

Social stress exacerbates the aversion to painful experiences in rats exposed to chronic pain: The role of the locus coeruleus

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ABSTRACT

Stressful experiences seem to negatively influence pain perception through as yet unknown mechanisms. As the noradrenergic locus coeruleus (LC) nucleus coordinates many components of the stress response, as well as nociceptive transmission, we evaluated whether the sensory and affective dimension of chronic neuropathic pain worsens in situations of stress due to adaptive changes of LC neurons. Accordingly, male rats were socially isolated for 5 weeks, and in the last 2 weeks, neuropathic pain was induced by chronic constriction injury. In this situation of stress, chronic pain selectively heightened the animal's aversion to painful experiences (affective pain), as measured in the place escape/avoidance test, although no changes were observed in the sensory dimension of pain. In addition, electrophysiological recordings of LC neurons showed a low tonic but exacerbated nociceptive-evoked activity when the injured paw was stimulated. These changes were accompanied by an increase in tyrosine hydroxylase and gephyrin expression in the LC. Furthermore, intra-LC administration of bicuculline, a γ -aminobutyric acid-A receptor antagonist, attenuated the negative affective effects of pain. These data show that changes in the LC are greater than those expected from the simple summation of each independent factor (pain and stress), revealing mechanisms through which stressors may exacerbate pain perception without affecting the sensorial dimension.

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1. Introduction

Chronic neuropathic pain is a common problem for which adequate treatments remain elusive. This is because the relationship between the nociceptive input that originates at the injured nerve and the resulting subjective pain experience is far from linear. Stress has consistently been shown to be a factor that contributes to the maintenance and amplification of the severity of pain [17,39]. However, most such reports are correlational and the underlying biological mechanisms remain unclear.

The main source of noradrenaline in the central nervous system is the pontine locus coeruleus nucleus (LC), a structure that is closely linked to pain, stress, and emotions. Indeed, ascending nociceptive inputs from the LC reach the forebrain, while its descending projections innervate the spinal cord [33]. In addition,

it is a structure that is essential to the cognitive branch of the stress response and it is thought to be dysregulated in mental disorders such as depression, anxiety, and posttraumatic stress disorder [24,40]. In this sense, we have recently shown that long-term neuropathic pain leads to anxio-depressive-like behaviour that is temporally accompanied by an impairment of the LC [2]. Moreover, antidepressants that inhibit the reuptake of noradrenaline can normalize the nociceptive-evoked responses of LC neurons in rats that experience neuropathic pain [3]. Thus, we hypothesized that stress may trigger functional and/or structural changes in the LC that amplify pain. Accordingly, in this study we have evaluated the effect of stress (social isolation) on the pain response and on the electrophysiological/neurochemical properties of LC in neuropathic rats.

2. Methods

2.1. Animals and experimental design

Male Sprague–Dawley rats (University of Cadiz [ES110120000210], Spain) were used in these studies, in accordance with the

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guidelines of the European Commission's directive (2010/63/EU) and the Spanish Law (RD 1201/2005). All the experimental protocols were approved by the Committee for Animal Experimentation at the University of Cadiz (Spain). Animals (weighing 200–220 g at the beginning of the experiment) were housed under standard laboratory conditions (water and food ad libitum, a constant room temperature of $22 \pm 1^\circ\text{C}$, and a 12-hour light/dark cycle). Neuropathic pain was induced by chronic constriction injury (CCI [5,6]) whereby the common left sciatic nerve was ligated. In sham, an identical dissection was performed in the left paw, but the sciatic nerve was not ligated. Stressed rats were housed individually in standard plastic cages ($22 \times 22 \times 14.5$ cm), while control animals were housed in groups of 4 in similar cages ($21.5 \times 46.5 \times 14.5$ cm). Control rats were housed according to their pain condition, that is, animals of the same cage belonged to the same group (sham or CCI). The stress regime lasted for 5 weeks and, where appropriate, neuropathic pain was induced during the third week of stress (Fig. 1A). Thus, 4 groups were evaluated: Sham-control, Sham-stress, CCI-control, and CCI-stress.

2.2. Nociceptive behavioural testing

Sensory pain was evaluated before, as well as 1 and 2 weeks after nerve injury (weeks 3, 4, and 5 of stress regime). The presence of mechanical allodynia was measured using an automatic apparatus to apply the von Frey test (Dynamic Plantar Aesthesiometer, Ugo Basile, Italy). This device uses a single nonflexible filament that applies increasing force (from 0 to 50 g) against the plantar surface of the hind paw over a 20-second period. The nocifensive paw withdrawal response automatically turns off the stimulus, and the mechanical pressure that evoked the response is recorded [8]. Two measurements were taken for each paw at 5-minute intervals and the mean value was considered the nociceptive threshold. Cold allodynia was evaluated applying a drop of acetone (100 μL) with a pipette to the centre of the ventral surface of the hind paw [16]. Acetone was applied alternately 5 times to each hind paw, at 5-minute intervals, and the responses were recorded over a 1-minute period according to the following scale: 0, no response; 1, quick withdrawal, flick, or stamp of the paw; 2, prolonged

