Archival Report

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Chemogenetic Silencing of the Locus Coeruleus–Basolateral Amygdala Pathway Abolishes Pain-Induced Anxiety and Enhanced Aversive Learning in Rats

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ABSTRACT

BACKGROUND: Pain affects both sensory and emotional aversive responses, often provoking anxiety-related diseases when chronic. However, the neural mechanisms underlying the interactions between anxiety and chronic pain remain unclear.

METHODS: We characterized the sensory, emotional, and cognitive consequences of neuropathic pain (chronic constriction injury) in a rat model. Moreover, we determined the role of the locus coeruleus (LC) neurons that project to the basolateral amygdala (BLA) using a DREADD (designer receptor exclusively activated by designer drugs).

RESULTS: Chronic constriction injury led to sensorial hypersensitivity in both the short term and long term. Otherwise, long-term pain led to an anxiety-like profile (in the elevated zero maze and open field tests), as well as increased responses to learn aversive situations (in the passive avoidance and fear conditioning tests) and an impairment of nonemotional cognitive tasks (in the novel object recognition and object pattern of separation tests). Chemogenetic blockade of the LC-BLA pathway and intra-BLA or systemic antagonism of beta-adrenergic receptors abolished both long-term pain-induced anxiety and enhanced fear learning. By contrast, chemogenetic activation of this pathway induced anxiety-like behaviors and enhanced the aversive learning and memory index in sham animals, although it had little effect on short- and long-term chronic constriction injury animals. Interestingly, modulation of LC-BLA activity did not modify sensorial perception or episodic memory.

CONCLUSIONS: Our results indicate that dimensions associated with pain are processed by independent pathways and that there is an overactivation of the LC-BLA pathway when anxiety and chronic pain are comorbid, which involves the activity of beta-adrenergic receptors.

Keywords: Anxiety, Aversive memory, Basolateral amygdala, Cognition, Locus coeruleus, Neuropathic pain

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The positive interaction between negative emotions (fear, anxiety, sadness, or depression) and pain has been well established. Epidemiological studies estimate that chronic pain affects more than 20% of the population in Europe and the United States (1–4) and that 20% to 30% of those individuals also suffer anxiety-related disorders (5,6). While this comorbidity is apparently clear, few studies have directly explored the interaction between pain and anxiety at the network level.

Current theories suggest that long-term (LT) pain triggers functional changes that would be responsible for affective and cognitive alterations (anxiety, depression, emotional decision making, and working memory). The locus coeruleus (LC) is a region that might be implicated in these changes (7–17). Indeed, we reported increased electrophysiological activity, tyrosine hydroxylase, and noradrenaline transporter expression in the LC and enhanced alpha2-adrenoceptor expression and sensitivity, which temporally coincides with the onset of anxiodepressive behavior in LT nerve-injured rats (8). Interestingly, new LC projection mapping data revealed that the LC noradrenaline system is anatomically and functionally heterogeneous and that its responses can be modulated by sensory inputs (18). Indeed, specific LC projections would appear to be modulated by painful input (18,19). One neural target of the LC is the basolateral amygdala (BLA), which integrates sensory information to encode and drive diverse and, at times opposing, affective behaviors, including anxiety, aversion, and reward behaviors (20). In fact, it was recently shown that the neuromodulatory LC system utilizes the BLA output to promote acute anxiety (21,22) and aversive learning (23). Together, these data suggest that LC-BLA projections are involved in mediating acute negative affective

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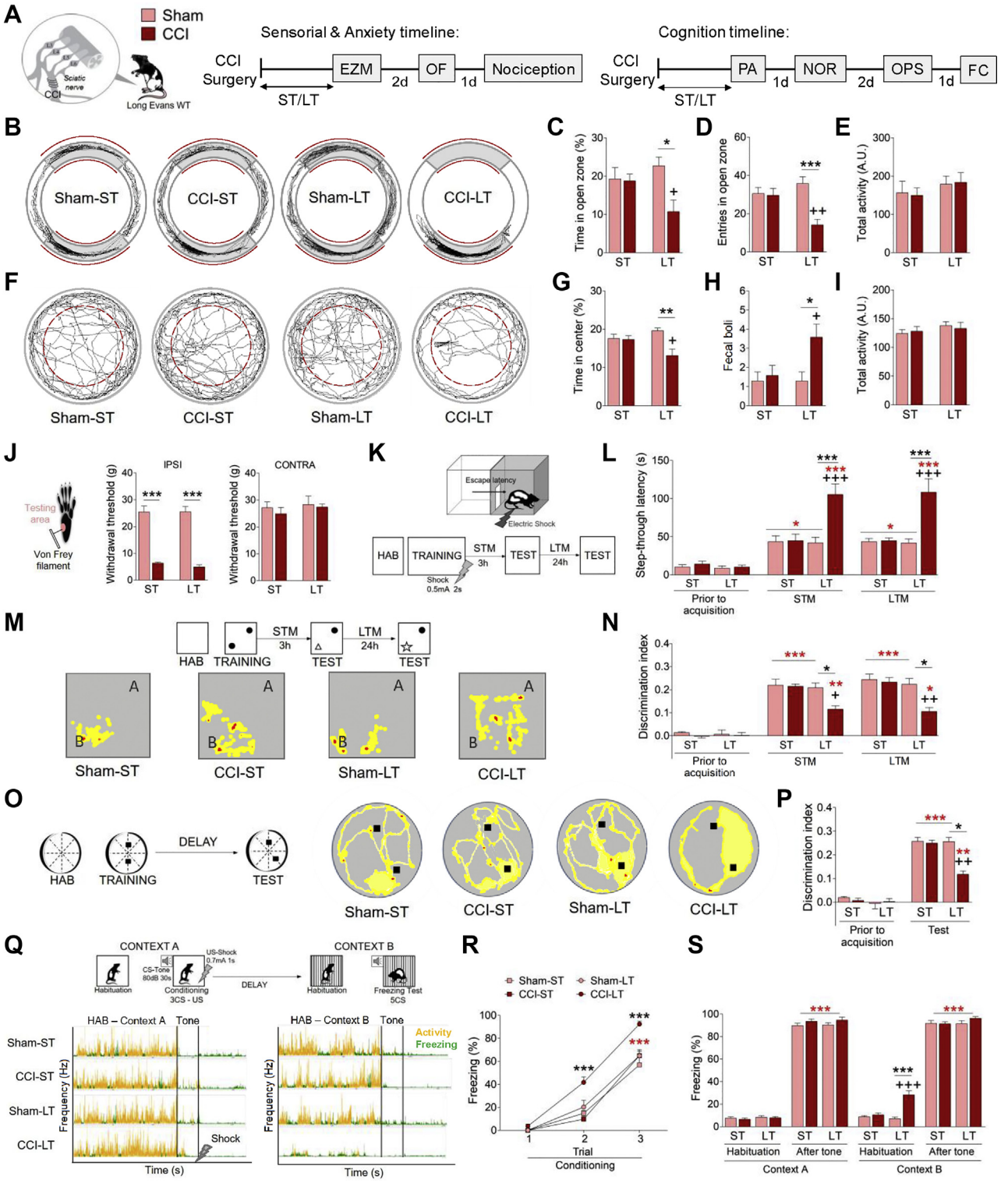

Figure 1. Sensory, affective, and cognitive characterization of the development of neuropathic pain. **(A)** Experimental timeline and schematic representation of chronic constriction injury (CCI) surgery for the behavioral experiments in male Long-Evans wild-type (WT) rats. Rats were tested 2–3 weeks (short-term [ST]) or 5–6 weeks (long-term [LT]) after surgery. **(B–E)** Effects of neuropathic pain on the elevated zero maze (EZM). **(B)** Representative activity traces, the graphs representing **(C)** the relative time spent in the open zone and **(D)** the number of entries into the open zone, and **(E)** the locomotor activity. **(F–I)** The effects of

behavior, although less is unknown about sustained aversive situations like chronic pain.

In the light of this situation, we hypothesized that noradrenergic LC projections to the BLA may be hyperactivate in chronic pain, driving processes like anxiety and increased aversive emotional learning. As such, we characterized the evolution of hypersensitivity and that of the anxiety and cognitive capacity in Long-Evans rats submitted to chronic constriction injury (CCI) of the sciatic nerve. These phenotypes where subsequently evaluated when the LC noradrenergic terminals in the BLA were chemogenetically modulated.

METHODS AND MATERIALS

Additional details of the materials and methods can be found in the figure legends and in the Supplement.

Animals

Male wild-type or tyrosine hydroxylase–Cre transgenic Long-Evans rats (350–450 g) were produced and maintained under standard laboratory conditions (University of Cádiz). All animal handling and use was carried out in accordance with the guidelines of the European Commission's directive (2010/63/EC) and Spanish law (RD 53/2013).

Surgical Procedures

Neuropathic Pain. CCI was used as a model of neuropathic pain (24,25), and short-term (ST) (2–3 weeks postsurgery) and LT (5–6 weeks postsurgery) experiments were performed.

Stereotaxic Surgery. Surgery was performed under ketamine (100 mg/kg) and xylazine (20 mg/kg) anesthesia. Rats were injected with a DREADD (designer receptor exclusively activated by designer drug). A DREADD adeno-associated virus 2 (AAV2)/hSyn-DIO-hM4D(Gi)-mCherry, AAV2/hSyn-DIO-rM3D(Gs)-mCherry or AAV2/hSyn-DIO-mCherry virus (Virus Vector Core, Gene Therapy Center Vector Core, University of North Carolina at Chapel Hill, Chapel Hill, NC) was injected unilaterally or bilaterally into the LC [1.4 μL/side (26): −3.2 anteroposterior from lambda, ±1.3 mediolateral, and 6.2 dorsoventral]. Unilateral administration was performed for testing the rM3D(Gs)-DREADD–mediated activation of LC neurons (c-Fos+ neurons). Alternatively, the BLA of rats (−2.9 anteroposterior from bregma, ±4.8 mediolateral, and −8.4 dorsoventral) was injected with the Fluoro-Gold retrograde tracer (4% w/v, 0.2 μL) (Fluorochrome, Denver, CO). The rats were allowed to recover for 3 weeks (DREADD virus) or 4 days (Fluoro-Gold tracer) before the behavioral studies were carried out, favoring robust expression in the target regions. A metal cannula was then implanted bilaterally into BLA 4 days before performing the behavioral studies.

