbed for one week before peripheral nerve injury.

Neuropathic Pain Model

Chronic neuropathic pain was induced by placing a cuff around the right common sciatic nerve (1). Before surgery, mice were assigned to experimental groups so that these groups did not initially differ for the mechanical nociceptive threshold or for body-weight. Surgery was performed under ketamine/xylazine anesthesia (ketamine 17 mg/ml, xylazine 2.5 mg/ml; intraperitoneal, 4 ml/kg) (Centravet, Taden, France). The common branch of the right sciatic nerve was exposed and a 2 mm section of split PE-20 polyethylene tubing (Harvard Apparatus, Les Ulis, France) was placed around it for the Cuff group. The Sham group underwent the same procedure without cuff implantation.

Preparation of Acute Slices

Mice, 9-12 weeks old, were killed by decapitation. The brain was removed and immediately immersed in cold (0-4°C) sucrose-based artificial cerebrospinal fluid containing (in mM): 248 sucrose, 11 glucose, 26 $NaHCO_3$, 2 KCl, 1.25 KH_2PO_4, 2 $CaCl_2$ and 1.3 $MgSO_4$ (bubbled with 95% O_2 and 5% CO_2). Transverse slices (400 µm thick) were performed with a vibratome (VT1000S, Leica, Nussloch, Germany). Slices were maintained at room temperature in a chamber filled with artificial cerebrospinal fluid containing (in mm): 126 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 2 $MgCl_2$ and 10 glucose (bubbled with 95% O_2 and 5% CO_2; pH 7.3; 310 mOsm measured).

Electrophysiological Recordings

Slices were transferred to a recording chamber and continuously superfused with oxygenated artificial cerebrospinal fluid. Pyramidal ACC neurons were recorded in the whole-cell configuration. Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, Edenbridge, UK) using a P-2000 puller (Sutter Instruments, Novato, CA, USA). They were filled with a solution containing the following (in mm): 145 KCl, 10 HEPES and 2 $MgCl_2$ (pH 7.3, adjusted with KOH; osmolarity 310 mOsm adjusted with sucrose) (3.5–4.5 MΩ). All recordings were performed in presence of CNQX (10 µM) and bicuculline (10 µM). Voltage-clamp and current-clamp recordings were performed with an Axopatch 200B amplifier (Molecular Devices, Union City, CA, USA) at a holding potential fixed at −60 mV or a holding current allowing maintaining the resting neuron at ca. -60 mV. Recordings were acquired with WinWCP 4.3.5 (courtesy of Dr. J. Dempster, University of Strathclyde, Glasgow, United Kingdom). All recordings were performed at 34°C. The ACC was illuminated with the same system used for the *in vivo* experiments (see below) triggered with WinWCP 4.3.5, the optic fiber being localized in the recording chamber at 3 mm from the recorded neuron.

Nociceptive Tests

The mechanical threshold of hindpaw withdrawal was evaluated using von Frey hairs (Bioseb, Chaville, France) (1). Mice were placed in clear Plexiglas® boxes (7 x 9 x 7 cm) on an elevated mesh screen and allowed to habituate for 15 minutes before testing. Filaments were applied to the plantar surface of each hindpaw in a series of ascending forces (0.16 to 15 grams). Each filament was tested five times per paw, being applied until it just bent, and the threshold was defined as 3 or more withdrawals observed out of the 5 trials. All animals were tested before and after the lesion and every week after the neuropathic pain induction. The latency for hindpaw withdrawal in

response to thermal stimulation was determined using the Hargreaves method (2). Mice were placed in clear Plexiglas boxes (7 cm × 9 cm × 7 cm) on a glass surface, and were allowed to habituate for 15 minutes before testing. The infrared beam of the radiant heat source (7370 Plantar Test; Ugo Basile, Comerio, Italy) was applied to the plantar surface of each hind paw. The cutoff to prevent damage to the skin was set at 15 seconds. Paw withdrawal latency were measured twice for each hind paw. The animals were tested 12 days and 46 days after the neuropathic pain induction.

Place Conditioning

All experiments were conducted by using the single trial conditioned place preference (CPP) protocol as described previously for rats (3). The apparatus (Imetronic, Pessac, France) consists of 3 Plexiglas chambers separated by manually operated doors. Two chambers (size 15 cm x 24 cm x 33 cm) distinguished by the texture of the floor and by the wall patterns are connected by a central chamber (size 15 cm x 11 cm x 33 cm). Eight to ten weeks after the Cuff/Sham surgery, mice went through a 3-day pre-conditioning period with full access to all chambers for 30 minutes each day. Time spent in each chamber was analyzed to control for the lack of spontaneous preference for one of the compartments. Animals spending more than 75% or less than 25% of the total time in one of the lateral chambers were removed from the study. On the conditioning day (day 4), mice first received intrathecal saline (10 µl) and were placed in a conditioning chamber. Four hours later, mice received intrathecal clonidine (10 µg / 10 µl) and were placed in the opposite chamber. Clonidine, an α2-adrenoceptor agonist, induces analgesia after intrathecal administration. Conditioning sessions lasted 15 minutes each, without access to the other chambers. On the test day (day 5, 20 hours after the last afternoon session), mice were placed in the center chamber with free access to all chambers and the time spent in each chamber was recorded for 30 minutes. With this procedure, CPP is the consequence from combined spontaneous pain-induced aversion to the unpaired side and spontaneous pain relief-induced reward in the clonidine paired side. The clonidine was administered intrathecally as described previously (4) for both CPP experiments and for testing its analgesic effect with von Frey filaments.

Locomotor Activity

Five to six weeks after induction of neuropathic pain, locomotor activity was monitored for both sham and neuropathic mice. Mice were individually placed in activity cages (32 x 20 cm floor area,

15 cm high) with 7 photocell beams. The number of beam breaks was recorded over 4 hours. The results were presented with 1 hour interval.

Anxiodepressive-Related Behavior

Behavioral testing was performed during the dark phase, under red light. While each mouse went through different tests, these were conducted according to the following rules: 1) at least one week separated 2 tests done on the same animal; 2) no mouse went through the same test twice; 3) the forced swimming test (FST) was always considered as terminal (i.e. no other test was done on mice after they went through forced swimming). Body weights were measured weekly.

Novelty Suppressed Feeding Test. The testing apparatus consisted of a 40 x 40 x 30 cm plastic box with the floor covered with 2 cm of sawdust. Twenty four hours prior to the test, food was removed from the home cage. At the time of testing, a single pellet of food was placed on a paper in the center of the box. An animal was then placed in a corner of the box and the latency to eat the pellet was recorded within a 5 minute period. This test induces a conflict between the drive to eat the pellet and the fear of venturing into the center of the box (5). The test was conducted 6 weeks after the peripheral nerve injury for the lesion study and one day after the last stimulation for the optogenetic study.

Splash Test. This test, based on grooming behavior, was performed as previously described (5, 1). The duration of grooming behavior was measured during 5 minutes after spraying a 10% sucrose solution on the dorsal coat of the mice. Grooming is an important aspect of rodent behavior and decreased grooming in this test may be related to the loss of interest in performing self-oriented minor tasks (6). The test was performed 7 weeks after the peripheral nerve injury for the lesion study and four days after the last stimulation for the optogenetic study.

Forced Swimming Test. FST (7) was conducted by gently lowering the mouse into a glass cylinder (height 17.5 cm, diameter 12.5 cm) containing 11.5 cm of water (23-25°C). Test duration was 6 minutes. The mouse was considered immobile when it floated in the water, in an upright position, and made only small movements to keep its head above water. Since little immobility was observed during the first 2 minutes, the duration of immobility was quantified over the last 4 minutes of the 6 minutes test. The test was done 8 weeks after the peripheral nerve injury.

Marble Burying Test. This test was done in Plexiglas cages (27 x 16 x 14 cm) containing 3 cm of fine sawdust. Twenty five glass marbles (1 cm diameter) were evenly spaced on top of the sawdust. Mice were placed individually into the cages and left undisturbed for 30 min. They were then removed and an observer blind to the condition of the animals counted the buried marbles. Marbles were considered buried if two-thirds, or more, of their surface was covered by sawdust. The number of buried marbles is a measure of animal anxiety (8-10). The test was performed 5 days after the last optogenetic stimulation.

Immunohistochemistry

For the lesion study, after the last behavioral testing the animals were perfused under deep sodium pentobarbital anesthesia (273.5 mg/kg; 0.15 ml) with 10 ml phosphate buffer (PB, 0.1 M, pH 7.4) followed by 100 ml of a paraformaldehyde solution (4% in phosphate buffer). Brains were removed and post-fixed overnight in the same fixative. Frontal sectioning of the brain (40 µm) was performed on a vibratome (Leica, Rueil-Malmaison, France). For NeuN immunostaining, sections were washed in PBS (3 x 10 minutes), incubated 15 minutes in a 1% H_2O_2 / 50% ethanol solution, washed in PBS (3 x 10 minutes) and pre-incubated in PBS containing Triton X-100 (0.3%) and 5% donkey serum for 45 minutes. Sections were then incubated overnight at room temperature in PBS containing Triton X-100 (0.3%), 1% donkey serum and a mouse anti-NeuN primary antibody (1:50000; Millipore, MAB377, Molsheim, France). Sections were then washed in PBS (3 x 10 minutes), incubated with a biotinylated horse anti-mouse secondary antibody (1:200 in PBS containing Triton X-100, 1% donkey serum) for 90 minutes, washed in PBS (3 x 10 minutes) and incubated with PBS containing the avidin-biotin-peroxidase complex (ABC kit; 0.2% A and 0.2% B; Vector laboratories) for 90 minutes. After being washed in Tris-HCl buffer, sections were incubated in 3,3'diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 in Tris-HCl for approximately 4 minutes and washed again. Sections were serially mounted on gelatine-coated slides, air dried, dehydrated in graded alcohols, cleared in Roti-Histol (Carl Roth, Karlsruhe, Germany) and coverslipped with Eukitt. Lesions were indicated by neuronal cell loss localized bilaterally and extended from 1.18 to 0.14 mm from the bregma for ACC lesion and from 0.38 to -1.22 mm from the bregma for pIC lesion.

