PAIN® 153 (2012) 1438–1449

www.elsevier.com/locate/pain

Analgesic antidepressants promote the responsiveness of locus coeruleus neurons to noxious stimulation: Implications for neuropathic pain

Cristina Alba-Delgado [a,b], Juan Antonio Mico [a,b], Pilar Sánchez-Blázquez [b,c], Esther Berrocoso [b,d,*]

[a] Neuropsychopharmacology Research Group, Department of Neuroscience, University of Cadiz, Cadiz, Spain
[b] Centro de Investigación Biomédica en Red de Salud Mental (CIBERSAM), Instituto de Salud Carlos III, Madrid, Spain
[c] Instituto Cajal CSIC, Madrid, Spain
[d] Neuropsychopharmacology Research Group, Psychobiology Area, Department of Psychology, University of Cadiz, Cadiz, Spain

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

ARTICLE INFO

Article history:
Received 18 August 2011
Received in revised form 8 March 2012
Accepted 29 March 2012

Keywords:
Locus coeruleus
Neuropathic pain
Duloxetine
Desipramine
Noxious stimulation
Electrophysiology

ABSTRACT

Antidepressants that block the reuptake of noradrenaline and/or serotonin are among the first-line treatments for neuropathic pain, although the mechanisms underlying this analgesia remain unclear. The noradrenergic locus coeruleus is an essential element of both the ascending and descending pain modulator systems regulated by these antidepressants. Hence, we investigated the effect of analgesic antidepressants on locus coeruleus activity in Sprague-Dawley rats subjected to chronic constriction injury (CCI), a model of neuropathic pain. In vivo extracellular recordings of locus coeruleus revealed that CCI did not modify the basal tonic activity of this nucleus, although its sensory-evoked response to noxious stimuli was significantly altered. Under normal conditions, noxious stimulation evokes an early response, corresponding to the activation of myelinated A fibers, which is followed by an inhibitory period and a subsequent late capsaicin-sensitive response, consistent with the activation of unmyelinated C fibers. CCI provokes an enhanced excitatory early response in the animals and the loss of the late response. Antidepressant administration over 7 days (desipramine, 10 mg/kg/day or duloxetine, 5 mg/kg/day, delivered by osmotic minipumps) decreased the excitatory firing rate of the early response in the CCI group. Moreover, in all animals, these antidepressants reduced the inhibitory period and augmented the late response. We propose that *N*-methyl-D-aspartate and alpha-2-adrenoceptors are involved in the analgesic effect of antidepressants. Antidepressant-mediated changes were correlated with behavioral effects indicative of analgesia in healthy and neuropathic rats.

1. Introduction

In addition to their effects on mood, antidepressants possess intrinsic analgesic properties, and for this reason, they have been used as analgesics for over 40 years. Indeed, although they form part of the first line of treatment for neuropathic pain, and despite their extensive clinical use for this purpose, the mechanisms underlying the analgesic action of antidepressants remain unclear. The classic hypothesis postulates that antidepressants block specific monoamine (noradrenaline or serotonin) transporters in the presynaptic membrane, maintaining or enhancing the activity of the descending inhibitory bulbospinal pain pathway that is frequently compromised in conditions of neuropathic pain [11,32,35]. However, direct evidence of the involvement of supraspinal areas in neuropathic pain and antidepressant-mediated analgesia, such as the noradrenergic locus coeruleus (LC), has not been forthcoming.

The role of the LC in acute pain has been studied extensively in healthy animals [13,26,27]. Inhibition of somatosensory information at the level of the spinal cord by the LC is triggered by nociceptive inputs to the latter, forming a "brainstem loop." LC neurons are activated by sensory stimuli and remarkably, by noxious stimuli such as foot or tail pinch, or sciatic nerve activation [4,13]. For both noxious and nonnoxious stimuli, brief bursting activity (early response) induced by the stimulus is followed by an inhibitory period (Fig. 3). Additionally, noxious stimuli that recruit C fibers also provoke a specific late response of LC neurons [26]. Thus, the LC electrical response to acute pain is the combined result of the late and early evoked responses. Both acute and chronic administration of antidepressants profoundly influences the electrical activity of LC neurons, and these alterations are thought to mediate their

* Corresponding author at: Department of Psychology, Campus Universitario Río San Pedro s/n, University of Cadiz, 11510-Puerto Real, Cádiz, Spain. Tel.: +34 956015224; fax: +34 956015225.
E-mail address: esther.berrocoso@uca.es (E. Berrocoso).

0304-3959/$36.00
http://dx.doi.org/10.1016/j.pain.2012.03.034

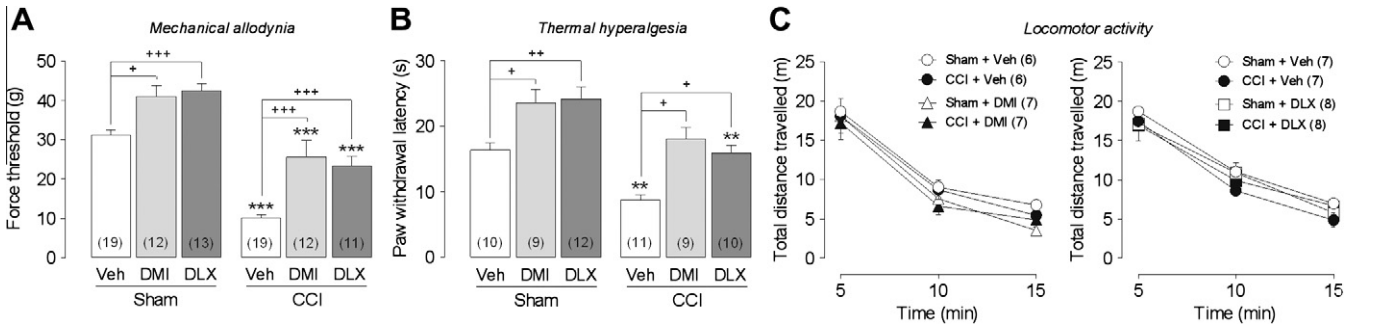

Fig. 1. Nociceptive behavioral assessment and locomotor activity 7 days after chronic constriction injury (CCI). (A, B) Effect of antidepressant treatment on the nociceptive threshold of the ipsilateral hind paw in sham and CCI rats. (A) The force value (grams) at which rats withdrew the paw in response to von Frey hair stimulation (0-50 g over 20 seconds) was determined as a measure of mechanical allodynia. (B) Paw-withdrawal latency in response to heat radiation (50 A, cutoff 30 seconds) was evaluated as a measure of thermal hyperalgesia. (C) Spontaneous horizontal ambulatory behavior was measured in a square box over 15 minutes and the total distance traveled was expressed as mean arbitrary units in 5-minute bins. Vehicle (Veh), desipramine (DMI, 10 mg/kg/day), or duloxetine (DLX, 5 mg/kg/day) were administered via osmotic minipumps for 1 week. Each column or symbol represents the mean ± SEM. The values in parentheses show the number of rats per group. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs respective sham-operated group; $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ vs respective vehicle-treated group (2-way analysis of variance test followed by Tukey-Kramer honestly significant difference posttest detailed in Table 1).

analgesic effects. However, it remains unclear if antidepressants modify the LC-evoked response, particularly in animals experiencing neuropathic pain in which pain pathways are modified.

Thus, the main aim of the present study was to investigate the response of LC neurons in neuropathic animals, both before and after nociceptive stimulation of the sciatic nerve. We also evaluated how antidepressant drugs, with demonstrated clinical analgesic effects, influenced this response [19,41]. To this end, duloxetine (DLX), a dual serotonin and noradrenaline reuptake inhibitor [16], and desipramine (DMI), a tricyclic noradrenaline reuptake inhibitor [16], were administered for 7 days. We demonstrate that neuropathic pain modifies the electrophysiological nociceptive-evoked responses of LC neurons, an effect that was normalized by the administration of antidepressants. Additionally, antidepressants also modified LC-evoked response in healthy animals. All these effects of antidepressants were closely correlated with the behavioral effects associated with analgesia.

2. Methods

2.1. Subjects

Male Sprague-Dawley rats (250-300 g) were housed under standard laboratory conditions (22°C, 12-hour light/dark cycle with lights on at 8 am, food and water ad libitum) in accordance with the European Community directive 86/609/EEC and Spanish Law (RD 1201/2005) regulating animal research. All the experimental protocols were reviewed and approved by the Ethical Committee committees of the University of Cádiz for animal care and use.

2.2. Model of neuropathic pain

Chronic constriction injury (CCI) was used as a model of neuropathic pain because it induces clinical signs and symptoms that mimic human conditions of neuropathic origin, such as spontaneous pain-related behavior, allodynia (a painful response to a normally innocuous stimulus), and hyperalgesia (increased response to a painful stimulus). CCI was induced after anaesthetizing rats with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg), as described previously [5,6]. The left sciatic nerve was exposed at the mid-thigh level proximal to the sciatic trifurcation. Four chromic gut (4/0) ligatures were tied loosely around the nerve 1.0-1.5 mm apart so as not to compromise the vascular supply, and the overlying muscle and skin was sutured with silk thread. Sham operations were performed in the same manner without ligating the nerve. Self-mutilation was presented in approximately 5% of the animals tested. Autotomy was never seen in the hind paw on the sham-operated animals.

2.3. Antidepressant treatment

CCI and sham-operated rats were treated for 7 days with DMI HCl (10 mg/kg/day; Sigma-Aldrich Chemicals, Madrid, Spain), DLX HCl (5 mg/kg/day; gifted from Eli Lilly, Indianapolis, IN, USA), or the vehicle alone (saline or 10% methyl-β-cyclodextrin, respectively). These doses were chosen on the basis of pilot studies performed in our laboratory and on the data available in the literature on the antinociceptive effect of DMI and DLX in neuropathic animals [28,36,40,50,54]. To ensure continuous delivery, drugs were administered via osmotic minipumps (Model 2ML1; Alzet Corp., Cupertino, CA, USA) implanted subcutaneously through a small incision between the scapulae at the time of CCI surgery. Drug concentrations were calculated based on the theoretical average weight of the rat during the experimentation period. All tests were performed 7 days after implanting the minipump to best mimic the clinical analgesic effects of antidepressant administration [19,41].

