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

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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 neurons relative to the site of injury: ipsilateral (LC_{ipsi}) or contralateral (LC_{contra}) locus coeruleus 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 behaviour 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 locus coeruleus modules was also evident as neuropathy develops. Hence, chemogenetic blockade of the $LC_{ipsi} \rightarrow$ 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 locus coeruleus-rostral anterior cingulate cortex pathway or the intra-rostral anterior cingulate cortex antagonism of alpha1- and alpha2-adrenoreceptors reversed long-term pain-induced depression. Furthermore, chemogenetic locus coeruleus 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 locus coeruleus modules promotes early restorative analgesia, as well as late depressive-like behaviour in chronic pain and depression comorbidity.

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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 pronociceptive 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 past 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_{ipsi}) or contralateral (LC_{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 and rostral anterior cingulate cortex (rACC).[6,19]

Materials and methods

A detailed description of the experimental procedure is provided in the Supplementary material.

Animals and pain model

Male transgenic Long–Evans tyrosine hydroxylase:Cre (TH:Cre) and wild-type Long–Evans rats (350–450 g) 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 tracer into rACC (AP: +3.0 mm, ML: ±1.0 mm and DV: 1.2 mm)[23] or spinal cord (L4–L6 segments).[24] See methodological validation of DREADD approach in Llorca-Torralba *et al.*[22] and Supplementary Figs. 1, 2 and 11.

Behavioural 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 centre of the ipsilateral and contralateral hind paw with a pipette. The acetone was applied four times to each hind paw alternately at 5-min 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; and 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), 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.) 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

Depressive-like behaviour was evaluated in the forced swimming test (FST) over two different sessions, a 15-min pretest was followed by a 5-min test performed 24 h later. The predominant behaviours were recorded (climbing, swimming, or immobility), 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, AU, %) was measured as an indicator of locomotor activity.[22]

Immunohistochemistry and histology

Immunohistochemistry was performed to evaluate the expression of DREADDs-mCherry, Fluoro-Gold tracer and c-fos in the LC, as well as dopamine beta-hydroxylase (DBH) in the spinal cord. The cannula placement in the rACC and spinal cord 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 ± standard error of the mean (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 on request to the corresponding author.

Results

Analgesia is driven by the activation of noradrenergic-LC$_{ipsi}$ 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: $P < 0.001$ versus sham; Fig. 1). By contrast, depressive-like behaviour was only observed in CCI-LT animals (immobility and climbing: $P < 0.01$ and $P < 0.05$ versus 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$_{ipsi}$ or LC$_{contra}$ 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$_{ipsi}$ blockade only significantly enhanced hypersensitivity in the ipsilateral hindpaw in the CCI-ST animals (acetone test: $P < 0.001$

versus the CCI-ST-saline; 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 min (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$_{contra}$ 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 and F and Supplementary Fig. 1E–H). CNO activation of the LC$_{ipsi}$ significantly reduced CCI evoked thermal and mechanical hypersensitivity in the ST, MT and LT animals relative to their respective CCI-saline 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$_{contra}$ neurons did not significantly modify CCI-induced pain at any time point (Fig. 2F). Furthermore, chemogenetic LC$_{ipsi/contra}$ 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$_{ipsi}$ or LC$_{contra}$ neurons relieves pain-induced depression

As long-term pain induces depressive-like behaviour (Fig. 1), we used the same chemogenetic approaches to explore the effect of silencing or activating the LC$_{ipsi}$ or LC$_{contra}$ in depressive CCI-LT rats. Unlike the sensory tests, chemogenetic silencing the LC$_{ipsi}$ or LC$_{contra}$ had a marked effect on emotional behaviour, reflected by the significant decrease in immobility (LC$_{ipsi}$ and LC$_{contra}$ $P < 0.001$) and the increased climbing (LC$_{ipsi}$ $P < 0.01$ and LC$_{contra}$ $P < 0.001$) behaviour of these animals (Fig. 3A and B). Alternatively, blocking either LC$_{ipsi}$ or LC$_{contra}$ had no effect on sham animals (Fig. 3A and B), although significant depressive behaviour was triggered in sham animals by rM3D(Gs)-DREADD LC activation (LC$_{ipsi}$ $P < 0.01$ and LC$_{contra}$ $P < 0.001$ versus sham-saline; 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 behaviours evaluated were affected by any locomotor dysfunction (Supplementary Fig. 4 A and B). In addition, a bilateral activation of the LC was evident through the increase in the rate-limiting enzyme in noradrenaline biosynthesis, TH (LC$_{ipsi}$ and LC$_{contra}$ $P < 0.05$ versus sham), and that of phosphorylated CREB (LC$_{ipsi}$ $P = 0.07$ and LC$_{contra}$ $P < 0.01$ versus sham; Supplementary Fig. 5).

Activation of the noradrenergic-LC$_{ipsi}$→spinal cord 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 spinal cord influences the pain-related phenotype. Unilateral administration of the retrograde fluorescent tracer Fluoro-Gold into the superficial dorsal horn of the ipsilateral spinal cord labelled a similar number of neurons in the LC$_{ipsi}$ and LC$_{contra}$ along the rostro-caudal axis, these neurons predominantly located in the central LC (Fig. 4A and B and Supplementary Fig. 6A). Hence, the LC appears to project bilaterally to the spinal cord and most of the spinally projecting neurons (around 80%) are situated in the ventral aspect of the nucleus (Fig. 4C).

