Pain and depression comorbidity causes asymmetric plasticity in the locus coeruleus neurons

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Abstract

There is strong comorbidity between chronic pain and depression, although the neural circuits and mechanisms underlying this association remain unclear. By combining immunohistochemistry, tracing studies and western-blotting, with the use of different DREADDs (Designer Receptor Exclusively Activated by Designer Drugs) and behavioural approaches in a rat model of neuropathic pain (chronic constriction injury), we explore how this comorbidity arises. To this end, we evaluated the time-dependent plasticity of noradrenergic-locus coeruleus (LC) neurons relative to the site of injury: ipsilateral (LC_ipsi) or contralateral (LC_contra) at three different time points: short- (2 days), mid- (7 days), and long-term (30-35 days from nerve injury). Nerve injury led to sensorial hypersensitivity from the onset of injury, whereas depressive-like behavior was only evident following long-term pain. Global chemogenetic blockade of the LC_ipsi system alone increased short-term pain sensitivity while the blockade of the LC_ipsi or LC_contra relieved pain-induced depression. The asymmetric contribution of LC-modules was also evident as neuropathy develops. Hence, chemogenetic blockade of the LC_ipsi→spinal cord projection, increased pain-related behaviours in the short-term. However, this lateralized circuit is not universal as the bilateral chemogenetic inactivation of the LC-rostral anterior cingulate cortex (rACC) pathway or the intra-rACC antagonism of alpha1- and alpha2-adrenoreceptors reversed long-term pain-induced depression. Furthermore, chemogenetic LC to spinal cord activation, mainly through LC_ipsi, reduced sensorial hypersensitivity irrespective of the time post-injury. Our results indicate that asymmetric activation of specific LC modules promotes early restorative-analgesia, as well as late depressive-like behavior in chronic pain and...
depression comorbidity.

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**Abbreviations:** A5 = Noradrenergic cell group; AAV= Adeno-associated virus, BLA= Basolateral amygdala; CCI= chronic constriction injury; CNO= Clozapine-n-oxide; contra= contralateral; DREADDs= Designer Receptor Exclusively Activated by Designer Drugs; FG= Fluoro-Gold; FST= forced swimming test; ipsi; ipsilateral; LC= locus coeruleus; LT= long-term; MT= mid-term, PFC= Prefrontal Cortex; rACC= rostral Anterior Cingulate Cortex; Sal= saline; SC= spinal cord; ST= short-term.

**Introduction**
Pain and depression represent two highly prevalent and deleterious disorders with high comorbidity rates (from 18 to 85%). Hence, pain is a major risk factor for depression, and depression can exacerbate chronic pain and obstruct effective therapies \(^1,\,2\) but the mechanisms underlying the overlap between these conditions remain unclear.

When pain induces depression, we speculate that the balance between the recruitment of restorative analgesia and harmful pro-nociceptive mechanisms determines whether pain following injury resolves or if it persists and becomes chronic, with the ensuing appearance of comorbid anxiodepressive symptoms. The noradrenergic locus coeruleus (LC) in the pontine brainstem is one of the brain regions that may mediate this neuroplasticity. Indeed, while descending inhibition of acute pain by the LC has been well established \(^3,\,4\), in the last decade there have been several reports of its role in facilitating or inhibiting chronic pain \(^5-\,7\). Furthermore, the role of the LC circuits in depression is still elusive, despite the many changes in the LC reported in animal models \(^8-\,10\), depressed patients \(^11,\,12\) and its key role in the action of antidepressants \(^13,\,14\). Therefore, here we will address how depression arises after the induction of pain to understand the mechanisms involved in pain-depression comorbidity. Specifically, we will focus on the pain caused by nerve injury (neuropathic pain), not least because neuropathic pain is a debilitating condition affecting around 7%–10% of the general population and it is notoriously resistant to currently available analgesic treatments \(^15,\,16\).

Here, we explored the time-dependent LC neural network reconfiguration in a rat model of neuropathic pain induced by chronic constriction injury (CCI), assessing the changes in the ipsilateral (LC\(_{\text{ipsi}}\)) or contralateral (LC\(_{\text{contra}}\)) pathways at three time points after nerve injury induction: short- (CCI-ST, 2 days), mid- (CCI-MT, 7 days), and long-term (CCI-LT, 30-35 days 4-5 weeks). Furthermore, as the LC is apparently composed of modules that might produce targeted neuromodulation \(^17,\,18\), we assessed the effects induced by the LC as a whole, as well as its specific efferent pathways to areas critical for sensory and unpleasant emotional experiences: the spinal cord (SC) and rostral anterior cingulate cortex (rACC) \(^6,\,19\).

**Materials and methods**
A detailed description of the experimental procedure is provided in Supplementary material.

Animals and pain model

Male transgenic Long-Evans tyrosine hydroxylase:Cre (TH:Cre) and wild-type Long-Evans rats (350-450g) were produced and maintained under standard laboratory conditions. CCI was used as a model of neuropathic pain.\(^{20, 21}\)

Virus and tracer injection

Rats were injected with a Designer Receptor Exclusively Activated by Designer Drugs (DREADD) virus (hM4D(Gi)-DREADD, rM3D(Gs)-DREADD or control-DREADD) into the LC (AP: -3.2 mm, ML: ±1.3 mm, DV: 6.2 mm)\(^{22}\) and Fluoro-Gold (FG) tracer into rACC (AP: +3.0 mm, ML: ±1.0 mm and DV: 1.2 mm)\(^{23}\) or SC (L4-L6 segments)\(^{24}\). See methodological validation of DREADD approach in\(^{22}\) and Supplementary Figures (Supplementary Figs. 1, 2 and 11).

Behavioral assessment

Acetone test: Cold hypersensitivity was evaluated using the acetone test. Animals were placed individually into Plexiglas chambers on a metal grid, and a drop of acetone (100 µl) was applied to the center of the ipsilateral and contralateral hind paw with a pipette. The acetone was applied 4 times to each hind paw alternately at 5 minute intervals and the responses were recorded over 1 min according to the following scale: 0, no response; 1, quick withdrawal, flick or stamping of the paw; 2, prolonged withdrawal or repeated flicking of the paw; 3, repeated flicking of the paw with persistent licking directed at the ventral side of the paw. The cumulative score for each rat was obtained by summing the score and dividing it by the number of assays.\(^{25}\)

Von Frey test: Mechanical hypersensitivity was measured with the von Frey test (Dynamic Plantar Aesthesiometer, Ugo Basile, Italy), applying a vertical force to the ipsilateral or contralateral hind paw that was increased from 0 to 50 g over a period of 20 s. Mechanical hypersensitivity was indicated by a reduction in the force that provokes paw withdrawal.\(^{26}\)
Cold plate test: Cold hypersensitivity was measured using the cold plate test. Animals were placed on a cold metal plate maintained at 4 ± 1 °C (Panlab S.L., Spain) and the number of times the animal briskly lifted its ipsilateral hind paw was measured over a period of 2 min 21.

Forced Swimming Test (FST): Depressive-like behavior was evaluated in the FST over two different sessions, a 15 min pre-test was followed by a 5 min test performed 24 hours later. The predominant behaviors were recorded (climbing -CL, swimming -SW, or immobility -IM), and scored in each 5 s period of the 300 s test session 27, 28.