2.4. Nociceptive behavioral assessment

The allodynia and hyperalgesia in rats were tested both before and 7 days after surgery. Prior to behavioral testing, the animals were habituated to their environment for 15 minutes, and they were rested for 30 minutes between each test. Mechanical allodynia was measured by an electronic version of von Frey test (Ugo Basile, Varese, Italy) [9]. Briefly, each rat was placed in a polycarbonate cage (20 × 10 × 14 cm) over an elevated metal grid that gave access to the plantar surface of the paws. An increasing vertical force (from 0 to 50 g) was applied to the operated hind paw over 20 seconds using a steel filament. Two measurements were recorded at 10-minute intervals to obtain the mean force at which a clear hind paw withdrawal response was evoked, reflecting the mechanical nociceptive threshold. Thermal hyperalgesia was evaluated by Hargreaves' method [23] whereby each rat was placed in a polycarbonate cage (20 × 10 × 14 cm) over an elevated transparent surface. Radiant heat was applied at a constant intensity (50 A) to the operated hind paw using a Plantar test device (Ugo Basile, Varese, Italy). A 30-second cut-off was established to avoid tissue damage when no reaction was observed. Two measurements were

made at 10-minute intervals and the mean latency of the voluntary hind paw withdrawal response was recorded as the thermal nociceptive threshold.

2.5. Locomotor activity

In a light-attenuated room, free exploratory ambulation was recorded individually over 15 minutes in a square transparent box ($40 \times 40 \times 40$ cm) using a SMART (Spontaneous Motor Activity Recording and Tracking) video system (v2.5, Panlab S.L., Barcelona, Spain). The total distance traveled (m) was measured and represented in 5-minute intervals as an indicator of locomotor activity.

2.6. Electrophysiological recordings

Single-unit extracellular recordings of LC neurons ipsilateral and contralateral to the operated hind paw were obtained 7 days after surgery as described previously [2,8]. Rats were anaesthetized with chloral hydrate (400 mg/kg, i.p.) and a catheter was inserted in the jugular vein for delivery of additional anesthetic. Rats were then placed in a stereotaxic frame (David Kopf, Tujunga, CA, USA) and the recording electrode was lowered into the LC (relative to lambda: AP −3.7 mm, LM ±1.1 mm, DV −8.2) [38]. The recording electrode was a single-barrel glass micropipette containing a 2% solution of Pontamine Sky Blue (Sigma-Aldrich Chemicals, Madrid, Spain). LC neurons were identified by standard criteria [12]: (1) long-lasting action potentials (>2 ms); (2) spontaneous firing at a regular rhythm; (3) a slow firing rate of 0.5-5 Hz; (4) characteristic spikes with a long-lasting positive-negative waveform (Fig. 3A); and (5) phasic activation by pressure applied to the hind paw followed by a longer period of postactivation inhibition. Once an action potential was isolated, firing patterns were analyzed offline using Spike2 software (Cambridge Electronic Design, Cambridge, UK).

A dose-response curve for clonidine (an alpha-2-adrenoceptor agonist: Sigma-Aldrich Chemicals, Madrid, Spain) was generated in a set of naïve rats treated with antidepressants. Briefly, increasing doses of clonidine (0.3-80 μg/kg, doubling dose) were administered (intravenously) for 1 minute until spontaneous LC activity ceased. Complete inhibition was achieved in all the cells tested. One dose-response curve was generated per rat and these dose-response curves were analyzed for the best nonlinear fit to the logistic 3-parameter equation [37]: $E = Emax\ [A]n/(ED50n + [A]n)$, where [A] is the intravenous dose of clonidine, E is the effect on the firing rate induced by A, Emax is the maximal percentage change at "infinite" dose (100%), ED50 is the effective dose eliciting 50% of Emax, and n is the slope factor of the dose-response curve.

2.7. Histological verification

At the end of each experiment, the location of the recording site was verified by iontophoresis of Pontamine sky blue (5 μA cathodic current, 10-15 minutes) through the recording electrode. The brain was fixed by transcardial perfusion of 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) and it was then removed. Brain sections (50 μm) were stained with Neutral Red and the site of recording was examined microscopically (Fig. 2B, C). Only measurements from cells within the LC were included in this study.

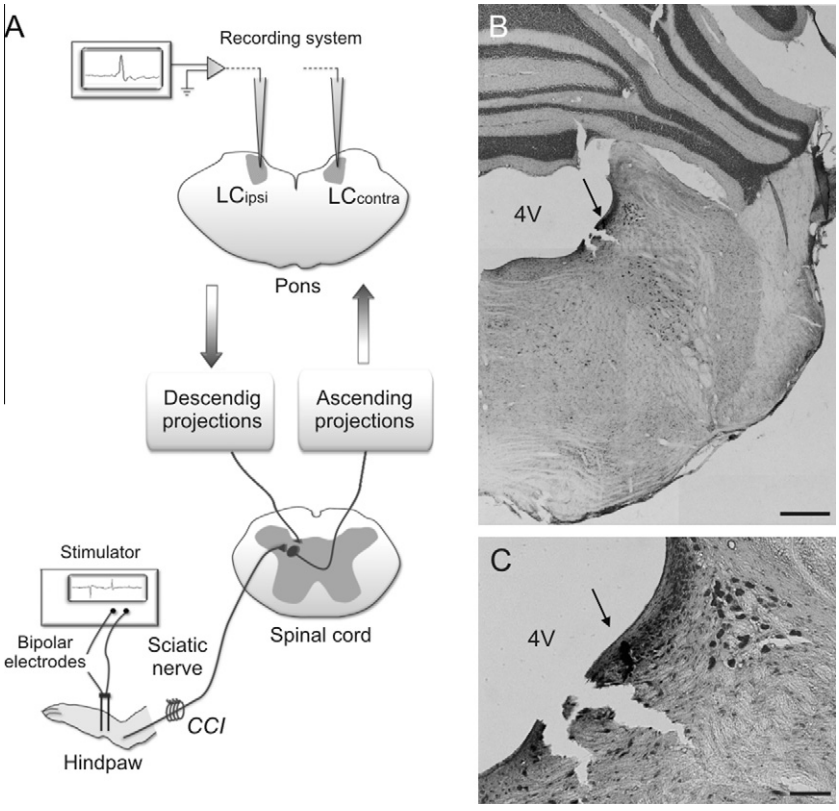

Fig. 2. Scheme of the experimental procedure to apply electrical footshock stimulation and the histological verification of recording side. (A) Schematic representation of the experimental procedure used to record electrophysiological activity in the locus coeruleus (LC). The sciatic nerve was stimulated using bipolar 26-gauge needles subcutaneously inserted into the left hind paw. Sensory-evoked activity of ipsilateral (ipsi) and contralateral (contra) LC neurons was recorded with a single-barrel glass micropipette. Chronic constriction injury (CCI) was inflicted 7 days before the electrophysiological recording. (B, C) Photomicrograph of a coronal section (Neutral Red stain) of the rat brainstem showing the recording site in the LC (black arrow). The dye staining was created by iontophoresis of Pontamine Sky Blue at the end of the recording session (scale bar = 500 μm; high magnification: scale bar = 100 μm). 4 V, 4th ventricle.

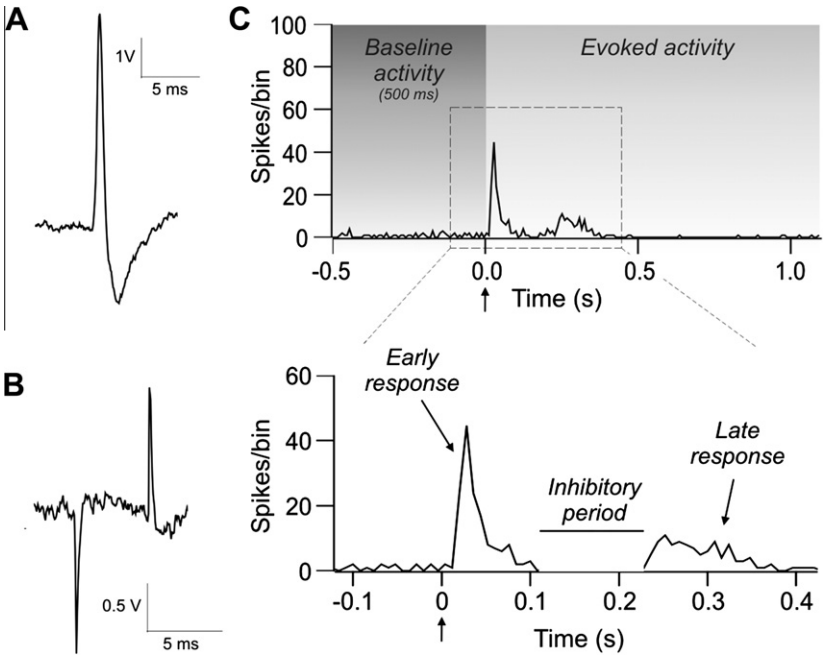

Fig. 3. Scheme of locus coeruleus (LC) sensory-evoked response to electrical footshock stimulation. (A) Representative oscillography trace of a typical action potential from an LC neuron recorded extracellularly. Note the pronounced initial segment and the broad waveform characteristic of LC action potentials. (B) Representative oscillography trace of a footshock artifact recorded extracellularly. (C) Schematic representation of a peristimulus time histogram constructed from the cumulative activity of a typical LC neuron during an electrical footshock train (up), and the magnification showing the excitatory response after the stimulus (down). *Y*-axis values indicate the number of spikes per 8-ms bin. Stimuli are presented at 0.0 s (black arrow) on the *X*-axis. Baseline activity corresponds to a 500-ms epoch preceding stimulation. Excitatory response was divided into two components: early (short-latency) and late (long-latency) responses separated by an inhibitory period (a period of null neuronal activity or below basal activity).

2.8. Peripheral footshock stimulation

Electrical pulse trains were applied using bipolar needle electrodes (26-gauge, 2-mm separation) inserted subcutaneously into the medial-external surface of the left hind paw corresponding to the zone of innervation by the sciatic nerve and contralateral to the LC being recorded (Fig. 2A). To evoke both late (C-fiber mediated) and early (A-fiber mediated) LC activity, footshock stimuli (5.0 ms in duration and 10 mA in intensity: Fig. 3B) were delivered every 2 seconds using a Stimulator CS-9 and a stimulus isolation unit (Cibertec, Madrid, Spain) with a total of 125 repetitions per train [26,27]. Thus, 8-9 peripheral footshock trains were applied in individual neurons in a total of 8-9 neurons per rat.

