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SHORT COMMUNICATION

Pain exacerbates chronic mild stress-induced changes in noradrenergic transmission in rats

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Abstract
Depression can influence pain and vice versa, yet the biological mechanisms underlying how one influences the pathophysiology of the other remains unclear. Dysregulation of locus coeruleus-noradrenergic transmission is implicated in both conditions, although it is not known whether this effect is exacerbated in cases of co-morbid depression and chronic pain. We studied locus coeruleus activity using immunofluorescence and electrophysiological approaches in rats subjected to unpredictable chronic mild stress (CMS, an experimental model of depression) and/or chronic constriction injury (CCI, a model of chronic neuropathic pain) for 2 weeks. CCI alone had no effect on any of the locus coeruleus parameters studied, while CMS led to a slight reduction in the electrophysiological activity of the locus coeruleus. Furthermore, CMS was associated with an increase in the number of tyrosine hydroxylase-positive cells in the locus coeruleus, although they were smaller in size. Interestingly, these effects of CMS were exacerbated when combined with CCI, even though no changes in the α2-adrenoreceptors or the noradrenaline transporter were observed in any group. Together, these findings suggest that CMS triggers several modifications in locus coeruleus-noradrenergic transmission that are exacerbated by co-morbid chronic pain.

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1. Introduction

Depression and chronic pain frequently co-exist, and they can mutually exacerbate one another, worsening the patient's prognosis and treatment response (Bair et al., 2004; Karp et al., 2005). It is unclear whether depression and chronic pain are causally related, or whether diagnosis with one condition implies a predisposition to the other. We previously investigated the complex relationship between these conditions in animal models of depression, pain, and co-morbid depression and pain (Bravo et al., 2012). In rats subjected to chronic mild stress (CMS), which induces depressive-like behavior, we observed minimal changes in sensorial pain perception. Moreover, depressive-like behavior in these animals was unaffected by chronic constriction injury (CCI), a commonly used model of neuropathic pain. However, we observed increased avoidance of painful stimuli in animals subjected to CMS. Moreover, we subsequently demonstrated that in rats with neuropathic pain, social stress exacerbates aversion to painful experiences through a locus coeruleus (LC)-related mechanism (Bravo et al., 2013). Moreover, LC impairment coincides with the onset of anxio-depressive-like symptoms in rats suffering chronic neuropathic pain (Alba-Delgado et al., 2013). Together, these findings point to a key role of the LC in the interaction between chronic pain and depression.

The LC is the main site of noradrenergic cell bodies in the CNS. Descending projections from the LC to the spinal cord block nociceptive inputs, while projections ascending to the forebrain regulate emotional responses (Haidkind et al., 2003). Although the involvement of the LC in both pain and depression has been well established, its role in comorbid pain and depression remains unclear. Accordingly, we have addressed this issue by analyzing LC activity in an animal model of co-morbid depression and chronic neuropathic pain.

2. Experimental procedures

2.1. Animals and experimental design

Experimental procedures were approved by the Committee for Animal Experimentation at the University of Cadiz in accordance with governmental guidelines and they complied with the International Association for the Study of Pain ethical guidelines (Zimmermann, 1983). After a period of habituation in standard conditions, male Sprague-Dawley rats (280–300 g) were subjected to chronic stress and/or neuropathic pain for 2 weeks (Figure 1A). Neuropathic pain was induced by chronic constriction injury (CCI) of the common left sciatic nerve (Figure 1B: (Bennett and Xie, 1988; Berrocoso and Mico, 2007). In sham-operated rats, an identical dissection was performed but the sciatic nerve was not ligated. To induce a depressive-like state, animals were individually and continuously subjected to CMS (Figure 1C) in sessions lasting 10-14 h (day and night). Control animals were not subjected to any stress.