Drug Administration

Clozapine-*N*-oxide (CNO) [3 μM/0.5 μL intra-BLA (27) or 1 mg/kg intraperitoneal (26)] (Enzo Life Sciences, Farmingdale, NY), propranolol [a beta-adrenergic receptor (β-AR) antagonist; 1 μg/0.5 μL intra-BLA (21) or 3 mg/kg intraperitoneal] (Sigma-Aldrich, Madrid, Spain), isoproterenol (a β-AR agonist; 30 μg/0.5 μL intra-BLA) (Sigma-Aldrich), or saline (ss) 0.9% was administered 20 minutes before behavioral testing. When administering both CNO and propranolol, CNO was injected 20 minutes before propranolol or saline.

Behavioral Assessment

Anxiety. For the elevated zero maze (EZM), the time spent in the open zones and the number of entries into these areas, as well as the total activity, were monitored during a 5-minute test (28,29).

For the open field test, the time spent in the central area, the total activity, and the number of fecal boluses was recorded.

Nociception. In the von Frey test, the withdrawal threshold (grams) of a hindpaw when subjected to a force increasing from 0 to 50 g over a period of 20 seconds was used as a measure of mechanical hypersensitivity (30).

The acetone test involves applying a drop of acetone (100 μL) to the surface in the middle of a hindpaw with a pipette (31).

In the cold plate test (4 ± 1°C), the times the animal briskly lifted its ipsilateral hindpaw was measured over a period of 2 minutes (32) as a measure of thermal hypersensitivity.

For dynamic weight bearing, spontaneous nociceptive behavior was measured as the weight (grams) or area (mm^2) of the hindpaw for 5 minutes (33).

neuropathic pain on the open field (OF) test. **(F)** Representative activity traces and graphs representing **(G)** the relative time spent in the central zone, **(H)** the number of fecal boluses, and **(I)** the locomotor activity. **(J)** Study of mechanical sensitivity of pain model. The graphs represent the withdrawal threshold (grams) of the ipsilateral (IPSI) and contralateral (CONTRA) hindpaw in response to von Frey hair stimulation (0–50 g, 20 seconds). **(K, L)** The effects of neuropathic pain on passive avoidance (PA). **(K)** Schematic representation of the PA procedure and the graph represents **(L)** the latency to enter the dark compartment (seconds) in the different phases of the paradigm: training (prior to acquisition), ST memory (STM) test (as learning index) and LT memory (LTM) test (as memory index). **(M, N)** The effects of neuropathic pain on the novel object recognition (NOR) test. **(M)** Schematic representation of the NOR procedure and representative heatmaps showing activity (yellow = low activity, and red = high activity) around the objects in memory tests (A = familiar object, B = novel object) and **(N)** the graph representing the discrimination index between objects in the different phases of the paradigm: training, STM, and LTM. **(O, P)** The effects of neuropathic pain on object pattern separation (OPS). **(O)** Schematic representation of the OPS procedure and representative heatmaps showing activity (yellow = low activity, red = high activity) around the columns in the OPS test session; **(P)** the graph representing the discrimination index between objects in the different phases of the paradigm: training and test. **(Q–S)** The effects of neuropathic pain on fear conditioning (FC). **(Q)** Schematic representation of the FC procedure and representative activity (yellow) and freezing (green) traces in the different phases of the FC test: habituation (HAB), during tone, and after tone in both contexts. The graphs represent **(R)** the relative freezing during conditioned stimulus (CS)–unconditioned stimulus (US) paired in context A over the trials (learning index) and **(S)** the relative freezing during HAB and after the tone sessions in contexts A and B. The data are represented as the mean ± SEM (n = 8 per group): $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ vs. sham; $+p < .05$, $++p < .01$, $+++p < .001$ vs. CCI as assessed by two-way analysis of variance followed by Newman-Keuls post hoc test. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ vs. prior to acquisition in each group as assessed by two-way analysis of variance with repeated measures followed by Newman-Keuls post hoc test. A.U., arbitrary units.

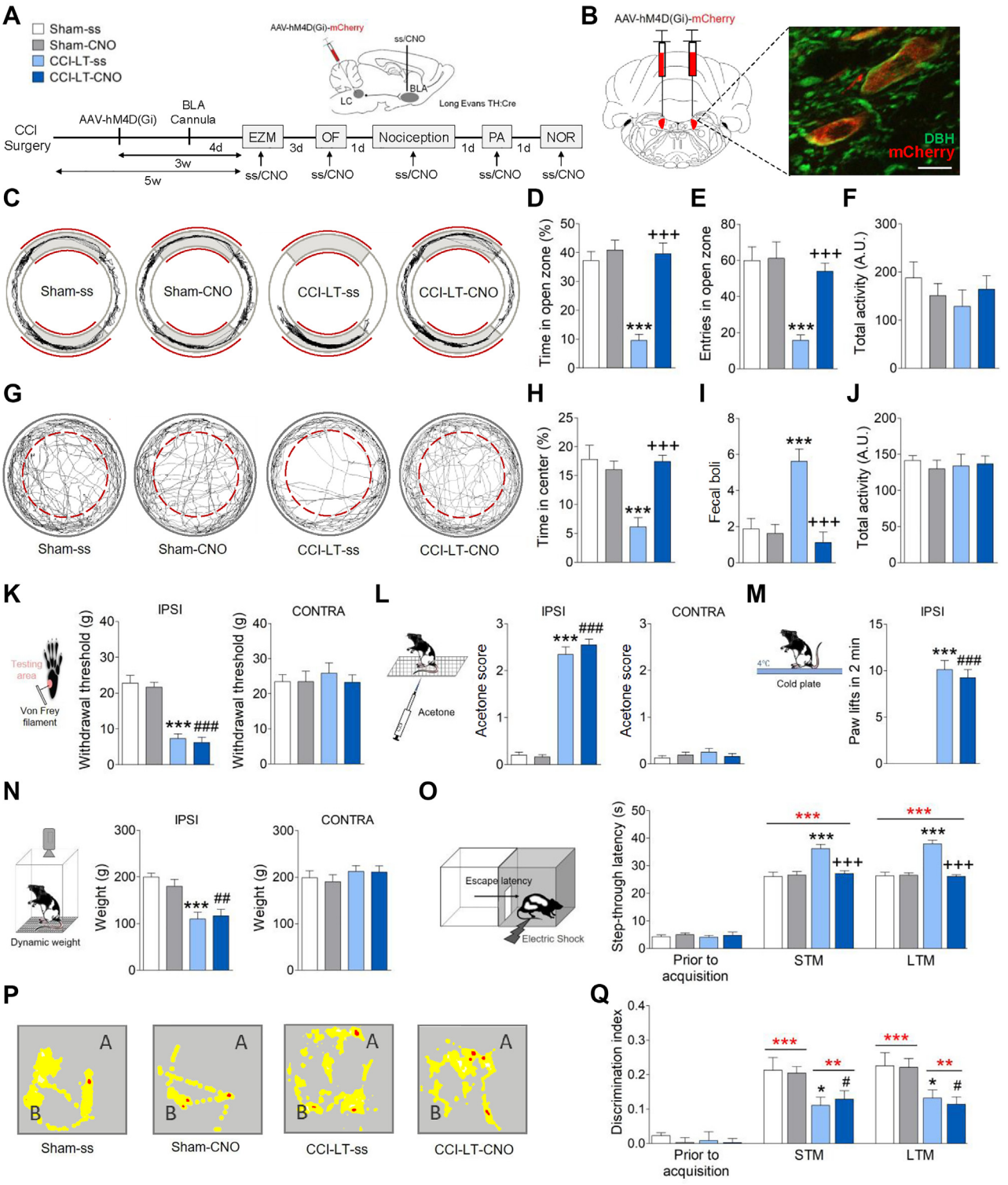

Figure 2. Selective inhibition of the locus coeruleus–basolateral amygdala (LC-BLA) pathway. **(A)** Experimental timeline and schematic representation of the hM4D(Gi)-DREADD (designer receptor exclusively activated by designer drugs) viral and cannula delivery in the behavioral experiments on male Long-Evans tyrosine hydroxylase–Cre (TH-Cre) rats. A Cre-dependent adeno-associated virus (AAV) fluorescently tagged with mCherry [AAV-hM4D(Gi)-mCherry] (1.4 μL/side) was injected bilaterally into the TH neurons of the LC of TH-Cre rats 2 weeks after chronic constriction injury (CCI). The rats were allowed to recover for 3 weeks and to achieve a robust expression of DREADD, and they were implanted bilaterally with metal cannulae into the BLA 4 days before the behavioral

Cognition. In the step-through passive avoidance (PA) test, electric shocks were applied via the grid floor in the dark chamber (2 seconds and 0.5-mA intensity), and the number of chamber changes in the habituation period and the latency of the animal to move into the dark chamber (step-through latency) were recorded in training session and two test sessions, 3 (ST memory [STM]) and 24 (LT memory [LTM]) hours after training (34).

In the novel object recognition test, the time spent exploring each object over 10 minutes was recorded, expressing the relative exploration of the novel object as a discrimination index [discrimination index = $(t_{novel} - t_{familiar})/(t_{novel} + t_{familiar})$, where t is time] (35). The task included a habituation session, training session, and two memory tests, 3 (STM as a learning index) and 24 (LTM as a memory index) hours after training.

The object pattern separation task is a modified version of the novel object recognition test and it was analyzed in the same way (36).

In fear conditioning, a tone (30 seconds, 5 kHz, 80 dB) was used as a conditioned stimulus, and a foot shock (1 second, 0.7 mA) was used as an unconditioned stimulus. The conditioned rats were tested for fear responses (freezing), defined as the absence of all movement except that necessary for respiration (37), and it was monitored throughout the procedure and represented as the percent freezing in each phase.