In a set of naïve animals, 1% capsaicin (Sigma-Aldrich Chemicals, Madrid, Spain), diluted in a solution containing 10% ethanol, 10% Tween-80, and 80% saline, was administered intraplantar in the same area of stimulation in order to characterize the sensory-evoked activity of the LC [26].

2.9. Immunoprecipitation and Western blotting

Seven days after surgery, another set of rats administered with cyclodextrin or DLX was anaesthetized with chloral hydrate (400 mg/kg i.p.) and exposed to 8-9 peripheral footshock trains once every 2 minutes, as described above. Two hours after the last footshock train, the rats were sacrificed by decapitation and the LC was removed bilaterally, homogenized, and centrifuged to obtain a crude synaptosomal pellet [11]. About 1 mg of protein in the pellet was solubilized by sonification in a solution containing 50 mM Tris-HCl [pH 7.7], as well as protease and phosphatase inhibitors (Sigma-Aldrich Chemicals, Madrid, Spain). The homogenates were divided into aliquots to assay alpha-2A-adrenoceptors by immunoprecipitation, or phospho(S890)-N-methyl-D-aspartate receptor R1 (pNMDAR1) and phospho(T286)-Ca2+/calmodulin-dependent protein kinase IIα (pCaMKII) in immunoblots. In order to evaluate the immunoprecipitation of alpha-2A-adrenoceptors [18], the membranes from LC homogenates were solubilized and incubated with biotin-conjugated primary antibodies (affinity-purified immunoglobulins: Fisher Scientific, Madrid, Spain) raised against the second extracellular loop of the receptor (KKGAGGGQQPAEPS: GenScript USA Inc., Piscataway, NJ, USA). At the end of this process, the pellets were heated to denature the proteins in the immunocomplexes recovered and they were resolved by SDS-PAGE gel (10–20%), as were the remaining LC homogenates. The separated proteins were transferred to 0.2-μm polyvinylidene difluoride membranes (162-0176; Bio-Rad, Madrid, Spain) that were probed with the corresponding primary antibodies: anti-alpha-2A-adrenoceptor (against aa: 189–202, GeneScript, Piscataway, NJ, USA), anti-pNMDAR1 (#3381; Cell Signaling, Danvers, MA, USA) and CaMKII (#3361; Cell Signaling, Boston, MA, USA). The primary antibodies were recognized by the corresponding horseradish peroxidase-conjugated secondary antibodies, which were detected with the Immobilon Western Chemiluminescent HRP substrate (#WBKLS0100; Millipore, Billerica, MA, USA) and visualized on a ChemiImager (IS-5500; Alpha Innotech, San Leandro, CA, USA). The Western blots were quantified by densitometry (average optical density of the pixels within the object area/mm2) using Quantity One Software (Bio-Rad, Madrid, Spain) and normalized to the signal for β-actin [39].

2.10. Data analysis

2.10.1. Tonic electrophysiological activity of LC neurons

The spontaneous tonic activity of LC neurons was evaluated in terms of: firing rate (Hz); coefficient of variation (%) (ratio of SD to the mean interval value of an interspike time-interval histogram); incidence of burst firing (expressed as the percentage of cells exhibiting burst firing) and mean percentage of spikes occurring in bursts (%). A neuron was considered to exhibit burst firing when it displayed at least 2 spikes with an initial interspike interval of <80 ms and subsequent interspike intervals of ⩾160 ms [20].

2.10.2. Sensory-evoked electrophysiological activity of LC neurons

Peristimulus time histograms (PSTH, 8-ms bin width) were constructed from the cumulative activity of each LC neuron during an electrical footshock train (Fig. 3C). The excitation threshold was defined as the mean prestimulus baseline activity (500-ms epoch preceding stimulation) by 2 SD. Thus, onset latency for an excitatory response to footshock stimuli was identified as the first 8-ms bin after the stimuli whose mean spiking exceeded the excitation threshold. The excitatory evoked response offset was determined as the time at which activity had returned to be consistently within 2 SD of the baseline. The excitation onset and offset times were used to define each "excitatory evoked epoch" in PSTHs. Thus, excitatory evoked activity was divided into 2 nonconsecutive epochs, considered as the early (short-latency) and late (longer-latency) responses [26] (Fig. 2B). Both evoked responses were assessed in terms of: (1) excitatory firing rate (Hz or spikes/s; calculated by averaging the 8-ms bins in each epoch whose value exceeded the excitation threshold); (2) incidence of response (expressed as the proportion of cells exhibiting an excitatory evoked response in each epoch in relation to total number of cells recorded); (3) latency of response (ms; onset of the excitatory evoked response for each epoch); and (4) duration of excitatory evoked response (ms; calculated from number of 8-ms bins that exceeded the excitation threshold). The inhibitory period was defined as the time between both evoked excitatory epochs in which there was no neuronal activity or it was below the basal activity.

All the data are presented as the mean ± SEM. The statistical significances between groups were analyzed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) and Statistica 10.0 (StatSoft Inc, Tulsa, OK, USA) software. As no differences were observed between the saline- and cyclodextrin-treated sham-operated groups, these data were pooled, as were the vehicle-treated CCI groups. For comparisons between 2 groups, an unpaired Student's *t*-test was used. For comparisons between more than 2 groups, 1- or 2-way analysis of variance followed by the Tukey-Kramer honestly significant difference posttest comparisons was performed. F-values with their associated degrees of freedom (*df*) (surgery, treatment, lateral, interaction, and residual) were expressed as Fs [*df* of surgery/residual], Ft [*df* of treatment/residual], FL [*df* of lateral/residual], or Fi [*df* of interaction/residual]. Fisher's exact test was used to analyze 2 categorical variables and Pearson's correlation test to measure the dependence between 2 variables. *P*-values of <0.05 were considered statistically significant.

3. Results

3.1. Effects of CCI and antidepressant treatment on nociceptive behavioral response

Seven days after experimental induction of neuropathy, CCI rats (n = 19) exhibited pronounced mechanical allodynia of the operated hind paw in response to the von Frey test ($P < 0.001$ vs sham, n = 19; Fig. 1A, Table 1). This allodynia was significantly reduced after treatment of DMI (n = 12) or DLX (n = 11) when compared with CCI rats that received the vehicle alone (DMI: $P < 0.001$ and DLX: $P < 0.001$; Fig. 1A, Table 1), increasing the mechanical threshold by 141.1% and 131.3%, respectively. In sham animals, the antidepressant administration also increased the mechanical threshold by 31.3% and 43.9% (n = 12 and n = 13 for DMI and DLX, respectively) compared with vehicle (DMI: $P < 0.05$ and DLX: $P < 0.001$, n = 19; Fig. 1A, Table 1).

Regarding thermal sensitivity, CCI animals (n = 11) showed thermal hyperalgesia when compared with sham-operated animals ($P < 0.01$, n = 12; Fig. 1B, Table 1). Antidepressant treatment significantly reduced it compared to CCI-vehicle group (n = 11), increasing the heat-induced withdrawal latency by 105.8% and 81.7%, respectively (DMI: $P < 0.05$, n = 9; and DLX: $P < 0.05$, n = 10; Fig. 1B, Table 1). In sham rats, antidepressant treatment in-

Table 1
Two-way ANOVA test summary of the behavioral assessment.

	Desipramine			Duloxetine		
	Surgery (F_s)	Treatment (F_t)	Interaction (F_i)	Surgery (F_s)	Treatment (F_t)	Interaction (F_i)
von Frey test	$F_{[1,58]} = 65.2^{***}$	$F_{[1,58]} = 31.1^{***}$	$F_{[1,58]} = 1.6$	$F_{[1,58]} = 177.4^{***}$	$F_{[1,58]} = 65.4^{***}$	$F_{[1,58]} = 0.4$
Plantar test	$F_{[1,35]} = 7.6^{**}$	$F_{[1,35]} = 12.0^{**}$	$F_{[1,35]} = 0.2$	$F_{[1,39]} = 22.7^{***}$	$F_{[1,39]} = 20.2^{***}$	$F_{[1,39]} = 0.0$
Locomotor activity	$F_{[1,22]} = 4.0$	$F_{[1,22]} = 0.3$	$F_{[1,22]} = 0.5$	$F_{[1,28]} = 0.1$	$F_{[1,28]} = 0.0$	$F_{[1,28]} = 0.0$

Data represent the F-values of 2-way analysis of variance (ANOVA) test of the nociceptive behavioral tests and locomotor activity 7 days after chronic constriction injury (CCI). Vehicle, desipramine (10 mg/kg/day), or duloxetine (5 mg/kg/day) were administered via osmotic minipumps for 1 week. The F-values with their associated degrees of freedom (*df*) were expressed as F_s [*df* of surgery/residual], F_t [*df* of treatment/residual], or F_i [*df* of interaction/residual]. The significance of factor was represented as $^{**}P < 0.01$ and $^{***}P < 0.001$.

Table 2
Tonic electrophysiological activity of locus coeruleus (LC) neurons.

	Vehicle				Desipramine		Duloxetine	
	$LC_{ipsilateral}$		$LC_{contralateral}$		$LC_{contralateral}$		$LC_{contralateral}$	
	Sham [9]	CCI [8]	Sham [7]	CCI [7]	Sham [6]	CCI [6]	Sham [6]	CCI [6]
Firing rate (Hz)	1.9 ± 0.2	1.7 ± 0.2	1.8 ± 0.1	1.7 ± 0.1	0.7 ± 0.0†††	0.5 ± 0.0†††	1.4 ± 0.1†	0.8 ± 0.1†
Variation coefficient (%)	37.62 ± 4.0	40.3 ± 2.9	37.3 ± 1.6	37.1 ± 2.1	40.0 ± 5.2	42.2 ± 4.9	41.3 ± 1.9	42.6 ± 7.6
Burst firing activity								
Incidence (%)	43.1 (6/14)	50.0 (7/14)	49.1(30/61)	42.1 (24/57)	29.7 (14/47)	12.5†† (6/48)	43.5 (24/55)	0.0*†† (0/45)
Number spikes/burst	2.0 ± 0.0	2.5 ± 0.3	2.1 ± 0.1	2.2 ± 0.1	2.5 ± 0.2	2.8 ± 0.4†	2.3 ± 0.2	∼
Spikes in burst (%)	2.3 ± 0.7	2.4 ± 0.6	2.1 ± 0.3	2.3 ± 0.5	5.3 ± 0.5†††	4.8 ± 0.8†	3.5 ± 0.5$^{\#}$	∼

Data represent the mean ± SEM. Chronic constriction injury (CCI) was inflicted 7 days before the electrophysiological studies were performed. The LC neuronal recordings were performed ipsilateral and contralaterally to the left hind paw in the vehicle group. Vehicle, desipramine (10 mg/kg/day), or duloxetine (5 mg/kg/day) were administered via osmotic minipumps for 1 week. The values in brackets represent the number of animals used, while the values in parentheses show the ratio of neurons exhibiting burst activity (incidence of response,%). ∼ not listed, as burst events was not observed. $^{*}P < 0.05$ vs duloxetine-treated sham group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, and $^{\dagger\dagger\dagger}P < 0.001$ vs respective vehicle-treated group (2-way analysis of variance test followed by Tukey-Kramer honestly significant difference post-test detailed in Table 1; Fisher's exact test for the incidence of response). $^{\#}P < 0.05$ vs vehicle-treated sham group (Student's *t*-test).

creased the thermal threshold by 36.3% and 47.4% after DMI (n = 9) and DLX (n = 12) treatment, respectively (DMI: $P < 0.05$ and DLX: $P < 0.01$; Fig. 1B, Table 1).