2.2. Behavioral tests

At the end of the habituation period and throughout the experimental phase, sensory pain was evaluated in all the experimental groups. Mechanical allodynia was measured using the von Frey test (Ugo Basile, Italy) by applying a vertical force to the paw, with a cut-off at 50 g (Berrocoso et al., 2011). The latency of paw withdrawal was recorded. Cold allodynia was measured using the acetone test, whereby a 100 μl drop of acetone was applied to the center of the hindpaw with a pipette, and the response was graded to a four-point scale: 0, no response; 1, paw withdrawal; 2, repeated paw flicking; 3, paw licking. The mean score value was calculated according to: (summation score value of each trials/number of total trials) (Bravo et al., 2012).

Behavioral despair was evaluated in one set of animals at the end of the experimental period using the modified forced swimming test (mFST). Rats were placed for 15 min in Plexiglas cylinders filled with 30 cm of water (pre-test session), and again for 5 min 24 h later (test session). The animal's behavior (immobility, swimming or climbing) was assessed throughout the test session (Bravo et al., 2012; Detke et al., 1995). Administration of the noradrenaline reuptake inhibitor, desipramine (20 mg/kg, i.p.; Sigma-Aldrich, USA), was used as a positive control in a parallel group and it was administered 23.5, 5 and 1 h before testing.

2.3. Electrophysiology

Single-unit extracellular recordings of LC neurons were obtained as described in Supplementary material (Alba-Delgado et al., 2012; Berrocoso and Mico, 2007).

2.4. Western blot

Rats were sacrificed by administering an overdose of chloral hydrate, and the LC was removed bilaterally to assess the presence of the noradrenaline transporter (NAT) (Supplementary material). Protein expression was detected using a LI-COR Odyssey® two-channel quantitative fluorescence imaging system (Bonsai Advanced Techonologies, Spain) and the digital images were analyzed by densitometry using ImageJ software (National Institutes of Health, USA).

2.5. Immunohistochemistry

Tyrosine hydroxylase (TH) immunohistochemistry of LC sections was performed as described in Supplementary material. The fluorescent signal was visualized on an Olympus BX60 microscope equipped with a U-MNU filter system and coupled to Olympus DP71 camera (Olympus, USA). TH-immunoreactive (TH-IR) cells were counted manually in an average of 6 LC sections per animal (n=4-5 rats per group). To detect changes in soma size, the mean soma area per group was calculated in 7-9 randomly selected TH-IR cells per section. Each TH-IR soma was outlined manually using a computer mouse and the cross-sectional area was calculated with ImageJ software.

2.6. Statistics

The data are represented as the mean ± SEM. The results were analyzed using either an unpaired Student t test or two- or three-way analysis of variance (ANOVA), with or without repeated measures, as appropriate. All post-hoc analyses were carried out using a Bonferroni post-hoc test. Burst incidence was analyzed using Fisher's exact test. The differences were considered significant at $p<0.05$.

3. Results

We first performed a behavioral analyses of each experimental groups and in agreement with our previous findings (Bravo et al., 2012) no differences in mechanical or cold allodynia in the injured hind-paw were observed between the two groups subjected to neuropathic pain (CCI-control and CCI-CMS; Figure 1D and F). In both CMS groups (sham-CMS and CCI-CMS), we detected cold but not mechanical