All behavioral assessments were monitored and analyzed using the SMART video 3.0 software (Panlab, Cornellà de Llobregat, Spain).

Histology and Immunohistochemistry

Immunohistochemistry was performed (12) to evaluate the expression of DREADD-mCherry, Fluoro-Gold tracer, and c-Fos in the LC. The cannula placement and CNO spread in the BLA was also verified.

Statistical Analysis

Data distribution was assumed to be normal and our analytic approaches were based on previously published work (8,19,23), but normality was not formally tested. The data are presented as the mean ± SEM and analyzed with the GraphPad Prism 5.0 software (GraphPad San Diego, CA) and Statistica 10.0 (StatSoft, Tulsa, OK). An unpaired Student *t* test was used to compare the values between the two groups. One- or two-way analyses of variance (with/without repeated measures) were followed by Newman-Keuls post hoc tests. In all cases, $p < .05$ was considered significant (Supplemental Tables S1–10).

RESULTS

Temporal Characterization of the Symptoms Produced by Pain

In these experiments, anxiety-like behaviors, nociceptive responses, and the cognitive capacity of CCI rats were evaluated at two time points after nerve injury, ST (2–3 weeks) and LT (5–6 weeks) (Figure 1A; Supplemental Figure S1A). As expected, anxiety was observed only in the CCI-LT animals (Figure 1B–I), with these rats spending less time in the open quadrant ($p < .05$) and entering the EZM fewer times ($p < .001$), as well as spending less time in the center of the open field ($p < .01$) and producing more fecal boluses ($p < .05$) in that test relative to the sham rats. By contrast, both CCI-ST and CCI-LT rats developed mechanical hypersensitivity in the ipsilateral hindpaw compared with the sham animals ($p < .001$) (Figure 1J), suggesting that pain arises after nerve injury and that it remains constant over time.

We next investigated whether the cognitive sphere is affected by the development of neuropathic pain, using strong emotional arousing (PA test) and weak nonemotional arousing (novel object recognition test) cognitive paradigms. Learning and memory involve two separate events, acquisition and storage, and thus STM was used as an index of learning and LTM as an index of memory. Step-through latencies in the PA test were significantly longer for CCI-LT rats than in the sham animals (STM $p < .001$; LTM $p < .001$) (Figure 1K–L), suggesting hypersensitivity to aversive experiences. By contrast, CCI-LT rats displayed poor discrimination in both novel object recognition tests relative to the sham rats (STM and LTM $p < .05$) (Figure 1M, N). These results were corroborated using the object pattern separation (nonemotional) and fear conditioning (emotional) paradigms. A cognitive deficit was evident in the CCI-LT animals with respect to the sham rats in the object pattern separation task ($p < .05$) (Figure 1O, P), and in the fear conditioning test (Figure 1Q), freezing behavior progressed more significantly during the conditioning phase in the CCI-LT

studies were carried out. Clozapine-*N*-oxide (CNO) (3 μM/0.5 μL) was administered 20 minutes before performing the behavioral tests. **(B)** Representative immunohistofluorescence images (scale bar = 10 μm) of hM4D(Gi)-mCherry expression in noradrenergic LC neurons (red = mCherry, green = dopamine beta hydroxylase). The rats were tested 5–6 weeks (long-term [LT]) after surgery. **(C–F)** The effects of inhibiting the LC-BLA pathway in the elevated zero maze (EZM). **(C)** The representative activity traces and the graphs represent **(D)** the relative time spent in the open zone and **(E)** the number of entries into that zone, or **(F)** the locomotor activity. **(G–J)** The effects of inhibiting the LC-BLA pathway in the open field (OF) test. **(G)** The representative activity traces and the graphs represent **(H)** the relative time spent in the center zone, **(I)** the number of fecal boluses, and **(J)** the locomotor activity. **(K–N)** Sensorial evaluation. The graphs represent **(K)** the withdrawal threshold (grams) of the ipsilateral (IPSI) and contralateral (CONTRA) hindpaws in response to von Frey hair stimulation (0–50 g, 20 seconds), **(L)** the response of IPSI and CONTRA hindpaws to acetone application (100 μL), **(M)** the number of lifts of the IPSI hindpaw in the cold plate test (2 minutes, 4°C), and **(N)** the weight distributed on the IPSI and CONTRA hindpaws in the dynamic weight-bearing test (5 minutes). **(O)** The effects of the inhibition of the LC-BLA pathway on passive avoidance (PA). The graph represents the latency to enter the dark compartment (seconds) in the different phases of the paradigm: training (prior to acquisition), short-term memory (STM) test (as learning index), and long-term memory (LTM) test (as memory index). **(P, Q)** The effects of inhibiting the LC-BLA pathway on the novel object recognition (NOR) test. **(P)** The representative heatmaps showing activity (yellow = low activity, red = high activity) around the objects in the memory tests (A = familiar object, B = novel object) and **(Q)** the graph represents the discrimination index between objects in the different phases of the paradigm: training, STM, and LTM. The data represent the mean ± SEM ($n = 8$ per group): $^{*}p < .05$,$^{***}p < .001$ vs. sham-saline (ss); $+++p < .001$ vs. CCI-LT-ss; $\#p < .05$, $\#\#p < .01$, $\#\#\#p < .001$ vs. sham-CNO; $^{**}p < .01$, $^{***}p < .001$ (red asterisks) vs. prior to acquisition of each group as assessed by two-way analysis of variance followed by Newman-Keuls post hoc test. A.U., arbitrary units.

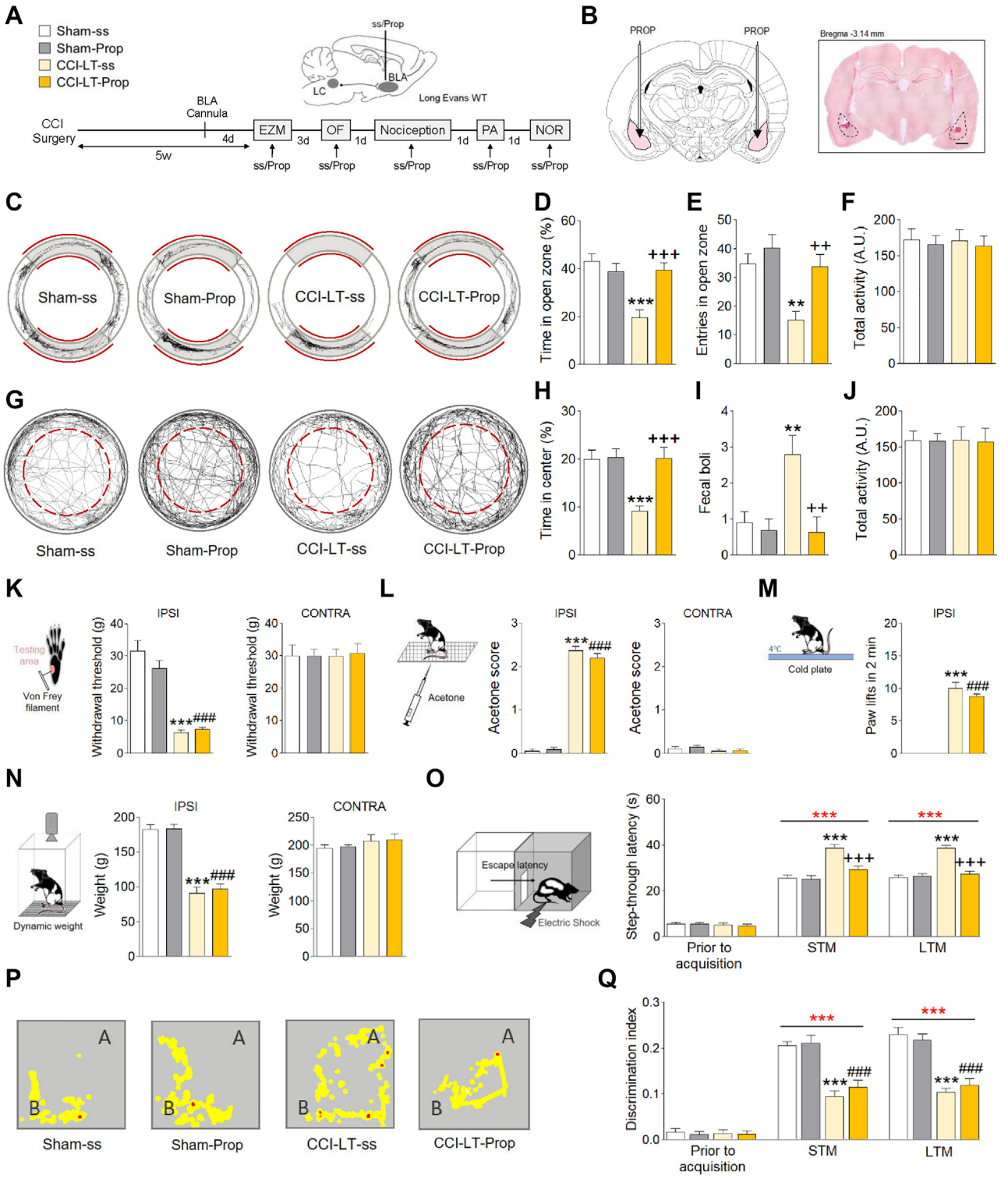

Figure 3. Pharmacological inhibition of beta-adrenergic receptors (β-ARs) in the basolateral amygdala (BLA). **(A)** Experimental timeline and schematic representation of cannula delivery for the behavioral experiments in male Long-Evans wild-type (WT) rats. A metal cannula was implanted bilaterally into the BLA 4 days before performing the behavioral studies. Propranolol (Prop) (1 μg/0.5 μL) was administered 20 minutes before behavioral testing. **(B)** Photomicrograph of a coronal section (neutral red stain) of the rat brainstem showing the tract of the cannula in the BLA (scale bar = 500 μm). The rats were tested 5–6 weeks (long-term [LT]) after chronic constriction injury (CCI). **(C–F)** The effects of inhibiting the β-ARs in the BLA in the elevated zero maze (EZM). **(C)** The representative activity traces and the graphs represent **(D)** the relative time spent in the open zone and **(E)** the number of entries into that zone, as well as **(F)**

rats than in the sham animals ($p < .001$) (Figure 1R). Surprisingly, CCI-LT animals also showed freezing behavior when habituating in another context (context B) ($p < .001$) (Figure 1S), while the rest of the groups remained unaffected. Finally, no significant differences were evident in the cognitive capacities between the CCI-ST and sham rats in any of the tests performed, and none of the behaviors evaluated were affected by any locomotor dysfunction (Figure 1E, I; Supplemental Figure S1B–G). These data show that neuropathy leads to ST and LT sensorial hypersensitivity, whereas anxiety and cognitive impairment develop only when nerve injury persists for long periods of time.