Locomotor spontaneous activity test: The total distance travelled (arbitrary units, A.U., %) was measured as an indicator of locomotor activity 22.

Immunohistochemistry and histology

Immunohistochemistry was performed to evaluate the expression of DREADDs-mCherry, FG-tracer and c-fos in the LC, as well as DBH in the SC. The cannula placement in the rACC and SC was also verified 22, 29.

Western blotting

Western blotting was performed to evaluate the expression of pCREB and TH proteins in the LC, which it was normalized to the level of β-actin 22.

Statistical Analysis

Results are presented as the means ± SEM. An unpaired Student’s T test was used to compare the values between the two groups. One- or two-way or repeated measures ANOVA followed by Newman-Keuls test were used to compare between more than two groups. p<0.05 was considered significant.

Data availability

Data are available upon request to the corresponding author.
Results

Analgesia is driven by the activation of noradrenergic-LC$_{\text{ipsi}}$ neurons

Sensorial hypersensitivity was evaluated through the acetone test and as expected, it appears immediately in the ipsilateral hindpaw after nerve damage and it remained constant over time (ST, MT and LT: p<0.001 vs sham, Fig. 1). By contrast, depressive-like behavior was only observed in CCI-LT animals (immobility and climbing: p<0.01 and p<0.05 vs sham, Fig. 1). This finding indicates that pain-induced depression evolves after pain has lasted at least 4-5 weeks $^{1, 22, 25, 27, 30, 31}$. To assess the pontine mechanisms underlying the time-dependence of this phenotype further, we selectively expressed hM4D(Gi)- or rM3D(Gs)-DREADD in the LC$_{\text{ipsi}}$ or LC$_{\text{contra}}$ to selectively manipulate LC activity at different time points from nerve injury. In agreement with our recent work $^{22}$, selective expression of hM4D(Gi)- or rM3D(Gs)-DREADD was achieved in noradrenergic LC neurons (Fig. 2A and Supplementary Fig. 1A-H). Thus, we assessed how hM4D(Gi)-DREADD-mediated LC inhibition affected sensorial hypersensitivity after systemic clozapine N-oxide (CNO) administration. LC$_{\text{ipsi}}$ blockade only significantly enhanced hypersensitivity in the ipsilateral hindpaw in the CCI-ST animals (Acetone test: p<0.001 vs the CCI-ST-Sal: Fig. 2B and Supplementary Fig. 2). Furthermore, this effect of CNO was also evident when exploring the number of lifts of the ipsilateral hindpaw in a different pain test (cold plate test: p<0.001, Fig. 1C). Enhancement of the cold plate response disappeared completely after 360 minutes (Fig. 1C), in line with the duration of the inactivation produced by CNO previously $^{32}$. Surprisingly, no significant effect on acetone test was seen at any time point post-injury when the LC$_{\text{contra}}$ was chemogenetically blocked (Fig. 1D).

As previous studies have shown that the pain threshold increases following acute electrical or chemogenetic stimulation of the LC $^{5, 33}$, we explored the effect of global noradrenergic LC activation using rM3D(Gs)-DREADDs at various phases of the development of neuropathy (Fig. 1E-F and Supplementary Fig. 1E-H). CNO activation of the LC$_{\text{ipsi}}$ significantly reduced CCI evoked thermal and mechanical hypersensitivity in the ST, MT and LT animals relative to their respective CCI-Sal controls (acetone test ST, MD and LT p<0.001; von Frey test ST p<0.01; MT p<0.05; and LT p<0.01: Fig. 2E). By contrast, CNO-mediated activation of LC$_{\text{contra}}$ neurons did not significantly...
modify CCI-induced pain at any time point (Fig. 2F). Furthermore, chemogenetic LC\textsubscript{ipsi/contra} inhibition/activation did not significantly modify the sensorial responses in the sham group or in the contralateral hindpaws of the CCI group (Fig. 2B-F and Supplementary Fig. 3A-D).

**Chemogenetic blockade of either the LC\textsubscript{ipsi} or LC\textsubscript{contra} neurons relieves pain-induced depression**

As long-term pain induces depressive-like behavior (Fig. 1), we used the same chemogenetic approaches to explore the effect of silencing or activating the LC\textsubscript{ipsi} or LC\textsubscript{contra} in depressive CCI-LT rats. Unlike the sensory tests, chemogenetic silencing the LC\textsubscript{ipsi} or LC\textsubscript{contra} had a marked effect on emotional behavior, reflected by the significant decrease in immobility (LC\textsubscript{ipsi} and LC\textsubscript{contra} p<0.001) and the increased climbing (LC\textsubscript{ipsi} p<0.01 and LC\textsubscript{contra} p<0.001) behavior of these animals (Fig. 3A-B). Alternatively, blocking either LC\textsubscript{ipsi} or LC\textsubscript{contra} had no effect on sham-animals (Fig. 3A-B), although significant depressive behavior was triggered in sham animals by rM3D(Gs)-DREADD LC activation (LC\textsubscript{ipsi} p<0.01 and LC\textsubscript{contra} p<0.001 vs Sham-Sal: Fig. 3C). Furthermore, LC activation in CCI-LT rats did not further augment the already elevated immobility time in the FST (Fig. 3C). None of the behaviors evaluated were affected by any locomotor dysfunction (Supplementary Fig. 4A-B). In addition, a bilateral activation of the LC was evident through the increase in the rate-limiting enzyme in noradrenaline biosynthesis, tyrosine hydroxylase (TH, LC\textsubscript{ipsi} and LC\textsubscript{contra} p<0.05 vs sham), and that of phosphorylated CREB (LC\textsubscript{ipsi} p=0.07 and LC\textsubscript{contra} p<0.01 vs sham: Supplementary Fig. 5).

**Activation of the noradrenergic-LC\textsubscript{ipsi}→SC pathway relieves pain**

To study the specific afferent pathways that modulate the evolution of neuropathy, we explored how the descending noradrenergic-LC pathway to the SC influences the pain-related phenotype. Unilateral administration of the retrograde fluorescent tracer FG into the superficial dorsal horn of the ipsilateral SC labeled a similar number of neurons in the LC\textsubscript{ipsi} and LC\textsubscript{contra} along the rostro-caudal axis, these neurons predominantly located in the central LC (Fig. 4A-B and Supplementary Fig. 6A). Hence, the LC appears to
project bilaterally to the SC and most of the spinally projecting neurons (around 80%) are situated in the ventral aspect of the nucleus (Fig. 4C).

In other experiment set, FG was injected unilaterally into the ipsilateral SC of CCI-ST and CCI-LT rats, and the expression of c-Fos in the LC was assessed (Fig. 4D-G). Nerve injury significantly increased the number of c-Fos labeled neurons in LC of CCI-ST rats bilaterally relative to the sham-ST rats (LC\textsubscript{ipsi}: p<0.001 and LC\textsubscript{contra}: p<0.001: Fig. 4E). Importantly, LC\textsubscript{ipsi} c-Fos expression was significantly higher than that in the LC\textsubscript{contra} of CCI-ST animals (p<0.01, Fig. 4E). This lateralized LC c-Fos expression was also evident in sham animals (p<0.05, Fig. 4E), suggesting an acute LC activation related to skin and muscle injury in sham animals. Furthermore, these c-Fos labeled neurons were preferentially located in the dorsal pole of the LC (around 90%, Fig. 4E).