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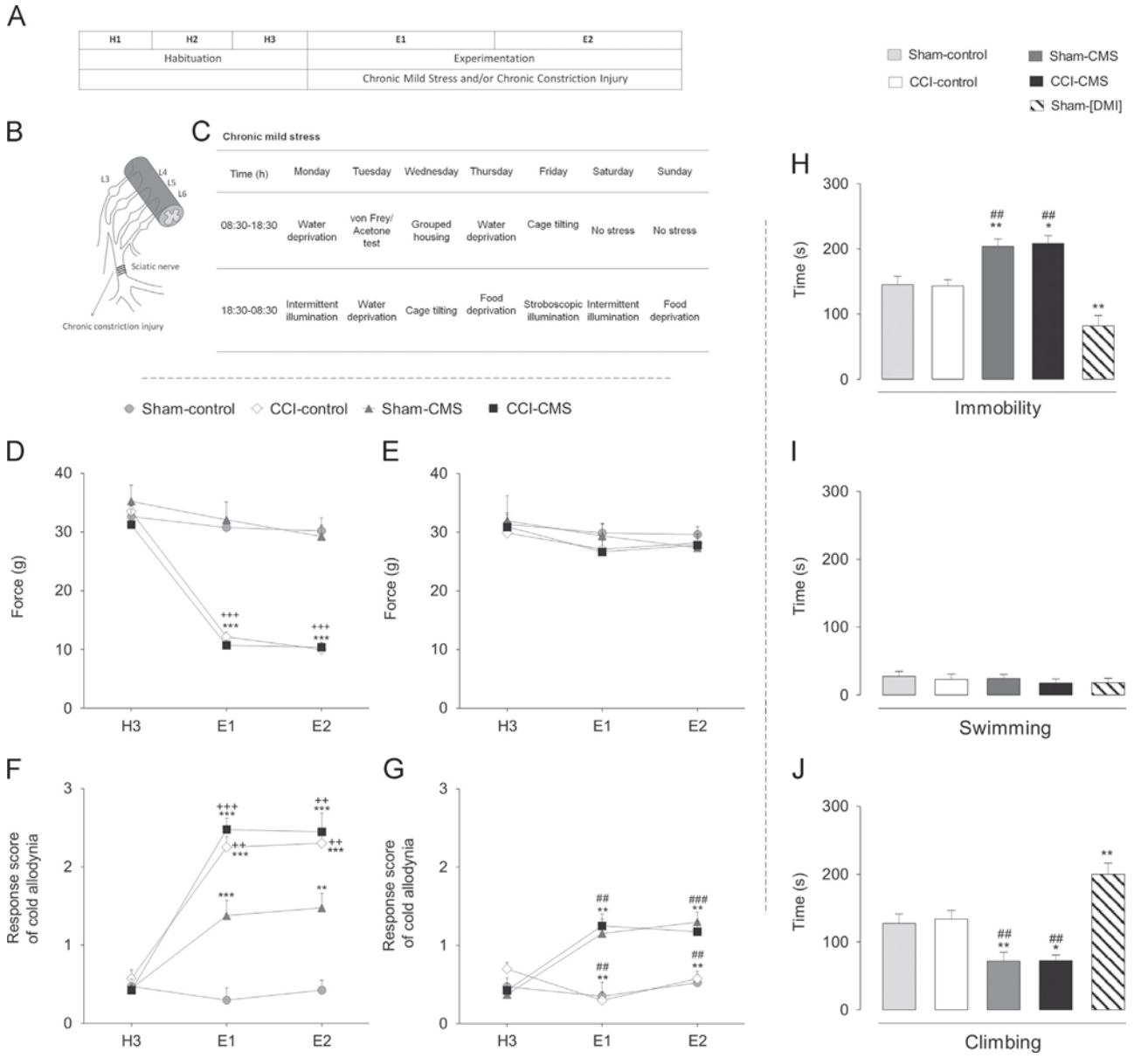

Figure 1 Experimental design and behavioral tests. *Top left*: schemes illustrating the (A) experimental schedule, and the protocols used for (B) chronic constriction injury and (C) chronic mild stress (animals were subjected to two unpredictable stressors each day for two weeks). *Bottom left*: withdrawal response of (D) the ipsilateral and (E) contralateral hindpaws in the von Frey test. Graphs show the cold allodynia score for the (F) ipsilateral and (G) contralateral hindpaws. *Right*: forced swimming test results showing the time spent (H) immobile, (I) swimming and (J) climbing. Data represent the mean+S.E.M. of 8-9 rats per group: $^{**}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ *vs.* sham-control; $^{++}p<0.01$, $^{+++}p<0.001$ *vs.* sham-CMS; $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.* CCI-control.

allodynia in the non-injured paw (Figure 1G and E). All CMS groups developed depressive-like behavior, as witnessed by an increase in immobility with respect to the sham-control and CCI-control (Figure 1H). This increase in immobility was offset by a decrease in the time spent climbing (Figure 1J). By contrast, sham operated animals treated with desipramine spent less time immobile and more time climbing than their untreated counterparts (Student *t* test: $p<0.01$; Figure 1H and J).