Selective Inhibition of the LC-BLA Pathway Relieves Pain-Induced Anxiety and Aversive Processing

We previously demonstrated in Sprague Dawley rats that c-Fos expression in the LC augments in LT neuropathic pain (38). Accordingly, there was a significant increase in the number of neurons labeled for c-Fos in the LC of CCI-LT with respect to the sham rats ($p < .05$) (Supplemental Figure S2A–F), confirming that LT nerve injury provokes LC hyperactivity.

One brain area innervated by LC neurons is the BLA, as seen by Fluoro-Gold tracing (Supplemental Figure S3A–L). Thus, to explore whether the activity of the LC-BLA pathway is involved in the phenotype observed in CCI-LT rats, we examined hM4D(Gi)-DREADD–mediated inhibition of this pathway (Figure 2A, B; Supplemental Figures S4A–M, S5A, S6A–D, S7, S8A–P, and S9A–H). CCI-LT-CNO animals exhibited a significant increase in the time spent and in the number of entries into the open zones relative to the CCI-LT-ss animals in the EZM ($p < .001$) (Figure 2C–E). Furthermore, more time was spent in the center of the open field, and there were fewer fecal boluses compared with CCI-LT-ss rats in this test ($p < .001$) (Figure 2G–I). Thus, inhibition of LC neurons projecting to the BLA appears to reverse anxiety in CCI-LT animals, reaching similar values to those found in sham-ss rats. Importantly, the hM4D(Gi) manipulation had no effect on the baseline anxiety of sham animals, and none of the manipulations affected locomotor activity (Figure 2F, J).

Subsequently, pain hypersensitivity was evaluated after CNO-mediated inhibition of the LC-BLA pathway in the von Frey, acetone, cold plate, and dynamic weight bearing tests. CNO administration did not produce any change in hypersensitivity-related responses in the CCI-LT-CNO with respect to CCI-LT-ss rats (Figure 2K–N; Supplemental Figure S5B–E). However, there was a significant decrease in the step-through latency of CCI-LT-CNO rats relative to CCI-LT-ss in the PA test (STM and LTM $p < .001$) (Figure 2O; Supplemental Figure S5F). Nevertheless, no differences were found in the novel object recognition test between CCI-LT-CNO and CCI-LT-ss rats (Figure 2P, Q; Supplemental Figure S5G–I). Moreover, inhibition of the LC-BLA pathway had no effect on the sham animals in any of the tests used. Hence, LT pain appears to activate the LC-BLA pathway, leading to anxiety and alterations in the processing of aversive information.

BLA β-ARs Are Involved in Pain-Induced Anxiety and Aversive Processing

To test whether blocking β-AR activity in the BLA would reverse the behaviors associated with LT pain, the effects of intra-BLA administration of the nonselective β-AR antagonist propranolol were tested (Figure 3A, B; Supplemental Figure S10A). Propranolol administration increased the time CCI-LT animals spent in the open zones of the EZM ($p < .001$) and their number of entries into this zone ($p < .01$) (Figure 3C–E). In addition, it also increased the time CCI-LT-propranolol animals spent in the center of the open field ($p < .001$), in conjunction with a decrease in the number of fecal boluses in this test ($p < .01$) relative to the CCI-LT-ss rats (Figure 3G–I). However, in terms of their sensorial evaluation, CCI-LT-propranolol rats performed similarly to CCI-LT-ss animals (Figure 3K–N; Supplemental Figure S10B–E). Propranolol did induce a significant decrease in the step-through latency of CCI-LT rats relative to the controls in the PA test ($p < .001$) (Figure 3O; Supplemental Figure S10F), although their performance in the novel object recognition test was no different (Figure 3P, Q; and Supplemental Figure S10G–I). We next determined the effect of intraperitoneal propranolol administration in the same tests. Interestingly, the systemic antagonism of β-ARs mimicked the effect of intra-BLA blockade of those receptors (Figure 4A–P; Supplemental Figure S11A–H). At the doses studied, propranolol also had no effect on baseline anxiety, nociception, and cognition in the sham animals, and none of the manipulations affected locomotor activity (Figures 3F, J and 4E, I; Supplemental Figures S10F, H–I and S11E, G, H). These data show that blocking β-ARs produced relief from pain-induced anxiety and that it reduced hypersensitivity to aversive experiences.

the locomotor activity. **(G–J)** The effects of β-ARs in the BLA in the open field (OF) test. **(G)** The representative activity traces and the graphs represent **(H)** the relative time spent in the central zone, **(I)** the number of fecal boluses, and **(J)** the locomotor activity. **(K–N)** Sensorial evaluation. The graphs represent **(K)** the withdrawal threshold (grams) of the ipsilateral (IPSI) and contralateral (CONTRA) hindpaws in response to von Frey hair stimulation (0–50 g, 20 seconds), **(L)** the response of the IPSI and CONTRA hindpaws to acetone application (100 μL), **(M)** the number of lifts of the ipsilateral hindpaw in the cold plate test (2 minutes, 4°C), and **(N)** the weight distributed on the IPSI and CONTRA hindpaws in the dynamic weight bearing test (5 minutes). **(O)** The effects of β-ARs in the BLA on passive avoidance (PA). The graph represents the latency to enter the dark compartment (seconds) in the different phases of the paradigm: training (prior to acquisition), short-term memory (STM) test (as learning index), and long-term memory (LTM) test (as memory index). **(P–Q)** The effects of β-ARs in the BLA in the novel object recognition (NOR) test. **(P)** The representative heatmaps showing activity (yellow = low activity, red = high activity) around the objects in the memory tests (A = familiar object, B = novel object) and **(Q)** the graph represents the discrimination index between objects in the different phases of the paradigm: training, STM, and LTM. The data represent the mean ± SEM (n = 8–9 per group): $^{**}p < .01$,$^{***}p < .001$ vs. sham-saline (ss); $++p < .01$, $+++p < .001$ vs. CCI-LT-ss; $###p < .001$ vs. sham-Prop as assessed by two-way analysis of variance followed by Newman-Keuls post hoc test. $^{***}p < .001$ (red asterisks) vs. prior to acquisition of each group as assessed by two-way analysis of variance with repeated measures followed by Newman-Keuls post hoc test. A.U., arbitrary units.