No differences were observed between the effects of DMI and DLX treatments in sham or CCI animals (von Frey test: sham: $t = 0.4$, $df = 22$, $P > 0.05$; CCI: $t = 0.4$, $df = 20$, $P > 0.05$; plantar test: sham: $t = 0.2$, $df = 17$, $P > 0.05$; CCI: $t = 1.0$, $df = 16$, $P > 0.05$).

In order to exclude the possibility that CCI and/or antidepressants affected locomotor activity of animals, the total distance traveled was evaluated at the same clock time as when the other experiments were conducted (Fig. 1C). No differences were found between sham and CCI animals, and no sedative effect was observed after antidepressant treatment.

3.2. Effects of CCI and antidepressant treatment on basal tonic activity of the LC

When the basal tonic neuronal activity (200 seconds before footshock stimulation start) of the ipsilateral and contralateral LC to operated hind paw was analyzed (Tables 2 and 3), neurons (n = 61 from contralateral LC and n = 14 from ipsilateral LC) from vehicle-treated sham animals (n = 7 and n = 9, respectively) had a similar tonic activity to that previously reported for the LC of anaesthetized rats [2,7,49]. CCI had no effect on the spontaneous firing characteristics of LC neurons contralateral and ipsilateral to the operated paw (n = 57 and n = 14 neurons from n = 7 and n = 8 animals, respectively), or on the mean basal firing rate, the regular firing pattern (variation coefficient), or burst firing compared to sham ($P > 0.05$; Table 2). No differences were found between both ipsilateral and contralateral LC activity in sham and CCI animals. In contrast, the administration of antidepressants diminished the basal activity and increased the percentage of spikes in burst of the LC in both sham-operated (DMI: n = 47 and DLX: n = 55 neurons from 6 rats) and CCI (DMI: n = 48 and DLX: n = 45 neurons from 6 rats) groups (see Tables 2 and 3 for detailed statistical analysis). Interestingly, this was accompanied by a lower incidence of neurons tonically firing in bursts in CCI rats (DMI: $P < 0.01$ and DLX: $P < 0.01$; Tables 1 and 2).

3.3. Effects of CCI and antidepressant administration on sensory-evoked activity of the LC

Firstly, we studied the effect of persistent electrical footshock stimulation on LC neurons in naïve animals (n = 22 neurons from 3 rats) [26]. This stimulation evoked a biphasic excitatory response (Fig. 3C), of which the first epoch had an average onset latency of 15.6 ± 1.3 ms, corresponding to the early LC response (Table 4). Assuming an approximate distance of 16 ± 1.0 cm from the mid-plantar surface of the hind paw to the contralateral LC, an underlying conduction velocity of 10.2 ± 0.2 m/s (linear distance/latency of sensory-evoked spikes) was calculated, consistent with conduction along thinly myelinated A fibers. After a period of inhibited activity (Fig. 3C), a late excitatory response of LC neurons was observed at 276.0 ± 16.9 ms (Table 4) with a mean conduction velocity of 0.5 ± 0.1 m/s. Intraplantar administration of 1% capsaicin in the operated hind paw consistently attenuated this late LC response, while it had no effect on the spikes in the first epoch in the same cells (Fig. 4G). Hence, this late response appeared to involve the contribution of unmyelinated C fibers [26,34].

When sensory-evoked discharge was analyzed in sham- and CCI-operated rats (Fig. 4A, D; Table 3), the excitatory responses of the LC were similar in vehicle-treated sham (both ipsilateral and contralateral LC recordings to left hind paw) and naïve rats, while CCI rats exhibited a distinct pattern of sensory-evoked activity both ipsilaterally and contralaterally. The early excitatory response was observed in both sham and CCI animals, but it was

Table 3
Two-way ANOVA test summary of the locus coeruleus (LC) electrophysiological activity.

	Response	Vehicle			Desipramine $LC_{contralateral}$			Duloxetine $LC_{contralateral}$			
		Surgery (F_s)	Lateral (F_L)	Interaction (F_i)	Surgery (F_s)	Treatment (F_t)	Interaction (F_i)	Surgery (F_s)	Treatment (F_t)	Interaction (F_i)
Tonic electrophysiological activity										
Firing rate (Hz)		$F_{[1,142]} = 1.0$	$F_{[1,142]} = 0.1$	$F_{[1,142]} = 0.0$	$F_{[1,209]} = 1.9$	$F_{[1,209]} = 126.1^{***}$	$F_{[1,209]} = 0.2$	$F_{[1,214]} = 4.1^{*}$	$F_{[1,214]} = 15.6^{***}$	$F_{[1,214]} = 1.4$
Variation coefficient (%)		$F_{[1,142]} = 0.2$	$F_{[1,142]} = 0.6$	$F_{[1,142]} = 0.4$	$F_{[1,209]} = 0.1$	$F_{[1,209]} = 1.2$	$F_{[1,209]} = 0.1$	$F_{[1,214]} = 0.0$	$F_{[1,214]} = 5.3^{*}$	$F_{[1,214]} = 0.4$
Number spikes/burst		$F_{[1,63]} = 2.3$	$F_{[1,63]} = 0.2$	$F_{[1,63]} = 1.4$	$F_{[1,70]} = 1.4$	$F_{[1,70]} = 9.7^{**}$	$F_{[1,70]} = 0.6$	~	~	~
Spikes in burst (%)		$F_{[1,63]} = 0.2$	$F_{[1,63]} = 0.0$	$F_{[1,63]} = 0.1$	$F_{[1,70]} = 0.1$	$F_{[1,70]} = 23.1^{***}$	$F_{[1,70]} = 0.4$	~	~	~
Sensory-evoked electrophysiological activity										
Latency (ms)	Early	$F_{[1,132]} = 1.1$	$F_{[1,132]} = 0.0$	$F_{[1,132]} = 2.4$	$F_{[1,209]} = 0.4$	$F_{[1,209]} = 1.4$	$F_{[1,209]} = 1.6$	$F_{[1,214]} = 2.6$	$F_{[1,214]} = 0.1$	$F_{[1,214]} = 4.6^{*}$
	Late	~	~	~	$F_{[1,117]} = 0.0$	$F_{[1,117]} = 30.7^{***}$	$F_{[1,117]} = 0.0$	$F_{[1,149]} = 3.4$	$F_{[1,149]} = 35.8^{***}$	$F_{[1,149]} = 5.4^{**}$
Excitatory firing rate (Hz)	Early	$F_{[1,132]} = 3.8$	$F_{[1,132]} = 1.0$	$F_{[1,132]} = 0.0$	$F_{[1,209]} = 0.5$	$F_{[1,209]} = 9.5^{**}$	$F_{[1,209]} = 13.6^{***}$	$F_{[1,214]} = 0.9$	$F_{[1,214]} = 0.7$	$F_{[1,214]} = 17.0^{***}$
	Late	~	~	~	$F_{[1,117]} = 0.8$	$F_{[1,117]} = 15.2^{***}$	$F_{[1,117]} = 1.0$	$F_{[1,149]} = 1.6$	$F_{[1,149]} = 4.6^{*}$	$F_{[1,149]} = 5.5^{*}$
Duration (ms)	Early	$F_{[1,132]} = = 0.1$	$F_{[1,132]} = 0.8$	$F_{[1,132]} = 0.7$	$F_{[1,209]} = 0.7$	$F_{[1,209]} = 6.2^{*}$	$F_{[1,209]} = 2.8$	$F_{[1,214]} = 0.5$	$F_{[1,214]} = 9.7^{**}$	$F_{[1,214]} = 2.1$
	Late	~	~	~	$F_{[1,117]} = 7.0^{*}$	$F_{[1,117]} = 8.2^{*}$	$F_{[1,117]} = 1.4$	$F_{[1,149]} = 13.6^{***}$	$F_{[1,149]} = 15.8^{***}$	$F_{[1,149]} = 1.8$
Inhibitory period (ms)		$F_{[1,132]} = 5.6^{***}$	$F_{[1,132]} = 15.4$	$F_{[1,132]} = 1.5$	$F_{[1,209]} = 86.2^{***}$	$F_{[1,209]} = 16.7^{***}$	$F_{[1,209]} = 0.5$	$F_{[1,214]} = 29.9^{***}$	$F_{[1,214]} = 35.7^{***}$	$F_{[1,214]} = 11.5^{**}$

Data represent the F-values of 2-way analysis of variance (ANOVA) test of tonic activity and sensory-evoked activity of LC neurons. Chronic constriction injury (CCI) was inflicted 7 days before the electrophysiological studies were performed. Vehicle, desipramine (10 mg/kg/day), or duloxetine (5 mg/kg/day) were administered via osmotic minipumps for 1 week. Sensory-evoked activity was divided into 2 nonconsecutive epochs: early and late responses (see Methods). The F-values with their associated degrees of freedom (*df*) were expressed as F_s [*df* of surgery/residual], F_t [*df* of treatment/residual], F_L [*df* of lateral/residual], or F_i [*df* of interaction/residual]. F_L compared the results obtained from LC neuronal recordings ipsilateral and contralateral to the left hind paw in the vehicle group. The significance of factor was represented as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. ~ not listed, as burst events were not observed.

Table 4
Sensory-evoked electrophysiological activity of locus coeruleus (LC) neurons to electrical footshock stimulation.