Concerning the optogenetic study, after the completion of the behavioral tests the animals were stimulated once with the same procedure as described before. Ninety minutes later the animals were perfused and c-Fos immunohistochemistry was performed. This was done to check the implant location and the presence of c-Fos, a biomarker for neuronal activity. For c-Fos immunostaining, the procedure used was the same as that described for NeuN immunostaining.

The primary antibody was a rabbit anti-c-Fos (1:10000; Santa Cruz Biotechnology, E1008) and a biotinylated donkey anti-rabbit secondary antibody (1:300 in PBS containing Triton X-100, 1% donkey serum). Animals having c-Fos induction outside of the ACC, for instance in the motor cortex, were excluded from analysis.

Figure S1. 3D representation of the lesions of the anterior cingulate cortex (ACC) and the posterior insular cortex (pIC). Borders of the brain are colored in green, corpus callosum and external capsule in yellow, anterior commissure and fornix in orange, lateral and third ventricles in blue and the lesions of the ACC and the pIC in magenta. ***These 3D images are available as separate supplemental files.***

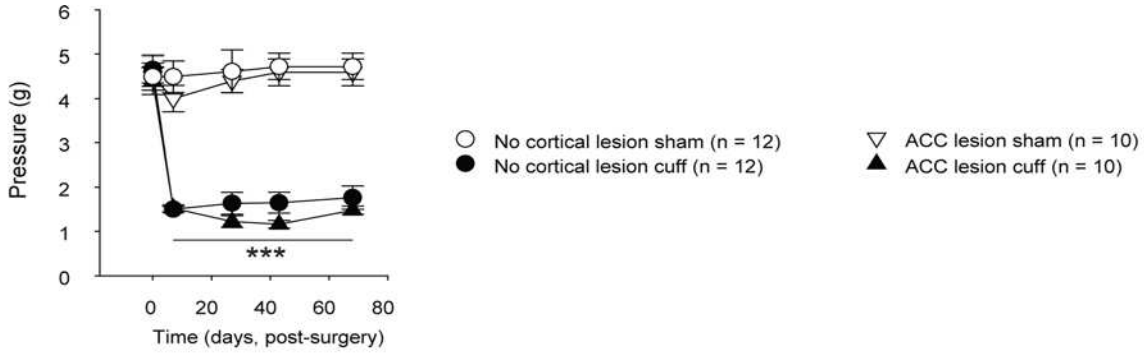

Figure S2. Influence of the anterior cingulate cortex (ACC) on the mechanical threshold. The lesion of the ACC has no effect on the increased mechanical sensitivity observed in neuropathic animals. This experiment was done independently from the one presented in Figure 2. Data are expressed as mean ± SEM. ****p* < .001 Neuropathy versus Sham.

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II. Hyperactivity of anterior cingulate cortex areas 24a/24b drives chronic pain-induced depression

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Introduction

Mood disorders, such as depression and anxiety, are frequently observed in patients suffering from chronic pain and add dramatically to the patients' pain burden (1). Preclinical studies showed that the anxiodepressive consequences of long term chronic pain, such as neuropathic pain, can be studied in murine models (2-4) and develop in a time-dependent manner (3, 9). Interestingly, mood disorders remain present 2 weeks after recovery from evoked hypersensitivity in an animal model of neuropathic pain (517), which raises the question whether the anxiodepressive consequences of pain are maintained independently from these sensory changes.

The anterior cingulate cortex (ACC), one of the brain regions implicated in chronic pain-induced mood disorders, is involved in the processing of both pain and mood-related information (6, 7). For instance, both the presence of ACC hyperactivity in depressed patients (315-317), and ACC transcriptome changes in mice with depressive-like behavior induced by the unpredictable chronic mild stress model (518), support the implication of the ACC in depression. On the other hand, clinical imaging studies show the recruitment of the ACC in pain processing (519), and preclinical studies more precisely associate the activation of ACC neurons with pain-like aversive behavior (9, 492). Furthermore, disinhibition (47) and increased excitability (520, 521) are also observed ex vivo in the ACC from rodent's models of chronic pain. The implication of the ACC is also supported by in vivo studies showing that a lesion of the ACC prevents both chronic pain-induced depression (9) and the aversiveness of spontaneous pain, without affecting mechanical sensitivity (9, 492, 513, 514). Lastly, optogenetic activation of pyramidal neurons within the ACC is sufficient to induce anxiety and depressive-like behavior in naive mice (9).

In the present study, we first aimed to characterize the long term evolution of the sensory, the aversive and the anxiodepressive consequences of neuropathic pain in mice. In addition,

within this 6 month time-course, we determined the in vivo electrophysiological alterations of the ACC accompanied by these various symptoms, and correlated them to different stages of the pathology.

Behaviorally, we show the existence of long-term symptom inertia of evoked hypersensitivity, aversions towards spontaneous pain, and anxiety/depressive-like consequences of neuropathic pain. These symptoms appear and disappear sequentially, with a temporal association between the aversive and depressive aspects. The in vivo electrophysiological recordings further show that ACC hyperactivity is correlated with the aversive and anxiodepressive consequences. This is further supported by ex vivo patch clamp recordings highlighting facilitation of excitatory, but not inhibitory, synaptic transmission onto ACC pyramidal neurons. Moreover, we show that the optogenetic inhibition of the ACC is sufficient to counteract the chronic pain-induced aversive and anxiodepressive-like phenotypes, which further supports a causal link between ACC hyperactivity and the affective aspects of neuropathic pain.

Methods

Animals

Experiments were conducted using male adult C57BL/6J (Charles River, L'Arbresle, France). All animals were group-housed with a maximum of five animals per cage and kept under a reversed 12-hour light/dark cycle. Only the animals used for the optogenetic experiments were single housed to avoid possible damage to the implant. All behavioral experiments were conducted during the dark phase under red light. The Chronobiotron animal facilities are registered for animal experimentation (Agreement A67-2018-38) and protocols were approved by the local ethical committee of the University of Strasbourg (CREMEAS, nº 02015021314412082).

Surgical procedures

Surgical procedures were done under ketamine/xylazine anesthesia (ketamine 17 mg/ml, xylazine 2.5 mg/ml; intraperitoneal, 4 ml/kg) (Centravet, Taden, France).

Neuropathic pain model

Neuropathic pain was induced by implanting a 2mm section of PE-20 polyethylene tubing (Harvard Apparatus, Les Ulis, France) around the main branch of the right sciatic nerve (522). Before the surgery, animals were assigned to experimental groups according to their initial mechanical nociceptive threshold, in order to even out the average mechanical threshold among groups. Animals in the sham condition underwent the same procedure without cuff implantation.

Virus injection

After anesthesia, C57BL/6J mice were placed in a stereotaxic frame (Kopf, Tujunga, CA) and 0.5 µl of AAV5-CaMKIIa-eArchT3.0-EYFP (UNC vector core) was injected bilaterally in the ACC

(areas 24a/24b) using a 5 µl hamilton syringe (0.05 µl/minute, coordinates for the ACC: +0.7 mm from bregma, lateral: ±0.3 mm, dorsoventral: -1.5 mm from the skull). After injection, the needle remained in place for 10 minutes before removal and then the skin was sutured.

Optic fiber cannula implantation

Four weeks (w) after virus injection, the animals underwent optic fiber cannula implantation. The mice were implanted unilaterally over the site of virus injection. Cannulas were implanted in the left hemisphere in half of each experimental group, whereas the other half received the implant in the right hemisphere. The optic fiber cannula was 1.7 mm long and 220 µm in diameter. The cannula was inserted 1.5 mm deep in the brain (MFC_220/250-0.66_1.7mm_RM3_FLT, Doric Lenses).

Optogenetic procedures

After a 3 to 7 days recovery period, we performed behavioral experiments. Green laser light (custom assembly, Green 520 nm, 50 mW, Miniature Fiber Coupled Laser Diode Module, Doric Lenses) was delivered through a 0.75 m long monofiber optic patch chord (MFP_240/250/2000-0.63_0.75m_FC-CM3, doric lenses) that was mounted to the optic fiber implant on the skull. Optogenetic inhibition was done either before or during behavioral testing, by emitting continuous light for 5 minutes with a power of 16 mW. Control animals underwent the same procedures but the light was turned off during stimulation procedures.

Behavioral analysis

Nociceptive testing

Von Frey filaments were used to determine the mechanical threshold of hindpaw withdrawal (Bioseb, Chaville, France). Mice were placed in Plexiglas® boxes (7 cm x 9 cm x 7 cm) on an elevated mesh screen. After 15 minute habituation, animals were tested by applying a series of ascending forces (0.16 to 15 grams) on the plantar surface of each hind paw. Each filament was tested 5 times per paw, applied until it just bent. The threshold was defined as 3 or more withdrawals observed out of the 5 trials. In order to characterize changes in mechanical thresholds during an extended period, we tested animals before and 1, 3, 5, 6, 12, 15, 16, 18, 19 and 21 w after sciatic nerve surgery. The animals used for optogenetic inhibition of the ACC were tested before sciatic nerve surgery and before the behavioral tests (8 and 14 w after the surgery). Finally, we tested the animals during the stimulation to see whether optogenetic inhibition affected mechanical thresholds.

Conditioned place preference

In order to evaluate spontaneous pain, the single trial conditioned place preference (CPP) paradigm was used (513). For this, we used an apparatus consisting of 3 Plexiglas® chambers separated by manually operated doors (Imetronic, Pessac, France). Two chambers (15cm x 24cm x 33cm), distinguishable by the texture of the floor and by the wall patterns, are connected by a central chamber (15cm x 11cm x 33cm). Animals went through a 3-day preconditioning period during which they had access to all chambers for 30 minutes each day. Time spent in each chamber was analyzed to control for the lack of spontaneous preference for one of the compartments. Animals that spent more than 75% or less than 25% of the total time in one of the chambers were removed from the study. On the conditioning day (day 4), mice first received intrathecal saline (10 µl) and were placed in a conditioning chamber. Four hours later, mice received clonidine (10 µg/10 µl), an α2-adrenoceptor agonist which induces analgesia after

intrathecal administration, and were placed in the opposite chamber. Conditioning lasted 15 minutes per compartment, without allowing the animal to access the other chambers. On the fifth day, mice were placed in the center chamber with free access to both conditioning chambers and the time spent in each chamber was recorded for 30 minutes. CPP was assessed for different batches of animals corresponding to 8, 14 and 22 w after the sciatic nerve injury.