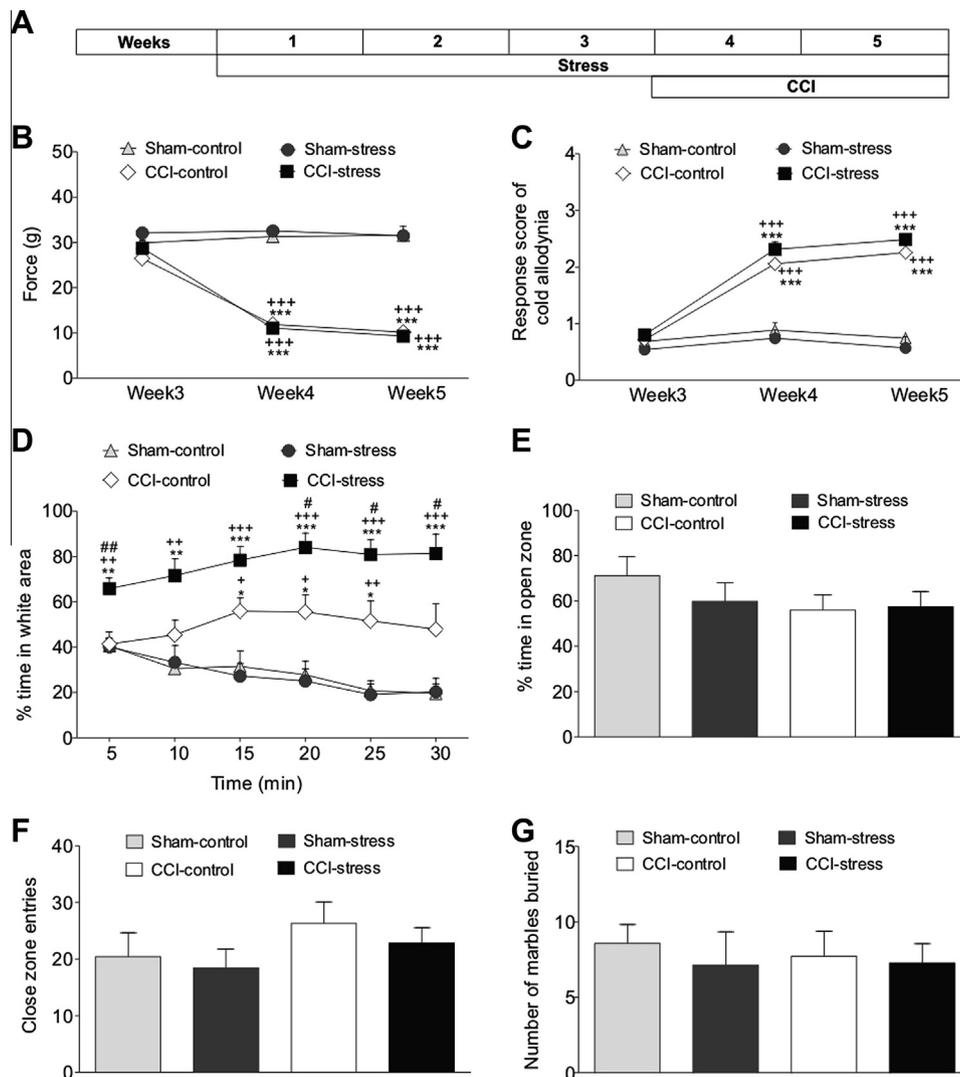


Fig. 1. Effect of neuropathic pain and stress on pain processing and anxiety-like behaviour. (A) Experimental design showing the programme of stress and chronic constriction injury (CCI). Mechanical (B) and cold allodynia (C) in response to a von Frey monofilament and acetone, respectively. (D) Emotional/cognitive component of pain measured by the place escape/avoidance test (PEAT). (E) Time spent in the open zone and (F) the number of entries into the closed zone in the elevated zero maze. (G) Number of buried marbles in the marble-burying test. The data are expressed as the mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs sham-control; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs sham-stress; # $P < 0.05$; ## $P < 0.01$, vs CCI-control ($n = 7-8$ per group).

withdrawal or repeated flicking of the paw; 3, repeated flicking of the paw with persistent licking directed at the ventral side of the paw. The cumulative score for each rat was obtained by summing the scores and dividing them by the number of assays.

2.3. Emotional behavioural testing

One set of animals was tested 5 weeks after stress in the place escape/avoidance test (PEAT) to explore the affective-cognitive dimension of pain [2,23]. Animals were placed in the centre of a 60 × 30 × 30 cm Plexiglas chamber on an elevated metal grid. One half of the chamber was white and the other half of the chamber was black. The rats were allowed to move freely throughout the chamber over the 30-minute test period, during which they were mechanically stimulated at 15-second intervals with a von Frey monofilament (60 g) on the plantar surface of the hind paw, depending on which area they were in. Thus, if the animals were in the black side of the chamber, the left (ipsilateral) paw was stimulated, whereas the right (contralateral) paw was stimulated when they were in the white side. Each 30-minute test session was recorded and subsequently analyzed with SMART (Spontaneous Motor Activity Recording and Tracking) software. We scored the proportion of time spent in the white area, binned into 5-minute time intervals.

To evaluate anxiety-like behaviour, the elevated zero maze test was performed after 5 weeks of stress [2]. After the animals were placed in a closed section, they were allowed to investigate the maze for 5 minutes, during which the time spent in the open zone was evaluated. Additionally, the number of close zone entries was recorded as indicative of motor behaviour. In addition, a marble-burying test was carried out [27] in which the rats were placed individually in a cage (21.5 × 46.5 × 14.5 cm) that contained 5-cm deep bedding and 20 marbles (1.5 cm in diameter), arranged in 5 by 4 rows. The test was performed over 30 minutes under white light, after which the rats were removed and the buried marbles counted. Marbles were considered buried if they were at least three-quarters covered with bedding. A decrease in the proportion of time spent in the open arms or an increase in the numbers of marbles buried was considered as an index of anxiety behaviour in the elevated zero maze test and marble buried test, respectively.

2.4. Intra-LC drug administration

At 4 days after CCI surgery, a parallel group of rats were anaesthetized and placed in a stereotaxic frame in order to implant a stainless steel guide cannula unilaterally into the right (contralateral) LC (relative to lambda and dural surface: anteroposterior –3.7 mm, midline –1.1 mm, and dorsoventral –6.2 mm; [32]). The contralateral LC was pharmacologically explored because most nociceptive inputs from periphery afferents decussate in the dorsal horn at spinal cord level and then; nociceptive information ascends to the contralateral LC neurons [33]. The cannula was fixed to the skull with polyacrylic cement, anchored with stainless steel screws, and it remained closed due to the insertion of a stainless steel wire. A dose of the γ -aminobutyric acid (GABA)-A receptor antagonist, 1(S), 9(R)-(–)-bicuculline methiodide (10 ng per 0.5 μ L; Sigma-Aldrich Chemicals, St. Louis, MO, USA), was dissolved in artificial cerebrospinal fluid and administered to the LC 5 minutes before the PEAT was performed. In addition, mechanical allodynia was evaluated using the von Frey test before and after the PEAT. To administer the drugs, animals were held gently while the wire was removed and a removable injector was inserted into the guide cannula, extending 1 mm beyond the guide tip. The injector was linked to a 10- μ L Hamilton syringe and the drug solution was injected over 30 seconds. The injector remained in the guide cannula for a further 1 minute after infusion to allow the

compound to spread. At the end of each experiment, 0.5 μ L of Pontamine Sky Blue was used to mark the cannula site. The location of the injection site was verified as being within the LC for all of the animals that were included in this study.

2.5. Electrophysiological LC recordings

Single-unit extracellular recordings of LC neurons contralateral to the operated hind paw were obtained 5 weeks after the start of the stress regime in a parallel set of animals [1,7]. The recording electrode was lowered into the LC (anteroposterior: –3.7 mm, mediolateral: –1.1 mm relative to lambda and 5.0–7.0 mm from brain surface after dura removal) [32]. LC neurons were identified on the basis of well-established criteria [12]: long-duration action potential (>2 ms), spontaneous firing at a regular rhythm, a slow firing rate between 0.5 and 5 Hz, and characteristic spikes with a long-lasting positive-negative waveform (Fig. 2B). Firing patterns were analyzed offline using the computer software Spike2 (Cambridge Electronic Design, Cambridge, UK). At the end of each experiment, the location of the recording site was verified (Fig. 2A). Only measurements from cells within the LC were included in this study.