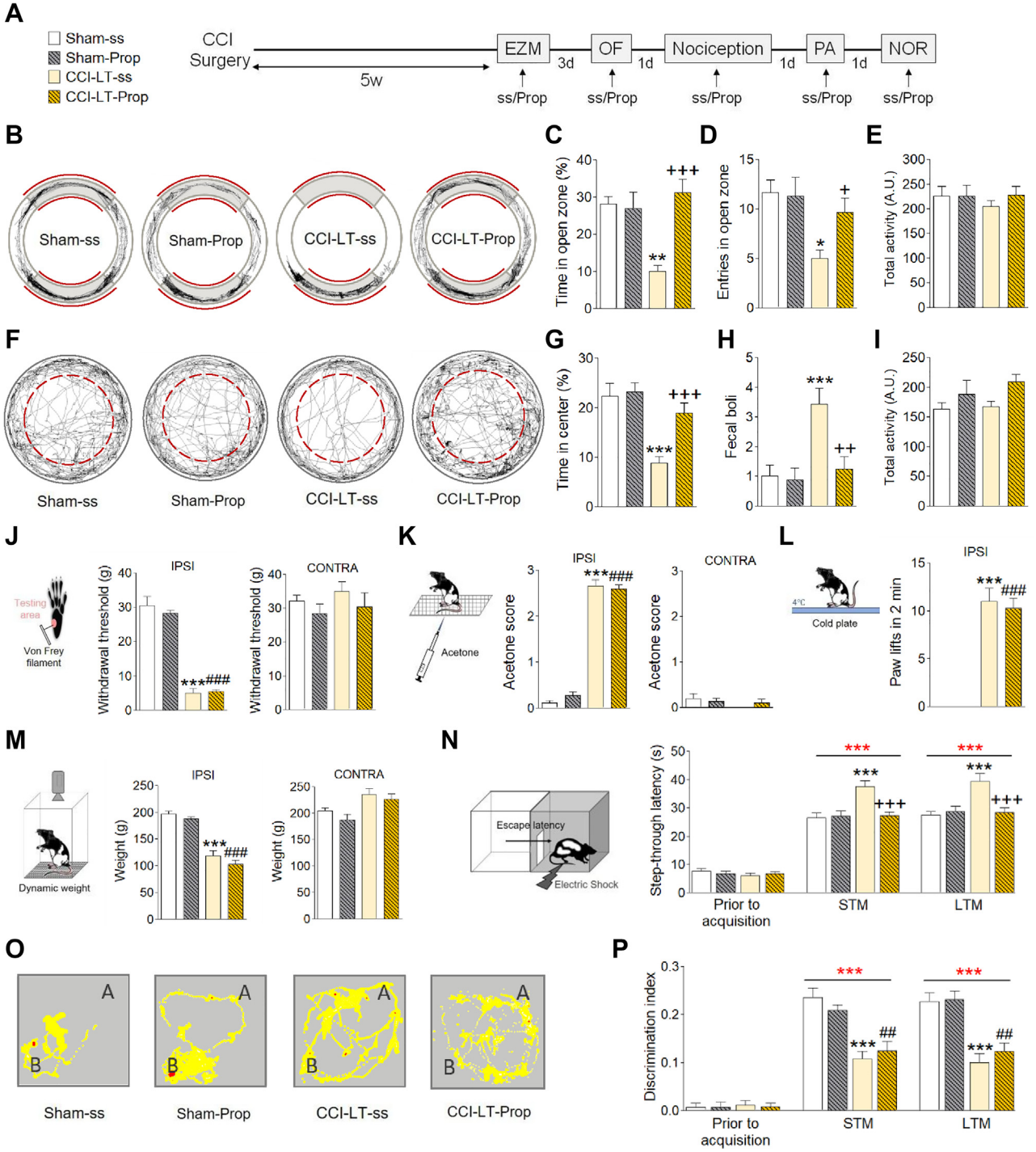

Figure 4. Systemic pharmacological inhibition of beta-adrenergic receptors (β-ARs). **(A)** Experimental timeline for the behavioral experiments in male Long-Evans wild-type rats. Propranolol (Prop) (3 mg/kg intraperitoneal) was administered 20 minutes before behavioral testing. The rats were tested 5–6 weeks (long-term [LT]) after chronic constriction injury (CCI). **(B–E)** The effects of the systemic inhibition of the β-ARs in the elevated zero maze (EZM). **(B)** The representative activity traces and the graphs represent **(C)** the relative time spent in the open zone and **(D)** number of entries into that zone, as well as **(E)** the locomotor activity. **(F–I)** The effects of the systemic inhibition of the β-ARs in the open field (OF) test. **(F)** The representative activity traces and the graphs represent **(G)** the relative time spent in the central zone, **(H)** the number of fecal boluses, and **(I)** the locomotor activity. **(J–M)** Sensorial evaluation. The graphs represent **(J)** the withdrawal threshold (grams) of the ipsilateral (IPSI) and contralateral (CONTRA) hindpaws in response to von Frey hair stimulation (0–50 g, 20 seconds), **(K)** the response of the IPSI and CONTRA hindpaws to acetone application (100 μL), **(L)** the number of lifts of the IPSI hindpaw in the cold plate test (2 minutes, 4°C), and **(M)** the weight distributed on the IPSI and CONTRA hindpaws in the dynamic weight-bearing test (5 minutes). **(N)** The effects of the

Selective Activation of the LC-BLA Pathway Induces Anxiety and Aversive Learning

To assess whether activation of the LC-BLA pathway would induce pain-related phenotypes, we examined the effects of rM3D(Gs)-DREADD–mediated activation of this pathway in sham and CCI-ST animals (Figure 5A, B; Supplemental Figures S12A–E, I–O, S13A). CNO-activated sham animals spent significantly less time in the open zones of the EZM and entered this zone less often than sham-ss rats ($p < .05$) (Figure 5C–F). This effect was significantly blocked by intra-BLA administration of propranolol ($p < .01$) (Supplemental Figure S14A–E). Moreover, similar results were obtained in the open field test (Figure 5G–J). Subsequent evaluation of the animal's cognitive abilities showed a significant increase in the step-through latency of sham-CNO rats relative to the sham-ss animals after STM ($p < .05$) and LTM ($p < .01$) (Figure 5O; Supplemental Figure S13F), which was significantly blocked by intra-BLA propranolol ($p < .05$) (Supplemental Figure S14A, F, G). Hence, LC-BLA activation appears to provoke an anxiogenic-like profile mediated by local β-AR activation, in agreement with previous optogenetic data (21). Strikingly, LC-BLA activation did not modify the parameters of CCI-ST rats, except that CNO decreased the number of entries into the open zones of the EZM relative to the CCI-ST-ss rats ($p < .05$) (Figure 5E). Furthermore, no significant change was observed between CCI-ST-CNO and CCI-ST-ss rats in the step-through latency (Figure 5O). We next explored the local effect of the nonselective β-AR agonist isoproterenol (Supplemental Figures S15A and S16A). At the dose studied, isoproterenol significantly reduced the time spent by CCI-ST animals in the open zone in the EZM ($p < .05$ vs. CCI-ST-ss) (Supplemental Figure S15B–E) but not in the open field test ($p = .06$ vs. CCI-ST-ss) (Supplemental Figure S15F–I). No significant change was observed in the step-through latency (Supplemental Figures S15N and S16F).

We also evaluated the activation of this LC-BLA pathway in LT pain (CCI-LT) (Figure 6A; Supplemental Figure S17A). While in these experiments sham-CNO animals performed similarly to those tested previously, CNO did not modify the pain-induced anxiety and aversive learning and memory of CCI-LT-CNO animals relative to the CCI-LT-ss rats (Figure 6B–I, N; Supplemental Figure S17F), although it did increase the number of fecal boluses recorded in the open field test (CCI-LT-CNO vs. CCI-LT-ss, $p < .01$) (Figure 6H). Importantly, CNO-activated CCI-LT rats did not further increase the already elevated expression of c-Fos neurons in the LC of CCI-LT-ss animals ($p < .05$ vs. sham-ss) (Supplemental Figure S12F–H).

No differences were found between groups in the sensorial evaluation (Figures 5K–N, 6J–M; Supplemental Figures S13B–E, M, S16B–E, and S17B–E) and novel object recognition test (Figures 5P–Q, 6O, P; Supplemental Figures S13G–I, S15O, P, S16G–I, and S17G–I). Thus, activation of the LC-BLA pathway through β-ARs promotes an anxiogenic-like state, as well as an increase in aversive learning and in the memory index in pain-free animals (sham), whereas minor changes are produced in ST and LT nerve-injured animals.

DISCUSSION

Unlike ST pain, LT neuropathic pain leads to an anxiety-like profile and cognitive alterations. Notably, chemogenetic blockade of LC neurons that project to the BLA can reverse such pain-induced anxiety, as well as enhancing PA learning and memory, suggesting that overactivity of this pathway involves β-AR. While activation of LC-BLA neurons leads to anxiety and to enhanced aversive learning and memory in pain-free animals, minor changes are produced in nerve-injured animals. Furthermore, LC-BLA neurons do not seem to modulate sensorial sensitivity or episodic memory.

It has been reported that sensorial hypersensitivity appears immediately in rodents following nerve injury and that after several weeks (3–8 weeks depending on the species and animal model), anxiodepressive and cognitive symptoms arise (7,39,40). Indeed, ST and LT nerve injury in Long-Evans rats lowers sensory thresholds, while only LT injury provokes an anxiogenic-like profile. An evaluation of aversive learning and memory performance shows that only CCI-LT enhances PA learning, as occurs in rodents previously submitted to acute distress, a process involving BLA activity (41). Alternatively, all animals demonstrate similar fear-conditioned acquisition in the fear conditioning test, as indicated previously (42), although the high rate of acquisition (around 90%) might limit the differences between the groups. Interestingly, CCI-LT animals display a higher percentage of freezing in the conditioning phase, developing stronger and earlier fear behavior when faced with an aversive stimulus. Furthermore, and somewhat surprisingly, CCI-LT animals freeze in the habituation phase in a different context (context B). Thus, it would appear that CCI-LT animals do not recognize context B to be different from context A and consequently do not extinguish contextual fear. Indeed, CCI-LT animals show deficits in visual attention, as well as in visuospatial recognition learning and memory, in the novel object recognition test (a nonemotional related test), as reported previously (43). Furthermore, CCI-LT rats also show a deficit in the object pattern separation test that aims to measure spatial pattern separation. Therefore, LT neuropathic pain appears to produce a cognitive bias affecting the processing of aversive stimuli rather than neutral stimuli, probably provoking and maintaining a state of anxiety as appears to occur in human disease-related anxiety (44).

systemic inhibition of the β-ARs on passive avoidance (PA). The graph represents the latency to enter the dark compartment (seconds) in the different phases of the paradigm: training (prior to acquisition), short-term memory (STM) test (as learning index), and long-term memory (LTM) test (as memory index). **(O–P)** The effects of the systemic inhibition of the β-ARs in the novel object recognition (NOR) test. **(O)** The representative heatmaps showing activity (yellow = low activity, red = high activity) around the objects in the memory tests (A = familiar object, B = novel object) and **(P)** the graph represents the discrimination index between objects in the different phases of the paradigm: training, STM, and LTM. The data represent the mean ± SEM ($n = 7–8$ per group): $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ vs. sham-saline (ss); $+p < .05$, $++p < .01$, $+++p < .001$ vs. CCI-LT-ss; $\#\#p < .01$, $\#\#\#p < .001$ vs. sham-Prop as assessed by two-way analysis of variance followed by Newman-Keuls post hoc test. $^{***}p < .001$ (red asterisks) vs. prior to acquisition of each group as assessed by two-way analysis of variance with repeated measures followed by Newman-Keuls post hoc test. A.U., arbitrary units.