In order to study, whether optogenetic inhibition of the ACC causes a preference, we used another version of the CPP test. For this test, we used a custom made box with 2 chambers (23cm x 22cm x 16cm), distinguishable by different wall patterns, connected with each other by a single sliding door. The test lasted four days. On the first day, animals were habituated to the testing box by allowing full access to both compartments for 5 minutes. During the second and third day, animals went through a conditioning period. For this purpose, during the mornings, the animals were placed in the compartment where they received no stimulation, while during the afternoon sessions they were stimulated following the above mentioned protocol. Control animals underwent the same procedures but during the afternoon phase the laser light remained off. On the fourth day we placed the animal at the level of the sliding door and measured the time spent in each compartment during 5 minutes.

Dark-light test

In order to measure anxiety-like behavior, we performed the dark-light test (523), with a two compartment testing box (18cm x 18cm x 14.5cm) connected by a dark tunnel (8.5cm x 7cm x 6cm). One compartment was brightly illuminated (1500 lux) whereas the other was dark. Mice were placed in the dark compartment in the beginning of the test and the time spent in the lit compartment was recorded for 5 minutes. This test was done 8 and 15 w after sciatic nerve surgery in different sets of animals.

Novelty suppressed feeding test

The novelty suppressed feeding (NSF) test was used to assess anxiodepressive-like behavior as it induces a conflict between the drive to eat and the fear of venturing into the center of the box (524). For this test, we used a 40cm x 40cm x 30cm plastic box with the floor covered with 2 cm of sawdust. Twenty-four hours before the test, we removed the food from the home cage. At the time of testing, a single pellet of food was placed on a square paper in the middle of the testing chamber. An animal was then placed in a corner of the box and the latency to eat the pellet was recorded within a 5 minute period. To evaluate this behavior in a time-dependent manner, the NSF test was performed 8, 11, 16 and 21 w after sciatic nerve surgery in independent sets of animals. For the optogenetic experiment, the NSF test was done directly after the inhibition procedure.

Splash test

This test was used to indirectly measure grooming behavior (3, 9). Duration of this behavior was measured for 5 minutes after spraying a 10% sucrose solution on the coat of the animals. Decreased grooming can be related to the loss of interest in performing self relevant tasks. To evaluate this behavior in a time-dependent manner, the splash test was performed on animals 11, 14 and 16 w after the peripheral nerve injury in independent sets of animals. For the optogenetic experiment, the splash test was done during the inhibition procedure.

Forced swimming test (FST)

This test was done to evaluate despair-like behavior in neuropathic animals (525). We lowered the mouse into a glass cylinder (height 17.5 cm, diameter 12.5 cm) containing 11.5 cm of water (23-25 °C). The test duration is 6 minutes, but since only little immobility is observed during the

first 2 minutes, we only quantify the duration of immobility during the last 4 minutes of the test. We considered the mouse to be immobile when it floated upright in the water, with only minor movements to keep its head above the water. This test was performed 7, 14, 17 and 21 w after the sciatic nerve surgery in different sets of animals.

Ex vivo electrophysiological recordings

Mice were killed by decapitation and the brain was removed, then immediately immersed in cold (0-4°C) sucrose-based artificial cerebrospinal fluid containing (in mM): 2 Kynurenic acid, 248 sucrose, 11 glucose, 26 NaHCO3, 2 KCl, 1.25 KH2PO4, 2 CaCl2 and 1.3 MgSO4 (bubbled with 95% O2 and 5% CO2). Transverse slices (400 µm thick) were performed with a vibratome (VT1000S, Leica, Nussloch, Germany). Slices were maintained at room temperature in a chamber filled with artificial cerebrospinal fluid containing (in mm): 126 NaCl, 26 NaHCO3, 2.5 KCl, 1.25 NaH2PO4, 2 CaCl2, 2 MgCl2 and 10 glucose (bubbled with 95% O2 and 5% CO2; pH 7.3; 310 mOsm measured). Slices were transferred to a recording chamber and continuously superfused with artificial cerebrospinal fluid saturated with 5% O2 and 95% CO2. Pyramidal ACC neurons were recorded in the whole-cell configuration. Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, Edenbridge, UK) using a P-1000 puller (Sutter Instruments, Novato, CA, USA). For optogenetic experiments performed in AAV5-CaMKIIa-eArchT3.0-EYFP injected animals, pipettes were filled with a solution containing the following (in mm): 145 KCl, 10 HEPES and 2 $MgCl_2$. For mIPSCs recordings, pipettes were filled with a solution containing the following (in mm): 75 Cs_2SO_4, 10 CsCl, 10 HEPES and 2 $MgCl_2$. The pH of intrapipette solutions was adjusted to 7.3 with KOH, and osmolarity to 310 mOsm with sucrose (3.5–4.5 MΩ). Recordings were performed in presence of CNQX (10 µM) and bicuculline (10 µM) for optogenetic experiments while mIPSCs were recorded with tetrodotoxin

(TTX, 0.5 µM) in the recording solution. For optogenetic experiments, the ACC was illuminated with the same system used for the in vivo experiments (see below) triggered with WinWCP 4.3.5, the optic fiber being localized in the recording chamber at 3 mm from the recorded neuron. For optogenetic experiments and mEPSC recordings, holding potential was fixed at −60 mV or at a holding current allowing maintaining the resting neuron at ca. -60 mV. For mIPSC recordings, the potential was fixed at 0 mV, corresponding to E_{Cl} in our experimental conditions. Recordings were acquired with WinWCP 4.3.5 (courtesy of Dr. J. Dempster, University of Strathclyde, Glasgow, United Kingdom). All recordings were performed at 34°C.

In vivo electrophysiological recordings

Animals were anesthetized in an induction box with a 2% isoflurane/air mixture (Vetflurane, Virbac) and after they were placed in a Kopf stereotaxic frame (KOPF 1730) equipped with a nose mask to continuously deliver the anesthesia.

A 1 x 1.4 mm cranial window was prepared directly anterior to bregma, ranging -0.7 to 0.7 mm lateral from the midline. The dura was opened to lower the glass electrode in the brain. Recordings of spontaneous activity were performed using sharp electrodes pulled from borosilicate micropipettes (1.2 mm outer and 0.69 mm inner diameters, Harvard Apparatus, 30-0044), with a Narashige pipette puller (tip diameter < 1 µm, resistance ±25 MΩ). The glass electrodes were filled with 0.5 M potassium acetate solution. The electrode signal was recorded through a silver wire, amplified with an operational amplifier (Neurodata IR-183A, Cygnus Technology inc.; gain x10), and then amplified further and filtered using a differential amplifier (Model 440, Brownlee Precision; gain x100; band pass filter 0.1-10kHz). The signal was then digitized with a CED digitizer (sampling rate: 20.8KHz) and recorded with Spike2 software

(Version 7.12b, Cambridge Electronic Design, Cambridge, UK). Raw data files were exported into Matlab and analyzed with custom Matlab scripts (Matlab 2015a).

During the recording procedure, isoflurane anesthesia was lowered to 0.5-0.75% and was monitored by regular paw pinching. The glass pipette was slowly lowered using a Scientifica one dimensional micromanipulator and recordings were done between 0.2 and 1.0 mm anterior to bregma ranging from -0.5 to 0.5 mm from the midline, which corresponds to layers 2/3 of the cortex. Neurons were recorded from the brain surface until 1500 µm deep. Once stable cell activity was detected, a 5 minute segment of spontaneous activity was recorded. Recording sites were marked by iontophoretically injecting a 4% Pontamine Sky blue dye (Sigma) in 0.5 M sodium-acetate solution (Sigma). At end of the recording, the mouse was perfused, the brain collected, and 40µm sections were cut on a cryostat. The position of the recorded cells was registered using microdrive reference point with respect to the Pontamine Sky blue dye deposit.

Single-Unit Analysis

Spike detection and spike sorting were done using Spike2. Further single-unit analysis was performed using custom Matlab scripts (Version 2014a, Matworks inc.). Firing rate and bursting activity were calculated. Bursts were defined as 3 or more spikes within a 50 msec time window. Bursting activity was analyzed by calculating the total number of bursting events within a 90 second data segment. The average number of spikes within a bursting event was also calculated. To assess the contribution of single spiking activity to the average firing rate, bursting events were artificially replaced by single spikes and firing rate was recalculated. Furthermore, cells were classified into four groups; regular single spiking, irregular single spiking, regular bursting and irregular bursting neurons.

Statistical Analysis

Data are expressed as mean ± SEM. Prior to comparing samples, data was tested for normality. When data were not normally distributed, the Kruskal-Wallis test was performed followed by Mann-Whitney U post hoc tests to compare the means. When data were normally distributed, groups were compared with ANOVA multiple group comparisons followed by Duncan post hoc analysis, or with the student t-test. Significance level was set to $p<0.05$. Statistical analyses were performed with Matlab 2015a (Matworks inc.) and STATISTICA 7.1 (Statsoft, Tulsa, Oklahoma).

Results

Inertia of affective and sensory symptoms of neuropathic pain

It has been previously shown that the affective consequences of neuropathic pain evolve over time (3, 94, 97). Indeed, while mechanical hypersensitivity is immediately present following nerve injury, mice develop anxiety-related behavior 3-4 w later, while depression-related behavior is observed after 6-8 w (3). In the longer term, we show that the mechanical hypersensitivity recovers spontaneously around 12 w postoperation (PO) ($F_{(47,308)}$=8.64, p<0.001, cuff<sham: 1st, 3rd, 5th, 6th and 12th w p <0.01, **Fig. 4A**). This raises the question of whether spontaneous pain and/or anxiodepressive consequences of chronic pain also recover or remain present.