When exploring which c-Fos neurons also expressed FG, we found that there was only a small population in the dorsal LC\textsubscript{ipsi} pole of the CCI-ST rats (p<0.05 vs sham: Fig. 4F). However, c-Fos/FG co-labeling was not found in either sham-LC\textsubscript{ipsi}/LC\textsubscript{contra} or CCI-ST LC\textsubscript{contra} (Fig. 4F). Regarding LT pain, significantly stronger c-Fos expression was evident in the dorsal LC\textsubscript{ipsi} and LC\textsubscript{contra} of CCI-LT animals relative to the respective LC area in the sham rats (LC\textsubscript{ipsi} p<0.001 and LC\textsubscript{contra} p<0.001), although there was no difference between sides (Fig. 4G). Moreover, no c-Fos/FG co-labelled LC neurons projected to the SC in CCI-LT rats. In CCI-ST and -LT rats, the expression of the noradrenaline synthesizing enzyme dopamine beta-hydroxylase (DBH) was also studied in the superficial dorsal horn of LC projecting segments (Supplementary Fig. 7). A bilateral increase in the expression of DBH fibres was evident in CCI animals just after nerve injury that was significant in the L5 and L6 lumbar dorsal horn sections (ST, p<0.05 vs sham: Supplementary Fig. 7A-B). By contrast, there was a loss of noradrenergic fibres after CCI-LT in the L6 segment (LT, p<0.05 vs sham animals: Supplementary Fig. 7A). These data demonstrate lateralized changes in the activation of the LC→SC pathway along neuropathy development.

DREADD-mediated inactivation of the noradrenergic LC\textsubscript{ipsi} or LC\textsubscript{contra}→SC pathway was explored behaviorally and like the global LC blockade (Fig. 2B and 2D), intrathecal CNO administration following hM4D(Gi)-DREADD administration to the LC\textsubscript{ipsi} enhanced thermal and mechanical hypersensitivity in the ipsilateral hindpaw of CCI-ST rats, yet not at later times (Fig. 5A). This was shown by the significant increase in the acetone score (p<0.001), a decrease in the mechanical withdrawal threshold (p<0.05)
and an increase in the number of ipsilateral paw lifts in the cold plate test of CCI-ST-CNO as opposed to CCI-ST-Sal animals (p<0.001). When exploring the outcome of blocking the LCcontra→SC pathway, no significant changes in evoked pain responses were observed (Fig. 5B). Like after global LCipsi or LCcontra inhibition, no significant effect was found in sham animals or in the response of the contralateral hindpaw of CCI animals after blocking the LCipsi or LCcontra→SC pathway (Fig. 5A-B and Supplementary Fig. 8A-B).

The rM3D(Gs)-DREADD activation of the noradrenergic LCipsi or LCcontra→SC pathway was also explored. Like global LC activation (Fig. 2E-F), DREADD-mediated activation of noradrenergic LCipsi neurons projecting to the SC reduced peripheral stimulus-evoked hypersensitivity in CCI-ST, -MT and -LT rats, as shown by the significant analgesic effects in the acetone, von Frey and cold plate tests (Fig. 6A). However, no change in thermal or mechanical allodynia was evident at any time point when the LCcontra to SC pathway was activated (Fig. 6B). To verify that the local activation of α2-adrenoreceptors is still able to produce analgesia in these animals, the α2-adrenoreceptor agonist clonidine was intrathecally administered producing a clear analgesic effect in CCI-LT rats (acetone test p<0.001, von Frey test p<0.05 vs CCI-LT-Sal: Fig. 6B). Finally, and like after global LCipsi or LCcontra activation, no change was found in sham animals or in the contralateral hindpaws of CCI animals (Fig. 6A-B and Supplementary Fig. 9A-C).

**Bilateral chemogenetic inhibition of the LC→rACC pathway relieves pain-induced depression**

One of the main LC targets involved in mood and pain-related emotions is the rostral anterior cingulate cortex (rACC) 19. Administration of FG into the ipsilateral rACC produced abundant labeling of neurons in the LCipsi (≈ 80%), suggesting that the projection of the LC to the rACC is lateralized. Most marked neurons were located in the central LC along the rostro-caudal axis (Fig. 7A-B and Supplementary Fig. 6B). Bilateral administration of FG to the rACC was also performed and as expected, there was a similar distribution of FG positive neurons bilaterally in the LC and preferentially in the dorsal pole (Fig. 7C-E and Supplementary Fig. 6C). The expression of c-Fos in the LC neurons of LT animals was also explored (Fig. 7F-H). As found previously (Fig.
4G), there was a similar bilateral increase in neurons expressing c-Fos in the dorsal LC pole of CCI-LT rats (LC\textsubscript{ipsi} and LC\textsubscript{contra} \(p<0.001\) vs sham: Fig. 7G). Following bilateral administration of the FG tracer to the rACC, more than 20% of c-Fos positive neurons projected specifically to the rACC and these projection neurons were located in the dorsal pole of the LC (Fig. 7H). No differences were found between the sides of the LC, demonstrating a bilateral over-activation of this pathway in CCI-LT animals in agreement with western blotting experiments about TH and phosphorylated CREB (Supplementary Fig. 5). Bilateral blockade of the LC-rACC pathway using hM4D(Gi)-DREADD had no significant effect on the evoked pain (acetone, von Frey and cold plate tests: Fig. 8A and Supplementary Fig. 10A-B). However, a reversion of the depressive phenotype was observed in CCI-LT rats (immobility and climbing \(p<0.01\) vs CCI-Sal: Fig. 8A). No impairment of spontaneous locomotion was found (Supplementary Fig. 10C).

In addition, reduced c-Fos expression (LC\textsubscript{ipsi} and LC\textsubscript{contra} \(p<0.001\) vs CCI-Sal: Supplementary Fig. 11A-C) and reduced c-Fos/FG co-labeling (LC\textsubscript{ipsi} \(p<0.01\) and LC\textsubscript{contra} \(p<0.05\) vs CCI-Sal: Supplementary Fig. 11D) was observed in the LC after chemogenetic inhibition of the LC→rACC pathway in CCI-LT rats.

**Blockade of \(\alpha\)-adrenoreceptors in the rACC relieves pain-induced depression**

To test whether blocking adrenoreceptor activity in the rACC might reverse pain-induced depression, we administered the \(\alpha\)1-adrenoreceptor antagonist prazosin (5 \(\mu\)g/0.5 \(\mu\)l), the \(\alpha\)2-adrenoreceptor antagonist idazoxan (9 \(\mu\)g/0.5 \(\mu\)l) or the \(\beta\)-adrenoreceptor antagonist propranolol (1 \(\mu\)g/0.5 \(\mu\)l) intra-rACC. Prazosin and idazoxan administration reversed depressive-like behavior (prazosin: immobility and climbing \(p<0.01\), idazoxan: immobility and climbing \(p<0.001\) vs CCI-Sal), although they did not change pain behaviors (acetone, von Frey and cold plate tests: Fig. 8B-C and Supplementary Fig. 12A-F). However, propranolol failed to modify pain- or depression-related behaviors in CCI-LT rats although it significantly increased depressive-like behavior in Sham (immobility and climbing \(p<0.01\) vs Sham-Sal: Fig. 8D and Supplementary Fig. 12G-I). Locomotor activity was not modified in any of the groups of rats evaluated (Supplementary Fig. 12B,E,H). These data suggest an involvement of \(\alpha\)-adrenoreceptors in pain-induced depression at the rACC level.
Discussion

We demonstrate that there are time-dependent plastic changes in the noradrenergic-LC system as pain develops from acute to chronic, and that these changes are associated with behavioral despair.