When spontaneous electrophysiological activity was studied to evaluate LC functionality (Table 2), the firing rates in the sham-control animals were similar to those in the neuropathic pain (CCI-control) and CMS (sham-CMS) (Figure 2A and Table 2). However, the combination of neuropathic pain and CMS (CCI-CMS) resulted in a decrease in firing activity with respect to both the sham-control and CCI-control ($p<0.01-p<0.05$; Figure 2A and B and Table 2). The histograms of the interspike interval (ISI) for sham-control and CCI-control revealed a similar Gaussian distribution of 0.5-0.6 s (Figure 2C and Table 2), reflecting a normal pattern of discharge. However, a shift to the right in the distribution (0.8 s) was observed in the sham-CMS and CCI-CMS groups (Figure 2D and Table 2). When the firing rate of one LC neuron had stabilized, UK14,304 or DMI was administered intravenously to evaluate α2-adrenoceptor

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Table 1 Statistics. The data were analyzed using a two-way or three-way analysis of variance (ANOVA), with or without repeated measures as appropriate. The independent variables were CCI (between-groups), CMS (between-groups) and Time (within-groups). Abbreviations: CCI, chronic constriction injury; CMS, chronic mild stress; I, ipsilateral; ISI, mean interspike interval; *C*, contralateral; mFST, modified forced swimming test; Immob, immobility; NAT, noradrenaline transporter; TH, tyrosine hydroxylase.

		CCI	CMS	CCI*CMS	Time	Time*CCI	Time*CMS	Time*CCI*CMS
Behavioral tests								
von Frey test	I	**$F_{(1,28)}=99.47^{***}$**	$F_{(1,28)}=0.00$	$F_{(1,28)}=0.58$	**$F_{(2,56)}=89.43^{***}$**	**$F_{(2,56)}=47.13^{***}$**	$F_{(2,56)}=0.01$	$F_{(2,56)}=1.06$
	C	$F_{(1,28)}=0.86$	$F_{(1,28)}=0.04$	$F_{(1,28)}=0.06$	$F_{(2,56)}=3.45$	$F_{(2,56)}=0.43$	$F_{(2,56)}=0.38$	$F_{(2,56)}=0.07$
Acetone test	I	**$F_{(1,28)}=108.80^{***}$**	**$F_{(1,28)}=16.47^{***}$**	**$F_{(1,28)}=10.71^{**}$**	**$F_{(2,56)}=89.76^{***}$**	**$F_{(2,56)}=35.11^{***}$**	**$F_{(2,56)}=8.62^{***}$**	$F_{(2,56)}=2.38$
	C	$F_{(1,28)}=0.25$	**$F_{(1,28)}=30.35^{***}$**	$F_{(1,28)}=0.16$	**$F_{(2,56)}=11.53^{***}$**	$F_{(2,56)}=0.54$	**$F_{(2,56)}=22.30^{***}$**	$F_{(2,56)}=0.61$
mFST	Immob.	$F_{(1,28)}=0.01$	**$F_{(1,28)}=27.99^{***}$**	$F_{(1,28)}=0.07$				
	Swimming	$F_{(1,28)}=0.95$	$F_{(1,28)}=0.19$	$F_{(1,28)}=0.00$				
	Climbing	$F_{(1,28)}=0.07$	**$F_{(1,28)}=22.37^{***}$**	$F_{(1,28)}=0.05$				
Protein quantification								
TH		$F_{(1,13)}=0.01$	**$F_{(1,13)}=19.76^{***}$**	$F_{(1,13)}=1.82$				
NAT		$F_{(1,16)}=0.15$	$F_{(1,16)}=0.44$	$F_{(1,16)}=0.84$				
Cell size		$F_{(1,13)}=2.42$	**$F_{(1,13)}=12.80^{**}$**	$F_{(1,13)}=3.23$				
Electrophysiological recordings								
Firing rate		$F_{(1,105)}=0.45$	**$F_{(1,105)}=14.35^{***}$**	$F_{(1,105)}=1.92$				
ISI		$F_{(1,105)}=0.02$	**$F_{(1,105)}=27.45^{***}$**	$F_{(1,105)}=2.24$				
Burst rate		$F_{(1,105)}=0.11$	**$F_{(1,105)}=11.27^{**}$**	$F_{(1,105)}=0.47$				
Spikes per burst		$F_{(1,28)}=0.45$	$F_{(1,28)}=0.31$	$F_{(1,28)}=0.45$				
% Spikes in burst		$F_{(1,28)}=0.47$	$F_{(1,28)}=1.62$	$F_{(1,28)}=0.03$				
$ED_{50\ UK14,304}$		$F_{(1,18)}=0.19$	$F_{(1,18)}=1.05$	$F_{(1,18)}=0.35$				
$ED_{50\ Desipramine}$		$F_{(1,23)}=0.23$	$F_{(1,23)}=0.78$	**$F_{(1,23)}=4.69^{*}$**				