To test the aversiveness of spontaneous pain, we performed the CPP test. Nerve injured animals displayed significant preference for the compartment associated with clonidine at 8 w PO ($H_{(3)}$=10.73, p<0.015, cuff saline vs cuff clonidine p<0.05), but also at 14 w PO (cuff saline vs cuff clonidine p<0.05, **Fig. 4C**), despite the absence of mechanical hypersensitivity at this time-point (**Supplementary Fig. 1A**). Interestingly, this preference disappeared after 22 w PO ($H_{(3)}$=0.89, p>0.8, **Fig. 4C**), suggesting a recovery from spontaneous pain.

We then tested whether anxiodepressive-like behavior evolved simultaneously with the somatosensory or the aversive components of neuropathic pain. In the dark-light test, neuropathic pain increased anxiety-like behavior as shown by a reduction of time spent in the lit compartment at 8 w PO (p<0.001, **Fig. 4G**). This anxiety-like behavior disappeared after 15 w PO, which coincides with the recovery of mechanical allodynia. In contrast, the splash test showed less grooming behavior during both 11 w (p<0.01) and 14 w PO (p<0.01) (**Fig. 4F**) despite the fact that hypersensitivity was no longer present. This difference in provoked-grooming behavior disappeared at 16 w PO (**Fig. 4C**). Recovery from anxiodepressive-like behavior measured by the

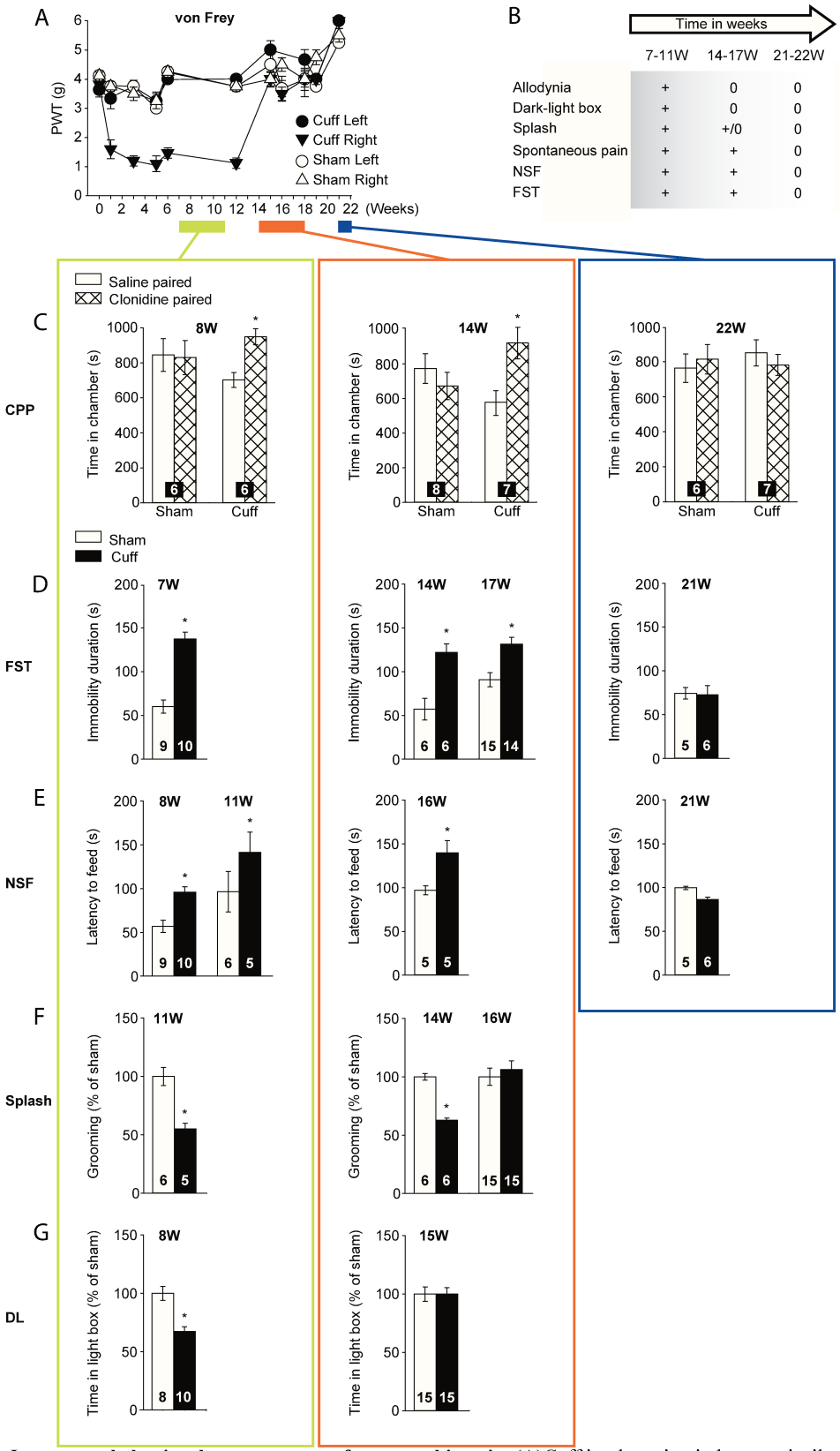
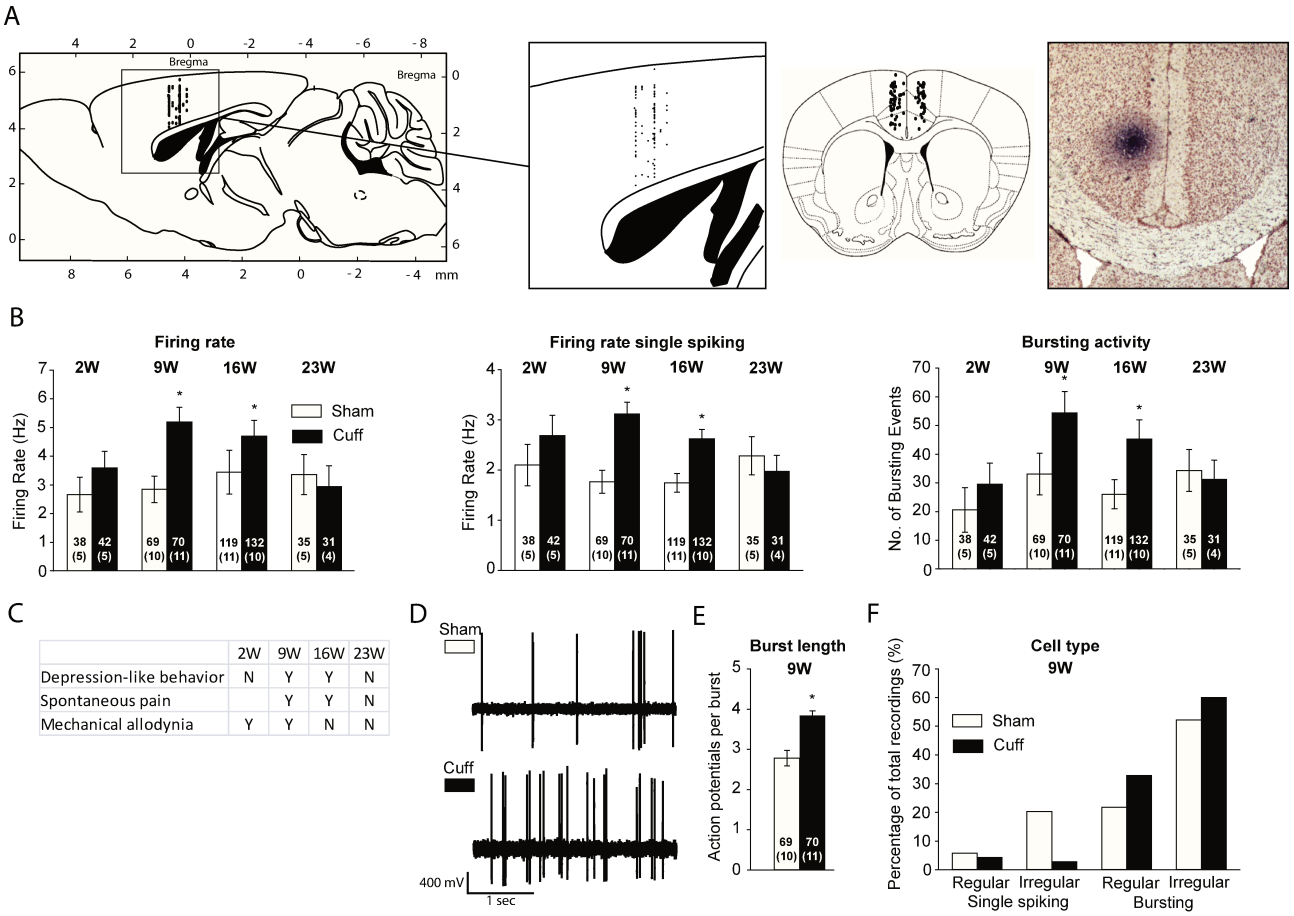


Figure 4. Long-term behavioral consequences of neuropathic pain. (A) Cuff implantation induces an ipsilateral long-lasting mechanical hypersensitivity in C57BL/6J mice. After 3 months, mechanical thresholds returns back to sham levels spontaneously. **(B)**Summary of tests used and the results obtained. 0, no phenotype; +, anxiety- or depression-like phenotype. **(C)**Neuropathic pain induces place preference for a clonidine (10 µg) paired chamber at 8 and 14 weeks (W) postoperation (PO) which disappears at 22 W PO. Neuropathic pain also induces anxiodepressive-like phenotypes which recovers in a time-dependent manner. Sciatic nerve injury significantly: increases immobility time at 7, 14, 17 but not 21 W PO in the forced swimming test (FST) **(D),** increases the latency to feed in the novelty suppressed feeding test (NSF) at 8, 11, 16 but not 21W PO **(E),** decreases grooming behavior in the splash test at 11 and 14 but not 16W PO **(F)** and decreases the time spent in the lit compartment of the dark–light box at 8 but not 15W PO **(G).** Data are expressed as mean ± SEM. Numbers in the bars represent the number of animals.