When a single unit was isolated, the tonic and the phasic activity of LC neurons were measured. The coefficient of variation (%) was expressed as the ratio of the SD to the mean interval value of an interspike time-interval histogram; the burst firing discharge was defined according to Grace and Bunney (1984) [20]; and histograms of mean interspike interval (ISI) were defined as the frequency of spike intervals for consecutive spikes. Phasic activation (burst of action potentials) was elicited by paw compression (ipsilateral or contralateral to injured paw) for 1 second between the ends of a pair of 15-cm surgical forceps (Ref. 501742-G; World Precision Instruments, Hertfordshire, UK) by applying pressure midway along the forceps such that the opposite sides of the forceps, at this midpoint, came into contact. This sensory-evoked discharge was followed by a postactivation inhibition or suppression period (Fig. 2C) [21,41].

In another set of rats, intravenous (i.v.) dose-response curves were constructed for UK14,304 (α_2 -adrenoceptor agonist; 2.5–80.0 μ g/kg; Tocris Bioscience, Bristol, UK) and desipramine (nor-adrenaline reuptake inhibitor; 0.05–1.6 mg/kg; Sigma-Aldrich Chemicals) in doubling dose. When complete inhibition was achieved, LC neuron activity was reversed by i.v. administration of the α_2 -adrenoceptor antagonists, RX821002 and idazoxan, respectively (Sigma Chemical, St. Louis, Missouri). The effective dose for eliciting 50% (ED₅₀) of E_{max} (100%) was analyzed for the best nonlinear fit to the logistic 3-parameter equation [31]. Only one dose-response curve was obtained per rat.

2.6. Immunohistochemistry

One set of animals was perfused at the end of the experiments [1] and 1 in each 4 free-floating sequential sections (30 μ m) were incubated with a sheep antityrosine hydroxylase (TH, 1:1000; Abcam, Cambridge, UK) or a mouse monoclonal antigeophysin (mAb7a, 1:400; Synaptic Systems, Goettingen, Germany). Subsequently, the sections were incubated with, respectively, a donkey antisheep Alexa Fluor 568 (1:1000; Invitrogen, Madrid, Spain), or with biotinylated donkey antimouse antibody (1:200; Jackson Immuno-research Lab, West Grove, PA, USA) followed by Streptavidin 488 (1:1000; Invitrogen), before they were washed and coverslipped with fluorogel aqueous mounting medium. TH-immunoreactivity (TH-IR) was visualised on an Olympus BX60 microscope (Olympus America, Center Valley, PA, USA) equipped with a U-MNU filter system, and the number of cell bodies labelled (TH-IR) were counted manually in an average of 6 sections of LC per animal (n = 4–5 rats per group), by an experimenter blind to the conditions. The