Sensorial hypersensitivity appears immediately after nerve injury, whereas anxiodepressive and cognitive symptoms arise after several weeks (4-6 weeks) 1, 22, 25, 27, 30, 31. To assess noradrenergic-LC changes when nociceptive signaling persists, LC activity in both hemispheres was explored at various times after unilateral nerve injury (from CCI-ST to CCI-LT). Immunohistochemistry revealed that there is increased c-Fos expression after nerve injury, both in the short-term and long-term. However, the expression of c-Fos is higher in the ipsilateral side of the LC than on the contralateral side at short-term, while the increase is of equal magnitude bilaterally in the LC of CCI-LT animals. These results show that the nerve injury-induced LC changes vary on either side of the body, as well as with the duration of the injury. Chemogenetic suppression of the LC activity shows that activity of LC ipsi neurons contributes to a milder pain phenotype, yet only in the early phase of nerve injury. However, LC contra blockade did not modify pain perception at any time point. Contrary to sensorial sensitivity, the blockade of either the LC ipsi or the LC contra completely reversed the depressive phenotype developed at CCI-LT. Furthermore, bilateral stronger expression of TH, pCREB and c-Fos of the LC was found at long-term, in agreement with previous data in major depression disorder patients (TH: 12) and in CCI-LT animals were both LCs were evaluated as a pool 22, 27, 30. Overall the results suggest an early lateralized activation of the LC that reduces the pain phenotype but a later bilateral activation that leads to behavioral despair in nerve-injured animals. Interestingly, hM4D(Gi)-DREADD-mediated inhibition of the LC did not affect sensorial exploration or depressive-like phenotype in sham animals, suggesting a weak tonic drive of LC activity in the uninjured state 4. We also explored the consequences of activating the LC globally. In sham animals, global LC activation had no effect on the sensory threshold, although significant depressive behavior was triggered when the LC ipsi or LC contra was activated chemogenetically, consistent with previous findings showing that the optogenetic/chemogenetic activation of LC neurons is itself anxiogenic 34, 35. In terms of
neuropathic pain, rM3D(Gs)-DREADD activation of LC neurons relieved pain at any time point, albeit with different contributions of the LC_{ipsi} and LC_{contra}. Indeed, global chemogenetic activation of the LC_{ipsi} produced a clear anti-neuropathic effect at all time points after nerve injury. However, global activation of LC_{contra} did not combat thermal and mechanical hypersensitivity at any time point. Furthermore, as depressive-like behavior is associated with long-term pain, the effect of LC activation was explored in the FST. Chemogenetic LC activation did not modify the pain-induced depression-like state, perhaps reflecting that maximal activation had already been achieved by long-term nerve injury.

In the light of these findings, we explored the role of LC→SC pathway along pain development. As occurred with global LC activity, hM4D(Gi)-DREADD blockade of the LC_{ipsi}→SC projection rather than the LC_{contra} projection reduced pain in the short-term. This was consistent with the c-Fos activation of the LC_{ipsi}-neurons projecting to the SC in the short-term and not at long-term (Fig. 2F). Accordingly, there are more DBH in the fibres of the SC in the CCI-ST, and fewer in the CCI-LT. Alterations in spinal DBH at different time points from nerve injury have been shown before in several rodent models. We extend these previous findings evaluating changes in spinal DBH along with spontaneous activity of LC→SC projecting neurons (c-Fos) and the behavioral effect of the chemogenetic LC blockade at two different time points from nerve injury. Overall, these changes would suggest an activation of the noradrenergic descending LC pathway to the SC at short-term, reducing the pain phenotype soon after injury but later failing when the insult persists. Furthermore, chemogenetic activation of both LC to SC neurons produces analgesia when neuropathy is well established (from 4 weeks after nerve injury), and we now show that this effect is mainly contributed by the LC_{ipsi} and can be triggered at any time point from injury. Our global evaluation of the LC and of the specific projection to the SC seems to refute the idea that the LC serves to generate pain under conditions of chronic pain (review in ). Indeed, intracerebroventricular (i.c.v.) administration of the neurotoxin anti-dopamine-β-hydroxylase saporin (anti-DβH-saporin) or intra-LC administration of lidocaine dampened the evoked pain in conditions of long-term nerve-injury. However, i.c.v. injection of anti-DβH-saporin disrupts all noradrenergic nuclei (A1-A7), some of which contribute to sensorial hypersensitivity (A5: , although our experiments focused only on the LC. On the other hand, lidocaine administered at the level of the LC will
block the activity of noradrenergic and non-noradrenergic cells \(42\), and our approach is to target only noradrenergic LC cells. Another important issue is the modular composition of the LC, whereby different projections may have opposing actions in a time-dependent manner. In this sense, the pain-inhibitory actions of noradrenaline in the spinal cord may be countered or even superseded by the activation of supraspinal facilitating pathways to the dorsal reticular nucleus (contributing to evoked pain: \(41\)), to the trigeminal spinal nucleus caudalis (evoked pain: \(43\)), or to the prefrontal cortex (PFC, spontaneous pain: \(5\)). Thus, further studies will be necessary to untangle the actions of the LC globally and in a modular fashion, also bearing in mind the strain differences that have been described in the noradrenergic pathways (see review in \(6\)).

One of the LC-related networks involved in affective dimensions and manifestations of pain-related expression of emotions is the rACC \(19, 44\). Accordingly, hyperactivity in the ACC is seen in depressed patients \(45\) and it has chronic neuropathic pain-induced anxiodepressive-like consequences \(46\). In this line, a bilateral increase of noradrenaline in the prefrontal cortex has been associated with long-term neuropathic pain (6 weeks after nerve injury) \(47\), suggesting an overactivation of the noradrenergic system in long-term pain. Furthermore, optogenetic activation of the LC-ACC projection enhances excitatory neurotransmission in vitro \(48\). As such, the FG and c-Fos co-labeling here reveals a significant bilateral increase in c-Fos expressing neurons that specifically project to the rACC in CCI-LT. The blockade of the LC\textsubscript{ipsi} and LC\textsubscript{contra} neurons projecting to the rACC completely reverses the depressive-like behavior, yet it did not modify stimulus-evoked pain responses. Furthermore, site-specific pharmacological blockade indicates that \(\alpha_1\)- and \(\alpha_2\)-adrenoreceptors within the rACC are necessary for this behavior. Mechanistically, electrophysiological studies in the medial PFC indicated that noradrenaline-persistent responses are mainly mediated by synergy between presynaptic \(\alpha_1\)-adrenoreceptor-mediated enhancement of glutamate release and postsynaptic \(\alpha_2\)-adrenoreceptor-mediated inhibition of hyperpolarization-activated cyclic nucleotide-gated cation channels \(49\). Thus, long-term pain overactivates the LC-ACC pathway (\(\alpha\)-adrenoreceptors), leading to behavioral despair, and overactivation of the LC-basolateral amygdala pathway (BLA) (\(\beta\)-adrenergic receptors) mediates anxiety and enhanced aversive learning \(22\). A similar phenomenon has been described when evaluating bilateral chemogenetic activation of the LC using resting-state functional MRI, which rapidly interrupts ongoing behavior, dampens exploratory activity and
augments anxiety, in conjunction with synchronized hyperconnectivity in the salient (that includes the ACC) and the amygdala networks. Zerbi’s finding together with other previous showing that chemogenetic activation of the LC→PFC pathway in neuropathic pain animals (without anxiety) increases anxiogenic-like behavior as well as spontaneous pain raise important questions about the involvement of the LC→ACC pathway in anxiety and that of non-eved pain in long-term neuropathic pain. Thus, experiments will be needed to determine if anxiety is triggered by different LC projections (LC→BLA and LC→ACC). Indeed, from a translational point of view it will be particularly relevant to determine if the blockade of β-adrenergic receptors, which has been shown to have an anxiolytic effect at BLA level also has a similar beneficial effect at the ACC level.