In another experiment set, Fluoro-Gold was injected unilaterally into the ipsilateral spinal cord of CCI-ST and CCI-LT rats, and
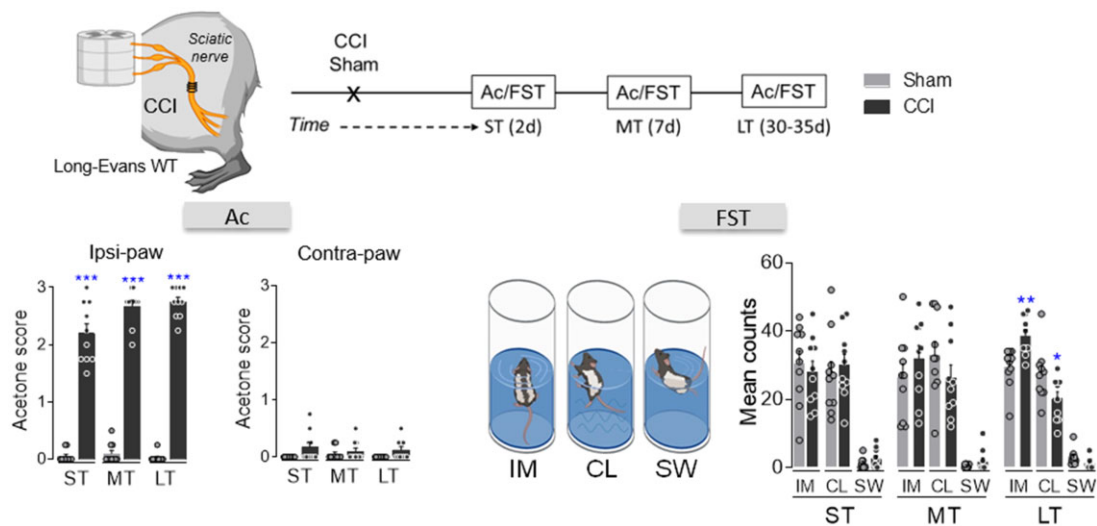
Figure 1 Sensory and affective characterization of the development of neuropathic pain. Scheme of the CCI model, timeline and graphs showing the response of the ipsilateral and contralateral paw in the acetone test and the predominant behaviour (immobility, IM; climbing, CL; swimming, SW) in the FST in the short (ST), mid (MT) and long term (LT) after CCI in wild-type rats ($n = 10$ animals/group): $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus sham, Student's t-test for each time point (ST, MT and LT). Ac = acetone; d = days; WT = wild-type.

the expression of c-Fos in the LC was assessed (Fig. 4D–G). Nerve injury significantly increased the number of c-Fos labelled neurons in LC of CCI-ST rats bilaterally relative to the sham-ST rats (LC_{ipsi}: $P < 0.001$ and LC_{contra}: $P < 0.001$; Fig. 4E). Importantly, LC_{ipsi} c-Fos expression was significantly higher than that in the LC_{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 labelled neurons were preferentially located in the dorsal pole of the LC ($\sim$90%, Fig. 4E). When exploring which c-Fos neurons also expressed Fluoro-Gold, we found that there was only a small population in the dorsal LC_{ipsi} pole of the CCI-ST rats ($P < 0.05$ versus sham; Fig. 4F). However, c-Fos/Fluoro-Gold colabelling was not found in either sham-LC_{ipsi}/LC_{contra} or CCI-ST LC_{contra} (Fig. 4F). Regarding long-term pain, significantly stronger c-Fos expression was evident in the dorsal LC_{ipsi} and LC_{contra} of CCI-LT animals relative to the respective LC area in the sham rats (LC_{ipsi} $P < 0.001$ and LC_{contra} $P < 0.001$), although there was no difference between sides (Fig. 4G). Moreover, no c-Fos/Fluoro-Gold colabelled LC neurons projected to the spinal cord in CCI-LT rats. In CCI-ST and -LT rats, the expression of the noradrenaline synthesizing enzyme 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, $P < 0.05$ versus sham; Supplementary Fig. 7A and B). By contrast, there was a loss of noradrenergic fibres after CCI-LT in the L6 segment (LT, $P < 0.05$ versus sham animals; Supplementary Fig. 7A). These data demonstrate lateralized changes in the activation of the LC→spinal cord pathway along neuropathy development.

DREADD-mediated inactivation of the noradrenergic-LC_{ipsi} or $LC_{contral}$→spinal cord pathway was explored behaviourally and like the global LC blockade (Fig. 2B and D), intrathecal CNO administration following hM4D(Gi)-DREADD administration to the LC_{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-saline

animals ($P < 0.001$). When exploring the outcome of blocking the LC_{contra}→spinal cord pathway, no significant changes in evoked pain responses were observed (Fig. 5B). Like after global LC_{ipsi} or LC_{contra} inhibition, no significant effect was found in sham animals or in the response of the contralateral hindpaw of CCI animals after blocking the LC_{ipsi} or LC_{contra}→spinal cord pathway (Fig. 5A and B and Supplementary Fig. 8A and B).