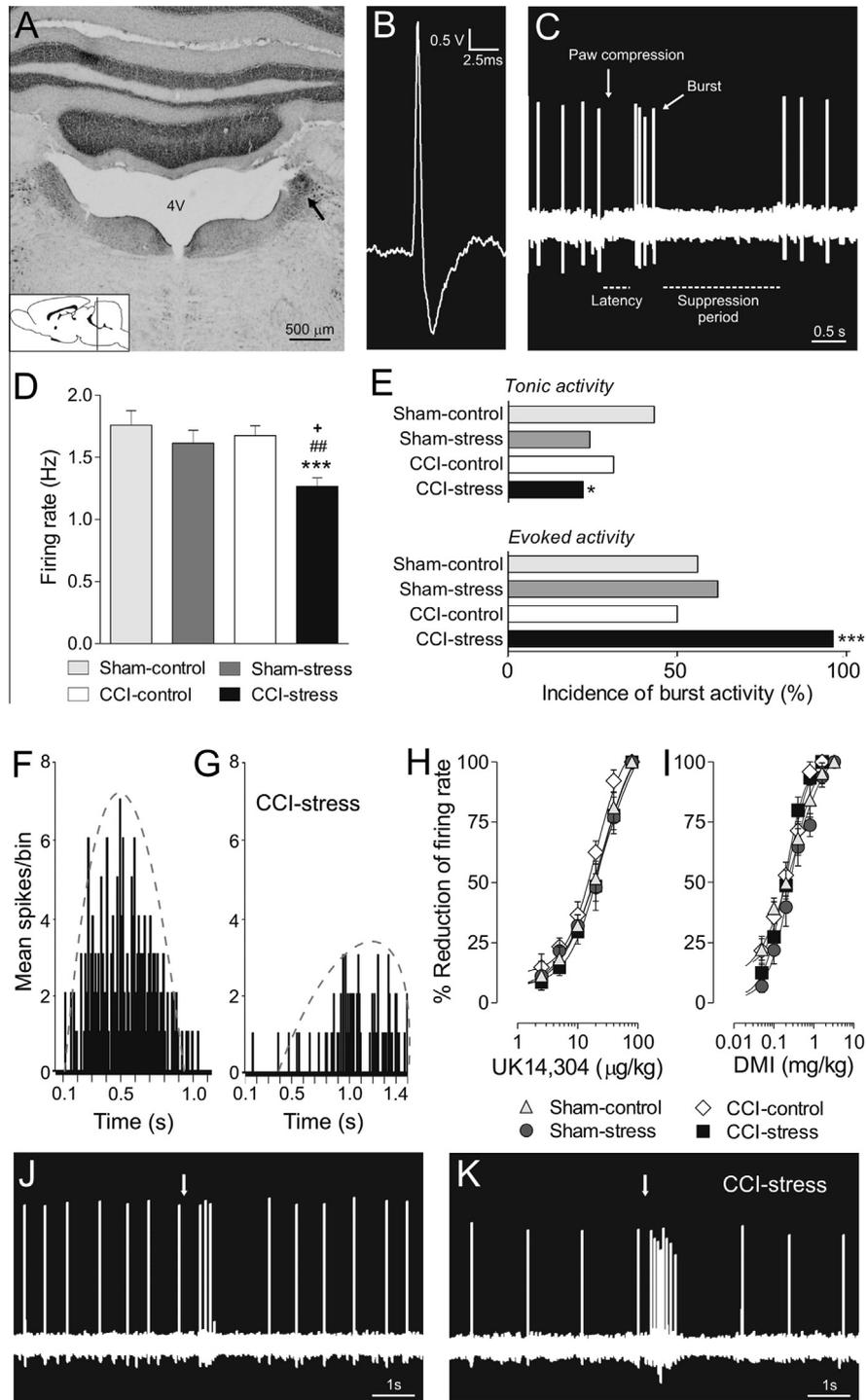


Fig. 2. Effect of neuropathic pain and stress on the electrophysiological activity of locus coeruleus (LC). (A) Photomicrograph of a coronal section (Neutral Red stain) from the rat brainstem showing the LC recording site (black arrow). 4V: 4th ventricle. (B) Representative oscillography trace of a typical LC neuron spike. (C) LC neuron typical response pattern to hind paw compression. Note an increase of firing activity after stimulus followed by a quiescence period (suppression period). The latency period was the onset of the sensory-evoked response (burst). (D) Spontaneous firing rate (Hz) of LC neurons in response to neuropathic pain and stress. CCI, chronic constriction injury. Data express the mean \pm SEM. * $P < 0.05$ and *** $P < 0.001$ vs sham-control; * $P < 0.05$ vs sham-stress; ** $P < 0.01$ vs CCI-control. (E) Percentage of incidence of burst firing in the spontaneous basal activity (tonic activity) and in response to contralateral hind paw compression (evoked activity). Data express the proportion (%) of neurons exhibiting burst activity respect to total recording neurons. * $P < 0.05$ and *** $P < 0.001$ vs sham-control. (F–G) Representative mean interspike interval (ISI) histograms showed regularity patterns of electrical discharge of LC neurons recorded from (F) sham-control, sham-stress, and CCI-control. X-axis values indicate the number of spikes per bin (10 ms). Y-axis values indicate the ISI distributions. (G) CCI-stress group did not show a Gauss distribution (dotted line) as seen in the rest of experimental groups. (H–I) Dose-response curves illustrating the inhibitory effect of (H) UK14,304 and (I) desipramine (DMI), respectively, on the firing rate of LC neurons. Symbol represents the mean \pm SEM of the reduction percentage from the basal firing rate. The horizontal axis represents the logarithmic cumulative drug doses. (J–K) Representative oscillography traces illustrating the sensory-evoked response of LC neurons to hind paw compression (black arrow) for (J) sham-control, sham-stress, CCI-control, and (K) CCI-stress groups. Note the increase of evoked response in CCI-stress group; 12–16 animals per group were used for the electrophysiological recordings (see details in Table 2).

Table 1
Analysis of variance (ANOVA) summary.

2-, 3- or 4-way ANOVA	von Frey test	Acetone test	PEAT	Bicuculline (10 ng/0.5 µL)	
				von Frey test	PEAT
CCI	F_(1,24) = 335.12***	F_(1,24) = 204.57***	F_(1,26) = 47.89***	F _(1,54) = 507.82***	F _(1,54) = 28.30***
Stress	F _(1,24) = 0.64	F _(1,24) = 0.06	F_(1,26) = 6.70*	F _(1,54) = 2.71	F _(1,54) = 4.93*
Treatment	~	~	~	F _(1,54) = 0.00	F _(1,54) = 17.57***
CCI × stress	F _(1,24) = 0.30	F_(1,24) = 5.19*	F_(1,26) = 7.69*	F _(1,54) = 0.02	F _(1,54) = 3.33
CCI × treatment	~	~	~	F _(1,54) = 0.02	F _(1,54) = 6.35*
Stress × treatment	~	~	~	F _(1,54) = 2.45	F _(1,54) = 0.05
CCI × stress × treatment	~	~	~	F _(1,54) = 3.00	F _(1,54) = 1.62
Time	F_(2,48) = 57.02***	F_(2,48) = 74.66***	F _(5,130) = 1.40	F _(1,54) = 0.79	F _(5,270) = 3.28**
Time × CCI	F_(2,48) = 66.83***	F_(2,48) = 56.64***	F_(5,130) = 8.97***	F _(1,54) = 3.24	F _(5,270) = 3.51**
Time × stress	F _(2,48) = 1.25	F _(2,48) = 0.16	F _(5,130) = 0.37	F _(1,54) = 0.11	F _(5,270) = 0.97
Time × treatment	~	~	~	F _(1,54) = 1.36	F _(5,270) = 0.18
Time × CCI × stress	F _(2,48) = 0.18	F _(2,48) = 0.20	F _(5,130) = 0.18	F _(1,54) = 0.60	F _(5,270) = 0.65
Time × CCI × treatment	~	~	~	F _(1,54) = 0.06	F _(5,270) = 3.11**
Time × stress × treatment	~	~	~	F _(1,54) = 1.86	F _(5,270) = 1.37
Time × CCI × stress × treatment	~	~	~	F _(1,54) = 1.26	F _(5,270) = 0.94
Two-way ANOVA	CCI	Stress	CCI × stress		
Behavioural tests					
Elevated zero maze	F _(1,24) = 1.36	F _(1,24) = 0.43	F _(1,24) = 0.71		
Marble-burying test	F _(1,24) = 0.05	F _(1,24) = 0.32	F _(1,24) = 0.09		
Electrophysiological recordings					
Tonic activity					
Firing rate	F_(1,194) = 6.15*	F_(1,194) = 9.81**	F _(1,194) = 2.48		
Variation coefficient	F _(1,194) = 0.65	F_(1,194) = 4.00*	F_(1,194) = 4.71*		
Burst rate	F _(1,194) = 0.54	F_(1,194) = 11.74***	F _(1,194) = 0.04		
Spikes per burst	F_(1,57) = 4.19*	F _(1,57) = 0.04	F _(1,57) = 0.04		
% Spikes in burst	F _(1,57) = 1.48	F _(1,57) = 1.93	F _(1,57) = 0.48		
Evoked activity after ipsilateral paw compression					
Latency	F_(1,85) = 11.3*	F _(1,85) = 0.46	F _(1,85) = 0.11		
Spikes/s per burst	F _(1,85) = 3.60	F _(1,85) = 2.80	F_(1,85) = 5.14*		
Suppression period	F _(1,85) = 3.60	F _(1,85) = 1.90	F _(1,85) = 2.90		
Evoked activity after contralateral paw compression					
Latency	F _(1,21) = 2.6	F _(1,21) = 0.1	F _(1,21) = 0.0		
Spikes/s per burst	F _(1,21) = 2.6	F _(1,21) = 0.1	F _(1,21) = 0.1		
Suppression period	F _(1,21) = 1.2	F _(1,21) = 0.0	F _(1,21) = 0.0		
Immunohistochemistry studies					
TH	F_(1,13) = 6.62*	F_(1,13) = 6.94*	F_(1,13) = 16.05**		
Gephyrin	F_(1,12) = 16.46**	F _(1,12) = 2.76	F _(1,12) = 1.02		

CCI, chronic constriction injury; TH, tyrosine hydroxylase; PEAT, place escape/avoidance test.