Pain induces plasticity in the brain and it potentiates communication between brain nuclei by enhancing specific pathways, at the same time silencing other nuclei and pathways. Our study shows short-term asymmetrical endogenous LC activation after nerve injury, as witnessed by the c-Fos activity, and by the fact that global LC and LC-SC pathway blockade attenuates injury-induced neuropathic pain ipsilaterally during the early post-injury period. However, this restorative LC analgesia fails over time, with the harmful bilateral activation of the LC and their projections to the rACC. These projections contribute to depression in the long-term through α1- and α2-adrenoceptor activity. These data suggest that pain-induced depression is not a direct consequence of the failure of the descending pain inhibition at LC level (LC→SC pathway). On the other hand, therapeutic approaches should focus on increasing noradrenaline exclusively at the SC level to avoid any deleterious effects at supraspinal sites. These findings explain why drugs that systemically increase noradrenaline availability are rarely used (e.g., reboxetine, desipramine), while drugs with complementary serotonergic or opioidergic actions (antidepressants (serotonin-noradrenaline reuptake inhibitors), tramadol, tapentadol) are more commonly chosen to treat pain or pain-depression comorbidity.

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**Competing interests**

The authors report no competing interests

**Supplementary material**

Supplementary material is available at *Brain* online.
References

1. Bravo L, Llorca-Torralba M, Suarez-Pereira I, Berrocoso E. Pain in neuropsychiatry: Insights from animal models. *Neurosci Biobehav Rev* 2020; 115: 96-115.

2. Doan L, Manders T, Wang J. Neuroplasticity underlying the comorbidity of pain and depression. *Neural Plast* 2015; 2015: 504691.

3. Millan MJ. Descending control of pain. *Prog Neurobiol* 2002; 66(6): 355-474.

4. Pertovaara A. Noradrenergic pain modulation. *Prog Neurobiol* 2006; 80(2): 53-83.

5. Hirschberg S, Li Y, Randall A, Kremer EJ, Pickering AE. Functional dichotomy in spinal- vs prefrontal-projecting locus coeruleus modules splits descending noradrenergic analgesia from ascending aversion and anxiety in rats. *Elife* 2017; 6.

6. Llorca-Torralba M, Borges G, Neto F, Mico JA, Berrocoso E. Noradrenergic Locus Coeruleus pathways in pain modulation. *Neuroscience* 2016; 338: 93-113.

7. Taylor BK, Westlund KN. The noradrenergic locus coeruleus as a chronic pain generator. *J Neurosci Res* 2017; 95(6): 1336-1346.

8. Bangasser DA, Valentino RJ. Sex differences in stress-related psychiatric disorders: neurobiological perspectives. *Front Neuroendocrinol* 2014; 35(3): 303-319.

9. Isingrini E, Perret L, Rainer Q, Amilhon B, Guma E, Tanti A et al. Resilience to chronic stress is mediated by noradrenergic regulation of dopamine neurons. *Nature neuroscience* 2016; 19(4): 560-563.

10. Zhang H, Chaudhury D, Nectow AR, Friedman AK, Zhang S, Juarez B et al. alpha1- and beta3-Adrenergic Receptor-Mediated Mesolimbic Homeostatic Plasticity Confers Resilience to Social Stress in Susceptible Mice. *Biological psychiatry* 2019; 85(3): 226-236.

11. Bernard R, Kerman IA, Thompson RC, Jones EG, Bunney WE, Barchas JD et al. Altered expression of glutamate signaling, growth factor, and glia genes in the locus coeruleus of patients with major depression. *Mol Psychiatry* 2011; 16(6): 634-646.

12. Ordway GA, Smith KS, Haycock JW. Elevated tyrosine hydroxylase in the locus coeruleus of suicide victims. *J Neurochem* 1994; 62(2): 680-685.

13. Belmaker RH, Agam G. Major depressive disorder. *N Engl J Med* 2008; 358(1): 55-68.
14. Perez-Caballero L, Torres-Sanchez S, Romero-Lopez-Alberca C, Gonzalez-Saiz F, Mico JA, Berrocoso E. Monoaminergic system and depression. Cell Tissue Res 2019; 377(1): 107-113.

15. Bouhassira D, Lanteri-Minet M, Attal N, Laurent B, Touboul C. Prevalence of chronic pain with neuropathic characteristics in the general population. Pain 2008; 136(3): 380-387.

16. Finnerup NB, Attal N, Haroutounian S, McNicol E, Baron R, Dworkin RH et al. Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. Lancet Neurol 2015; 14(2): 162-173.

17. Chandler DJ, Jensen P, McCall JG, Pickering AE, Schwarz LA, Totah NK. Redefining Noradrenergic Neuromodulation of Behavior: Impacts of a Modular Locus Coeruleus Architecture. The Journal of neuroscience : the official journal of the Society for Neuroscience 2019; 39(42): 8239-8249.

18. Uematsu A, Tan BZ, Ycu EA, Cuevas JS, Koivumaa J, Junyent F et al. Modular organization of the brainstem noradrenaline system coordinates opposing learning states. Nature neuroscience 2017; 20(11): 1602-1611.

19. Barthas F, Sellmeijer J, Hugel S, Waltisperger E, Barrot M, Yalcin I. The anterior cingulate cortex is a critical hub for pain-induced depression. Biological psychiatry 2015; 77(3): 236-245.

20. Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 1988; 33(1): 87-107.

21. Berrocoso E, De Benito MD, Mico JA. Role of serotonin 5-HT1A and opioid receptors in the antiallodynic effect of tramadol in the chronic constriction injury model of neuropathic pain in rats. Psychopharmacology (Berl) 2007; 193(1): 97-105.

22. Llorca-Torralba M, Pilar-Cuellar F, Bravo L, Bruzos-Cidon C, Torrecilla M, Mico JA et al. Opioid Activity in the Locus Coeruleus Is Modulated by Chronic Neuropathic Pain. Mol Neurobiol 2019; 56(6): 4135-4150.