The rM3D(Gs)-DREADD activation of the noradrenergic-LCipsi or LC_{contra}→spinal cord pathway was also explored. Like global LC activation (Fig. 2E and F), DREADD-mediated activation of noradrenergic-LCipsi neurons projecting to the spinal cord 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 LC_{contra} to spinal cord 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 $P < 0.05$ versus CCI-LT-saline; Fig. 6B). Finally, and like after global LC_{ipsi} or LC_{contra} activation, no change was found in sham animals or in the contralateral hindpaws of CCI animals (Fig. 6A and 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 rACC.[19] Administration of Fluoro-Gold into the ipsilateral rACC produced abundant labelling of neurons in the LC_{ipsi} ($\approx$80%), suggesting that the projection of the LC to the rACC is lateralized. Most marked neurons were located in the central LC along the rostro-caudal axis (Fig. 7A and B and Supplementary Fig. 6B). Bilateral administration of Fluoro-Gold to the rACC was also performed and as expected, there was a similar distribution of Fluoro-Gold-positive neurons bilaterally in the LC and preferentially in the dorsal pole (Fig. 7C–E and Supplementary Fig. 6C). The expression of c-Fos in the LC neurons of LT animals was also explored (Fig. 7F–H). As found previously (Fig. 4G), there was a similar bilateral increase in neurons
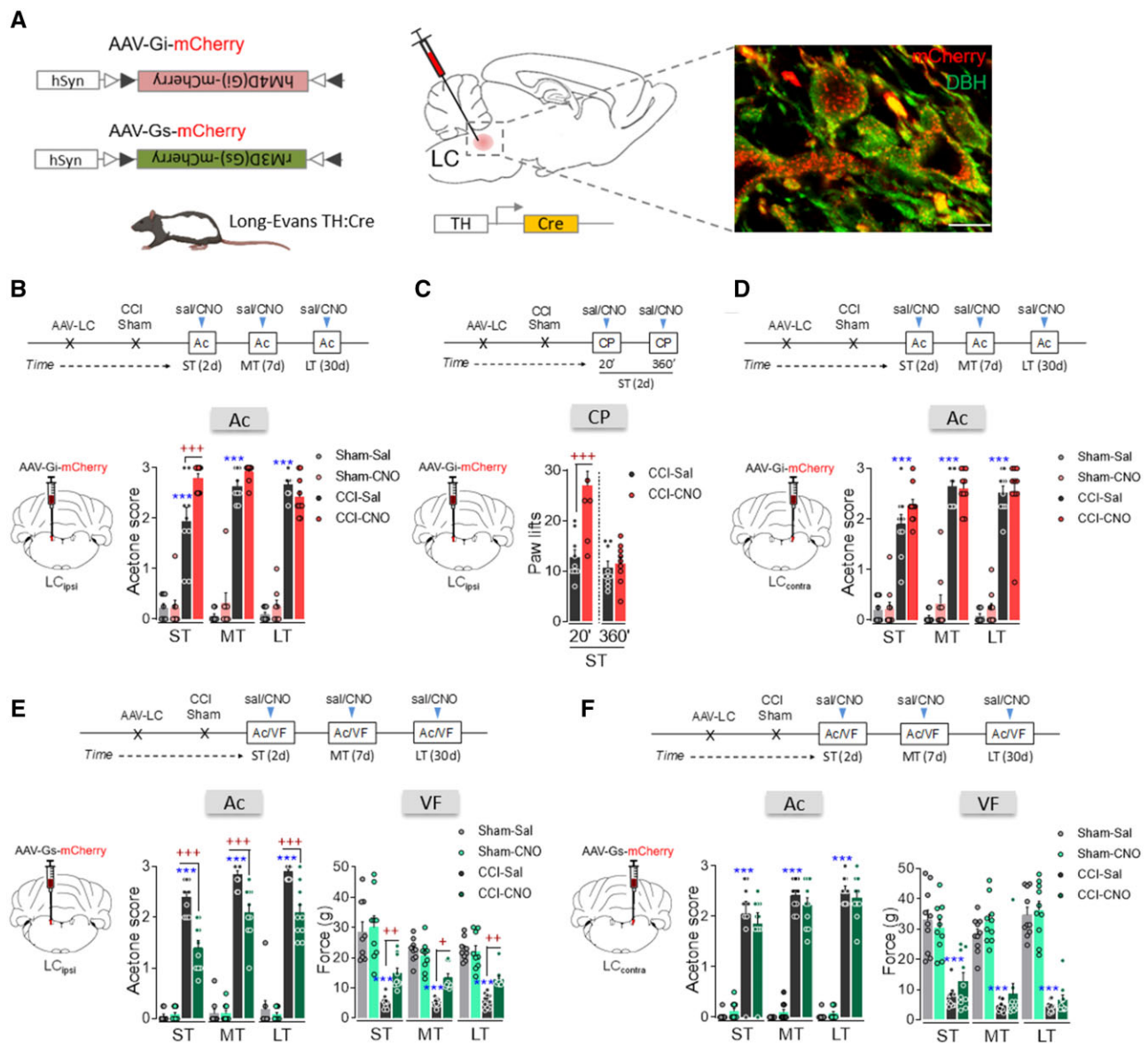
Figure 2 Effect of chemogenetic inhibition and activation of ipsilateral and contralateral LC on nociceptive behaviour after nerve injury. (A) Scheme of noradrenergic-LC inhibition or activation using the DREADD strategy in TH:Cre rats and representative immunohistofluorescence image (scale bar = 10 μm) of mCherry expression in noradrenergic-LC neurons (red = mCherry, green = DBH). **(B)** Response of the ipsilateral paw of CCI-ST, -MT and -LT rats in the acetone test after ipsilateral LC (LC$_{ipsi}$) noradrenergic inhibition by CNO (1 mg/kg, i.p.) (n = 8–10 animals/group: ***$P < 0.001$ versus sham-sal; +++$P < 0.01$ versus 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: +++$P < 0.001$ versus 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: ***$P < 0.001$ versus sham-sal). **(E)** Response of the ipsilateral paw of CCI-ST, -MT and -LT rats in the acetone and von Frey tests after noradrenergic-LCipsi activation by CNO (1 mg/kg, i.p.) (n = 9–10 animals/group: ***$P < 0.001$ versus sham-sal; +$P < 0.05$, ++$P < 0.01$, +++$P < 0.001$ versus 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: ***$P < 0.001$ versus sham-sal). One- or two-way ANOVA with repeated measures, Newman–Keuls *post hoc* test. Ac = acetone test; CP = cold plate test; d = days; i.p. = intraperitoneal; VF = von Frey test.

expressing c-Fos in the dorsal LC pole of CCI-LT rats (LC$_{ipsi}$ and LC$_{contra}$ $P < 0.001$ versus sham: Fig. 7G). Following bilateral administration of the Fluoro-Gold tracer to the rACC, >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 overactivation 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 and B). However, a reversion of the depressive phenotype was observed in CCI-LT rats (immobility and climbing $P < 0.01$ versus CCI-saline; Fig. 8A). No impairment of spontaneous locomotion was found (Supplementary Fig. 10C).
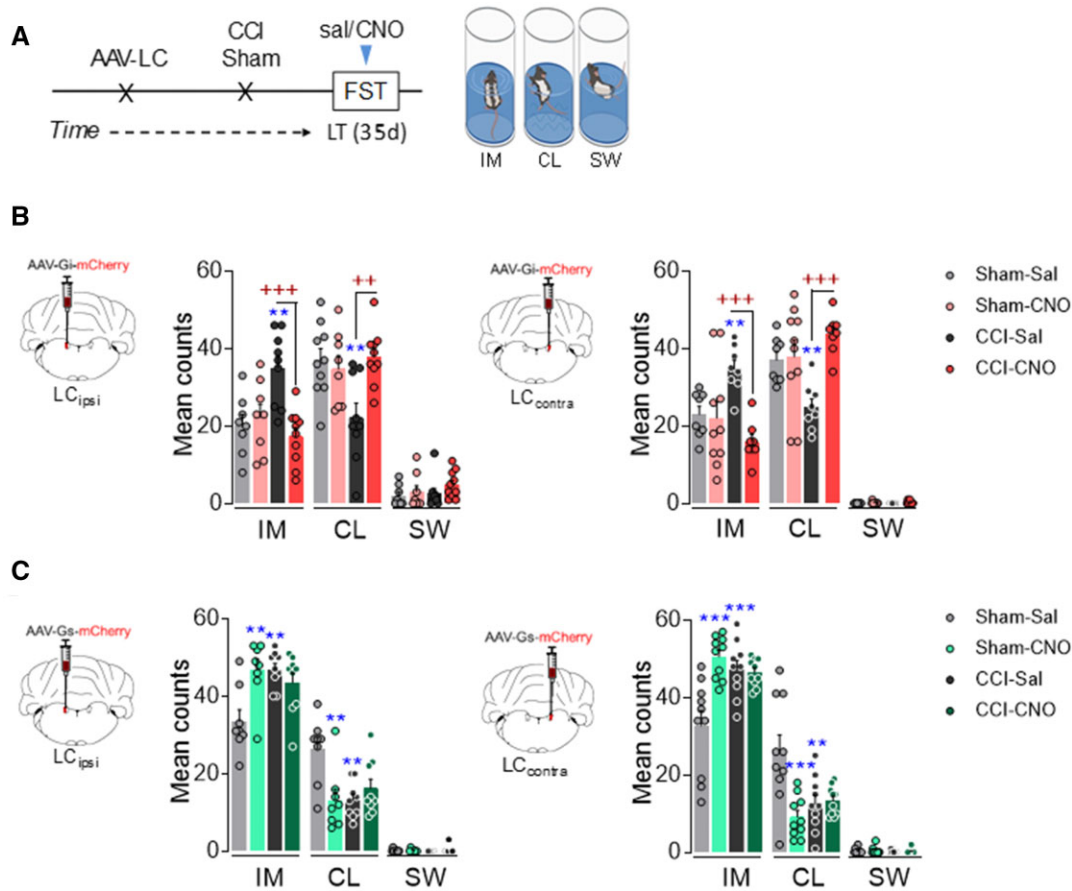
Figure 3 Effect of chemogenetic inhibition and activation of ipsilateral and contralateral LC on depressive-like behaviour after long-term neuropathy. (**A**) Timeline of noradrenergic-LC inhibition or activation using the DREADD strategy in TH:Cre rats. (**B**) Representative image of noradrenergic-LC$_{ipsi}$ or LC$_{contra}$ inhibition and predominant behaviour in the FST after CNO (1 mg/kg, i.p.) (n = 8–10 animals/group: **$P < 0.001$ versus sham-sal; ++$P < 0.01$, +++$P < 0.001$ versus CCI-sal). (**C**) Representative image of noradrenergic-LC$_{ipsi}$ or LC$_{contra}$ activation and predominant behaviour in the FST after CNO (1 mg/kg, i.p.) (n = 8–10 animals/group: **$P < 0.001$, ***$P < 0.001$ versus sham-sal). Two-way ANOVA, Newman–Keuls *post hoc* test. CL = climbing; d = days; IM = immobility; i.p. = intraperitoneal; sal = saline; SW = swimming.