Data represent the F-values of 2-, 3-, and 4-way analysis of variance (ANOVA) of behavioural, electrophysiological, and immunohistochemistry studies. Ipsilateral and contralateral terms refer to the injured paw. Significant results are indicated in bold. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$

average number of TH-IR cells in each LC was used for statistical purposes. The expression of gephyrin was detected by using a Leica Spectra Confocal microscope (Leica Microsystems Inc, Buffalo Grove, IL, USA). A Z-stack consisting of 5–6 optical sections was acquired with a 63X oil immersion from 2 randomized LC per rat ($n = 2$ rats per group). Each z plane was analyzed by using a Macro generated from ImageJ software (National Institutes of Health, Bethesda, MD, USA), which converted the image to 8 bits and normalized the background in respect to the control. Quantification of gephyrin expression was performed as described elsewhere [35]. Thus, after normalizing the background, the number of clusters was quantified (minimal cluster size of $0.1 \mu\text{m}^2$) by using a threshold (70 on a 0–255 greyscale) for signal intensity that maximized the selection of puncta and minimized the background noise. The results were expressed as the number of clusters/100 μm^2 .

2.7. Statistical analysis

Data represent the mean \pm SEM, and all the results were analyzed using either 2-, 3-, or 4-way analysis of variance, with or without repeated measures, as appropriate. The factors of variance were: CCI (between-groups), stress (between-groups), treatment (bicuculline treatment, between-groups), and time (within-groups) (see Table 1 for detailed statistical analysis). All further post hoc analyses were carried out using a Bonferroni post hoc test.

The burst incidence was analyzed by Fisher's exact test. The level of significance considered was $P < 0.05$.

3. Results

3.1. Nociceptive and emotional behavioural response

Animals subjected to 3 weeks of stress exhibited similar pain thresholds when compared with nonstressed animals (control vs stress groups: Fig. 1B–C). However, neuropathic rats (CCI-control and CCI-stress) exhibited pronounced mechanical allodynia of the operated hind paw in the von Frey test at week 4 and 5 of the stress regime ($P < 0.001$; Fig. 1B). Moreover, they scored significantly higher than the sham-control or sham-stress in the acetone test ($P < 0.001$; Fig. 1C), reflecting cold allodynia. Overall, the sensorial nociceptive responses of the ipsilateral hind paw were similar in both CCI groups (CCI-control and CCI-stress), and no decrease in the pain threshold was observed in sham-stress. We evaluated the affective-cognitive dimension of pain using the PEAT at week 5 (2 weeks after neuropathy induction) (Fig. 1D). As expected, CCI-control animals demonstrated a greater tendency to escape from the dark side than the sham-operated rats. Interestingly, the CCI-stress rats spent even more time within the white area than the CCI-control animals, with significance at 5, 20, 25, and 30 minutes ($P < 0.01$, $P < 0.05$, $P < 0.05$, $P < 0.05$,

Table 2
Electrophysiological properties of locus coeruleus neurons.

	Sham-control {16}	CCI-control {12}	Sham-stress {16}	CCI-stress {16}
Tonic activity				
Firing rate (Hz)	1.8 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.2 ± 0.1^{****#}
Variation coefficient (%)	37.3 ± 1.6	39.4 ± 1.4	37.5 ± 1.6	33.0 ± 1.5[#]
Burst firing activity				
Incidence (%)	42.3 (22/52)	30.7 (16/52)	28.6 (12/42)	21.2 (11/52)[*]
Burst rate (burst/ms)	11.0 ± 2.0	10.0 ± 3.0	5.0 ± 1.0	3.0 ± 1.0[#]
Spikes per burst	2.1 ± 0.1	2.0 ± 0.0	2.2 ± 0.1	2.0 ± 0.0
Spikes in burst (%)	2.1 ± 0.3	2.9 ± 0.4	1.8 ± 0.4	2.1 ± 0.4
Evoked activity after ipsilateral paw compression				
Incidence (%)	56.3 (31/55)	50.0 (20/40)	61.9 (13/21)	96.1 (25/26)^{***}
Latency (ms)	581.9 ± 64.9	400.8 ± 32.3	561.1 ± 76.5	315.8 ± 34.1^{***}
Spikes/s per burst	3.4 ± 0.2	4.3 ± 0.3	3.2 ± 0.3	4.6 ± 0.4^{**#}
Suppression period (s)	1.4 ± 0.1	1.4 ± 0.1	1.4 ± 0.0	1.1 ± 0.0
Evoked activity after contralateral paw compression				
Incidence (%)	50.0 (8/16)	54.5 (6/11)	54.5 (6/11)	45.5 (5/11)
Latency (ms)	478.3 ± 31.5	419.3 ± 33.8	472.7 ± 34.0	402.2 ± 63.9
Spikes/s per burst	3.6 ± 0.3	4.0 ± 0.5	3.5 ± 0.2	4.0 ± 0.3
Suppression period (s)	1.1 ± 0.1	1.3 ± 0.2	1.1 ± 0.2	1.3 ± 0.1
Effective dose 50				
UK14,304 (μg/kg)	21.8 ± 2.9 [6]	16.6 ± 3.0 [4]	21.0 ± 3.3 [6]	21.2 ± 3.9 [5]
Reversion (%)	81.4 ± 5.3 [6]	87.7 ± 26.6 [3]	96.5 ± 12.6 [6]	128.2 ± 26.5 [4]
Desipramine (mg/kg)	0.3 ± 0.1 [7]	0.2 ± 0.0 [5]	0.3 ± 0.1 [7]	0.2 ± 0.0 [8]
Reversion (%)	33.7 ± 5.0 [4]	35.1 ± 8.2 [4]	40.6 ± 11.4 [7]	40.8 ± 4.7 [7]

CCI, chronic constriction injury.

Data represent the mean ± SEM of tonic and sensory-evoked activity of locus coeruleus (LC) neurons in response to neuropathic pain and stress in (n) rats per group. Ipsilateral and contralateral terms refer to the injured paw. The values in parentheses represent the LC neurons ratio exhibiting burst activity. The values in brackets represent the number of pharmacologically tested neurons. The drug effective dose 50 was estimated by Parker and Waud's equation. Complete inhibition of LC activity by UK14,304 and desipramine was reversed by intravenous administration of RX821002 and idazoxan, respectively. The factor significance was represented as **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 vs sham-control; **P* < 0.05 vs sham-stress, #*P* < 0.05 and ##*P* < 0.01 vs CCI-control. The burst incidence was analyzed by Fisher's exact test: **P* < 0.05 vs sham-control.