23. Johansen JP, Fields HL, Manning BH. The affective component of pain in rodents: direct evidence for a contribution of the anterior cingulate cortex. Proc Natl Acad Sci U S A 2001; 98(14): 8077-8082.

24. David-Pereira A, Sagalajev B, Wei H, Almeida A, Pertovaara A, Pinto-Ribeiro F. The medullary dorsal reticular nucleus as a relay for descending pronociception induced by the mGluR5 in the rat infralimbic cortex. Neuroscience 2017; 349: 341-354.
25. Bravo L, Mico JA, Rey-Brea R, Perez-Nievas B, Leza JC, Berrocoso E. Depressive-like states heighten the aversion to painful stimuli in a rat model of comorbid chronic pain and depression. *Anesthesiology* 2012; 117(3): 613-625.

26. Berrocoso E, Mico JA, Vitton O, Ladure P, Newman-Tancredi A, Depoortere R *et al.* Evaluation of milnacipran, in comparison with amitriptyline, on cold and mechanical allodynia in a rat model of neuropathic pain. *Eur J Pharmacol* 2011; 655(1-3): 46-51.

27. Alba-Delgado C, Llorca-Torralba M, Horrillo I, Ortega JE, Mico JA, Sanchez-Blazquez P *et al.* Chronic pain leads to concomitant noradrenergic impairment and mood disorders. *Biological psychiatry* 2013; 73(1): 54-62.

28. Detke MJ, Rickels M, Lucki I. Active behaviors in the rat forced swimming test differentially produced by serotonergic and noradrenergic antidepressants. *Psychopharmacology (Berlin)* 1995; 121(1): 66-72.

29. Llorca-Torralba M, Suarez-Pereira I, Bravo L, Camarena-Delgado C, Garcia-Partida JA, Mico JA *et al.* Chemogenetic Silencing of the Locus Coeruleus-Basolateral Amygdala Pathway Abolishes Pain-Induced Anxiety and Enhanced Aversive Learning in Rats. *Biological psychiatry* 2019; 85(12): 1021-1035.

30. Alba-Delgado C, Llorca-Torralba M, Mico JA, Berrocoso E. The onset of treatment with the antidepressant desipramine is critical for the emotional consequences of neuropathic pain. *Pain* 2018; 159(12): 2606-2619.

31. Bravo L, Torres-Sanchez S, Alba-Delgado C, Mico JA, Berrocoso E. Pain exacerbates chronic mild stress-induced changes in noradrenergic transmission in rats. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology* 2014; 24(6): 996-1003.

32. Tervo DGR, Proskurin M, Manakov M, Kabra M, Vollmer A, Branson K *et al.* Behavioral variability through stochastic choice and its gating by anterior cingulate cortex. *Cell* 2014; 159(1): 21-32.

33. Viisanen H, Pertovaara A. Influence of peripheral nerve injury on response properties of locus coeruleus neurons and coeruleospinal antinociception in the rat. *Neuroscience* 2007; 146(4): 1785-1794.

34. McCall JG, Al-Hasani R, Siuda ER, Hong DY, Norris AJ, Ford CP *et al.* CRH Engagement of the Locus Coeruleus Noradrenergic System Mediates Stress-Induced Anxiety. *Neuron* 2015; 87(3): 605-620.
35. Sciolino NR, Plummer NW, Chen YW, Alexander GM, Robertson SD, Dudek SM et al. Recombinase-Dependent Mouse Lines for Chemogenetic Activation of Genetically Defined Cell Types. *Cell Rep* 2016; 15(11): 2563-2573.

36. Hayashida K, Clayton BA, Johnson JE, Eisenach JC. Brain derived nerve growth factor induces spinal noradrenergic fiber sprouting and enhances clonidine analgesia following nerve injury in rats. *Pain* 2008; 136(3): 348-355.

37. Hughes SW, Hickey L, Hulse RP, Lumb BM, Pickering AE. Endogenous analgesic action of the pontospinal noradrenergic system spatially restricts and temporally delays the progression of neuropathic pain following tibial nerve injury. *Pain* 2013; 154(9): 1680-1690.

38. Ma W, Eisenach JC. Chronic constriction injury of sciatic nerve induces the up-regulation of descending inhibitory noradrenergic innervation to the lumbar dorsal horn of mice. *Brain Res* 2003; 970(1-2): 110-118.

39. Brightwell JJ, Taylor BK. Noradrenergic neurons in the locus coeruleus contribute to neuropathic pain. *Neuroscience* 2009; 160(1): 174-185.

40. Marques-Lopes J, Pinho D, Albino-Teixeira A, Tavares I. The hyperalgesic effects induced by the injection of angiotensin II into the caudal ventrolateral medulla are mediated by the pontine A5 noradrenergic cell group. *Brain research* 2010; 1325: 41-52.

41. Martins I, Carvalho P, de Vries MG, Teixeira-Pinto A, Wilson SP, Westerink BH et al. Increased noradrenergic neurotransmission to a pain facilitatory area of the brain is implicated in facilitation of chronic pain. *Anesthesiology* 2015; 123(3): 642-653.

42. Martin JH. Autoradiographic estimation of the extent of reversible inactivation produced by microinjection of lidocaine and muscimol in the rat. *Neuroscience letters* 1991; 127(2): 160-164.

43. Kaushal R, Taylor BK, Jamal AB, Zhang L, Ma F, Donahue R et al. GABA-A receptor activity in the noradrenergic locus coeruleus drives trigeminal neuropathic pain in the rat; contribution of NAalpha1 receptors in the medial prefrontal cortex. *Neuroscience* 2016; 334: 148-159.

44. Shackman AJ, Salomons TV, Slagter HA, Fox AS, Winter JJ, Davidson RJ. The integration of negative affect, pain and cognitive control in the cingulate cortex. *Nature reviews Neuroscience* 2011; 12(3): 154-167.

45. Mayberg HS, Liotti M, Brannan SK, McGinnis S, Mahurin RK, Jerabek PA et al. Reciprocal limbic-cortical function and negative mood: converging PET findings in depression and normal sadness. *Am J Psychiatry* 1999; 156(5): 675-682.
46. Sellmeijer J, Mathis V, Hugel S, Li XH, Song Q, Chen QY et al. Hyperactivity of Anterior Cingulate Cortex Areas 24a/24b Drives Chronic Pain-Induced Anxio depressive-like Consequences. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2018; 38(12): 3102-3115.

47. Suto T, Eisenach JC, Hayashida K. Peripheral nerve injury and gabapentin, but not their combination, impair attentional behavior via direct effects on noradrenergic signaling in the brain. *Pain* 2014; 155(10): 1935-1942.

48. Koga K, Yamada A, Song Q, Li XH, Chen QY, Liu RH et al. Ascending noradrenergic excitation from the locus coeruleus to the anterior cingulate cortex. *Mol Brain* 2020; 13(1): 49.

49. Zhang Z, Cordeiro Matos S, Jego S, Adamantidis A, Seguela P. Norepinephrine drives persistent activity in prefrontal cortex via synergistic alpha1 and alpha2 adrenoceptors. *PLoS One* 2013; 8(6): e66122.