$^{*}p<0.05$.
$^{**}p<0.01$.
$^{***}p<0.001$.

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Table 2 Electrophysiological properties of LC neurons. Spontaneous activity in response to CCI and/or CMS (mean±S.E.M.). Values in parentheses represent the proportion of neurons exhibiting burst activity. Values in square brackets represent the number of neurons pharmacologically tested. A total of 12–15 rats per group were used: $^{*}p<0.05$, $^{**}p<0.01$ *vs.* sham-control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.* CCI-control. ISI, mean interspike interval.

	Sham-control	CCI-control	Sham-CMS	CCI-CMS
Spontaneous activity				
Firing rate (Hz)	1.8±0.1	1.8±0.1	1.5±0.1	**1.3±0.1**$^{**\#\#}$
ISI (s)	0.6±0.0	0.5±0.0	**0.8±0.1**$^{\#\#}$	**0.8±0.0**$^{**\#\#\#}$
Burst firing activity				
Incidence (%)	42.9 (12/28)	38.5 (10/26)	**19.2 (5/26)***	**13.8 (4/29)**$^{*\#}$
Burst rate (burst/ms)	7.1±1.8	8.1±2.4	3.1±1.4	**1.4±0.7**$^{*\#}$
Spikes per burst	2.3±0.2	2.1±0.1	2.0±0.0	2.0±0.0
Spikes in burst (%)	2.0±0.3	2.0±0.4	1.6±0.4	1.6±0.3
Effective dose 50				
UK14,304 (μg/kg)	21.8±2.9 [6]	16.6±3.0 [4]	24.0±6.1 [5]	24.7±5.6 [7]
Desipramine (mg/kg)	0.3±0.1 [6]	0.2±0.0 [5]	0.2±0.0 [7]	0.3±0.0 [8]