In addition, reduced c-Fos expression (LC$_{ipsi}$ and LC$_{contra}$ $P < 0.001$ versus CCI-sal: Supplementary Fig. 11A–C) and reduced c-Fos/Fluoro-Gold colabelling (LC$_{ipsi}$ $P < 0.01$ and LC$_{contra}$ $P < 0.05$ versus CCI-saline; Supplementary Fig. 11D) was observed in the LC after chemogenetic inhibition of the LC→rACC pathway in CCI-LT rats.

Blockade of α-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 α1-adrenoreceptor antagonist prazosin (5 μg/0.5 μl), the α2-adrenoceptor antagonist idazoxan (9 μg/0.5 μl) or the β-adrenoreceptor antagonist propranolol (1 μg/0.5 μl) intra-rACC. Prazosin and idazoxan administration reversed depressive-like behaviour (prazosin: immobility and climbing $P < 0.01$, idazoxan: immobility and climbing $P < 0.001$ versus CCI-saline), although they did not change pain behaviours (acetone, von Frey and cold plate tests; Fig. 8B and C and Supplementary Fig. 12A–F). However, propranolol failed to modify pain- or depression-related behaviours in CCI-LT rats although it significantly increased depressive-like behaviour in sham (immobility and climbing $P < 0.01$ versus sham-saline; Fig. 8D and Supplementary Fig. 12G–I). Locomotor activity was not modified in any of the groups of rats evaluated (Supplementary

Fig. 12B, E and H). These data indicate an involvement of α-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 behavioural 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 signalling 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 and long term. However, the expression of c-Fos is higher in the ipsilateral side of the LC than on the contralateral side in the 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
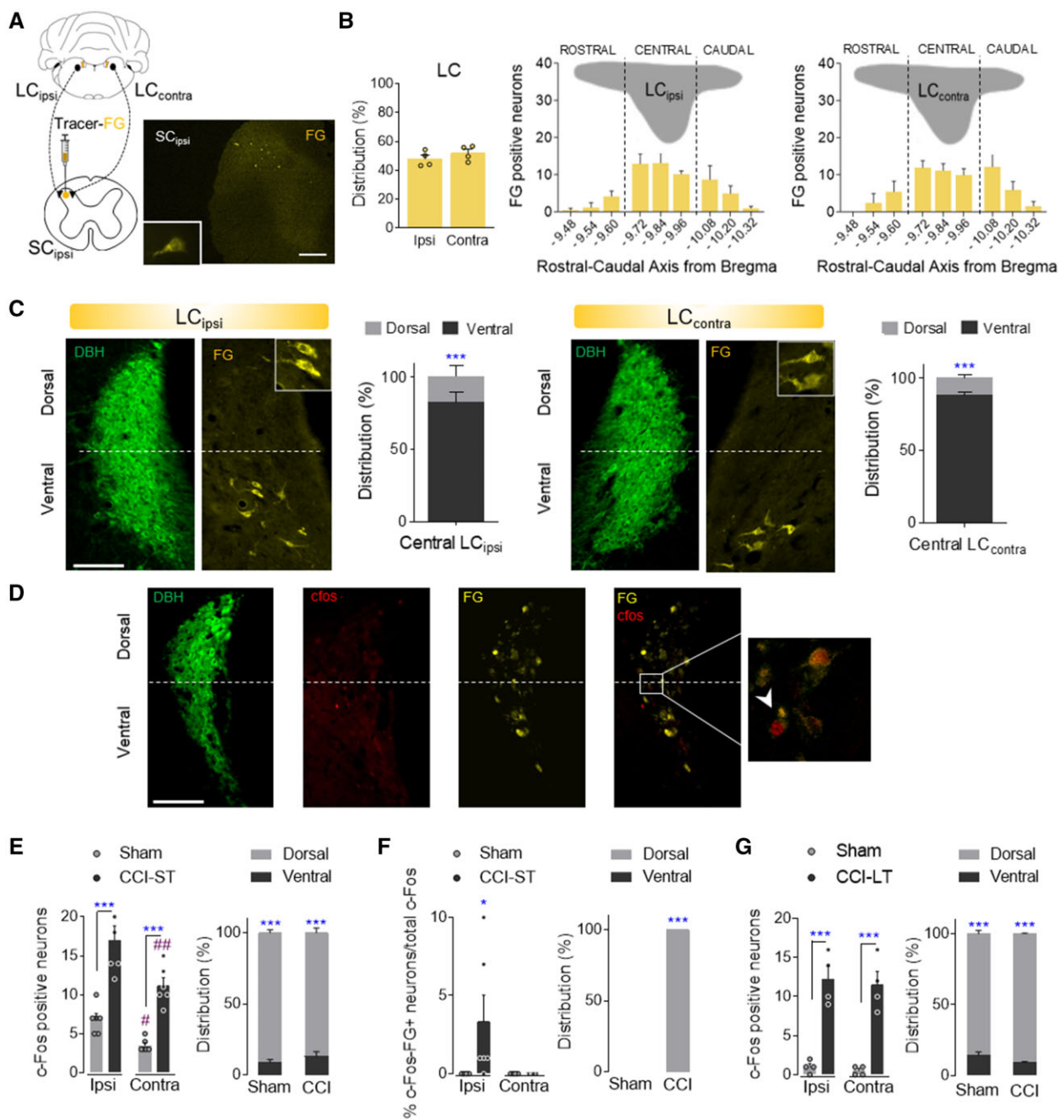
Figure 4 Study of the LC projections to the spinal cord. (**A** and **B**) Retrograde Fluoro-Gold tracer strategy and representative image (scale bar = 500 μm) to target LC neurons that project to the ipsilateral spinal cord in wild-type rats. Ipsilateral and contralateral distribution of Fluoro-Gold (FG) labelled LC neurons and Fluoro-Gold-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 dorsoventral 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 ipsilateral spinal cord (SC$_{ipsi}$) ($n = 4$ animals: ***$P < 0.001$ versus ventral LC, Student's *t*-test). (**D**) Representative immunofluorescence of the central LC showing the DBH, c-Fos, Fluoro-Gold expression and merged image. The *inset* shows an example of c-Fos+FG-positive neurons (scale bar = 100 μm). (**E** and **F**) Numbers of *c*-Fos and c-Fos+FG-positive neurons of central LC$_{ipsi}$ and LC$_{contra}$, as well as the dorsoventral distribution in sham and CCI-ST rats (mean + SEM of the number of neurons per slice, $n = 6$ animals/group: *$P < 0.05$, ***$P < 0.001$ versus Sham; #$P < 0.05$, ##$P < 0.01$ versus LCipsi, two-way ANOVA, Newman–Keuls *post hoc* test and ***$P < 0.001$ versus ventral LC, Student's *t*-test). (**G**) Numbers of c-Fos positive neurons of central LC$_{ipsi}$ and LC$_{contra}$, as well as the dorsoventral distribution in sham and CCI-LT rats (mean + SEM neurons per slice, $n = 4$ animals/group: ***$P < 0.001$ versus sham, two-way ANOVA, Newman–Keuls *post hoc* test and ***$P < 0.001$ versus ventral LC, Student's *t*-test). c-Fos (red); DBH = dopamine beta-hydroxylase (green); FG = Fluoro-Gold (yellow).