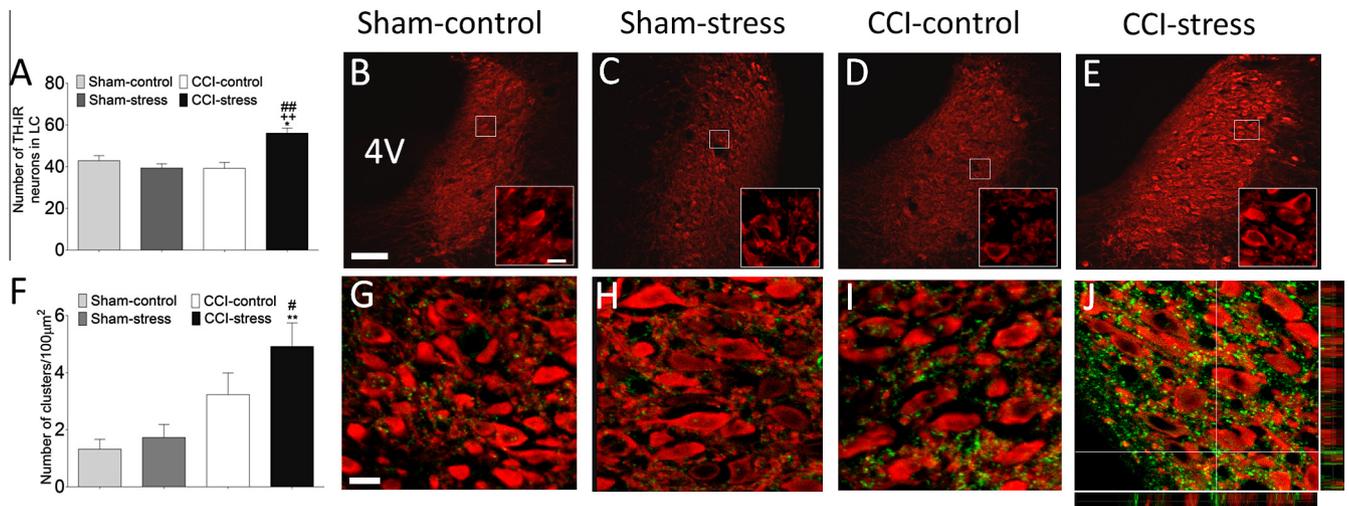


Fig. 3. Effect of neuropathic pain and stress on tyrosine hydroxylase (TH) and gephyrin expression by locus coeruleus (LC) neurons. (A) Graph depicting the number of TH immunoreactive (TH-IR) neurons in LC and (B–E) representative photomicrographs of TH expression (red colour) in sections from (B) sham-control, (C) sham-stress, (D) CCI-control, and (E) CCI-stress animals (n = 4–5 per group). The TH was found in the perikarya of LC neurons (magnification box). (F) The histogram shows the number of clusters in each 100 μm². (G–J) Representative photomicrographs of gephyrin expression (green colour) in sections containing the LC of (G) sham-control, (H) sham-stress, (I) CCI-control, and (J) CCI-stress animals (n = 2 per group). Scale bars: (B–E) 100 μm and high magnification box: 15 μm, (G–J) 20 μm. Abbreviations: 4V, fourth ventricle; CCI, chronic constriction injury. The data are expressed as the mean ± SEM. **P* < 0.05 vs sham-control; ***P* < 0.01 vs sham-stress; #*P* < 0.05 and ##*P* < 0.01 vs CCI-control.

respectively). Nevertheless, anxiety-like behaviour was not modified in any group, because there were no differences in the time spent in the open zone in the elevated zero maze (Fig. 1E) and the number of close zone entries was similar in all groups, precluding a motor component in the observed effects (Fig. 1F). Similarly, no differences were found in the marble-burying test (Fig. 1G).

3.2. Electrophysiological activity of LC neurons

We evaluated any possible noradrenergic dysfunction by exploring the tonic (spontaneous) firing of contralateral LC neurons

(Table 2). Neither nerve injury (CCI-control) nor social isolation (sham-stress) modified LC firing activity. However, when pain and stress were combined (CCI-stress), they induced a lower tonic firing rate and burst activity than that displayed by sham-operated and CCI-controls (*P* < 0.001, *P* < 0.05, and *P* < 0.01, respectively: Table 2 and Fig. 2D), suggesting a decrease in activity of LC neurons. Furthermore, ISI histograms from sham-control, sham-stress, and CCI-control groups showed a similar Gaussian distribution of about 0.5 ms (Fig. 2F), reflecting a normal discharge pattern. However, the CCI-stress group presented an obvious rightward shift in this distribution with a greater number of ISI at intervals of about 1.0–1.5 ms (Fig. 2G). This abnormal distribution of ISI was

corroborated by a reduction of the variation coefficient of CCI-stress (Table 2).

Alpha2-adrenoceptors play an important role in controlling the firing activity of LC neurons, and consequently, in noradrenaline release from noradrenergic fibres. Indeed, i.v. administration of UK14,304 or desipramine inhibited spontaneous activity in a dose-dependent manner, producing a similar ED₅₀ and dose-response curve in all experimental groups (Fig. 2H-I). Complete inhibition of LC neuron activity by UK14,304 and desipramine ($E_{max} = 100\%$) was reversed by i.v. administration of the α_2 -adrenoceptor antagonists, RX821002 (100%) and idazoxan (30%), respectively. Therefore, no differences were found regarding α_2 -adrenoceptors function among groups (Table 2).

When we evaluated the sensory-evoked (phasic) activity of the LC during ipsilateral paw compression, evoked discharge was slightly enhanced in CCI animals when measured through the number of spikes per burst. However, in CCI animals also subjected to stress, both spikes per burst and the incidence of the response augmented significantly (Fig. 2E). This effect was accompanied by a shortening of the latency (Table 2 and Fig. 2J-K). These differences were not found when the contralateral hind paw was compressed (Table 2).

3.3. TH and gephyrin expression at the LC Level

In another group of animals, we explored TH expression in the LC by immunofluorescence, revealing that there was a significant upregulation in the expression of TH in the LC (bilaterally) of the CCI-stress animals with respect to the sham-control, sham-stress, and CCI-control ($P < 0.05$, $P < 0.01$, $P < 0.01$ respectively: Fig. 3A, B-E). By contrast, the animals that experienced just pain or stress did not show any changes in the number of TH-IR neurons when compared to the sham-control group. When we evaluated the expression of gephyrin, a multifunctional protein selectively located at inhibitory synapses [15] (Fig. 3F, G-J), there was also a significant increase expression in CCI-stress when compared to the sham-control or sham-stress rats ($P < 0.01$ and $P < 0.05$, respectively, Fig. 3F). The expression of gephyrin was restricted to the dendritic domains of TH-IR (Fig. 3J), which are specifically GABAergic inhibitory innervations [14].