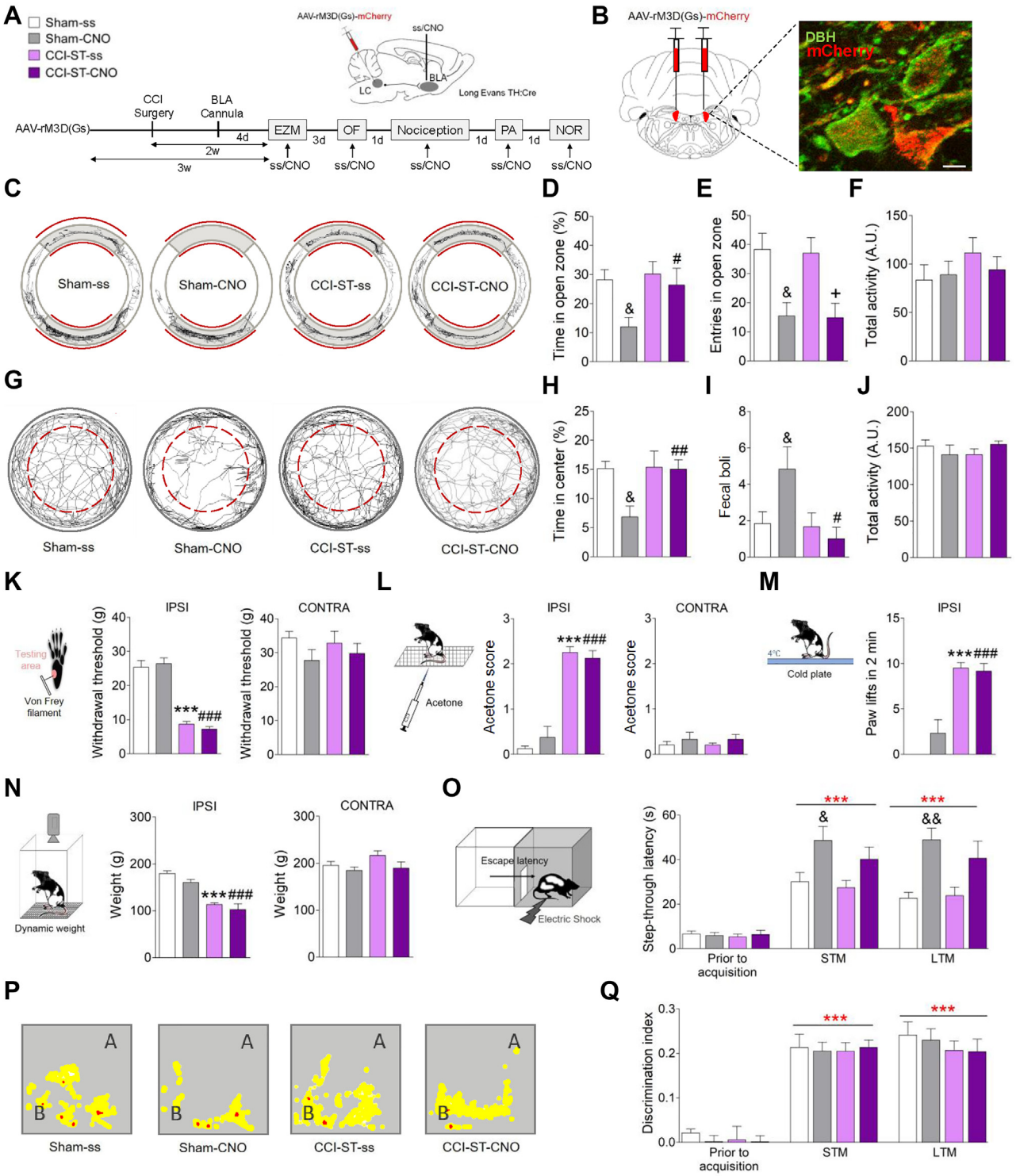

Figure 5. Selective activation of the locus coeruleus–basolateral amygdala (LC-BLA) pathway (short-term [ST] neuropathic pain). **(A)** Experimental timeline and schematic representation of the viral and cannula delivery of rM3D(Gs)-DREADD (designer receptor exclusively activated by designer drugs) for behavioral experiments in male Long-Evans tyrosine hydroxylase (TH)–Cre rats. A Cre-dependent adeno-associated virus (AAV), fluorescently tagged with mCherry [AAV-rM3D(Gs)-mCherry] (1.4 μL/side), was injected bilaterally into the TH neurons of the LC of TH-Cre rats 1 week before inducing chronic constriction injury (CCI). The rats were implanted bilaterally with a metal cannula into the BLA 4 days before the behavioral studies were performed. Clozapine-*N*-oxide (CNO) (3 μM/0.5 μL) was administered 20 minutes before performing the behavioral tests. **(B)** Representative immunofluorescence images (scale bar = 10 μm) of

The activity of LC-noradrenergic neurons is required to elicit acute stress-induced anxiety, and optogenetic/chemogenetic activation of LC neurons itself is anxiogenic (45,46). One of the LC-related networks involved in the expression of emotions is the BLA. Stress augments the noradrenaline in the BLA (47,48), this noradrenaline coming from the LC and from other structures (20). Later studies showed that photostimulation of LC-noradrenergic fibers in the BLA evokes noradrenaline release in this structure, altering BLA neuronal activity and condition aversion, and increasing anxiety-like behavior in control animals (45). Accordingly, selective chemogenetic activation of tyrosine hydroxylase–positive LC neurons that project to the BLA elicits anxiety-like behavior and aversive memory in sham animals, specifically linking the LC-BLA network with negative emotions in pain free animals. Furthermore, it is notable that LC-BLA inhibition does not modify any of the parameters evaluated in sham, suggesting that inhibition of the LC-noradrenergic system is not necessarily anxiolytic.

In the light of these findings, we hypothesize that pain-induced overactivation of the LC-BLA pathway increases the salience of aversive sensory events in LT nerve-injured animals. Blocking this pathway produces robust recovery of the anxiogenic and cognitive-aversive thresholds, which is mediated by beta-adrenergic activity in the BLA. In fact, site-specific pharmacological blockade indicates that β-ARs within the BLA are necessary for this behavior and that they mediate the anxiety-like phenotype associated with increased noradrenaline release in the BLA (21). Furthermore, systemic administration of a β-AR antagonist also reverses the anxiogenic phenotype of animals experiencing LT pain. This effect may have translational value, opening the possibility to treat pain-induced anxiety with brain penetrant beta-blockers. By contrast, chemogenetic LC-BLA inhibition had no effect on a low-arousing behavioral task (novel object recognition test). Previous studies revealed that noradrenergic activation of BLA modulates hippocampal consolidation in the novel object recognition test, although emotional arousal was enhanced in this test and the role of the BLA was potentiated in all these studies (49–52). By contrast, our protocol produces little emotional arousal, minimizing the influence of the LC-BLA pathway in this paradigm. Indeed, if this nonemotional task only includes strong novelty-related emotional arousal, plastic changes in the hippocampus are mediated by the BLA (53). Furthermore, deficits evident in the novel object recognition test have been ascribed to enhanced noradrenergic input to the prefrontal cortex following nerve injury (54). This suggests that other LC brain targets (the hippocampus or prefrontal cortex) might be involved in the novel object recognition test deficits in CCI-LT animals. Alternatively, no change in sensorial hypersensitivity arises when the LC-BLA noradrenergic pathway is inhibited, suggesting that this pathway is not directly related to nociception itself.

We also wondered about the consequences of activating LC-BLA neurons in nerve-injured animals. As expected, stronger activity of this pathway leads to an anxiogenic profile and, consequently, to an increase in the processing of aversive information mediated by β-AR in pain-free animals. Strikingly, nerve injured animals that do not develop secondary anxiety-related symptoms do not display a robust enhancement of their anxiety-related profile when LC-BLA neurons are chemogenetically activated. However, the pharmacological activation of β-AR in the BLA with isoproterenol did intensify the anxiogenic phenotype of these animals to some extent. This was unexpected and might be due to changes in AR expression, with opposing effects of β-ARs and β_2-ARs (55,56). Alternatively, these behavioral manifestations may be counteracted by the activity of other pathways that impede the noradrenergic activation of the BLA after ST injury. Perhaps, these opposing effects are overridden after a period of time, leading to the anxiogenic phenotype and the stronger aversive memory performance evident in CCI-LT rats. Further studies into the different ARs and the expression of c-Fos–mediated rM3D(Gs)-DREADD will be necessary to determine what plastic changes take place in this phase of pain. Interestingly, it was recently shown that the chemogenetic activation of LC neurons projecting to the prefrontal cortex provokes anxiety and aversion in naïve animals, as well as anxiety, aversion, and spontaneous pain in animals with neuropathic pain but without comorbid anxiety (19). This demonstrates that LC neurons can produce distinct responses and that prefrontal cortex–projecting ensemble is susceptible to full activation in conjunction with pain, provoking anxiety. rM3D(Gs)-DREADD was not able to further intensify anxiety or the already elevated c-Fos expression in LT nerve-injured animals, suggesting that LT pain leads to maximal activation of the LC-BLA pathway. Bearing in mind previous findings (19), it would be

rM3D(Gs)-mCherry expression in noradrenergic LC neurons (red = mCherry; green = dopamine beta hydroxylase). The rats were tested 2–3 weeks (ST) after surgery. **(C–F)** The effects of activating the LC-BLA pathway in the elevated zero maze (EZM). **(C)** The representative activity traces and the graphs represent **(D)** the relative time spent in the open zone and **(E)** the number of entries into that zone, as well as **(F)** the locomotor activity. **(G–J)** The effects of activating the LC-BLA pathway in the open field (OF) test. **(G)** The representative activity traces and the graphs represent **(H)** the relative time spent in the central zone, **(I)** the number of fecal boluses, and **(J)** the locomotor activity. **(K–N)** Sensorial evaluation. The graphs represent **(K)** the withdrawal threshold (grams) of the ipsilateral (IPSI) and contralateral (CONTRA) hindpaws in response to von Frey hair stimulation (0–50 g, 20 seconds), **(L)** the response of the IPSI and CONTRA hindpaws to acetone application (100 μL), **(M)** the number of lifts of the ipsilateral hindpaw in the cold plate test (2 minutes, 4°C), and **(N)** the weight distributed on the IPSI and CONTRA hindpaws in the dynamic weight bearing test (5 minutes). **(O)** The effects of activating the LC-BLA pathway on passive avoidance (PA). The graph represents the latency to enter the dark compartment (seconds) in the different phases of the paradigm: training (prior to acquisition), short-term memory (STM) test (as learning index), and long-term memory (LTM) test (as memory index). **(P–Q)** The effects of activating the LC-BLA pathway on the novel object recognition (NOR) test. **(P)** The representative heatmaps showing activity (yellow = low activity, red = high activity) around the objects in the memory tests (A = familiar object, B = novel object) and **(Q)** the graph represents the discrimination index between objects in the different phases of the paradigm: training, STM, and LTM. The data represent the mean ± SEM (n = 5–6 per group): $^{**}p < .01$, $^{***}p < .001$ vs. sham-saline (ss); $+p < .05$ vs. CCI-ST-ss; $\#p < .05$, $\#\#p < .01$, $\#\#\#p < .001$ vs. sham-CNO; $\&p < .05$, $\&\&\ p < .01$ vs. sham-ss as assessed by two-way analysis of variance followed by Newman-Keuls post hoc test. $^{***}p < .001$ (red asterisks) vs. prior to acquisition of each group as assessed by two-way analysis of variance with repeated measures followed by Newman-Keuls post hoc test. A.U., arbitrary units.