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 in the 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 indicate an early lateralized activation of the LC that reduces the pain phenotype but a
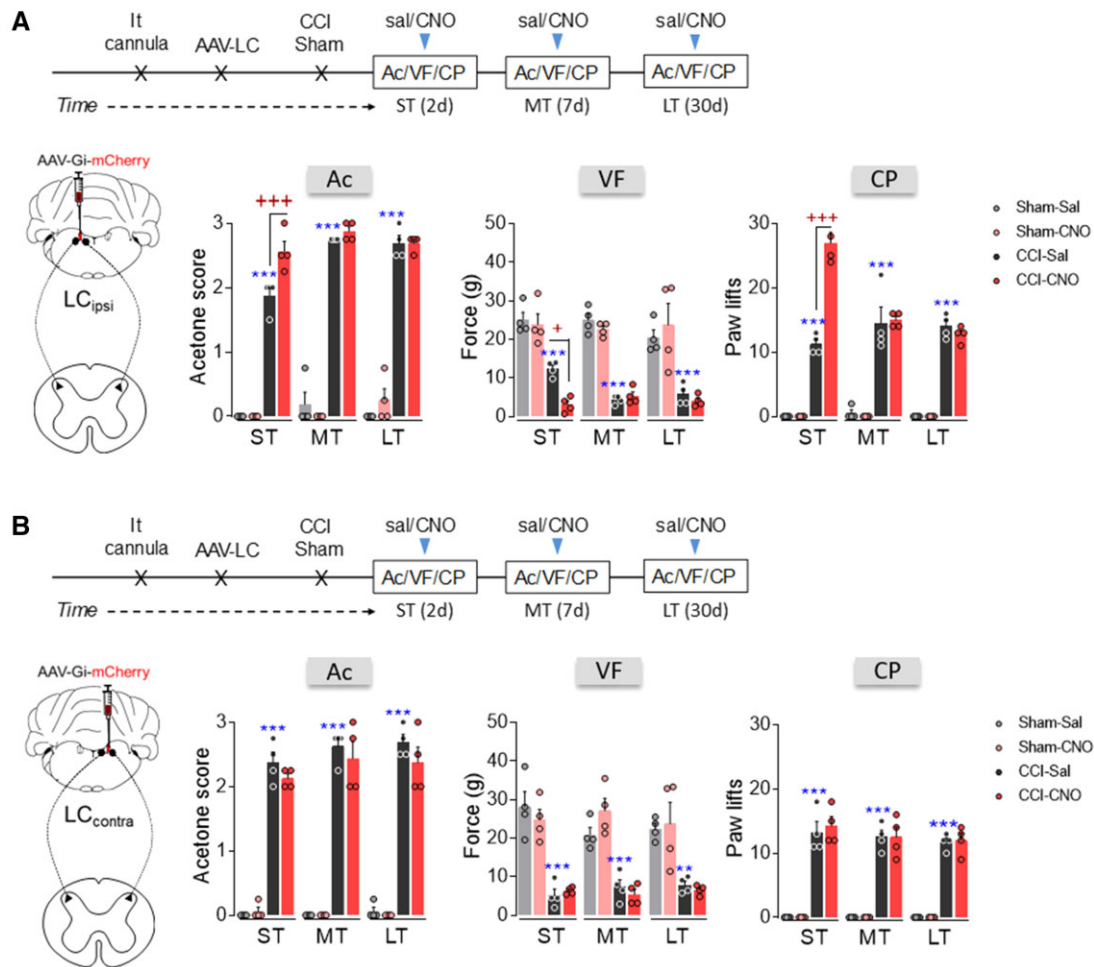
Figure 5 Effect of chemogenetic inhibition of the LC$_{ipsi/contra}$→spinal cord pathway on the time course of nociceptive behaviour after nerve injury. (A and B) Timeline and representative image of noradrenergic-LC$_{ipsi}$→spinal cord or LC$_{contra}$→spinal cord pathway inhibition using the AAV-Gi-mCherry 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: **$P < 0.01$, ***$P < 0.001$ versus sham-sal; +$P < 0.05$, +++$P < 0.001$ versus CCI-sal, two-way ANOVA with repeated measures, Newman–Keuls *post hoc* test). Ac = acetone test; CP = cold plate test; d = days; i.t. = intrathecal; sal = saline; SC = spinal cord; VF = von Frey test.