NSF test was delayed, for which increased latency to feed was present at 8 ($p<0.001$), 11 ($p<0.05$), and 16 w PO ($p<0.05$), with recovery at 21 w PO (**Fig 4E**). The presence of depressive-like behavior, as tested through FST, was also long-lasting. Indeed, nerve injured mice spent more time immobile, showing increased helplessness behavior at 7 ($p<0.0001$), 14 ($p<0.01$) and 17 w PO ($p<0.01$) (**Fig. 4D**), which recovered only at 21 w PO (**Fig. 4D**). Together, these data show that some anxiodepressive consequences of neuropathic pain persist for over 2 months after the spontaneous recovery from mechanical hypersensitivity, which suggests that chronic pain / depression-like comorbidity follows a time course similar to the affective component of neuropathic pain rather than to its sensory component (**Fig. 4B**).

ACC hyperactivity coincides with the emotional and anxiodepressive consequences of neuropathic pain

ACC activity might be implicated in the anxiodepressive-like consequences of pain, since ACC lesions prevent such consequences and optogenetic stimulation can induce depression-like behavior (9). Indeed, ACC neurons from nerve-injured animals have a significantly higher in vivo spontaneous firing rate and display an increased number of bursting events at 9 ($p<0.01$) and 16 w PO ($p<0.01$, **Fig 5B**). Additionally, they show an increased number of action potentials per burst at 9 w PO ($p<0.01$, **Fig. 5E**). When classifying neurons in four categories (regular spiking, irregular spiking, regular bursting and irregular bursting), sham animals showed more irregular spiking neurons (**Fig. 5F**), while an increase in bursting neurons was observed in nerve-injured animals at 9 w PO (**Fig. 5F**). In the absence of anxiodepressive-like phenotypes, i.e. at 2 w PO (before affective symptoms develop) and at 23 w PO (after affective symptoms recovered), the firing rate or bursting activity remain similar between sham and peripheral nerve-injured animals (**Fig. 5B**). To evaluate whether ACC hyperactivity is a consequence of increased bursts only, we



	2W	9W	16W	23W
Depression-like behavior	N	Y	Y	N
Spontaneous pain		Y	Y	N
Mechanical allodynia	Y	Y	N	N

Figure 5. Electrophysiological changes in ACC single-unit activity as a consequence of neuropathic pain surgery. (A) Representative illustration of recording sites. **(B)**Overview of the development and recovery of different aspects of neuropathic pain. **(C)**Single-unit firing rate and bursting activity are increased at 9 and 16 W PO but not at 2 and 23 W PO. When bursting activity is replaced by single spikes for further analysis, firing rate remains increased at 9 and 16 W PO. **(D)**Example of representative recordings from sham and cuff animals at 9 W PO **(E)** An increased number of action potentials per burst at 9 w. **(F)**Percentage of neurons classified as regular single spiking, irregular single spiking, regular bursty, irregular bursty.

replaced them with single action potentials, which showed that the increased firing rate is also caused by non bursting activity at 9 ($p<0.01$) and 16 w PO ($p<0.01$, **Fig 5B**).

Even though the cuff was placed on the right sciatic nerve only, there was no lateralization effect in terms of firing rate and bursting activity, whatever the considered time-point (**Supplementary Fig. 1D**).

Facilitation of excitatory synaptic transmission in the ACC coincides with emotional and anxiodepressive consequences of neuropathic pain

To assess the impact of neuropathic pain on the synaptic transmission of pyramidal neurons, we recorded miniature synaptic currents at 8 w, when nerve-injured animals displayed depressive behavior. Both the frequency and amplitude of excitatory miniature synaptic currents (mEPSCs) ($p<0.05$, **Fig. 6A**) were significantly increased in nerve-injured mice, indicating facilitation of excitatory synaptic transmission onto pyramidal ACC neurons involving both pre- and postsynaptic changes. We did not find a change, however, in mIPSC frequency and amplitude in nerve-injured mice (**Fig. 6B**).

Temporal inhibition of the ACC relieves the affective consequences of neuropathic pain

Based on results demonstrating hyperactivity of the ACC in mice displaying anxiodepressive-like behavior, we studied whether the optogenetic temporal inhibition of the ACC may counteract these consequences.

The delivery of AAV5-CaMKIIa-eArchT3.0-EYFP resulted in reliable virus transfection in the ACC, which was confirmed by evaluation of EYFP fluorescence in the target structure (**Fig. 7A**). In order to characterize the effect of green laser light illumination on transfected ACC neurons, we performed ex vivo electrophysiological recordings. Patch clamp recordings showed

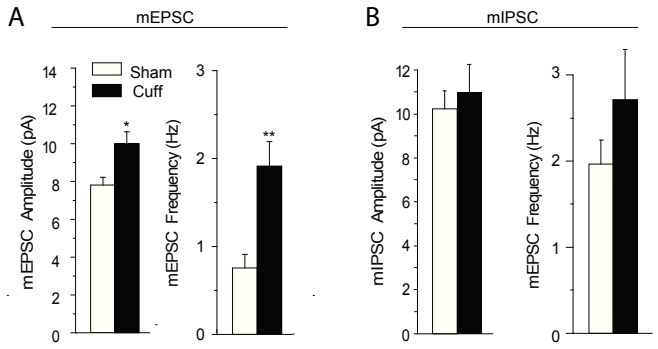


Figure 6. Increased excitatory synaptic transmission after neuropathic pain surgery. (A) mEPSC amplitude and frequency of pyramidal neurons are increased in nerve-injured mice, whereas mIPSC amplitude and frequency are unchanged **(B)**.

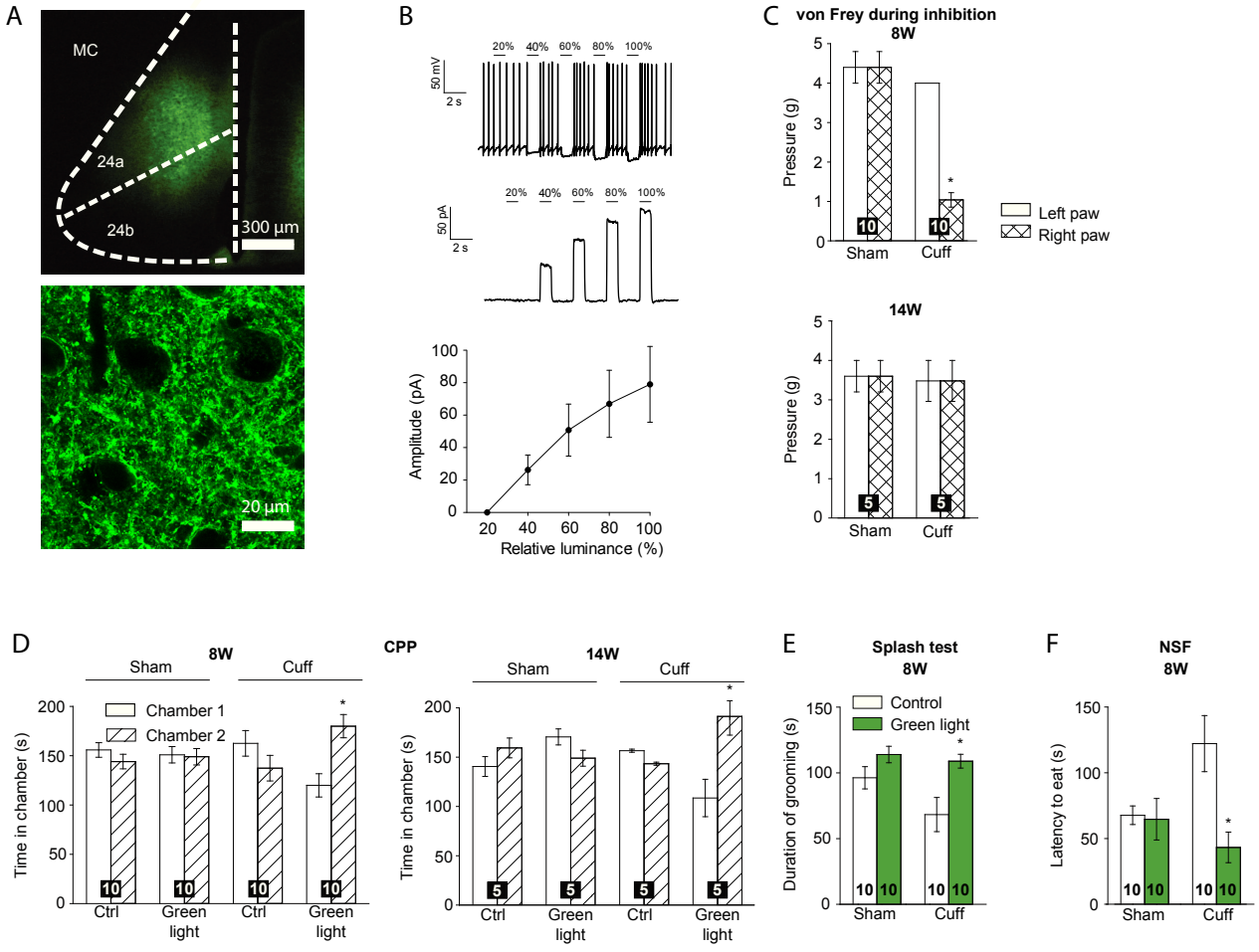


Figure 7. Optogenetic ACC inhibition blocks spontaneous pain and anxiodepressive consequences of neuropathic pain. (A) Representative picture of the AAV5-CaMKII-eArchT3.0-EYFP sites 5 weeks after the injection. **(B)** Light-evoked effects recorded in ACC pyramidal neurons of AAV5-CaMKIIa-eArchT3.0-EYFP transfected mice. Top: representative trace recorded in the current clamp mode, note the full inhibition of spikes at all luminances tested; middle: representative trace of light-evoked currents recorded in the voltage clamp mode; bottom: luminanceresponse curve of light-evoked currents (n=6); the maximal luminance corresponds to 16 mW. **(C)**Mechanical hypersensitivity is not affected by ACC inhibition. **(D)**Optogenetic inhibition of the ACC induces a place preference at 8 and 14 W PO in cuff implanted animals for the compartment in which they are stimulated. **(E)**ACC temporal inhibition during the splash test reverses the decreased grooming behavior observed in cuff non stimulated animals. **(F)**ACC temporal inhibition prior to NSF test blocks the cuff induced increased latency to feed.

that illumination with green light inhibits action potential firing reliably, by inducing an inward current (**Fig. 7B**).