	Response	Vehicle					Desipramine		Duloxetine	
		$LC_{contralateral}$	$LC_{ipsilateral}$		$LC_{contralateral}$		$LC_{contralateral}$		$LC_{contralateral}$	
		Naïve [3]	Sham [3]	CCI [3]	Sham [7]	CCI [7]	Sham [6]	CCI [6]	Sham [6]	CCI [6]
Incidence of response (%)	Early	100.0 (22/22)	100.0 (9/9)	100.0 (9/9)	100.0 (61/61)	100.0 (57/57)	100.0 (47/47)	100.0 (48/48)	100.0 (55/55)	100.0 (45/45)
	Late	100.0 (22/22)	100.0 (9/9)	0.0*** (0/9)	100.0 (61/61)	15.8*** (9/57)	82.9 (39/47)	25.0***† (12/48)	92.7(51/55)	71.1†††† (32/45)
Latency (ms)	Early	15.6 ± 1.3	20.0 ± 3.7	15.0 ± 1.3	16.5 ± 1.2	15.9 ± 0.9	14.0 ± 0.8	16.0 ± 1.1	13.6 ± 0.8	18.0 ± 1.3
	Late	276.0 ± 16.9	250.0 ± 20.1	~	268.3 ± 10.1	274.7 ± 21.2	165.3 ± 12.3†††	164.8 ± 12.5†	221.4 ± 13.2†	168.0 ± 7.3**†††
Excitatory firing rate (Hz)	Early	18.3 ± 2.8	17.4 ± 1.0	23.4 ± 1.4**	18.4 ± 1.6	26.9 ± 1.7*	19.7 ± 2.7	10.1 ± 1.2*†††	20.1 ± 2.4	14.9 ± 1.9**††
	Late	7.5 ± 1.2	7.3 ± 0.4	~	7.5 ± 0.7	8.3 ± 1.5	4.3 ± 0.5††	3.0 ± 0.7†	7.6 ± 0.7	4.4 ± 0.6*†
Duration (ms)	Early	35.5 ± 5.0	32.0 ± 3.0	38.0 ± 2.5	41.7 ± 5.6	38.3 ± 2.9	45.1 ± 3.9	55.6 ± 3.7†	49.6 ± 5.8	60.0 ± 4.8††
	Late	45.6 ± 9.5	44.0 ± 4.5	~	44.7 ± 6.7	12.8 ± 3.2*	131.6 ± 17.5†††	49.1 ± 3.2*†	117.7 ± 18.8†††	48.6 ± 8.0***†
Inhibitory period (ms)		444.3 ± 58.3	428.5 ± 74.3	1400.0 ± 64.2***	434.5 ± 98.4	1360.0 ± 77.9***	123.5 ± 12.5††	914.9 ± 171.1**	164.0 ± 19.7†	380.7 ± 135.9†††

Data represent the mean ± SEM. The sciatic nerve was stimulated using bipolar 26-gauge needles subcutaneously inserted into the left hind paw and the sensory-evoked activity of ipsilateral and contralateral LC neurons was recorded. The naïve group corresponds to rats neither operated nor treated. Chronic constriction injury (CCI) was inflicted 7 days before the electrophysiological studies were performed. Vehicle, desipramine (10 mg/kg/day), or duloxetine (5 mg/kg/day) were administered via osmotic minipumps for 1 week. The values in brackets represent the number of animals used, and those in parentheses show the ratio of neurons exhibiting excitatory evoked activity (incidence of response,%). Sensory-evoked activity was divided into two non-consecutive epochs: early and late responses (see Methods). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs respective sham-operated group; †$P < 0.05$, ††$P < 0.01$, and †††$P < 0.001$ vs respective vehicle-treated group (2-way analysis of variance test followed by Tukey-Kramer honestly significant difference posttest detailed in Table 1; Fisher's exact test for the incidence of response).

significantly greater in the latter ($P < 0.01$ for ipsilateral LC and $P < 0.05$ for contralateral LC; Tables 3 and 4). By contrast, the proportion of neurons displaying the longer-latency response dropped significantly (0.0% and 15.8%, respectively; $P < 0.001$, Fisher's exact test, Tables 3 and 4) in CCI rats, and it persisted less in those neurons in which it was observed ($P < 0.05$; Tables 3 and 4). Moreover, LC recording from CCI animals showed an increase of inhibitory period after early response three times greater than that observed in sham animals ($P < 0.001$; Table 4).

Antidepressant treatment contributed to a recovery of the LC response in CCI rats (DMI: n = 48 and DLX: n = 45 neurons from 6 rats; Fig. 4E-F, Table 4). Both DMI and DLX reduced the magnitude (DMI: $P < 0.001$ and DLX: $P < 0.01$; Table 4) and increased the duration of the early excitatory response (DMI: $P < 0.05$ and DLX: $P < 0.01$; Table 4) compared to vehicle-CCI group. This effect was accompanied by a higher incidence (DMI: $P < 0.05$ and DLX: $P < 0.001$, Fisher's exact test, Table 4) and duration of the late response than in animals that received the vehicle alone (DMI: $P < 0.05$ and DLX: $P < 0.05$; Table 4). Interestingly, antidepressants also increased the duration of the late response in the sham-operated group (DMI: $P < 0.001$, n = 47 and DLX: $P < 0.001$, n = 55 neurons from 6 rats; Table 4, Fig. 4B, C). Furthermore, they reduced the latency of the late response and consequently the inhibitory period in both sham-operated and CCI rats (Table 4), although the average conduction velocity was kept within the range previously reported for the A and C fiber evoked response. As before, the long-latency response was sensitive to 1% capsaicin, suggesting a C fiber-mediated effect (Fig. 4H, I).

These changes in the sensory-evoked neuronal activity of the LC correlated well with the analgesic effect of antidepressant administration both in healthy and neuropathic rats (Fig. 5). Indeed, a significant negative correlation was evident between the excitatory firing rate of the early response in nerve-injured rats and the antineuropathic effect when the DMI and DLX data were pooled (von Frey test: $r = -0.7$, $P < 0.0001$; plantar test: $r = -0.7$, $P < 0.001$; Fig. 5A, B). Conversely, a positive correlation was found for the duration of the early response in CCI rats (von Frey test: $r = 0.7$, $P < 0.0001$; plantar test: $r = 0.7$, $P < 0.001$; Fig. 5C, D). In addition, the duration of the late responses in LC neurons was positively correlated with the increase in the nociceptive threshold in the von Frey and plantar tests after antidepressant treatment in CCI (von Frey test: $r = 0.8$, $P < 0.0001$; plantar test: $r = 0.7$, $P < 0.001$) and sham-operated rats (von Frey test: $r = 0.9$, $P < 0.0001$; plantar test: $r = 0.9$, $P < 0.0001$; Fig. 5E, F). All other correlations between the analgesic effect of antidepressant and LC evoked-activity were not statistically significant (data not shown).

3.4. Effects of CCI and antidepressant treatment on NMDAR activation

There is abundant evidence that NMDAR are crucial in mediating pain hypersensitivity [30]. In the LC, the early-evoked response to a noxious stimulus appears to result from the release of excitatory amino acids, such as glutamate, via activation of NMDAR and other receptors within the LC or from afferent nerves from the nucleus paragigantocellularis (PGi) [42]. Immunohistochemistry studies demonstrated NMDAR1 expression in non-tyrosine hydroxylase axon terminals in the LC, which may derive from PGi projections [48]. To evaluate the role of NMDAR activation in the effects observed in sham and CCI animals before and after antidepressant treatment, NMDAR1 phosphorylation [39] was quantified in unstimulated animals and after electrical footshock stimulation of the sciatic nerve. No NMDAR phosphorylation was observed in any nonstimulated animals (Fig. 6A), indicating that NMDAR are activated only upon stimulation. Indeed, electrical stimulation of CCI animals promoted protein kinase C-mediated phosphorylation of NMDAR1 Ser890 to a similar extent in both