later bilateral activation that leads to behavioural 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 behaviour 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.[35,36] 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 antineuropathic 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 behaviour 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→spinal cord pathway along pain development. As occurred with global LC activity, hM4D(Gi)-DREADD blockade of the LC$_{ipsi}$→spinal cord 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 spinal cord in the short term but not the long term (Fig. 2F). Accordingly, there are more DBH in the fibres of the spinal cord 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.[37–39] We extend these previous findings evaluating changes in spinal DBH along with spontaneous activity of LC→spinal cord projecting neurons (c-Fos) and the behavioural 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 spinal cord in the short term, reducing the pain phenotype soon after injury but later failing when the insult persists. Furthermore, chemogenetic activation of both LC to spinal cord neurons produces analgesia when neuropathy is well established (from 4 weeks after nerve injury),[5] 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 spinal cord seems to refute the idea that
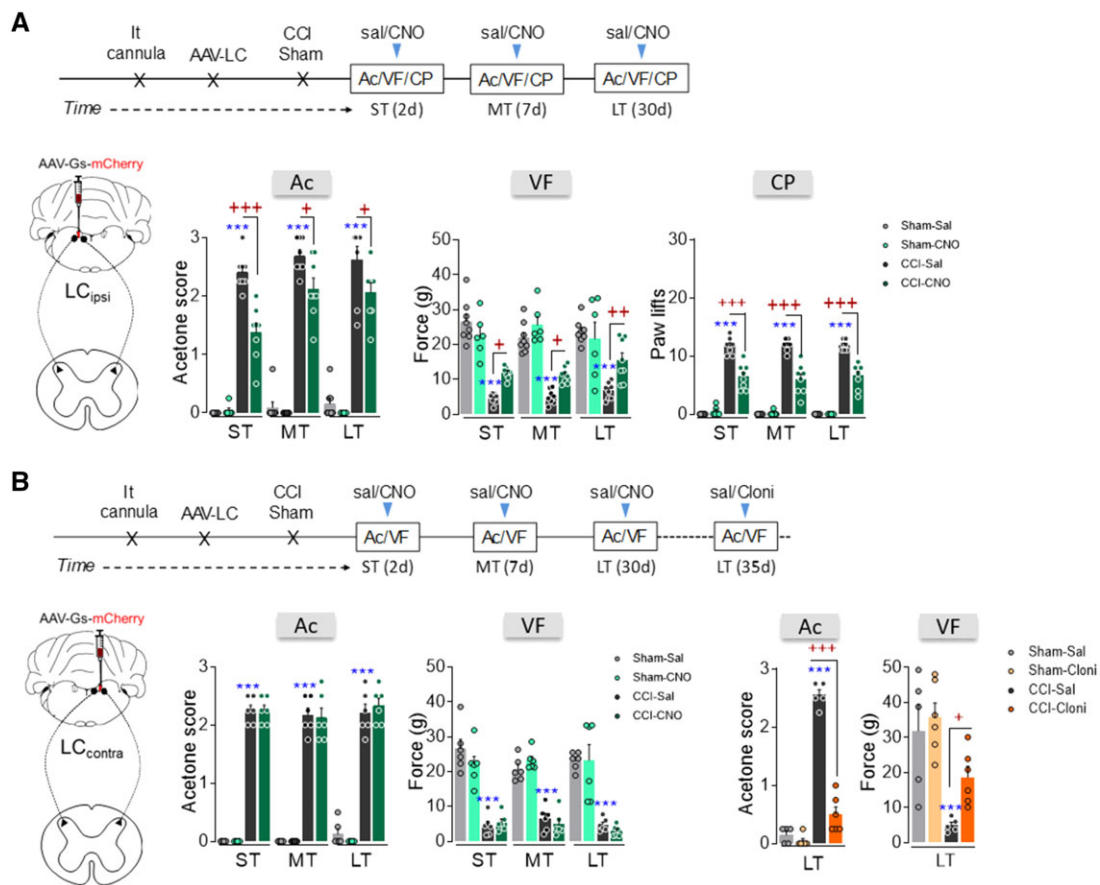
Figure 6 Effect of chemogenetic activation of the LC$_{ipsi/contra}$→spinal cord pathway on the time-course of nociceptive behaviour after nerve injury. (A and B) Timeline and representative image of noradrenergic-LC$_{ipsi}$→spinal cord or LC$_{contra}$→spinal cord pathway activation using the AAV-Gs-mCherry 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 ras in the short term (ST), mid-term (MT) and long term (LT) after CCI (n = 6–8 animals/group: ***P < 0.001 versus sham-sal; +P < 0.05, ++P < 0.01, +++P < 0.001 versus 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 versus 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 versus sham-sal; +P < 0.05, +++P < 0.001 versus CCI-sal). Two-way ANOVA with or without repeated measures, Newman–Keuls *post hoc* test. Ac = acetone test; Cloni = clondine; CP = cold plate test; d = days; i.t. = intrathecal; sal = saline; SC = spinal cord; VF = von Frey test.

the LC serves to generate pain under conditions of chronic pain (review in ref. 7) Indeed, intracerebroventricular 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.[40] However, intracerebroventricular injection of anti-DβH-saporin disrupts all noradrenergic nuclei (A1–A7), some of which contribute to sensorial hypersensitivity (A5),[41,42] 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,[43] 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[42]) to the trigeminal spinal nucleus caudalis (evoked pain[44]) 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 Llorca-Torralba *et al.*[6] for a review).