50. Zerbi V, Floriou-Servou A, Markicevic M, Vermeiren Y, Sturman O, Privitera M et al. Rapid Reconfiguration of the Functional Connectome after Chemogenetic Locus Coeruleus Activation. *Neuron* 2019; 103(4): 702-718 e705.

51. Apkarian AV, Hashmi JA, Baliki MN. Pain and the brain: specificity and plasticity of the brain in clinical chronic pain. *Pain* 2011; 152(3 Suppl): S49-64.

52. Bravo L, Llorca-Torralba M, Berrocoso E, Mico JA. Monoamines as Drug Targets in Chronic Pain: Focusing on Neuropathic Pain. *Front Neurosci* 2019; 13: 1268.

53. Hughes S, Hickey L, Donaldson LF, Lumb BM, Pickering AE. Intrathecal reboxetine suppresses evoked and ongoing neuropathic pain behaviours by restoring spinal noradrenergic inhibitory tone. *Pain* 2015; 156(2): 328-334.

54. Mico JA, Ardid D, Berrocoso E, Eschalier A. Antidepressants and pain. *Trends Pharmacol Sci* 2006; 27(7): 348-354.
Figure legends

Figure 1. Sensory and affective characterization of the development of neuropathic pain. (A) Scheme of the chronic constriction injury (CCI) model, timeline and graphs showing the response of the ipsilateral and contralateral paw in the acetone test and the predominant behavior (immobility, IM; climbing, CL; swimming, SW) in the forced swimming test (FST) in the short-term (ST), mid-term (MT) and long-term (LT) after CCI in wild-type rats (n=10 animals/group): *p<0.05, **p<0.01, ***p<0.001 vs Sham, Student’s t-test for each time point (ST, MT and LT). d, days.

Figure 2. Effect of chemogenetic inhibition and activation of ipsilateral and contralateral LC on nociceptive behavior after nerve-injury. (A) Scheme of noradrenergic LC inhibition or activation using the DREADD strategy in TH:Cre rats and representative immunohistofluorescence image (scale bar = 10 µm) of mCherry expression in noradrenergic LC neurons (red = mCherry, green = DBH). (B) Response of the ipsilateral paw of CCI-ST, -MT and -LT rats in the acetone test after ipsilateral LC (LC_ipsi) noradrenergic inhibition by CNO (1 mg/kg, i.p.) (n = 8-10 animals/group: ***p<0.001 vs Sham-Sal; +++p<0.01 vs CCI-Sal). (C) Response of the ipsilateral paw to thermal stimulus 20 and 360 min after LC_ipsi inhibition by CNO (1 mg/kg, i.p.) (n = 9 animals/group: +++p<0.001 vs CCI-Sal). (D) Response of the ipsilateral paw of CCI-ST, -MT and -LT rats in the acetone test after noradrenergic contralateral LC (LC_contra) inhibition by CNO (1 mg/kg, i.p.) (n = 9-10 animals/group: ***p<0.001 vs Sham-Sal). (E) Response of the ipsilateral paw of CCI-ST, -MT and -LT rats in the acetone and von Frey tests after noradrenergic LC_ipsi activation by CNO (1 mg/kg, i.p.) (n = 9-10 animals/group: ***p<0.001 vs Sham-Sal; +p<0.05, ++p<0.01, +++p<0.001 vs CCI-Sal). (F) Response of the ipsilateral paw of CCI-ST, -MT and -LT rats in the acetone and von Frey tests after noradrenergic LC_contra activation by CNO (1 mg/kg, i.p.) (n = 10 animals/group: ***p<0.001 vs Sham-Sal). One-way or two-way ANOVA with repeated measures, Newman-Keuls post-hoc test. CCI, chronic constriction injury; ST, short-term, MT, mid-term; LT, long-term; Sal, saline; CNO, Clozapine-n-oxide; LC, locus coeruleus; i.p., intraperitoneal; Ac, acetone test; CP, Cold Plate test; VF, von Frey test; d, days; DBH, dopamine beta-hydroxylase.
Figure 3. Effect of chemogenetic inhibition and activation of ipsilateral and contralateral LC on depressive-like behavior after long-term neuropathy. (A) Timeline of noradrenergic LC inhibition or activation using the DREADD strategy in TH:Cre rats. (B) Representative image of noradrenergic LC_{ipsi} or LC_{contra} inhibition and predominant behavior in the FST after CNO (1 mg/kg, i.p.) (n = 8-10 animals/group: **p<0.001 vs Sham-Sal; ++p<0.01, +++p<0.001 vs CCI-Sal). (C) Representative image of noradrenergic LC_{ipsi} or LC_{contra} activation and predominant behavior in the FST after CNO (1 mg/kg, i.p.) (n = 8-10 animals/group: **p<0.001, ***p<0.001 vs Sham-Sal). Two-way ANOVA, Newman-Keuls post-hoc test. CCI, chronic constriction injury; FST, forced swimming test; IM, immobility; CL, climbing; SW, swimming; Sal, saline; CNO, Clozapine-n-oxide; LC, locus coeruleus; i.p., intraperitoneal; d, days.

Figure 4. Study of the LC projections to the SC. (A-B) Retrograde FG tracer strategy and representative image (scale bar = 500 μm) to target LC neurons that project to the ipsilateral SC in wild-type rats. Ipsilateral and contralateral distribution of FG labeled LC neurons and FG positive neurons along the rostral-caudal axis of the ipsilateral (LC_{ipsi}) and contralateral (LC_{contra}) LC (mean + SEM of the number of neurons per slice, n = 4 animals: Student’s t-test and one-way ANOVA, Newman-Keuls post-hoc test). (C) Representative immunofluorescence of the central LC_{ipsi} and LC_{contra} showing the dorso-ventral aspect (half height relative to DBH expression) (scale bar = 200 μm). The graphs show the dorso-ventral distribution of the central LC_{ipsi} and LC_{contra} projections to the SC_{ipsi} (n = 4 animals: ***p<0.001 vs Ventral LC, Student’s t-test). (D) Representative immunofluorescence of the central LC showing the DBH, c-Fos, FG expression and merged image. The inset shows an example of c-Fos+FG positive neurons (scale, bar = 100 μm). (E-F) Numbers of c-Fos and c-Fos+FG positive neurons of central LC_{ipsi} and LC_{contra}, as well as the dorso-ventral distribution in Sham and CCI-ST rats (mean + SEM of the number of neurons per slice, n = 6 animals/group: *p<0.05, ***p<0.001 vs Sham; #p<0.05, ##p<0.01 vs LC_{ipsi}, two-way ANOVA, Newman-Keuls post-hoc test; and ***p<0.001 vs Ventral LC, Student’s t-test). (G) Numbers of c-Fos positive neurons of central LC_{ipsi} and LC_{contra}, as well as the dorso-ventral distribution in Sham and CCI-LT rats (mean + SEM neurons per slice, n = 4 animals/group: ***p<0.001 vs Sham, two-way ANOVA, Newman-Keuls post-hoc test; and ***p<0.001 vs Ventral LC, Student’s t-test). CCI, chronic constriction injury;
LC, locus coeruleus; SC, spinal cord; FG, Fluoro-Gold (yellow); DBH, dopamine beta-hydroxylase (green); c-Fos (red).