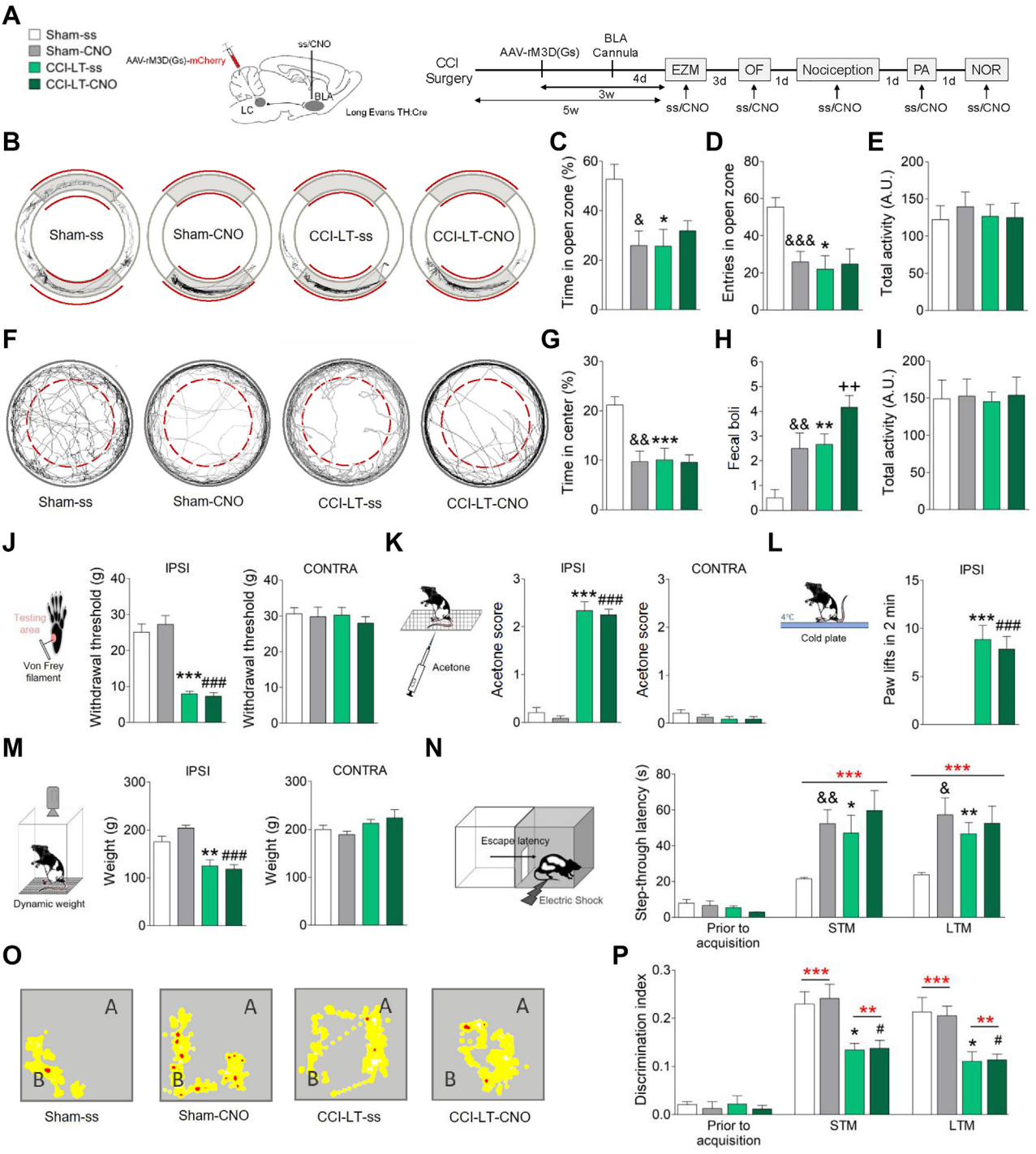

Figure 6. Selective activation of the locus coeruleus–basolateral amygdala (LC-BLA) pathway (long-term [LT] neuropathic pain). **(A)** Experimental timeline and schematic representation of viral and cannula delivery of rM3D(Gs)-DREADD (designer receptor exclusively activated by designer drugs) for behavioral experiments in male Long-Evans tyrosine hydroxylase (TH)–Cre rats. A Cre-dependent adeno-associated virus (AAV), fluorescently tagged with mCherry [AAV-rM3D(Gs)-mCherry] (1.4 μL/side), was injected bilaterally into the TH neurons of locus coeruleus (LC) of TH-Cre rats 2 weeks after chronic constriction injury (CCI). The rats were allowed to recover for 3 weeks to achieve robust expression of DREADD, and a metal cannula was implanted bilaterally into the BLA 4 days before carrying out the behavioral studies. Clozapine-*N*-oxide (CNO) (3 μM/0.5 μL) was administered 20 minutes before the behavioral testing. The rats were tested 5–6 weeks (LT) after surgery. **(B–E)** The effects of activating the LC-BLA pathway in the elevated zero maze (EZM). **(B)** The representative activity traces

very interesting to explore the LC–prefrontal cortex activity in animals experiencing neuropathic pain in conjunction with comorbid anxiety. Stronger activity of the LC–medial prefrontal cortex projecting neurons is required during extinction of aversive learning (23). Weaker activity in this circuit may underlie the inability to achieve extinction of aversive experiences described in LT pain animals that also display anxiety (57). Finally, neither mechanical sensory nor low-arousal task behavior is modified in these animals, indicating that the sensorial, affective, and cognitive dimensions are independent. Accordingly, one dimension can deteriorate without affecting the other.

Using ethological tests and models that involve learned/punished responses, the data presented here show that overactivation of the LC-BLA pathway provoked by chronic pain leads to anxiety and enhanced processing of aversive stimuli, which in turn might exaggerate vulnerability when confronted by stress. Specifically, pain would augment the noradrenaline in the BLA, enhancing the memorization of negative events. The inhibition of this projection, or that of the local blockade of β-ARs, inhibits pain-induced anxiety. This beneficial effect on anxiety was also found when beta-blockers were administered systemically, which may open new interventional options for the treatment of comorbid pain and anxiety. Finally, our study builds on previous work showing the circuit-selective modular organization of the LC system and its role in regulating brain function in the healthy and disease states (19,21,23).

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ARTICLE INFORMATION

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REFERENCES

1. Reid KJ, Harker J, Bala MM, Truyers C, Kellen E, Bekkering GE, *et al.* (2011): Epidemiology of chronic non-cancer pain in Europe: Narrative review of prevalence, pain treatments and pain impact. Curr Med Res Opin 27:449–462.
2. Goldberg DS, McGee SJ (2011): Pain as a global public health priority. BMC Public Health 11:770.
3. Johannes CB, Le TK, Zhou X, Johnston JA, Dworkin RH (2010): The prevalence of chronic pain in United States adults: Results of an Internet-based survey. J Pain 11:1230–1239.
4. Breivik H, Collett B, Ventafridda V, Cohen R, Gallacher D (2006): Survey of chronic pain in Europe: Prevalence, impact on daily life, and treatment. Eur J Pain 10:287–333.
5. Maletic V, Raison CL (2009): Neurobiology of depression, fibromyalgia and neuropathic pain. Front Biosci (Landmark Ed) 14:5291–5338.
6. McWilliams LA, Cox BJ, Enns MW (2003): Mood and anxiety disorders associated with chronic pain: An examination in a nationally representative sample. Pain 106:127–133.
7. Alba-Delgado C, Cebada-Aleu A, Mico JA, Berrocoso E (2016): Comorbid anxiety-like behavior and locus coeruleus impairment in diabetic peripheral neuropathy: A comparative study with the chronic constriction injury model. Prog Neuropsychopharmacol Biol Psychiatry 71:45–56.