One of the LC-related networks involved in affective dimensions and manifestations of pain-related expression of emotions is the rACC.[19,45] Accordingly, hyperactivity in the ACC is seen in depressed patients[46] and it has chronic neuropathic pain-induced anxiodepressive-like consequences.[47] 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),[48] suggesting an overactivation of the noradrenergic system in long-term pain. Furthermore, optogenetic activation of the LC-ACC projection enhances excitatory neurotransmission *in vitro*.[49] As such, the Fluoro-Gold and c-Fos co-labelling 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$_{ipsi}$ and LC$_{contra}$ neurons projecting to the rACC completely reverses the depressive-like behaviour, yet it did not modify stimulus-evoked pain responses. Furthermore, site-specific pharmacological blockade indicates that α1- and α2-adrenoreceptors within the rACC are necessary for this behaviour. Mechanistically, electrophysiological studies in the medial PFC indicated that noradrenaline-persistent responses are mainly mediated by synergy between presynaptic α1-adrenoreceptor-mediated enhancement of glutamate release and postsynaptic α2-adrenoreceptor-mediated inhibition of hyperpolarization-activated cyclic nucleotide-gated cation channels.[50]
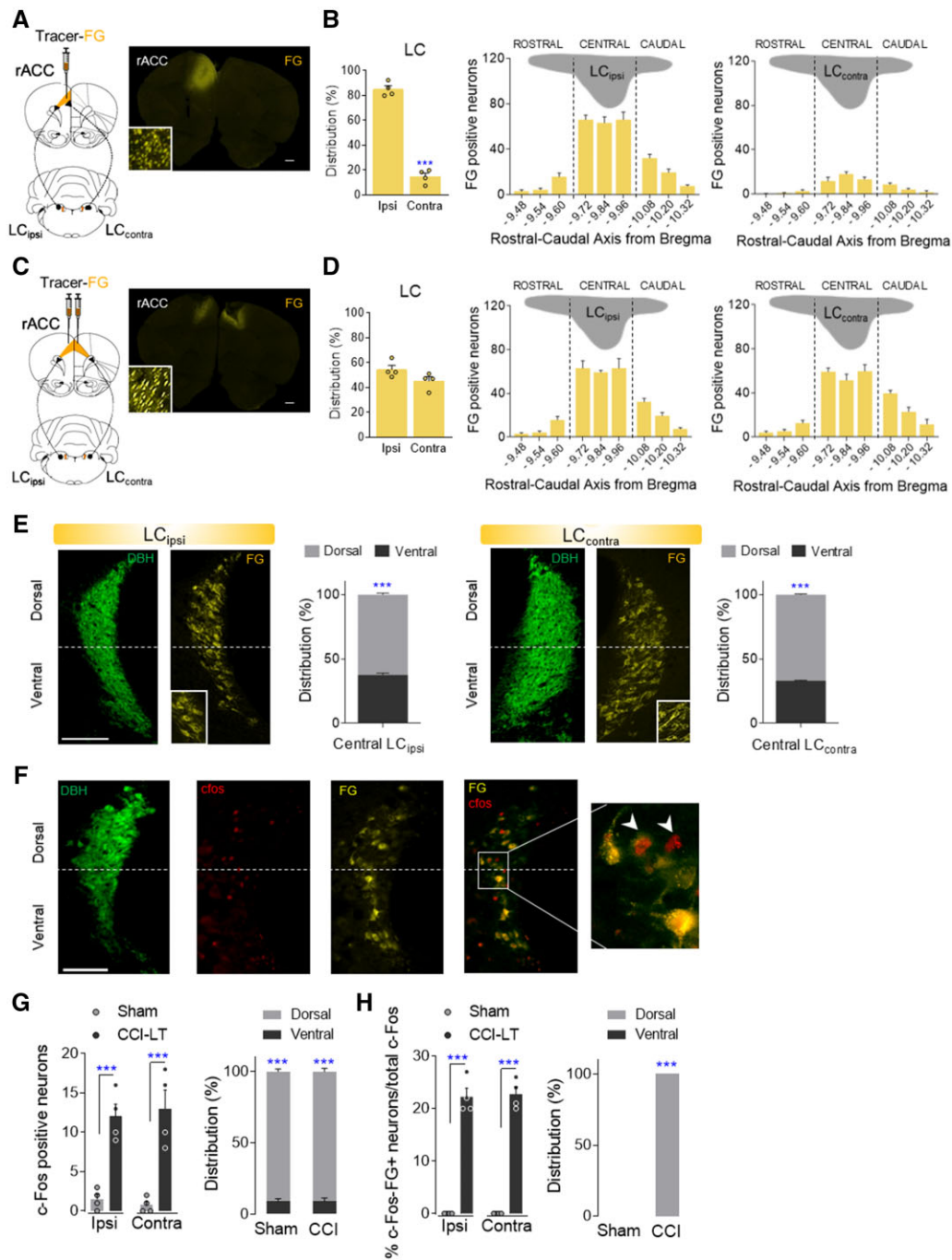
Figure 7 Neuroanatomical study of the LC projections to the rACC. (A and B) Retrograde Fluoro-Gold tracer strategy to target LC neurons that project to the ipsilateral rACC in wild-type rats and a representative image (scale bar = 500 μm). The ipsilateral and contralateral distribution of Fluoro-Gold-labelled LC neurons, and of Fluoro-Gold-positive neurons along the rostral-caudal axis of ipsilateral (LC_{ipsi}) and contralateral (LC_{contra}) LC (mean + SEM of the number of neurons per slice, $n = 4$ animals: ***$P < 0.001$ versus Ipsi, Student's t-test; one-way ANOVA, Newman–Keuls *post hoc* test). (C and D) Retrograde Fluoro-Gold tracer strategy to target LC neurons that project to the bilaterally rACC and representative image (scale bar = 500 μm). Ipsilateral and contralateral distribution of Fluoro-Gold-labelled LC neurons and Fluoro-Gold-positive neurons along the rostral-caudal axis of LC_{ipsi} and LC_{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_{ipsi} and LC_{contra} (–9.84 from bregma[34]) showing the dorsoventral aspect (half height relative to DBH expression) (scale bar = 100 μm). The graphs show the dorsoventral distribution of the central LC_{ipsi} and LC_{contra} projection to the rACC ($n = 4$ animals: ***$P < 0.001$ versus ventral LC, Student's t-test). (F) Representative immunofluorescence of the central LC showing the DBH, c-Fos, Fluoro-Gold expression and merged image (c-Fos+FG). The inset shows an example of c-Fos+FG- positive neurons (scale bar = 100 μm). (G and H) Numbers of c-Fos and c-Fos+FG-positive neurons of central LC_{ipsi} and LC_{contra} as well as the dorsoventral distribution in sham and CCI-LT rats (mean + SEM neurons per slice, $n = 4$ animals/group: ***$P < 0.001$ versus sham, two-way ANOVA, Newman–Keuls *post hoc* test; and ***$P < 0.001$ versus ventral LC, Student's t-test). c-Fos (red); DBH = dopamine beta-hydroxylase (green); FG = Fluoro-Gold (yellow).
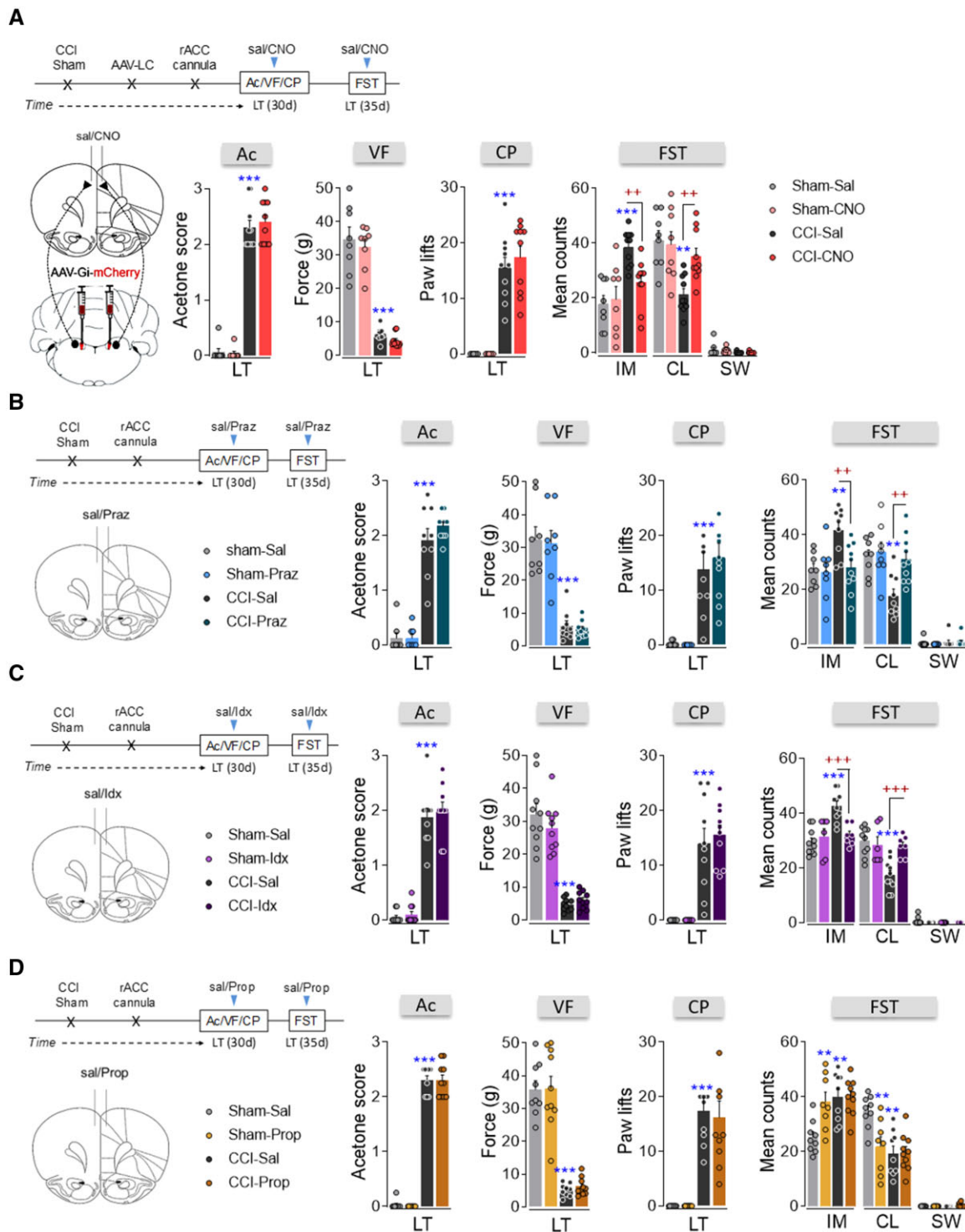
Figure 8 Effect of the chemogenetic inhibition of the LC-rACC pathway and the pharmacological blockade of adrenoreceptor activity in the rACC after long-term neuropathy. (A) Timeline and cartoon of the bilateral AAV-Gi-mCherry injection into the LC, and intra-rACC CNO (3 μM) administration to inhibit 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 behaviour in the FST (IM = immobility; CL = climbing; SW = swimming) was evaluated (*n* = 8–10 animals/group: **$P < 0.01$, ***$P < 0.001$ versus sham-sal; ++$P < 0.01$ versus CCI-sal, two-way ANOVA, Newman–Keuls *post hoc* test). **(B–D)** Timeline and cartoon of the pharmacological inhibition of α1-adrenoceptors (prazosin, 5 μg; *n* = 8–10 animals/group), α2-adrenoceptors (idazoxan, 9 μg; *n* = 6–10 animals/group) or β-adrenoceptors (propranolol, 1 μg; *n* = 7–10 animals/group) in the rACC of CCI-LT wild-type rats: **$P < 0.01$, ***$P < 0.001$ versus sham-sal; ++$P < 0.01$, +++$P < 0.001$ versus CCI-sal, two-way ANOVA, Newman–Keuls *post hoc* test. Ac = acetone test; CP = cold plate test; d = days; Idx = idazoxan; Praz = prazosin; Prop = propranolol; sal = saline; VF = von Frey test.