In vivo, our results demonstrate that mechanical hypersensitivity was not affected by ACC inhibition ($p<0.05$) (**Fig. 7C**). However, inhibition of the CaMKIIa ACC neurons induced a place preference in cuff implanted animals at 8 w PO (i.e. when hypersensitivity is still present) ($H_{(3)}=9.45$, $p=0.02$, cuff stimulated vs cuff control $p<0.05$) (**Fig. 7D**), as well as at 14 w PO (i.e. when hypersensitivity spontaneously recovered) ($H_{(3)}=11.54$, $p=0.009$, cuff stimulated vs cuff control $p<0.05$) (**Fig. 7D**; **Supplementary Fig. 1E**); without affecting sham animals. These findings support the idea that inhibition of the ACC relieved the aversiveness of spontaneous pain in nerve-injured mice.

Finally, we show that the optogenetic inhibition of the ACC also suppressed the anxiodepressive phenotype in nerve-injured animals, as observed by a normalization of grooming duration in the splash test (**Fig 7E**) and latency to eat in the NSF test (**Fig. 7F**). Unstimulated nerve-injured animals, however, did still show the characteristic phenotypes (sham control vs cuff control $p<0.05$, **Fig. 7E, F**).

Discussion

In the present study, we show that different symptoms of neuropathic pain, including evoked hypersensitivity, aversiveness of spontaneous pain and anxiodepressive-like consequences, are segregated in a time-dependent manner since they develop and cease at different time points after nerve injury. The in vivo electrophysiological recordings show ACC hyperactivity, which coincides with the time window of pain aversion and anxiodepressive-like behavior. Ex vivo patch clamp recordings further support ACC hyperactivity, as shown by increased excitatory synaptic transmission. Finally, our results show that temporal inhibition of the ACC hyperactivity can alleviate the aversive and anxiodepressive aspects of neuropathic pain.

A growing number of clinical and preclinical studies show that the comorbidity of chronic pain and mood disorder changes over time (3, 4, 9), which raises the question of whether the various symptoms of neuropathic pain are interdependent or develop separately. While animals develop mechanical hypersensitivity immediately after the nerve injury, they spontaneously recover from this symptom after 3 months, which allows for the study of the behavioral consequences of neuropathic pain in the presence and absence of hypersensitivity. Patients with chronic pain usually experience spontaneous or ongoing pain also, which is rarely evaluated in preclinical studies. In animals, this symptom can be unmasked by alleviating the pain-related tonic aversive state in a CPP procedure (9, 513). For instance, injection of lidocaine into the rostral ventromedial medulla, a brain area that mediates descending modulation of pain (526), or spinal injection of clonidine (9, 513), induce place preference only in nerve injured animals. In this study, we detected the presence of spontaneous pain until 22 w, i.e. over 2 months after hypersensitivity disappeared, which is the first pathophysiological evidence of naturally-occurring dichotomy between evoked hypersensitivity and spontaneous pain in an animal model. The hypersensitivity and the spontaneous pain that follow nerve injury have been proposed to

share some mechanistic features. Indeed, both may imply an upregulation of voltage gated Nav1.8 channels in primary afferent neurons (527), an alteration of descending pathways (526) and of spinal NK-1 positive ascending projections (528). However, studies also point out that they can be distinguished mechanistically as well as neuroanatomically. Indeed, the lesion of the ACC can block the aversiveness of spontaneous pain in both neuropathic (9, 514) and inflammatory pain (492) models without affecting mechanical hypersensitivity (5,15, 17); while the lesion of the posterior insular cortex can suppress the maintenance of mechanical hypersensitivity (9, 529) without affecting spontaneous pain (9). In addition, large-diameter fibers of the dorsal column were proposed to mediate mechanical hypersensitivity but not spontaneous pain (528). This dichotomy should thus be taken into consideration for drug development, since evoked hypersensitivity, rather than spontaneous pain, is presently used for target validation. As it is more often spontaneous pain that leads patients to seek treatment, this may in part explain why the development of new treatments has not always provided translational satisfaction.

While mechanical hypersensitivity is no longer present after 3 months, we still observe aversive and depressive-like behavior until 22 weeks. Previously, it has been reported in rats that anxiodepressive-like behavior persists at least 2 weeks after normalization of mechanical sensitivity following cuff removal (517). However, longer term consequences were not addressed. Our results in mice confirm and further extend this sensory/affective dichotomy by showing that depressive-like symptoms persist more than 2 months after cessation of hypersentivity. However, we also show that recovery from anxiety-like behavior is faster and coincide with the disappearance of mechanical hypersensitivity. It is then possible to hypothesize that anxiety-like behavior may be associated with the somatosensory component of chronic pain, while depressive-like aspects are caused by the aversive state induced by spontaneous pain.

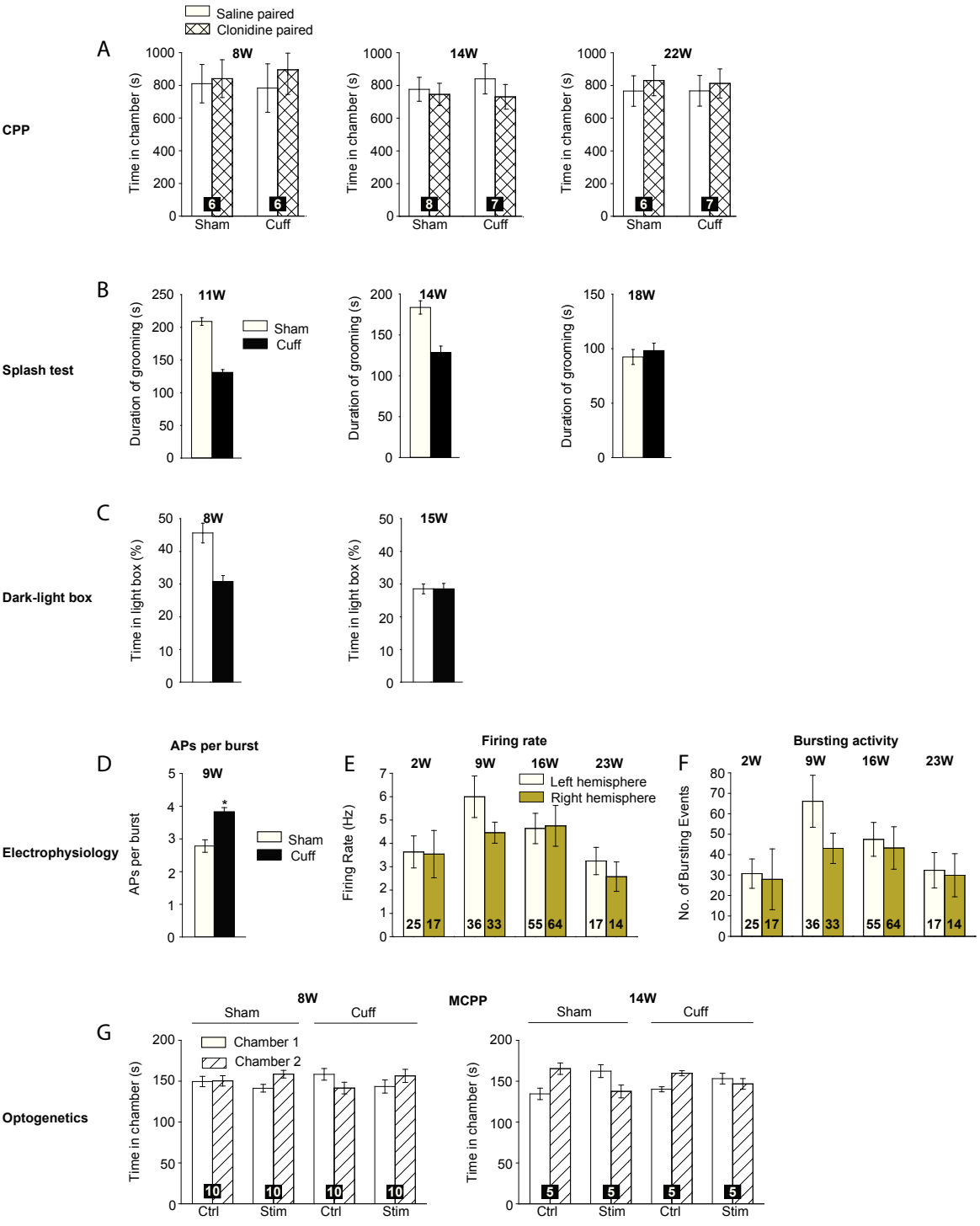
These prolonged emotional consequences thus point out the presence of long-term plastic changes in the brain, secondary to a peripheral nerve injury. One of the cortical regions where such morphological (47, 522) and functional plasticity (391, 521) has been reported is the ACC.

In this study, in vivo single unit extracellular recordings evidenced increased activity of the ACC at 9 and 16 week PO, which coincides with aversive and depressive-like behavior. In humans, fMRI studies have shown that the ventral part of the ACC, which is involved in emotional processing (249), is hyperactive in depressed patients (315, 316), and that activity patterns in ACC subregions correlated with symptom clusters such as sadness and depressed mood (315). This role is further supported by an animal study showing increased cingulate activity, as shown by c-Fos overexpression, accompanied by depressive-like behavior in a social-defeat-paradigm (356). Such hyperactivity may rely on alterations in neurotransmitter systems, such as an imbalance between excitatory and inhibitory transmission (47). Indeed, a loss of inhibitory synapses onto excitatory pyramidal neurons, and a loss of the excitatory drive unto inhibitory fast-spiking interneurons, have been proposed to underlie local disinhibition of the cortical network in a context of neuropathic pain (47). This disinhibition might explain the increased activity observed in the ACC of patients with nerve injury (67). Studies from the depression field further support this imbalance. Indeed, Northoff and Sibille (318), hypothesized that abnormal ACC activity in depression can be associated with changes in GABA interneurons. Indeed, somatostatin (341) and parvalbumin levels (342), neuropeptides expressed in GABA neurons, are low in patients with major depressive disorders (318). Our data show that ACC hyperactivity observed in neuropathic pain-induced depressed animals might also be linked to long-term increase in excitatory synaptic transmission.