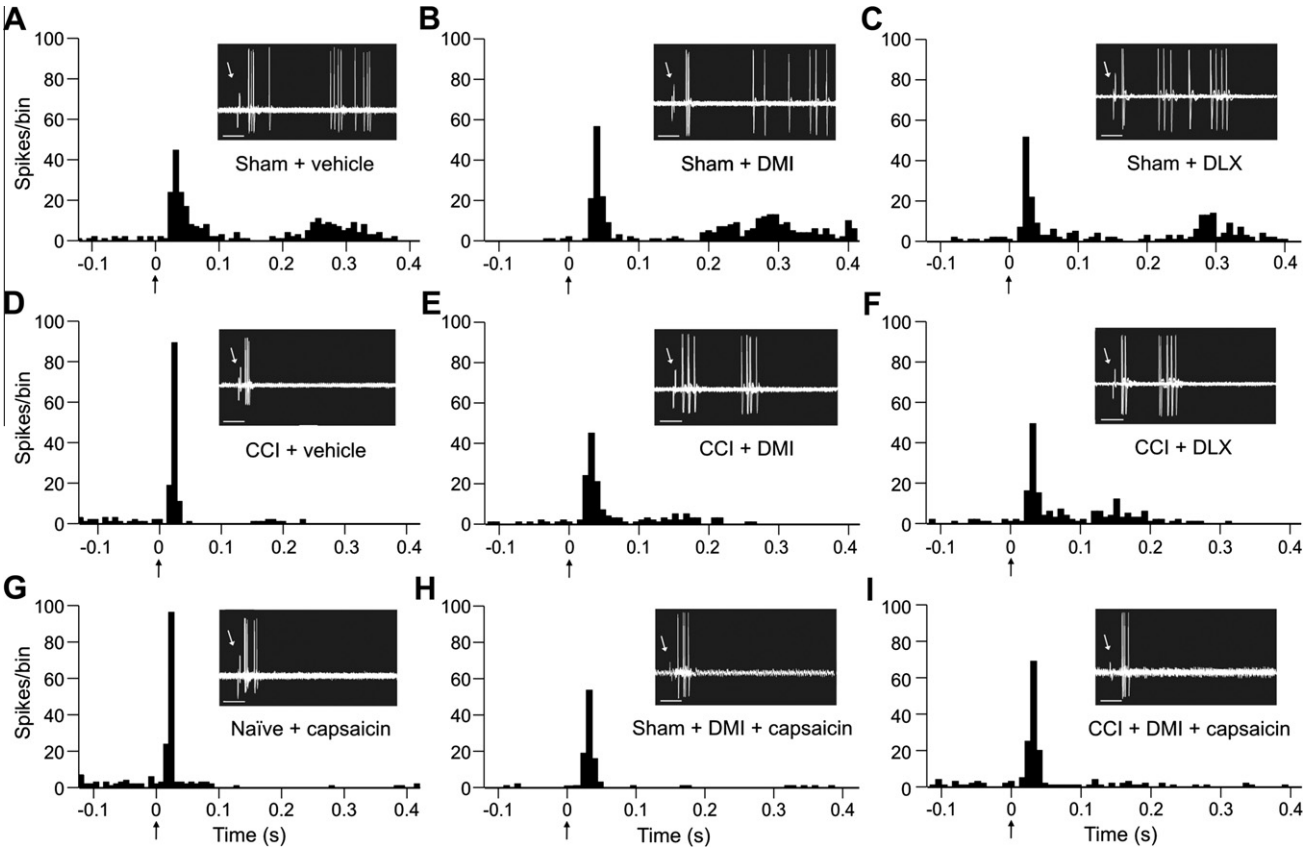

Fig. 4. Representative peristimulus time histograms (PSTHs) illustrating the sensory-evoked response of locus coeruleus (LC) neurons for each experimental group. (A-F) PSTHs were constructed from the cumulative activity of a characteristic LC neuron of each group during an electrical footshock train. *Y*-axis values indicate the number of spikes per 8-ms bin. Stimuli are presented at 0.0 s (black arrow) on the *X*-axis. Oscillography traces (small image) representing footshock-evoked spikes of LC neurons to electrical stimuli (footshock artifact denoted by the arrow; scale bar = 50 ms) for each experimental group. The recording contains 5 superimposed pulses with their corresponding neuronal responses. Desipramine (DMI, 10 mg/kg/day) or duloxetine (DLX, 5 mg/kg/day) were administered via osmotic minipumps for 1 week. Intraplantar administration of 1% capsaicin in the left hind paw (G-I) reduced the late-evoked response of LC neurons. CCI, chronic constriction injury.

LC (Fig. 6A). This suggests there is an overactivation of NMDAR function in CCI animals in response to nociceptive electrical stimulation, which may account for the enhanced excitatory early response observed in these animals. Moreover, in agreement with our electrophysiological findings, DLX treatment blocked NMDAR1 subunit phosphorylation in CCI animals (Fig. 6A).

Moreover, we investigated the activation of CaMKII. When NMDAR are activate, different kinases, including protein kinase C and Src, enhance NMDAR function by acting on specific cytosolic residues on the NMDAR subunits. Subsequently, CaMKII becomes activated [39]. Thus, to evaluate the possible involvement of an NMDAR potentiation in the observed effects, the phosphorylation of CaMKII was also determined. Similar to NMDAR results, no CaMKII phosphorylation expression was observed in nonstimulated animals, either sham or CCI (Fig. 6B). However, electric stimulation, more significantly in CCI animals, promoted an increase in the activating Thr286 autophosphorylation of CaMKII. In contrast, DLX in CCI significantly reduced the pCaMKII levels (Fig. 6B).

3.5. Effects of CCI and antidepressant treatment on alpha-2-adrenoceptor function

LC activity is regulated by alpha-2-adrenoceptors, which control electrical activity and noradrenaline release in the LC and in terminal areas like the spinal cord. To study the possible role of these receptors in the evoked-response of LC in sham and CCI animals after footshock stimulation, immunoprecipitations of alpha-2-adrenoceptors were performed. No differences were found among all groups (Fig. 6C). In addition, we obtained dose-response curves for clonidine (an alpha-2-adrenoceptor agonist) in sham animals treated with vehicle alone (n = 9) or antidepressants (DMI, n = 6 or DLX, n = 7) to determine the effect of both drugs on activity of alpha-2-adrenoceptors of LC. The inhibitory effect of clonidine on the firing rate of LC neurons in vehicle-treated rats was similar to a previous report (ED50 = 2.0 ± 0.3 μg/kg) [2]. However, the antidepressants shift to the right the dose-effect curve for clonidine (Fig. 6D), and the ED50 was increased after both DMI (12.5 ± 2.4 μg/kg; Ft[2,19] = 20.1, $P < 0.001$) and DLX (3.7 ± 0.6 μg/kg; Ft[2,19] = 20.1, $P < 0.01$) treatments. Moreover, DMI ED50 was more elevated than observed for DLX ($t = 3.7$, $df = 11$, $P < 0.01$).

4. Discussion

The findings presented here demonstrate that chronic neuropathic pain disrupts electrophysiological LC-evoked responses to a train of noxious stimuli. Moreover, our study shows that antidepressants with analgesic effects (DMI and DLX) promote the activation of the LC upon nociceptive stimulation, leading to analgesia in both healthy and neuropathic animals.

CCI has no effect on the spontaneous tonic activity of LC neurons [49] either contralaterally or ipsilaterally, and indeed, CCI of the sciatic nerve in rats does not alter extracellular noradrenaline levels or alpha-2-adrenoceptor function in the LC 7 days after surgery [2]. These findings indicate that nerve injury, which produces a significant decrease in the pain threshold, has no effect on tonic LC activity, at least at this specific time point. By contrast, when

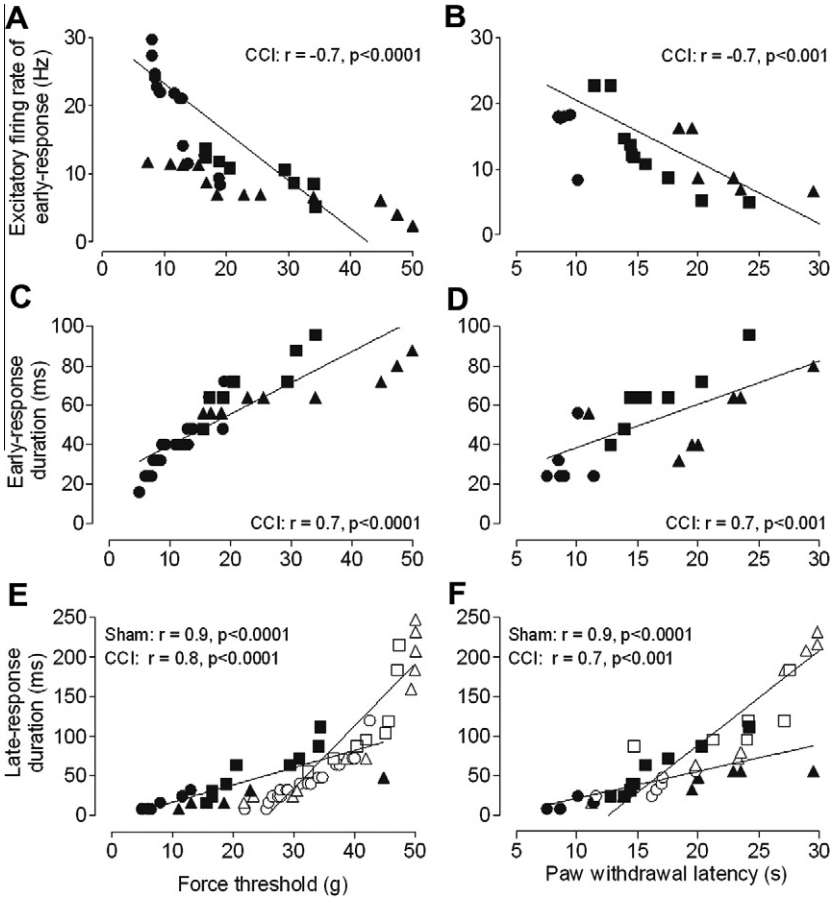

Fig. 5. Correlation between the sensory-evoked activity of the locus coeruleus (LC) and the analgesic effects of antidepressants in sham-operated (white) and chronic constriction injury (CCI) (black) rats. The nociceptive threshold (*X*-axis) was measured 7 days after CCI. Vehicle (circle), desipramine (triangle; 10 mg/kg/day) and duloxetine (square; 5 mg/kg/day) were administered via osmotic minipumps for 1 week. Excitatory LC activity (*Y*-axis) was evoked by electrical footshock stimulation of the operated hind paw (See Methods). (A) Correlation between the excitatory firing rate of the early-response and the mechanical threshold (grams) in response to von Frey hair stimulation in CCI rats. (B) Correlation between the excitatory firing rate of the early-response and the thermal threshold (seconds) in response to plantar test in CCI rats. (C) Correlation between the duration of the sensory-evoked early-response and the mechanical threshold in CCI rats. (D) Correlation between the duration of the early-response and the thermal threshold in CCI rats. (E) Correlation between duration of the sensory-evoked late-response and the mechanical threshold in sham-operated and CCI rats. (F) Correlation between duration of the sensory-evoked late-response and the thermal threshold in sham and CCI rats. Statistic: Pearson's correlation test.

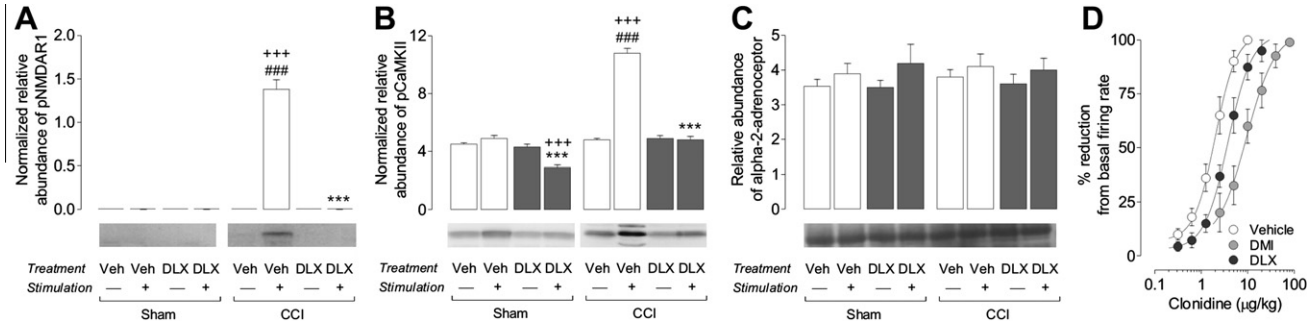

Fig. 6. Effect of antidepressants on *N*-methyl-D-aspartate receptor 1 (NMDAR1) activation and alpha-2-adrenoceptor function in the locus coeruleus (LC) 7 days after chronic constriction injury (CCI). (A) Representative blots and densitometric quantification of NMDAR1 (pSer890) subunit phosphorylation from LC homogenates of sham and CCI animals. (B) Representative blots and densitometric quantification of Ca^{2+}/calmodulin-dependent protein kinase IIα (CaMKII, pThr286) phosphorylation from LC homogenates of sham and CCI animals. (C) Representative blots and densitometric quantification of alpha-2A-adrenoceptor immunoprecipitation from the LC of sham and CCI animals. The sciatic nerve was stimulated using bipolar 26-gauge needles subcutaneously inserted into the left hind paw (see Methods). Each column represents the mean in arbitrary values of 3 assays performed on LC samples obtained from two independent groups of rats. Equal loading was verified by assaying β-actin. $^{***}P < 0.001$ vs respective vehicle group (1-way analysis of variance test) and $^{++}P < 0.01$, $^{+++}P < 0.001$ vs respective sham group (Student's *t*-test). (D) Dose-response curves illustrating the inhibitory effect of clonidine on the LC firing rate in sham rats after antidepressant treatment. Vehicle, duloxetine (DLX, 5 mg/kg/day) or desipramine (DMI, 10 mg/kg/day) were administrated via osmotic minipumps for 1 week. Increasing doses of clonidine were administered (intravenously) until the spontaneous activity ceased. Complete inhibition was achieved in all the cells tested. Each symbol represents the mean ± SEM of the percentage reduction of the basal firing rate.