Thus, long-term pain overactivates the LC-ACC pathway (α-adrenoreceptors), leading to behavioural despair and overactivation of the LC-basolateral amygdala (BLA) pathway (β-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 behaviour, dampens exploratory activity and augments anxiety, in conjunction with synchronized hyperconnectivity in the salient (that includes the ACC) and the amygdala networks.[51] Zerbi's findings, together with other previous studies showing that chemogenetic activation of the LC→PFC pathway in neuropathic pain animals (without anxiety) increases anxiogenic-like behaviour as well as spontaneous pain,[5] raise important questions about the involvement of the LC→ACC pathway in anxiety and that of non-evoked pain in long-term neuropathic pain. Thus, experiments will be needed to determine whether 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 whether the blockade of β-adrenergic receptors, which has been shown to have an anxiolytic effect at BLA level,[29] 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.[52] 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-spinal cord 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 indicate that pain-induced depression is not a direct consequence of the failure of the descending pain inhibition at LC level (LC→spinal cord pathway). On the other hand, therapeutic approaches should focus on increasing noradrenaline exclusively at the spinal cord level to avoid any deleterious effects at supraspinal sites.[5] These findings explain why drugs that systemically increase noradrenaline availability are rarely used (e.g. reboxetine, desipramine), while drugs with complementary serotoninergic or opioidergic actions [antidepressants (serotonin-noradrenaline reuptake inhibitors), tramadol, tapentadol] are more commonly chosen to treat pain or pain–depression comorbidity.[53–55]

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Competing interests

Supplementary material

Supplementary material is available at *Brain* online.

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