To leap from correlative analyses to a causal link between the ACC hyperactivity and behavioral outputs of neuropathic pain, we performed optogenetic inhibition of the ACC. We

show that the inhibition of the ACC suppresses the aversiveness of the spontaneous pain and depressive-like consequences of neuropathic pain without affecting the mechanical hypersensitivity. These results further reinforce the sensory/affective dichotomy, as well as the links on one hand between pain aversiveness and depressive-like consequences, and on the other hand between ACC hyperactivity and these affective aspects of chronic pain.

In conclusion, our results emphasize that mood disorders comorbid with chronic pain are temporally segregated from the evoked hypersensitivity whereas they follow the same time course as the aversiveness of spontaneous pain. This should be taken into consideration to improve the translational feature of preclinical models, and for preclinical target validation of relevant potential therapies. The fact that the emotional aspects of chronic pain are driven by ACC hyperactivity further highlights the ACC and its circuitry as critical neuroanatomical substrates to further explore mood disorder mechanisms.



Supplementary figure 1. (A)CPP results before conditioning for 8, 14 and 22 W PO. No significant preference was present before conditioning. **(B)**Absolute values for the splash test for 11, 14 and 18 W PO. **(C)**Percentage of time spent in the light compartment for 8 and 16 W PO. **(D)**Average number of APs per burst event from ACC neurons 9 W PO. No lateralization effect was measured **(E)**firing rate and **(F)**bursting activity for ACC neurons recorded 2, 9, 16 and 23 W PO. **(G)**CPP results before conditioning with optogenetic inhibition for 8 and 14 W PO. No significant preference was present before conditioning.

General discussion

In the next section, we first discuss the methodological considerations of the cuff model, excitotoxic lesions, electrophysiology and optogenetics. In the second part, we discuss the results of two papers in a broader perspective.

I. Methodological considerations

Modeling neuropathic pain can be done in a variety of ways. For a number of reasons we chose the approach as described in the previous chapters.

A. Cuff model: modeling neuropathic pain in rodents

Even though neuropathic pain includes both somatosensory and emotional components, most preclinical studies focus on mechanical allodynia since it's the easiest symptom to evaluate in rodents. The most frequently used approach to evaluate mechanical allodynia is using von Frey filaments which helps evaluate static mechanical allodynia by applying static pressure on a single point of the hindpaw. In the clinic however, dynamic mechanical allodynia is usually measured by brushing a cotton bud or brush against the affected area (530). While it is possible to measure dynamic mechanical allodynia in rats, even though it is not well characterized (531), it is very challenging to adapt this method in mice since they are more active and their paws are too small to be reliably stimulated with a brush. Therefore, in this study, we mainly used von Frey filaments.

In our model, mechanical allodynia is observed from the first day until 3 months after surgery. Interestingly, cuff implanted animals spontaneously recover from mechanical allodynia after 3 months. Although reasons as to why this happens are of yet unclear, possible mechanisms at the level of the nerve, spinal and supraspinal areas could potentially provide an explanation. For instance, one cause of neuropathic pain is the loss of peripheral neurons caused by injury or

metabolic disease such as diabetes. Therefore, recruitment of cellular survival strategies which prevent neuronal apoptosis such as synaptic remodeling, and remyelination can all counterbalance the loss of neuronal input that normally accompanies peripheral neuropathic pain (532, 533). In addition, ectopic activity in primary sensory neurons by the increased translation and trafficking of receptors and ion channels under the influence of neurotrophic factors also contribute to increased sensory sensitivity (36). Therefore, receptor endocytosis or a reduction of receptor trafficking can potentially underlie the recovery from mechanical allodynia in our model. On the other hand, nerve injury has been shown to induce an immune response by recruiting immune cells including macrophages and lympocytes (534) and by increasing proinflammatory cytokines (534) which have been shown to increase excitability (535, 536). Therefore, decrease of proinflammatory cytokines could further decrease the excitability of peripheral nerves and lead to decreased mechanical hypersensitivity.

At the spinal level GABAergic inhibition is reduced after nerve injury which could be due to an decrease inhibitory neurons (40, 42) or due to a decrease of input from peripheral excitatory peripheral neurons which decreases excitability or burst firing pattern of GABAergic neurons (537). Therefore, recovery of GABAergic activity might reduce neuropathic symptoms (40, 42, 537).

Finally, supraspinal descending control could contribute to the recovery from mechanical allodynia. Serotonergic and adrenergic innervation from the antinociceptive pathway which includes the rostral ventromedial medulla (538, 539), the dorsal raphe nucleus (539) and the locus coeruleus (539) has been shown to induce GABA release in the spinal dorsal horn (540) which reduces excitability. A potential mechanism might lie in the increase sprouting of descending fibers as it has been shown that nerve injury induces spinal BDNF which in turn increases the amount descending noradrenergic fibers that originate from the locus coeruleus (541).

Besides mechanical allodynia, hyperalgesia is another common symptom of neuropathic pain. Previous studies have shown that hyperalgesia exists during the first 15 days after cuff surgery and can be tested with the "Radiant heat paw-withdrawal test" (8). Considering that the aim of our studies was to expose the long-term consequences of neuropathic pain, this test would not have been of much of our interest.

Even though spontaneous pain is a more debilitating symptom than the evoked nociceptive response in neuropathic pain patients, it remained elusive in preclinical studies. Methods to examine spontaneous pain in mice include the use of mouse facial expressions, vocalizations and conditioned place preference. In the next part I will provide the advantageous and disadvantageous for each of these techniques.

The monitoring of facial expression of mice has been proposed as a potential to measure spontaneous pain (542) in which a three point scale is used to distinguish five facial features (542, 543). A major advantage of this technique is that spontaneous pain can be measured several times and directly without any intervention. However, it seems to be mandatory that white animals with red eyes are used as the lack of contrast on an overall black animal make the recording of facial features too difficult. For this reason there are, as of now, no studies published using this technique on non-white coated animals (544). Considering that this method is not well standardized in mice, we could not use facial expressions to monitor spontaneous pain.

Another proposed technique to directly measure spontaneous pain is the use of rodent ultrasound vocalization (USV). USVs are recorded as they are found more consistently during acute application of nociceptive stimuli (544) in contrast to audible vocalizations. However, whether USV communication is directly linked to pain is unclear as the majority of studies failed to measure USVs in chronic pain models (544). A major disadvantage of this technique are the

fact that, in order to measure USVs, the mouse has to be placed in a mouse holder which can cause stress (545) and therefore cause problems in detecting stimuli dependent USVs. Other problems are the sensitivity to background noise, which can cause false positive detection, and the lack of standardization. Variations in vocalization frequencies due to age, animal strain and sex and the use of different frequencies and recording techniques by different groups all cause difficulties in standardizing this technique (544).

In this thesis work, spontaneous pain was measured by performing the CPP test in which the preference of the compartment coupled with an intrathecal injection of a non-rewarding analgesic (clonidine) is an indirect measurement for the presence of spontaneous pain. CPP is due to the rewarding effects of pain relief as well as the aversive effect of spontaneous pain. In rats, the analgesics could be injected through a permanently implanted catheter (513) which allows successive injections in animals without causing pain due to the injection needle. In mice, however, the space between vertebras is too small to allow the placement of a catheter. Additionally, placing the catheter takes a lot of time and injecting the analgesic through this method can damage the spinal cord which impairs the possibility of retesting.

CPP has been validated substantially in the literature (3, 9, 544) and can be done semi-automatically with the use of automated CPP testing chambers. A disadvantage of this test is that, as we use the indirect measure of preference, the test is highly dependent on memory which means that lesioning of inhibition of a brain region related to memory could impair the test results. Finally, it is likely that animals feel pain due to entering of injection needle, however, this confound is possibly nullified by the use of a sham injection group as they show no preference for the clonidine coupled compartment.

B. Excitotoxic lesion: Permanent ablation

In order to initiate the project we needed a global understanding of the role of the pIC and the ACC in neuropathic pain-induced depression. For this reason we decided to start the project with a global ablation by using ibotenic acid. Our results show that the role of the ACC and pIC are functionally distinct. For instance, lesioning the pIC blocked the maintenance of mechanical allodynia whereas the ACC is critical for the emotional component and anxiodepressive consequences of neuropathic pain. Caution should be taken however, with oversimplification of the results. For instance, the ACC as well as the pIC are heterogeneous regions which can be divided between dorsal and ventral or agranular, granular and dysgranular, respectively. The lesioning technique used in our study lacks the precision to cater to such anatomical nuances, especially when the region is not clearly delimited as is the case with the IC. Two other disadvantages of excitotoxic lesioning are that the brain is lesioned indiscriminately of cell type and permanently. In order to gain cell type specificity and inhibit the ACC temporarily, we designed an experiment using optogenetic inhibition which will be discussed in part IV of this chapter.

Even though our results suggest a segregation of functions of these regions, we should keep in mind that the ACC and the pIC project to each other and project in part to the same brain regions. Therefore, one might suggest that the lesion of either of these regions influences the other either by direct or indirect connectivity.