both uninjured and injured animals receive antidepressants, there is a decrease in the tonic firing rate of LC neurons. This suggests that antidepressant administration increases the availability of noradrenaline, resulting in the activation of alpha-2-adrenoceptors that exert a tonic inhibitory effect on the firing rate of LC neurons [21,22,29,33,44,52]. Accordingly, a similar increase would be expected in the terminals of descending LC neurons that project to the spinal cord, which will therefore contribute to analgesia. Furthermore, antidepressants increased the percentage of spikes occurring in burst, which is one of the most reliable indicators of robust enhancement of noradrenaline release [17]. Interestingly, antidepressants produced a decrease in the number of neurons tonically bursting (incidence) in neuropathic animals; none was found in DLX-CCI, and there was a tendency to lower firing rate in these animals. Thus, this lower tonic electrophysiological activity may reflect an even larger increase in availability of noradrenaline (in the LC and its terminal areas) in CCI-treated animals. This hypothesis would imply that antidepressants are more efficient in inducing the release of noradrenaline in conditions of neuropathic pain, as suggested for gabapentin and pregabalin [24,45].

Long-lasting nociceptive pulses in control animals effectively evoke the well-documented early LC response, corresponding to rapidly conducting peripheral nerve activation (A fiber). After a period of neuronal inhibition, this fast response was followed by the late response that appears to correspond to C fiber activation [26]. However, in CCI animals, this biphasic response was significantly altered, with an increase in the excitatory firing rate and almost total loss of the later LC response. Previous studies have shown that the early response and the subsequent postactivation inhibition are triggered by glutamate release from the PGi in the medulla that conveys nociceptive inputs from the periphery [13]. The effect of glutamate is dose dependent and thus, higher glutamate concentrations produce a stronger early excitatory effect followed by a more prolonged inhibitory response [55]. Interestingly, we previously found that neuropathic pain increases pERK1/2 expression in the PGi, a marker of cell activity [2]. Now we show that there is an increase in NMDAR1 activity following a noxious stimulus in a model of neuropathic pain. Therefore, overactivation of the NMDAR1 may underlie the enhanced early response and the longer inhibitory period that subsequently blocks the late response in CCI animals. It has also been shown that the LC inhibitory period is mediated by Ca2+-dependent K+ conductance [3] and recurrent LC collateral inhibition is mediated by alpha-2-adrenoceptors [1,15]. However, in CCI animals, alpha-2-adrenoceptors are not modified, even after nociceptive stimulation (see also [2]). Overall, our data show that neuropathic pain leads to functional and molecular changes in noradrenergic terminals in the spinal cord [31] but also within the ipsilateral and contralateral LC. In fact, the LC-evoked response is similar in both LC, consistent with data indicating that ascending and descending information is driven bilaterally [14,24,47]. We propose that neuropathic pain enhances the effects of the glutamatergic system at both LC, dampening the descending noradrenergic inhibition.

The biphasic firing response was recovered by administering antidepressants to CCI animals, mimicking the LC-evoked response seen in control animals. In fact, both DLX and DMI attenuate the excessive excitatory firing of the early-response in CCI animals, reducing the inhibitory period and the latency of the late response, thereby increasing the incidence and duration of the second excitatory epoch. Furthermore, antidepressant administration blocked NMDAR1 phosphorilation in CCI animals, in agreement with the decrease in both the excitatory peak of the early-response and the inhibitory period. These findings are consistent with the down-regulation of NMDA function produced by antidepressant treatment [10,43,46]. Accordingly, intra-LC injection of the glutamate transporter activator, riluzole, in nerve-injured rats reduced pain hypersensitivity [25]. In sham-operated animals, antidepressant administration did not affect the early response, but decreased the inhibitory period and prolonged the late-response duration, highlighting the mechanisms that might potentially contribute to the analgesic effects of these drugs in uninjured animals. As previously mentioned, the inhibitory period in the LC is thought to be mediated by alpha-2-adrenoceptors. Indeed, it has been suggested that drugs that limit the LC inhibitory period (eg, alpha-2-adrenoceptors LC blockers [1,15,51]) may selectively promote a substantially enhanced late response of the LC to noxious stimuli enhancing noradrenergic descending analgesia. Given that the administration of antidepressants attenuates the inhibitory effects of clonidine in uninjured animals, it seems plausible that this desensitization may account for the shorter inhibitory period in sham-operated rats. Accordingly, DMI produced a more pronounced desensitization of alpha-2-adrenoceptors and a greater reduction in the late response latency than DLX in sham-operated animals. As the inhibitory period is also shorter in CCI-treated animals, alpha-2-adrenoceptor desensitization may also be responsible for the analgesic effect of antidepressants in neuropathic pain. It should be noted that alpha-2 expression in membranes did not appear to differ among the groups of animals tested here, although these immunoprecipitation experiments do not distinguish the expression in intracellular vesicular membranes, and thus it is possible that antidepressants promote alpha-2-adrenoceptors internalization. Our study suggests that antidepressants modify LC-evoked activity at two different levels: (1) decreasing the excessive excitatory firing rate of the early response in CCI; and (2) blocking the inhibitory period, and consequently increasing the late response in both CCI and sham-operated animals. The early response may be mediated by NMDAR and the late response by NMDAR in conjunction with alpha-2-adrenoceptors. Thus, antidepressants sum the early and longer late responses, producing a substantially stronger response that would increase noradrenaline release in the spinal cord, both in neuropathic and control animals, thereby enhancing the contribution of the descending noradrenergic pathway to analgesia.

It should be noted that antidepressants were administered systemically in order to mimic a clinical setting. As such, the possibility that the effects of antidepressants on LC activity are part of a circuit in the ascending and descending pain pathways cannot be excluded. In addition, nociceptive inputs to the LC were examined with electrical stimulation rather than more natural stimuli, such as mechanical or thermal. The temporal precision of electrical stimulation allows C fiber activity to be clearly separated from A fiber activity, and therefore, the effects of CCI and/or antidepressants on each of these responses can be unambiguously analyzed. Additional studies employing more natural stimuli will be of interest to further determine the role of LC-evoked response in neuropathic pain, and in the analgesic effects of antidepressants. Finally, our study evaluates the effect of two mechanistically different antidepressants with analgesic effect: DMI, which preferentially inhibits the reuptake of noradrenaline [33], and DLX, inhibitor of the reuptake of both noradrenaline and serotonin [53]. Thus, although the effects found were in general similar, we cannot discard the possible contribution of the serotoninergic system in the effect of DLX. Therefore, future mechanistic studies about the effect of different antidepressants on the two phases of LC-evoked activity in healthy and neuropathic pain animals would lead us to understand better the analgesic mechanism of the action of antidepressants.

In summary, the study presented here demonstrates that LC activity upon nociceptive stimulation is altered in the CCI model of chronic neuropathic pain. Furthermore, we show that antidepressants with antineuropathic effects restore LC-evoked activity in parallel with their behavioral analgesic effects. These studies provide novel insight as to inhibition of descending pain is

regulated and the analgesic mode of action of antidepressants in the treatment of chronic neuropathic pain.

Conflict of interest statement

The authors do not have any conflict of interest.

Acknowledgements

We would like to thank Mrs. Raquel Rey-Brea, Mr. Jesus Gallego-Gamo, and Mrs. Beatriz Fraile for their excellent technical assistance. This study was supported by grants from the Fondo de Investigacion Sanitaria (PI10/01221, PI1101704); CIBERSAM G09 and G18; Junta de Andalucía, Consejería de Innovación, Ciencia y Empresa (CTS-510 and CTS-4303); Catedra Externa del Dolor Grünenthal-Universidad de Cadiz, FP7-PEOPLE-2010-RG (268377) and an FPU fellowship (AP2007-02397).