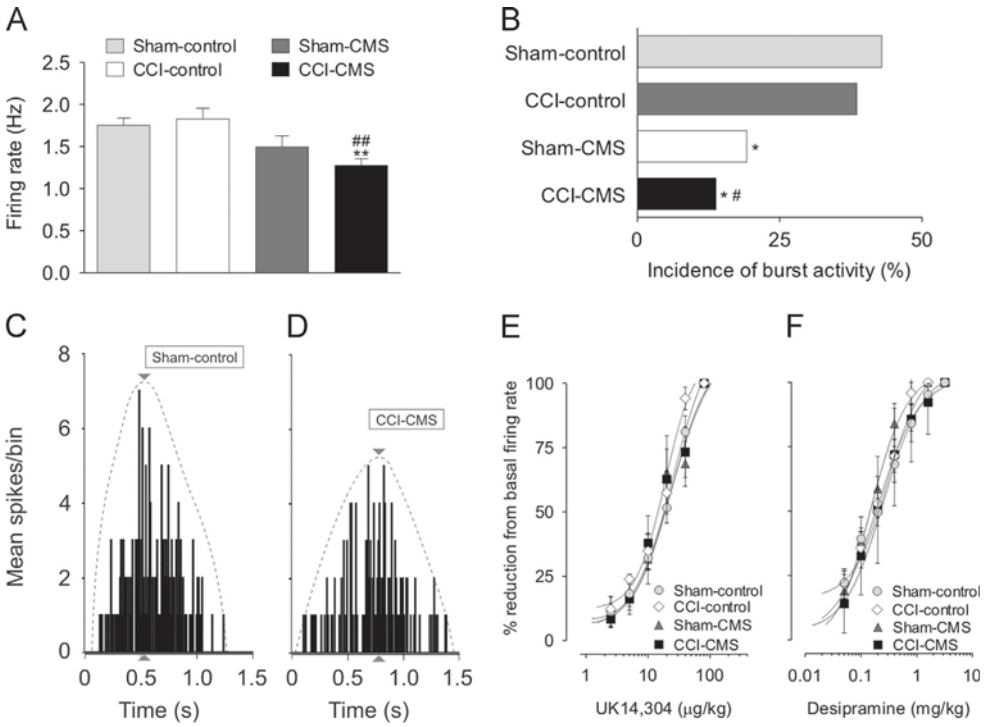

Figure 2 Electrophysiological activity. (A) Spontaneous firing rate of LC neurons in response to CCI and/or CMS. (B) Percentage burst firing during spontaneous activity (12–15 rats per group were evaluated (mean+S.E.M.): $^{*}p<0.05$, $^{**}p<0.01$ *vs.* sham-control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs.* CCI-control. (C,D) Representative ISI histograms showing the patterns of electrical discharge recorded in (C) sham-control and (D) CCI-CMS (bin=10 s). (E,F) Dose-response curves illustrating the inhibitory effect of (E) UK14,304 and (F) desipramine. The data represent the mean±S.E.M. of the percentage decrease from the basal firing rate (4–8 neurons pharmacologically tested).

and NAT function, respectively. Both drugs inhibited spontaneous activity in a dose-dependent manner, producing similar dose-response curves in all experimental groups (Figure 2E and F and Table 2). NAT expression in the LC was also determined in another set of animals and two-way ANOVA revealed no differences between groups (Figure 3I and Table 1), consistent with our electrophysiological data.

Finally, there was no change in the number of TH-IR neurons in animals that experienced pain alone *vs.* sham-control (Figure 3A–H). By contrast, there was a tendency for the number of TH-IR neurons to increase in sham-CMS with respect to the sham-control group, an increase that was significant when compared to the CCI-control ($p<0.05$). Interestingly, this difference was even more pronounced in

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the CCI-CMS animals with respect to the sham-control and CCI-control ($p<0.05$, $p<0.01$ respectively; Figure 3G). Further analyses revealed significant changes in the frequency distribution of cell size. Around 90% of the cells in the control groups had a well-defined oval soma and a large, easily visible nucleus, and they were larger than 200 μm^2 (Figure 3H, C and D). However, we found fewer cells of this size range in the CMS groups, with an increase in the proportion of smaller cells (50–200 μm^2; Figure 3H). These smaller neurons had a more irregular and rounded morphology and in some cases their nucleus was barely visible (Figure 3E and F).

4. Discussion

We investigated the changes in noradrenergic transmission associated with co-morbid depression and pain using a combination of two well established animal models. Our findings suggest that pain exacerbates the changes in LC-noradrenergic transmission associated with depression.

Chronic pain was induced in rats by constriction of the sciatic nerve, which results in a rapid, long lasting and stable decrease in the pain threshold, while depressive-like behavior was induced by the CMS protocol, whereby a depressive-like state gradually develops over time in response to unpredictable stresses. By combining these approaches, we sought to mimic the clinical situation in which patients with chronic pain must cope with stressful events. In these conditions, our electrophysiological analyses revealed a general decrease in LC activity in CCI-CMS compared with their corresponding controls, and as witnessed by a decrease in firing rate and burst activity. Lower electrical activity has been correlated with reduced noradrenaline release in the LC and terminal fields in rats (Berridge and Abercrombie, 1999; Florin-Lechner et al., 1996). Although no marked electrophysiological changes were detected in CMS rats, we observed a clear trend