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8. Alba-Delgado C, Llorca-Torralba M, Horrillo I, Ortega JE, Mico JA, Sanchez-Blazquez P, *et al.* (2013): Chronic pain leads to concomitant noradrenergic impairment and mood disorders. Biol Psychiatry 73:54–62.
9. Alba-Delgado C, Mico JA, Sanchez-Blazquez P, Berrocoso E (2012): Analgesic antidepressants promote the responsiveness of locus coeruleus neurons to noxious stimulation: Implications for neuropathic pain. Pain 153:1438–1449.
10. Borges G, Miguelez C, Neto F, Mico JA, Ugedo L, Berrocoso E (2017): Activation of extracellular signal-regulated kinases (ERK 1/2) in the locus coeruleus contributes to pain-related anxiety in arthritic male rats. Int J Neuropsychopharmacol 20:463.
11. Borges GP, Mico JA, Neto FL, Berrocoso E (2015): Corticotropin-releasing factor mediates pain-induced anxiety through the ERK1/2 signaling cascade in locus coeruleus neurons. Int J Neuropsychopharmacol 18:pyv019.
12. Bravo L, Alba-Delgado C, Torres-Sanchez S, Mico JA, Neto FL, Berrocoso E (2013): Social stress exacerbates the aversion to painful experiences in rats exposed to chronic pain: The role of the locus coeruleus. Pain 154:2014–2023.
13. Bravo L, Mico JA, Rey-Brea R, Camarena-Delgado C, Berrocoso E (2016): Effect of DSP4 and desipramine in the sensorial and affective component of neuropathic pain in rats. Prog Neuropsychopharmacol Biol Psychiatry 70:57–67.
14. Bravo L, Torres-Sanchez S, Alba-Delgado C, Mico JA, Berrocoso E (2014): Pain exacerbates chronic mild stress-induced changes in noradrenergic transmission in rats. Eur Neuropsychopharmacol 24:996–1003.
15. Llorca-Torralba M, Borges G, Neto F, Mico JA, Berrocoso E (2016): Noradrenergic Locus Coeruleus pathways in pain modulation. Neuroscience 338:93–113.
16. Llorca-Torralba M, Mico JA, Berrocoso E (2018): Behavioral effects of combined morphine and MK-801 administration to the locus coeruleus of a rat neuropathic pain model. Prog Neuropsychopharmacol Biol Psychiatry 84:257–266.
17. Torres-Sanchez S, Borges GDS, Mico JA, Berrocoso E (2018): Opioid and noradrenergic contributions of tapentadol to the inhibition of locus coeruleus neurons in the streptozotocin rat model of polyneuropathic pain. Neuropharmacology 135:202–210.
18. Martins AR, Froemke RC (2015): Coordinated forms of noradrenergic plasticity in the locus coeruleus and primary auditory cortex. Nat Neurosci 18:1483–1492.
19. Hirschberg S, Li Y, Randall A, Kremer EJ, Pickering AE (2017): Functional dichotomy in spinal- vs. prefrontal-projecting locus coeruleus modules splits descending noradrenergic analgesia from ascending aversion and anxiety in rats. Elife 6:e29808.
20. Robertson SD, Plummer NW, de Marchena J, Jensen P (2013): Developmental origins of central norepinephrine neuron diversity. Nat Neurosci 16:1016–1023.
21. McCall JG, Siuda ER, Bhatti DL, Lawson LA, McElligott ZA, Stuber GD, *et al.* (2017): Locus coeruleus to basolateral amygdala noradrenergic projections promote anxiety-like behavior. Elife 6:e18247.
22. Siuda ER, Al-Hasani R, McCall JG, Bhatti DL, Bruchas MR (2016): Chemogenetic and optogenetic activation of galphas signaling in the basolateral amygdala induces acute and social anxiety-like states. Neuropsychopharmacology 41:2011–2023.
23. Uematsu A, Tan BZ, Ycu EA, Cuevas JS, Koivumaa J, Junyent F, *et al.* (2017): Modular organization of the brainstem noradrenaline system coordinates opposing learning states. Nat Neurosci 20:1602–1611.
24. Bennett GJ, Xie YK (1988): A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 33:87–107.
25. Berrocoso E, De Benito MD, Mico JA (2007): 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) 193:97–105.
26. Vazey EM, Aston-Jones G (2014): Designer receptor manipulations reveal a role of the locus coeruleus noradrenergic system in isoflurane general anesthesia. Proc Natl Acad Sci U S A 111:3859–3864.
27. Tervo DGR, Proskurin M, Manakov M, Kabra M, Vollmer A, Branson K, *et al.* (2014): Behavioral variability through stochastic choice and its gating by anterior cingulate cortex. Cell 159:21–32.
28. Cryan JF, Kelly PH, Chaperon F, Gentsch C, Mombereau C, Lingenhoehl K, *et al.* (2004): Behavioral characterization of the novel GABAB receptor-positive modulator GS39783 (N,N'-dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine): Anxiolytic-like activity without side effects associated with baclofen or benzodiazepines. J Pharmacol Exp Ther 310:952–963.
29. Bleickardt CJ, Mullins DE, Macsweeney CP, Werner BJ, Pond AJ, Guzzi MF, *et al.* (2009): Characterization of the V1a antagonist, JNJ-17308616, in rodent models of anxiety-like behavior. Psychopharmacology (Berl) 202:711–718.
30. Berrocoso E, Mico JA, Vitton O, Ladure P, Newman-Tancredi A, Depoortere R, *et al.* (2011): Evaluation of milnacipran, in comparison with amitriptyline, on cold and mechanical allodynia in a rat model of neuropathic pain. Eur J Pharmacol 655:46–51.
31. Bravo L, Mico JA, Rey-Brea R, Perez-Nievas B, Leza JC, Berrocoso E (2012): Depressive-like states heighten the aversion to painful stimuli in a rat model of comorbid chronic pain and depression. Anesthesiology 117:613–625.
32. Jasmin L, Kohan L, Franssen M, Janni G, Goff JR (1998): The cold plate as a test of nociceptive behaviors: Description and application to the study of chronic neuropathic and inflammatory pain models. Pain 75:367–382.
33. Tetreault P, Dansereau MA, Dore-Savard L, Beaudet N, Sarret P (2011): Weight bearing evaluation in inflammatory, neuropathic and cancer chronic pain in freely moving rats. Physiol Behav 104:495–502.
34. Suarez-Pereira I, Canals S, Carrion AM (2015): Adult newborn neurons are involved in learning acquisition and long-term memory formation: The distinct demands on temporal neurogenesis of different cognitive tasks. Hippocampus 25:51–61.
35. Romero-Granados R, Fontan-Lozano A, Delgado-Garcia JM, Carrion AM (2010): From learning to forgetting: Behavioral, circuitry, and molecular properties define the different functional states of the recognition memory trace. Hippocampus 20:584–595.
36. van Hagen BT, van Goethem NP, Lagatta DC, Prickaerts J (2015): The object pattern separation (OPS) task: A behavioral paradigm derived from the object recognition task. Behav Brain Res 285:44–52.
37. Fanselow MS (1980): Conditioned and unconditional components of post-shock freezing. Pavlov J Biol Sci 15:177–182.
38. Llorca-Torralba M, Pilar-Cuéllar F, Bravo L, Bruzos-Cidon C, Torrecilla M, Mico JA, *et al.* (2019): Opioid activity in the locus coeruleus is modulated by chronic neuropathic pain. Mol Neurobiol 56:4135–4150.
39. Barthas F, Sellmeijer J, Hugel S, Waltisperger E, Barrot M, Yalcin I (2015): The anterior cingulate cortex is a critical hub for pain-induced depression. Biol Psychiatry 77:236–245.
40. Yalcin I, Barthas F, Barrot M (2014): Emotional consequences of neuropathic pain: Insight from preclinical studies. Neurosci Biobehav Rev 47:154–164.
41. Ito W, Erisir A, Morozov A (2015): Observation of distressed conspecific as a model of emotional trauma generates silent synapses in the prefrontal-amygdala pathway and enhances fear learning, but ketamine abolishes those effects. Neuropsychopharmacology 40:2536–2545.
42. Moriarty O, Roche M, McGuire BE, Finn DP (2012): Validation of an air-puff passive-avoidance paradigm for assessment of aversive learning and memory in rat models of chronic pain. J Neurosci Methods 204:1–8.
43. Kodama D, Ono H, Tanabe M (2011): Increased hippocampal glycine uptake and cognitive dysfunction after peripheral nerve injury. Pain 152:809–817.
44. Burman OH, Parker RM, Paul ES, Mendl MT (2009): Anxiety-induced cognitive bias in non-human animals. Physiol Behav 98:345–350.
45. McCall JG, Al-Hasani R, Siuda ER, Hong DY, Norris AJ, Ford CP, *et al.* (2015): CRH engagement of the locus coeruleus noradrenergic system mediates stress-induced anxiety. Neuron 87:605–620.
46. Sciolino NR, Plummer NW, Chen YW, Alexander GM, Robertson SD, Dudek SM, *et al.* (2016): Recombinase-dependent mouse lines for

chemogenetic activation of genetically defined cell types. Cell Rep 15:2563–2573.
47. Galvez R, Mesches MH, McGaugh JL (1996): Norepinephrine release in the amygdala in response to footshock stimulation. Neurobiol Learn Mem 66:253–257.
48. Hatfield T, Spanis C, McGaugh JL (1999): Response of amygdalar norepinephrine to footshock and GABAergic drugs using in vivo microdialysis and HPLC. Brain Res 835:340–345.
49. Mello-Carpes PB, da Silva de Vargas L, Gayer MC, Roehrs R, Izquierdo I (2016): Hippocampal noradrenergic activation is necessary for object recognition memory consolidation and can promote BDNF increase and memory persistence. Neurobiol Learn Mem 127:84–92.
50. Mello-Carpes PB, Izquierdo I (2013): The nucleus of the solitary tract–> nucleus paragigantocellularis–> locus coeruleus–> CA1 region of dorsal hippocampus pathway is important for consolidation of object recognition memory. Neurobiol Learn Mem 100:56–63.
51. Barsegyan A, McGaugh JL, Roozendaal B (2014): Noradrenergic activation of the basolateral amygdala modulates the consolidation of object-in-context recognition memory. Front Behav Neurosci 8:160.
52. Roozendaal B, Castello NA, Vedana G, Barsegyan A, McGaugh JL (2008): Noradrenergic activation of the basolateral amygdala modulates consolidation of object recognition memory. Neurobiol Learn Mem 90:576–579.
53. McReynolds JR, Anderson KM, Donowho KM, McIntyre CK (2014): Noradrenergic actions in the basolateral complex of the amygdala modulate Arc expression in hippocampal synapses and consolidation of aversive and non-aversive memory. Neurobiol Learn Mem 115:49–57.
54. Suto T, Eisenach JC, Hayashida K (2014): Peripheral nerve injury and gabapentin, but not their combination, impair attentional behavior via direct effects on noradrenergic signaling in the brain. Pain 155:1935–1942.
55. Bashiri H, Rezayof A, Sahebgharani M, Tavangar SM, Zarrindast MR (2016): Modulatory effects of the basolateral amygdala alpha2-adrenoceptors on nicotine-induced anxiogenic-like behaviours of rats in the elevated plus maze. Neuropharmacology 105:478–486.
56. Buffalari DM, Grace AA (2007): Noradrenergic modulation of basolateral amygdala neuronal activity: Opposing influences of alpha-2 and beta receptor activation. J Neurosci 27:12358–12366.
57. Mutso AA, Radzicki D, Baliki MN, Huang L, Banisadr G, Centeno MV, *et al.* (2012): Abnormalities in hippocampal functioning with persistent pain. J Neurosci 32:5747–5756.