References

[1] Aghajanian GK, Cedarbaum JM, Wang RY. Evidence for norepinephrine-mediated collateral inhibition of locus coeruleus neurons. Brain Res 1977;136:570–7.
[2] Alba-Delgado C, Borges G, Sanchez-Blazquez P, Ortega JE, Horrillo I, Mico JA, Meana JJ, Neto F, Berrocoso E. The function of alpha-2-adrenoceptors in the rat locus coeruleus is preserved in the chronic constriction injury model of neuropathic pain. Psychopharmacology (Berl) 2011 [Epub ahead of print].
[3] Andrade R, Aghajanian GK. Locus coeruleus activity in vitro: intrinsic regulation by a calcium-dependent potassium conductance but not alpha 2-adrenoceptors. J Neurosci 1984;4:161–70.
[4] Aston-Jones G, Cohen JD. An integrative theory of locus coeruleus-norepinephrine function: adaptive gain and optimal performance. Annu Rev Neurosci 2005;28:403–50.
[5] Bennett GJ, Xie YK. A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. Pain 1988;33:87–107.
[6] Berrocoso E, De Benito MD, Mico JA. Role of serotonin 5-HT1A and opioid receptors in the antiallodynic effect of tramadol in the chronic constriction injury model of neuropathic pain in rats. Psychopharmacology (Berl) 2007;193:97–105.
[7] Berrocoso E, Mico JA. In vivo effect of venlafaxine on locus coeruleus neurons: role of opioid, alpha(2)-adrenergic, and 5-hydroxytryptamine(1A) receptors. J Pharmacol Exp Ther 2007;322:101–7.
[8] Berrocoso E, Mico JA, Ugedo L. In vivo effect of tramadol on locus coeruleus neurons is mediated by alpha2-adrenoceptors and modulated by serotonin. Neuropharmacology 2006;51:146–53.
[9] Berrocoso E, Mico JA, Vitton O, Ladure P, Newman-Tancredi A, Depoortere R, Bardin L. Evaluation of milnacipran, in comparison with amitriptyline, on cold and mechanical allodynia in a rat model of neuropathic pain. Eur J Pharmacol 2011;655:46–51.
[10] Bobula B, Hess G. Antidepressant treatments-induced modifications of glutamatergic transmission in rat frontal cortex. Pharmacol Rep 2008;60:865–71.
[11] Brightwell JJ, Taylor BK. Noradrenergic neurons in the locus coeruleus contribute to neuropathic pain. Neuroscience 2009;160:174–85.
[12] Cedarbaum JM, Aghajanian GK. Noradrenergic neurons of the locus coeruleus: inhibition by epinephrine and activation by the alpha-antagonist piperoxane. Brain Res 1976;112:413–9.
[13] Chiang C, Aston-Jones G. Response of locus coeruleus neurons to footshock stimulation is mediated by neurons in the rostral ventral medulla. Neuroscience 1993;53:705–15.
[14] Clark FM, Yeomans DC, Proudfit HK. The noradrenergic innervation of the spinal cord: differences between two substrains of Sprague-Dawley rats determined using retrograde tracers combined with immunocytochemistry. Neurosci Lett 1991;125:155–8.
[15] Ennis M, Aston-Jones G. Evidence for self- and neighbor-mediated postactivation inhibition of locus coeruleus neurons. Brain Res 1986;374:299–305.
[16] Finnerup NB, Otto M, McQuay HJ, Jensen TS, Sindrup SH. Algorithm for neuropathic pain treatment: an evidence based proposal. Pain 2005;118:289–305.
[17] Florin-Lechner SM, Druhan JP, Aston-Jones G, Valentino RJ. Enhanced norepinephrine release in prefrontal cortex with burst stimulation of the locus coeruleus. Brain Res 1996;742:89–97.
[18] Garzon J, Rodriguez-Munoz M, Lopez-Fando A, Sanchez-Blazquez P. Activation of mu-opioid receptors transfers control of Galpha subunits to the regulator of G-protein signaling RGS9-2: role in receptor desensitization. J Biol Chem 2005;280:8951–60.
[19] Goldstein DJ, Lu Y, Detke MJ, Lee TC, Iyengar S. Duloxetine vs. placebo in patients with painful diabetic neuropathy. Pain 2005;116:109–18.
[20] Grace AA, Bunney BS. The control of firing pattern in nigral dopamine neurons: burst firing. J Neurosci 1984;4:2877–90.
[21] Grandoso L, Pineda J, Ugedo L. Comparative study of the effects of desipramine and reboxetine on locus coeruleus neurons in rat brain slices. Neuropharmacology 2004;46:815–23.
[22] Grant MM, Weiss JM. Effects of chronic antidepressant drug administration and electroconvulsive shock on locus coeruleus electrophysiologic activity. Biol Psychiatry 2001;49:117–29.
[23] Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain 1988;32:77–88.
[24] Hayashida K, Obata H, Nakajima K, Eisenach JC. Gabapentin acts within the locus coeruleus to alleviate neuropathic pain. Anesthesiology 2008;109:1077–84.
[25] Hayashida K, Parker RA, Eisenach JC. Activation of glutamate transporters in the locus coeruleus paradoxically activates descending inhibition in rats. Brain Res 2010;1317:80–6.
[26] Hirata H, Aston-Jones G. A novel long-latency response of locus coeruleus neurons to noxious stimuli: mediation by peripheral C-fibers. J Neurophysiol 1994;71:1752–61.
[27] Hirata H, Aston-Jones G. Long-latency responses of brain noradrenergic neurons to noxious stimuli are preferentially attenuated by intravenous morphine. Brain Res 1996;714:9–18.
[28] Jett MF, McGuirk J, Waligora D, Hunter JC. The effects of mexiletine, desipramine and fluoxetine in rat models involving central sensitization. Pain 1997;69:161–9.
[29] Lacroix D, Blier P, Curet O, de Montigny C. Effects of long-term desipramine administration on noradrenergic neurotransmission: electrophysiological studies in the rat brain. J Pharmacol Exp Ther 1991;257:1081–90.
[30] Liu XJ, Gingrich JR, Vargas-Caballero M, Dong YN, Sengar A, Beggs S, Wang SH, Ding HK, Frankland PW, Salter MW. Treatment of inflammatory and neuropathic pain by uncoupling Src from the NMDA receptor complex. Nat Med 2008;14:1325–32.
[31] Ma W, Eisenach JC. Chronic constriction injury of sciatic nerve induces the up-regulation of descending inhibitory noradrenergic innervation to the lumbar dorsal horn of mice. Brain Res 2003;970:110–8.
[32] Mao J, Mayer DJ, Price DD. Patterns of increased brain activity indicative of pain in a rat model of peripheral mononeuropathy. J Neurosci 1993;13:2689–702.
[33] Mateo Y, Fernandez-Pastor B, Meana JJ. Acute and chronic effects of desipramine and clorgyline on alpha(2)-adrenoceptors regulating noradrenergic transmission in the rat brain: a dual-probe microdialysis study. Br J Pharmacol 2001;133:1362–70.
[34] Mendell LM. Physiological properties of unmyelinated fiber projection to the spinal cord. Exp Neurol 1966;16:316–32.
[35] Mico JA, Ardid D, Berrocoso E, Eschalier A. Antidepressants and pain. Trends Pharmacol Sci 2006;27:348–54.
[36] Ortega-Alvaro A, Gibert-Rahola J, Mellado-Fernandez ML, Chover AJ, Mico JA. The effects of different monoaminergic antidepressants on the analgesia induced by spinal cord adrenal medullary transplants in the formalin test in rats. Anesth Analg 1997;84:816–20.
[37] Parker RB, Waud DR. Pharmacological estimation of drug-receptor dissociation constants. Statistical evaluation. I. Agonists. J Pharmacol Exp Ther 1971;177:1–12.
[38] Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 6th ed. Amsterdam: Elsevier; 2009.
[39] Sanchez-Blazquez P, Rodriguez-Munoz M, Garzon J. Mu-opioid receptors transiently activate the Akt-nNOS pathway to produce sustained potentiation of PKC-mediated NMDAR-CaMKII signaling. PLoS One 2010;5:e11278.
[40] Sands SA, Reisman SA, Enna SJ. Effect of antidepressants on GABA(B) receptor function and subunit expression in rat hippocampus. Biochem Pharmacol 2004;68:1489–95.
[41] Sindrup SH, Gram LF, Skjold T, Grodum E, Brosen K, Beck-Nielsen H. Clomipramine vs desipramine vs placebo in the treatment of diabetic neuropathy symptoms. A double-blind cross-over study. Br J Clin Pharmacol 1990;30:683–91.
[42] Singewald N, Schneider C, Philippu A. Effects of neuroactive compounds, noxious and cardiovascular stimuli on the release of amino acids in the rat locus coeruleus. Neurosci Lett 1994;180:55–8.
[43] Skolnick P, Popik P, Trullas R. Glutamate-based antidepressants: 20 years on. Trends Pharmacol Sci 2009;30:563–9.
[44] Svensson TH, Usdin T. Feedback inhibition of brain noradrenaline neurons by tricyclic antidepressants: alpha-receptor mediation. Science 1978;202:1089–91.
[45] Tanabe M, Takasu K, Takeuchi Y, Ono H. Pain relief by gabapentin and pregabalin via supraspinal mechanisms after peripheral nerve injury. J Neurosci Res 2008;86:3258–64.
[46] Tokarski K, Bobula B, Wabno J, Hess G. Repeated administration of imipramine attenuates glutamatergic transmission in rat frontal cortex. Neuroscience 2008;153:789–95.
[47] Tsuruoka M, Maeda M, Nagasawa I, Inoue T. Spinal pathways mediating coeruleospinal antinociception in the rat. Neurosci Lett 2004;362:236–9.
[48] Van Bockstaele EJ, Colago EE. Selective distribution of the NMDA-R1 glutamate receptor in astrocytes and presynaptic axon terminals in the nucleus locus coeruleus of the rat brain: an immunoelectron microscopic study. J Comp Neurol 1996;369:483–96.

[49] Viisanen H, Pertovaara A. Influence of peripheral nerve injury on response properties of locus coeruleus neurons and coeruleospinal antinociception in the rat. Neuroscience 2007;146:1785–94.
[50] Wattiez AS, Libert F, Privat AM, Loiodice S, Fialip J, Eschalier A, Courteix C. Evidence for a differential opioidergic involvement in the analgesic effect of antidepressants: prediction for efficacy in animal models of neuropathic pain? Br J Pharmacol 2011;163:792–803.
[51] Wei H, Pertovaara A. Spinal and pontine alpha2-adrenoceptors have opposite effects on pain-related behavior in the neuropathic rat. Eur J Pharmacol 2006;551:41–9.
[52] West CH, Weiss JM. Effects of chronic antidepressant drug administration and electroconvulsive shock on activity of dopaminergic neurons in the ventral tegmentum. Int J Neuropsychopharmacol 2011;14:201–10.
[53] Wong DT. Duloxetine (LY 248686): an inhibitor of serotonin and noradrenaline uptake and an antidepressant drug candidate. Expert Opin Investig Drugs 1998;7:1691–9.
[54] Yalcin I, Tessier LH, Petit-Demouliere N, Doridot S, Hein L, Freund-Mercier MJ, Barrot M. Beta2-adrenoceptors are essential for desipramine, venlafaxine or reboxetine action in neuropathic pain. Neurobiol Dis 2009;33:386–94.
[55] Zamalloa T, Bailey CP, Pineda J. Glutamate-induced post-activation inhibition of locus coeruleus neurons is mediated by AMPA/kainate receptors and sodium-dependent potassium currents. Br J Pharmacol 2009;156:649–61.