3.4. Effect of intra-LC bicuculline administration

As gephyrin expression was augmented in CCI-stress animals and since the inhibitory modulation of LC neurons is thought to be mediated mainly through GABA-A receptors, we evaluated the effect of intra-LC (contralateral) bicuculline administration (Fig. 4D). Bicuculline significantly decreased the time spent by CCI-stressed animals in the white area (Fig. 4C), while it did not modify the time spent in the white area of any of the sham-operated animals (Fig. 4B). Additionally, this effect seemed to be specific because no motor or behavioural impairment was evident in this test (Speed in the white area [cm/s]: Vehicle: Sham-control: 4.4 ± 0.8 , Sham-stress: 7.3 ± 1.6 , CCI-control: 5.9 ± 1.1 , CCI-stress: 4.3 ± 0.7 ; Bicuculline: Sham-control: 5.9 ± 1.1 , Sham-stress: 3.1 ± 1.3 , CCI-control: 4.8 ± 1.0 , CCI-stress: 4.1 ± 0.4). Moreover, intra-LC administration of bicuculline did not modify the mechanical nociceptive pain threshold in any group (Fig. 4A).

4. Discussion

Stress is considered a psychobiological factor that may have an important adverse influence on the course of chronic pain. The present study demonstrates that socially isolated neuropathic rats suffer worse affective pain and that this effect is modulated by LC neurons.

We have explored the effect of being socially isolated on the different dimensions of neuropathic pain perception. In the

sensory-discriminative component, our results show that CCI rats developed the same level of allodynia irrespective of whether they were stressed by isolation. To evaluate the affective-cognitive elements, we have used the PEAT. This test evaluates the aversive nature of a noxious stimulus based on the avoidance of a preferred location where the stimulus is delivered. Thus, the animals have to choose between a dark nonanxiogenic area in which the injured paw is stimulated and the white mildly anxiogenic region in which the noninjured paw is stimulated [23]. As expected, we found that CCI-controls spend more time in the white area than the sham-control or sham-stress groups. However, the CCI-stress group spent even more time in the white area, with the maximum avoidance score. It is interesting to note that this effect was already evident within 5 minutes after starting the test, suggesting that the animals are highly focused on pain avoidance, and that stress increases the aversion to noxious stimulation.

It appears that anxiety did not compromise the results obtained in the PEAT, as all the animals tested showed similar levels of anxiety when evaluated in the elevated zero maze and marble-burying tests. We previously found that 2 weeks of neuropathic pain and chronic mild stress selectively increased the aversion to painful stimuli, without affecting classic depressive-like behaviours measured through the forced swimming and anhedonia test [10]. Thus, these and previous data corroborate that it takes approximately 4 weeks for rats to develop affective disorders (anxiety and depression) in association with neuropathic pain [2,19,37,43]. Nevertheless, further studies will be necessary to explore the effect of stress on the pain dimensions and secondary affective disorders over longer periods.

Regarding LC function, tonic LC discharge activity was not modified by either CCI or stress, consistent with previous data [1,11,22]. However, the basal firing rate and bursting activity highlighted a significant decrease in the spontaneous activity of LC neurons in CCI-stress. As the decrease of tonic activity could be mediated by indirect activation of inhibitory α_2 -adrenoceptors [25], we checked for possible overactivation of these receptors. However, the dose-response curves of the α_2 -adrenoceptor agonist UK14,304 and the noradrenaline reuptake inhibitor desipramine were similar in all groups, ruling out such a hypothesis. When we evaluated the expression of TH, the rate-limiting enzyme of noradrenaline synthesis, there was no change after 5 weeks in isolation or 2 weeks of neuropathic pain, consistent with the electrophysiological data. An increase in TH has previously been seen in the LC after stressful stimuli [26]. However, in other studies no such changes were detected (using chronic footshock or restraint stress [36]). This might also be the case in our model of stress, possibly because this short period of isolation alone is insufficient to trigger a stress response in adult animals or alternatively, because adaptive changes had already developed. On the other hand, while we did not find any change in TH expression 2 weeks after CCI, an increase was evident in group-housed rats after 4 weeks [2]. This suggests that isolation might advance the changes observed 4 weeks after neuropathy is induced. However, it is important to note that although both conditions lead to an increase in TH expression, the electrophysiological situation is quite different. A decrease in tonic and burst firing was observed in CCI-stressed rats after 2 weeks, while there was no change in tonic activity, but bursting activity and α_2 -adrenoceptors sensitivity was enhanced in CCI-group-housed rats after 4 weeks [2]. Whereas behaviourally isolated rats did not show any change in anxiety, their pain experience appeared to be heightened. By contrast, after 4 weeks, CCI group-housed rats consistently showed anxio-depressive-like behaviours [2] that were in accordance with the LC changes in the brain tissue of depressed patients seen postmortem [28,29,45]. Together, these data indicate that specific LC dysregulation might be linked to different behavioural outcomes. It will be interesting to

determine if isolated CCI animals at 4 weeks will develop anxiety-depressive disorders similar to those already described for CCI-grouped housed rats, and whether they are accompanied by comparable alterations in the LC. Such information will help to link behavioural alterations to specific brain signatures.

Neuropathic pain was previously shown to be associated with higher glutamic acid decarboxylase expression and GABA release in the LC [44]. Accordingly, gephyrin expression was slightly enhanced by nerve injury, but this expression was significantly robust when nerve injury was associated with stress, and it was accompanied by functional changes such as lower and irregular tonic discharge. In addition, when a noxious mechanical stimulus was applied to the injured paw, there was a trend in the CCI-control animals to increase the burst firing rate of the contralateral LC, consistent with our previous data using an electrical train stimulus [3]. As with the other parameters studied, this effect was very pronounced in the CCI-stress group. Interestingly, no differences among groups were found when the noninjured paw was stimulated, suggesting that the exaggerated evoked response is restricted to the nociceptive input from the injury (ascending pain pathway). Therefore, CCI-stress altered the pattern of LC discharge to noxious stimuli such that the ratio of evoked/tonic activity increased, especially when the injured paw is stimulated. We propose that this might effectively enhance the LC-forebrain

signalling temporally linked to noxious events, increasing the attention to pain perception in CCI-stress. Indeed, studies in patients have shown that attention to pain enhances perceived pain [34,42]. It has been shown in awake animals that the LC facilitates different behavioural outcomes by shifting between tonic and phasic modes of discharge [4,40]. Thus, CCI-stress may shift the physiological response of the LC-noradrenergic system toward a phasic state when the injured paw is stimulated. This displacement would augment the activation of the LC by a noxious stimulus and consequently produce hyperarousal to perceived pain and stronger avoidance to re-experience pain in conditions of CCI-stress in the PEAT. That is, CCI-stress group has a more negative interpretation of painful experiences in spite of similar sensorial pain and anxiety threshold. Interestingly, it has been suggested in animal models that the enhanced ratio of evoked/tonic activity of LC neurons is causing the exaggerated response to trauma-related stimuli of posttraumatic stress disorder [18]. A similar pattern was found in the LC of adult rats subjected to resident-intruder social stress model [9].