**Figure 5. Effect of chemogenetic inhibition of the LC_{ipsi/contra}→SC pathway on the time-course of nociceptive behavior after nerve-injury.** (A-B) Timeline and representative image of noradrenergic LC_{ipsi}→SC or LC_{contra}→SC pathway inhibition using the AAV-Gi-mCherry strategy in TH:Cre rats. Response of the ipsilateral paw in the acetone, von Frey and cold plate tests after CNO (3 μM, i.t) administration to rats in the short-term (ST), mid-term (MT) and long-term (LT) after CCI (n = 4 animals/group: **p<0.01, ***p<0.001 vs Sham-Sal; +p<0.05, +++p<0.001 vs CCI-Sal, two-way ANOVA with repeated measures, Newman-Keuls post-hoc test). CCI, chronic constriction injury; LC, locus coeruleus; SC, spinal cord; Sal, saline; CNO, clozapine-n-oxide; i.t., intrathecal; Ac, acetone test; VF, von Frey test; CP, cold plate test; d, days.

**Figure 6. Effect of chemogenetic activation of the LC_{ipsi/contra}→SC pathway on the time-course of nociceptive behavior after nerve-injury.** (A-B) Timeline and representative image of noradrenergic LC_{ipsi}→SC or LC_{contra}→SC pathway activation using the AAV-Gs-mCherry strategy in LE-TH:Cre rats. (A) Response of the ipsilateral paw in the acetone, von Frey and cold plate tests after CNO (3 μM, i.t) administration to rats in the short-term (ST), mid-term (MT) and long-term (LT) after CCI (n= 6-8 animals/group: ***p<0.001 vs Sham-Sal; +p<0.05, ++p<0.01, +++p<0.001 vs CCI-Sal). (B) Response of the ipsilateral paw of CCI-ST, -MT and -LT rats in the acetone and von Frey tests after CNO (3 μM, i.t) administration (n= 6 animals/group: ***p<0.001 vs Sham-Sal). In addition, the response of the ipsilateral paw of CCI-LT rats was evaluated in the acetone and von Frey tests after clonidine (20 μg, i.t) administration (n= 5-6 animals/group: ***p<0.001 vs Sham-Sal; +p<0.05, +++p<0.001 vs CCI-Sal). Two-way ANOVA with or without repeated measures, Newman-Keuls post-hoc test). CCI, chronic constriction injury; LC, locus coeruleus; SC, spinal cord; Sal, saline; CNO, clozapine-n-oxide; Cloni, clonidine; i.t., intrathecal; Ac, acetone test; VF, von Frey test; CP, cold plate test; d, days.
Figure 7. Neuroanatomical study of the LC projections to the rACC. (A-B) Retrograde FG tracer strategy to target LC neurons that project to the ipsilateral rACC in wild-type rats and a representative image (scale bar = 500 μm). The ipsilateral and contralateral distribution of FG labeled LC neurons, and of FG positive neurons along the rostral-caudal axis of ipsilateral (LC\textsubscript{ipsi}) and contralateral (LC\textsubscript{contra}) LC (mean + SEM of the number of neurons per slice, n = 4 animals: ***p<0.001 vs Ipsi, Student’s t-test; one-way ANOVA, Newman-Keuls post-hoc test). (C-D) Retrograde FG tracer strategy to target LC neurons that project to the bilaterally rACC and representative image (scale bar = 500 μm). Ipsilateral and contralateral distribution of FG labeled LC neurons and FG positive neurons along the rostral-caudal axis of LC\textsubscript{ipsi} and LC\textsubscript{contra} (mean + SEM of the number of neurons per slice, n = 4 animals: Student’s t-test and one-way ANOVA, Newman-Keuls post-hoc test). (E) Representative immunofluorescence images of central LC\textsubscript{ipsi} and LC\textsubscript{contra} (~9.84 from Bregma: Paxinos and Watson, 2007) showing the dorso-ventral aspect (half height relative to DBH expression) (scale bar = 100 µm). The graphs show the dorso-ventral distribution of the central LC\textsubscript{ipsi} and LC\textsubscript{contra} projection to the rACC (n = 4 animals: ***p<0.001 vs Ventral LC, Student’s t-test). (F) Representative immunofluorescence of the central LC showing the DBH, c-Fos, FG expression and merged image (c-Fos+FG). The inset shows an example of c-Fos+FG positive neurons (scale, bar = 100 µm). (G-H) Numbers of c-Fos and c-Fos+FG positive neurons of central LC\textsubscript{ipsi} and LC\textsubscript{contra} projection to the rACC (n = 4 animals/group: ***p<0.001 vs Sham, two-way ANOVA, Newman-Keuls post-hoc test; and ***p<0.001 vs Ventral LC, Student’s t-test. CCI, chronic constriction injury; LT, Long-term; LC, locus coeruleus; rACC, rostral Anterior Cingulate Cortex; FG, Fluoro-Gold (yellow); DBH, dopamine beta-hydroxylase (green); c-Fos (red).

Figure 8. Effect of the chemogenetic inhibition of the LC-rACC pathway and the pharmacological blockade of adrenoreceptor activity in the rACC after long-term neuropathy. (A) Timeline and cartoon of the bilateral AAV-Gi-mCherry injection into the LC, and intra-rACC CNO (3 μM) administration to inhibit the noradrenergic LC→rACC pathway of TH:Cre rats. Response of the ipsilateral hindpaw of CCI-LT rats in the acetone, von Frey and cold plate tests. The predominant behavior in the forced swimming test (FST: immobility, IM; climbing, CL; swimming, SW) was evaluated (n=
8-10 animals/group: **p<0.01, ***p<0.001 vs Sham-Sal; ++p<0.01 vs CCI-Sal, two-way ANOVA, Newman-Keuls post-hoc test). (B-D) Timeline and cartoon of the pharmacological inhibition of \( \alpha_1 \)-adrenoceptors (prazosin, 5 \( \mu \)g; \( n = 8-10 \) animals/group), \( \alpha_2 \)-adrenoceptors (idazoxan, 9 \( \mu \)g; \( n = 6-10 \) animals/group) or \( \beta \)-adrenoceptors (propranolol, 1 \( \mu \)g; \( n = 7-10 \) animals/group) in the rACC of CCI-LT wild-type rats: **p<0.01, ***p<0.001 vs Sham-Sal; ++p<0.01, +++p<0.001 vs CCI-Sal, two-way ANOVA, Newman-Keuls post-hoc test. CCI, chronic constriction injury; LC, locus coeruleus; rACC, rostral anterior cingulate cortex; Sal, saline; CNO, Clozapine-N-oxide; Praz, prazosin; Idx, Idazoxan; Prop, propranolol; Ac, acetone test; VF, von Frey test; CP, cold plate test; LT, Long-term; d, days.
Figure 1

222x90mm (150 x 150 DPI)
Figure 2

204x200mm (150 x 150 DPI)
Figure 3

150x122mm (150 x 150 DPI)
Figure 4

195x203mm (150 x 150 DPI)
Figure 5

177x162mm (150 x 150 DPI)
Figure 6

190x160mm (150 x 150 DPI)
Figure 7
190x256mm (150 x 150 DPI)
Figure 8

185x238mm (150 x 150 DPI)