C. Considerations of electrophysiological recordings under anesthesia

The main debate surrounding our electrophysiological experiments involves the use of anesthetics. In our study, we performed our electrophysiological recording under isoflurane anesthesia which has been shown to decrease regional activity in cortical brain areas (546, 547). Besides its main effect on the voltage gated sodium channels, isoflurane's anesthetic properties are, at least partly, caused by its enhancing effects on postsynaptic GABA-A receptor gating in the presence of submaximal GABA concentrations (548). Isoflurane also increases Ca^{2+} release from intracellular stores, which facilitates the release of GABA and activates Ca^{2+} dependent K^{+} channels, and inhibits voltage-gated Na^{+} channels, all of which reduce neuronal excitability (549). An fMRI study showed that isoflurane decreases the amplitude of low frequency fluctuations (ALFF), which is a measurement of spontaneous regional activity at rest, in a number of regions including the somatosensory cortex and the thalamus (547) which further supports the inhibitory role of isoflurane. Additionally, they found that functional connectivity in the cortico-thalamo-cerebellar circuit and sensory areas was reduced. Although these structures are part of the pain matrix and provide input to the ACC, no difference in ACC activity or functional connectivity was reported under the influence of isoflurane. On the other hand, in this study besides the induction period, the isoflurane administered less than 1% in which no significant EGG alterations was observed (data not shown). Accordingly, follow up studies in awake as well as in freely moving animals can help us to confirm the role of the ACC in neuropathic pain condition and to correlate the electrophysiological recordings with the behavioral alterations.

D. Optogenetics: cell specific temporal excitation and inhibition

Optogenetic is a technique that allows cell specific excitation or inhibition. In order to stimulate the ACC, we used Thy1-ChR2-YFP mice which express ChR2-YFP in Thy1 expression neurons. What should be noted is that; all neurons that express Thy1 also express vGlut1 and CaMKII however only a subset of neurons expressing vGlut1 or CaMKII express Thy1 (550). This means that optogenetic stimulation of ChR2-YFP containing neurons stimulates glutamatergic neurons however not all glutamatergic neurons are stimulated.

For optogenetic inhibition of the ACC we used a transfection method by injecting AAV5-CaMKIIa-eArchT3.0-EYFP into the ACC. This genetic construct allows the expression of inhibitory opsin eArchT3.0 coupled to yellow fluorescent protein. CaMKIIa is present in 32-35% of all cortical neurons and is only present in glutamatergic cells (551, 552).

A major advantage of optogenetics is that it allows for temporary excitation or inhibition of specific neurons and the coupling with the YFP allowed us to check whether the viral transfection was in the right location. A disadvantage, however, was that our glass cannulas were too big to be implanted bilaterally. For this reason, left and right side implantations were divided equally among all experimental groups. Verification of c-Fos expression showed that even though implantation was unilateral, expression of c-Fos was bilateral (9). This could be due to the light reaching from one hemisphere to the other.

II. Functional differentiation between the ACC and pIC

The aim of our first study was to elucidate the ACC's and pIC's roles in the affective and sensory components of neuropathic pain as well as in the neuropathic pain mood disorder comorbidity. Our lesioning results show that the pIC is essential for the sensory component of chronic pain whereas the emotional as well as the anxiodepressive component depends on the ACC. ACC stimulation helped to further support this notion, as ACC stimulation in naive mice led to anxiodepressive-like behavior.

Many previous studies have implicated the ACC in depression (see chapter V). The dACC has been shown to be hypoactive in depressed patients whereas the vACC has been shown to be hyperactive (553). A preclinical study, using the social-defeat paradigm further confirmed ACC hyperactivity in an animal depression model. In the present work, we showed that optogenetic ACC stimulation can induce anxiodepressive-like behavior which persists several days after the end of the stimulation and that ACC inhibition in neuropathic mice alleviates anxiodepressive behavior. Together this further suggests that anxiodepressive –like behavior is triggered by a sustained disadaptation of the affective system not the somatosensory system, even in the presence of neuropathic pain.

The pIC has been shown to play a role in the processing of pain intensity information (554, 555). This was further confirmed in our studies which showed that pIC lesioning had no effect on the aversiveness of spontaneous pain and anxiodepressive consequences but did alleviate mechanical allodynia, showing that that the pIC is not involved in the affective component of chronic pain but is involved in the sensory component especially the maintenance of mechanical allodynia.

Some studies relate the IC with anxiety and depression (556, 557). However, this discrepancy might underlie methodological differences as the mentioned studies either study

acute pain (555) instead of chronic pain or target a different IC subregion. This notion is supported by studies indicating that not the pIC but the anterior IC, which was studied in (556), is involved in cognition/emotion related processing whereas the pIC is involved in somatosensory processing (558, 559).

III. ACC hyperactivity and neuropathic pain mood disorder comorbidity

Our in vivo electrophysiological recordings show ACC (24a/24b) hyperactivity when animals with peripheral nerve injured display aversive as well as anxiodepressive-like behaviors. In MDD patients, a number of studies showed that ACC hyperactivity is in part caused by a reduction of GABAergic activity which in turn causes a shift in excitation/inhibition balance which causes increased perigenual ACC activity (336, 560, 561). The perigenual ACC is part of the default mode network (DMN) and has been shown to have a negative reciprocal connection with the DLPFC (562), which is part of the executive network (561, 563). Activity changes in the DMN and executive networks have been hypothesized to underlie depression symptoms such as excessive worrying (564-566).

The executive network is responsible for cognitive-executive functions including generation of goal-orientation and the processing of external stimuli stemming from exteroceptive sensory inputs (567). The DMN however, has been associated with various inner mental functions like mind wandering (568, 569), undirected thoughts (570), consciousness (571, 572) and, most importantly, self-referential activity (573-575). Increase in the DMN/ perigenual ACC causes a decrease in the executive network / DLPFC which directs the mental awareness towards internal content which causes excessive brooding, rumination and increased self-referential processing in MDD patients (564-566).

Another MDD symptom is anhedonia; the loss of interest or pleasure in previously rewarding activities (American Psychiatric Association, 2013). Anhedonia is characterized by decreased responsiveness of the mesocorticolimbic reward circuitry (576-578). As a part of this circuitry the ACC plays a role in detecting the salience of external stimuli and in reward feedback monitoring (579, 580) by altering dopamine release in the VTA in order to direct behavior towards rewarding or away from harmful stimuli (581). ACC hyperactivity therefore, could alter its influence on the VTA by decreasing dopamine release and cause anhedonic behavior by decreasing the urgency of a rewarding stimulus.

The ACC also modulates the generation of fear by integrating sensory and affective information (582). Hyperactivity of the ACC could be responsible for the generation of anxiety in the neuropathic pain condition since projections between the ACC and BLA have been shown to be glutamatergic (583). Additionally, a recent study showed that pain-related hyperactivity of the BLA plays an important role in emotional-affective aspects of pain (584).

In our study the inhibition of the ACC resulted in the alleviation of depressive-like symptoms. As has been mentioned before, depression symptoms are often associated with an excitatory/inhibitory imbalance. Therefore, we propose that inhibition of the structure could be a potential way to treat depression. Inhibition of the ACC can be accomplished through a number of ways. DBS uses electrical stimulation which has been hypothesized to inhibit through; creating a depolarization block, inactivate voltage-gated currents or activation of inhibitory afferents (585). Indeed ACC-DBS has been used successfully in the treatment of both neuropathic pain (446) and depression (324, 586). Another way to alter the excitatory/inhibitory balance is through the use of drugs that act on glutamergic systems. A wide range of antidepressive agents have shown to reduce glutamatergic action through competitive NMDA antagonism or blockage such

as; phencyclidine, dizocilpine and ketamine (587) . However, increasing GABAergic activity through drugs, such as benzodiazepines, in order to skew the excitatory/inhibitory balance towards inhibition did not alleviate depression symptoms in most studies (588).

IV. Perspectives

Future work should focus on expanding our techniques and direct focus towards a more computational approach in order to gain a clearer picture of what ACC hyperactivity really constitutes. As mentioned before, future work done in awake animals could further deepen the understanding of the ACC's contribution in anxiodepressive-like behavior and affective processes by allowing recordings during behavioral tasks. In turn, computational methods could use ACC activity in order to predict animal behavior.

Additionally, using permanently implanted electrodes will allow us to record, and differentiate between, multiple neurons simultaneously. In turn, this could allow us to correlate single-unit activity between different cells in the same and other cell layers in order to generate an ACC network model of affective and emotional processing. This is important because "hyperactivity" is likely only a symptom of an underlying meaningful change of processing within the broader network of the ACC. By doing recordings of a single neuron at a time, we miss this underlying information.

Performing in vivo electrophysiological recordings and optogenetics simultaneously could provide a lot of information. For instance, network analysis could be done by exciting or inhibiting the ACC and recording activity in output regions such as the BLA. When using permanently implanted electrodes all three of these facets (behavior, electrophysiology and optogenetic stimulation) can be combined and provide a more complete understanding of hyperactivity, network connectivity and behavioral consequences.

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Jim SELLMEIJER

Le cortex cingulaire antérieur : une structure clé dans les conséquences émotionnelles de la douleur neuropathique

Résumé

Outre le stress chronique, la douleur chronique représente une cause majeure de dépression. En effet, environ 50% des patients qui souffrent d'une douleur chronique développent des troubles de l'humeur. Les perturbations des structures cérébrales impliquées dans la perception de la douleur pourraient contribuer à cette comorbidité, dont les mécanismes restent pourtant mal compris. Nous avons étudié l'implication du cortex cingulaire antérieur (CCA) dans les conséquences sensorielles et émotionnelles de la douleur neuropathique dans un modèle murin. Nous avons montré qu'une lésion du CCA ou une inhibition des neurones pyramidaux du CCA préviennent l'émergence des désordres émotionnels dans notre modèle. De plus, nos résultats indiquent que ces conséquences émotionnelles coïncident avec une hyperactivité neuronale dans le CCA. En conclusion, nous montrons que le CCA est une structure clé pour la dépression induite par la douleur neuropathique.

Résumé en anglais

Besides chronic stress, chronic pain is one of the prevalent determinants for depression. Indeed, around 50% of chronic pain patients develop mood disorders. Alterations in brain regions implicated in pain processing may also be involved in affective processing, thus potentially be responsible of mood disorders. However, the underlying mechanisms of this comorbidity are not yet elucidated. Here, we studied the role of the anterior cingulate cortex (ACC) in the somatosensory, aversive and anxiodepressive consequences of neuropathic pain. We showed that a permanent lesion or temporal inhibition of ACC pyramidal neurons blocked the development or suppressed the expression of an anxiodepressive phenotype in neuropathic mice. In addition, anxiodepressive-like behavior coincided with ACC hyperactivity. In conclusion we show that the ACC is a critical hub for neuropathic pain-induced depression.