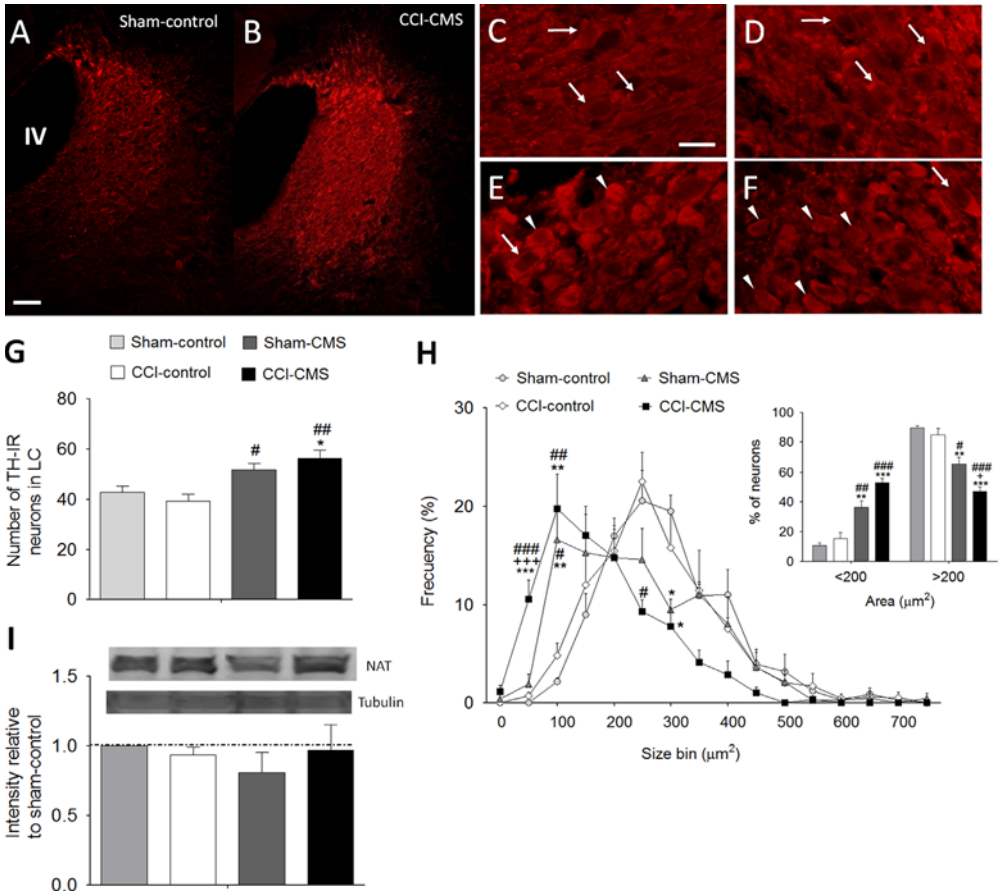

Figure 3 Effect of neuropathic pain and CMS on TH, soma size and NAT expression in LC neurons. (A,B) Representative photomicrographs showing TH expression (in red) in sections from (A) sham-control and (B) CCI-CMS ($n=4$-5 per group). Scale bar: 100 μm. (C–F) Representative photomicrographs showing large neurons (arrows) in (C) sham-control and (D) CCI-control. Note the high proportion of small neurons (arrowheads) in the (E) sham-CMS and (F) CCI-CMS. Scale bar: 20 μm. (G,H) Graphs depicting (G) the number of TH-IR neurons in the LC and (H) the frequency distribution of TH-IR soma size for each experimental group. Note the larger proportion of small neurons (<200 μm^2) in sham-CMS and CCI-CMS (inset). The data are expressed as the mean+S.E.M.: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs.* sham-control; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.* CCI-control; $^{+}p<0.05$, $^{+++}p<0.001$ *vs.* sham-CMS. (I) Quantitative analysis of NAT expression. The data represent the mean+S.E.M. of 5 assays performed on LC samples from 6 rats per group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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towards diminished spontaneous LC activity in stressed animals, an effect that became evident when neuropathic pain was combined with CMS. This decrease in electrical activity was accompanied by an increase in the number of TH-IR cells in CCI-CMS and sham-CMS animals with respect to their controls. This increase in TH expression would suggest higher concentration of noradrenaline because TH is the rate-limiting enzyme in its synthesis. However, it also might reflect a compensatory response to the decrease in noradrenaline induced by CMS (reduced electrical activity). For example, noradrenaline synthesis (downstream TH) or catabolism (elevated monoamine oxidase levels (Meyer et al., 2006)) might be altered. Although the effect was slightly higher in CCI-CMS animals, it was not significantly different to the increase in the sham-CMS group. Hence, neuropathic pain does not apparently modify TH expression in animals subjected to CMS, or alternatively, a ceiling threshold may exist. Surprisingly, TH-IR cells had a smaller soma in sham-CMS than in control animals, an effect that was further exaggerated in CCI-CMS. In animal models of neurodegenerative disorders, the shrinkage of TH-IR cells in the LC has often been described (German et al., 2005; Polak et al., 2011). Other microstructural changes in the LC and degeneration of noradrenergic axons have also been demonstrated in the frontal cortex in rats subjected to long-term stress (Kitayama et al., 1997; Kitayama et al., 2008). Together and waiting for additional morphological studies, these observations suggest that CCI exacerbates the neuronal damage induced by stress. Although the absolute number of TH-IR neurons increased in CMS animals, our electrophysiological