Although several neurotransmitters have been shown to modulate tonic and sensory-evoked LC activity (eg, excitatory amino acids, GABA, corticotropin-release-factor), our data as well as previous data point to an enhanced GABAergic neurotransmission in the LC in neuropathic pain [38,44]. Thus, we explored the effect

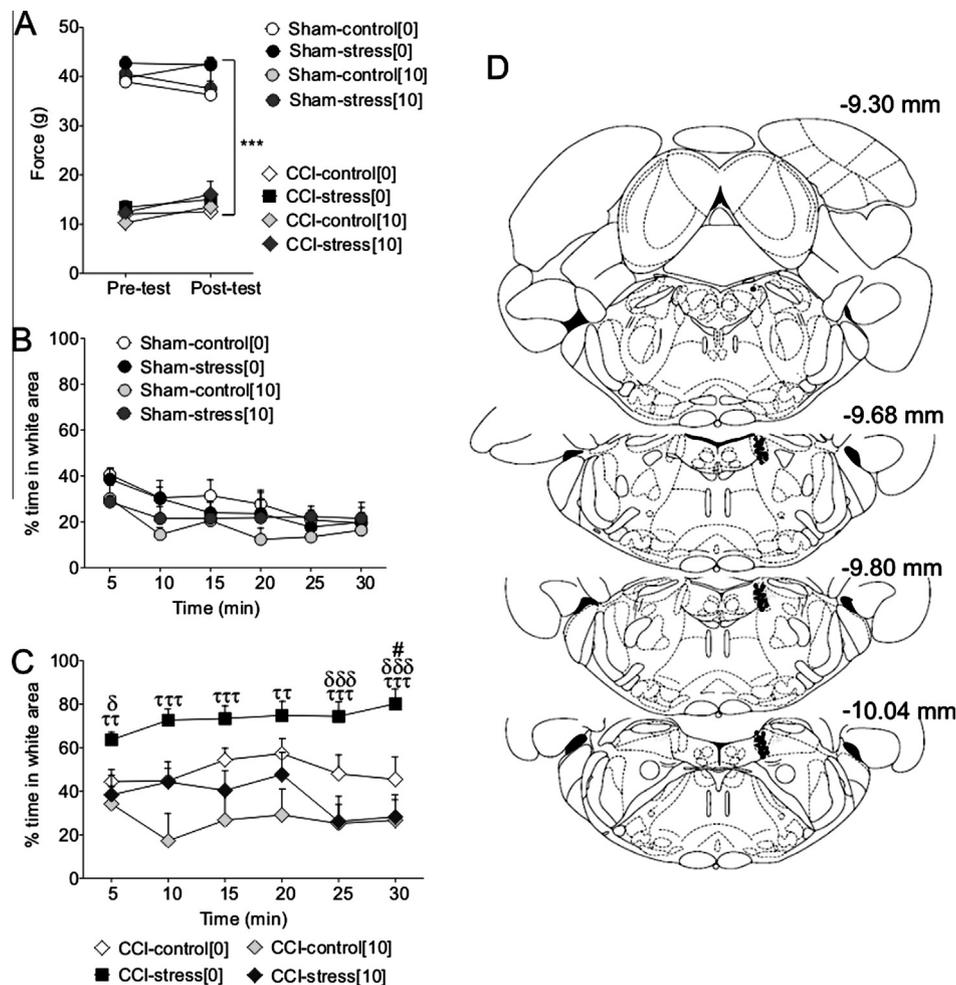


Fig. 4. Behavioural testing after bicuculline administration: (A) Mechanical allodynia in response to a von Frey monofilament before and after performing the place escape/avoidance test (PEAT). (B-C) Emotional/cognitive component of pain measured by the PEAT in sham (B) and CCI groups. (C) [n] shows the dose of bicuculline in ng/0.5 μ L. (D) Histological representation of the unilateral injection sites within the LC where the tip of the injection cannula is represented by a black dot (-9.30 to -10.04 mm posterior to Bregma [32]). The data are expressed as the mean \pm SEM. [#] $P < 0.05$ vs CCI-control. ^{*} $P < 0.05$, ^{††} $P < 0.01$, ^{†††} $P < 0.001$ vs CCI-control [10]; [°] $P < 0.05$, ^{°°} $P < 0.001$ vs CCI-stress [10] ($n = 7-11$ per group).

of GABA-A receptors blockade in the contralateral LC because GABA signals mainly through these receptors [14,30]. Results showed that the intra-LC administration of bicuculline, a GABA-A receptor antagonist, diminished the time spent in the white area in the PEAT. Altogether, this suggests that GABA blockade at LC level attenuated the negative affective effects of pain inputs, probably due to a decrease of evoked/tonic activity ratio in the LC. Further studies in the ipsilateral LC are necessary to make a definitive statement about the role of GABA neurotransmission in the ascending and descending pain pathway mediated by LC.

Current conceptualization of chronic pain is toward the integration of the obvious sensorial aspects, with other more diffuse, such as psychological and social, variables that may explain different patients' outcomes [13]. In the current study, we have used animals to model pain perception when social network is removed and could be translated to those neuropathic pain patients that have or perceive altered social support (lack of supportive relationships [hostility from family and/or friends], marital problems, lack of financial support, belonging to disadvantaged groups [homeless]). Our results show that when neuropathic pain is associated with social isolation stress, there is a modified LC reaction to noxious stimuli that exacerbate the response. As a consequence, this greater reactivity to pain stimulus compromises the ability of an individual to adapt to new contingencies. This might be an adaptive LC response that aims to enhance the negative pain experience as a defensive reaction. These data, jointly with previous ones about anxiety-depressive-like disorders developed at long-term neuropathic pain, are placing the brain noradrenaline system as a key area mediating mood alterations in chronic pain.

Conflict of interest

The authors declare no conflict of interest.

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