data revealed a decrease in their activity, a finding that should be confirmed in further studies measuring noradrenaline levels in the LC and projection areas (frontal cortex and spinal cord) in basal and evoked conditions (after a painful or stressful event). Furthermore, we detected no changes in NAT or α2-adrenoceptor expression, indicating that the changes observed in spontaneous activity are probably caused by another neurotransmitter, possibly GABA (Bravo et al., 2013; Dimitrov et al., 2013).

Taken together, our immunohistochemical and electrophysiological findings suggest that CMS induces several modifications at the level of the LC that are exacerbated by chronic pain. The increased impairment of the LC seen in CCI-CMS may be explained by alterations in the emotional component of pain interpretation, as pain perception remained constant throughout the experimental period. In the mFST, similar immobility and climbing scores were recorded for sham-CMS and CCI-CMS animals, suggesting that CMS dysregulates the noradrenergic system and subsequently impairs the capacity to cope in stressful situations. However, although no differences were detected between groups, it is likely that depressive-like behavior developed earlier in the co-morbid situation. We previously demonstrated increased avoidance of painful paw stimulation in animals exhibiting depressive-like behavior (Bravo et al., 2012), suggesting that LC impairment might contribute to the augmented perception of pain in CCI-CMS *vs.* CCI-control.

In conclusion, our findings in an animal model of co-morbid pain and depression demonstrate that neuropathic pain exacerbates the changes induced by CMS at the level of the LC. CMS results in an increase in the number of TH-IR cells and a concomitant decrease in the size of these cells, effects that were exacerbated by neuropathic pain. Thus, functional impairment of these cells appears to contribute to a decrease in spontaneous LC activity, which in turn might impair the appropriate emotional response to harmful stimuli, such as pain and stress.

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Contributors

Dr. Lidia Bravo performed the behavioral, inmunohistochemistry and western blots experiments and contributed to the experimental design, analysis of the data, and writing of the first draft of the manuscript. Ms. Sonia Torres and Dr. Cristina Alba-Delgado performed the electrophysiological experiments and contributed to the analysis of the data, and writing the first draft of the manuscript. Dr. Juan A. Mico contributed to the discussion of the experimental results and writing the first draft of the manuscript. Dr. Esther Berrocoso contributed to the experimental design, analysis of the data, and writing of the manuscript. All authors have approved the final manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.euroneuro.2014.01.011.

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