Short communication

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In vivo stimulation of locus coeruleus: effects on amygdala subnuclei

Elisa Rodríguez-Ortega[1], Fernando Cañadas[1], Francisca Carvajal[2], and Diana Cardona[3]*

[1] Departamento de Psicología, Universidad de Almería, Spain. Ctra. Sacramento s/n, Almeria, Spain, [2] Centro de Investigaciones Biomédicas, Universidad Autónoma de Chile. Llano Subercaseaux, Santiago de Chile, Chile, [3] Departamento de Enfermería, Fisioterapia y Medicina, Universidad de Almería, Spain. Ctra. Sacramento s/n, Almeria (Spain),
** Email: dcardona@ual.es*

The locus coeruleus (LC) is the major noradrenergic nucleus and sends projections to almost all brain areas. A marked increase in norepinephrine release has been demonstrated in several brain areas in response to exposure to acute stressful stimuli, especially those innervated by LC projections. One of the brain areas innervated by LC neurons is the amygdala, a structure highly involved in emotional processes and memory formation. The aim of this study was to increase knowledge of the functional connectivity between the LC and the amygdala subnuclei. To reach this objective, we evaluated c-fos immunoreactive cells in amygdala nuclei following direct electrical stimulation of the LC in conscious animals. This analysis of *c-fos* immunoreactivity could inform whether there are differences in activity of the amygdala subnuclei related to LC electrical stimulation in conscious animals. Our results showed a marked increase in c-fos activity in these amygdala subnuclei both ipsilateral and contralateral to LC electrical stimulation *in vivo*. Therefore, our study provides evidence that *in vivo* electrical stimulation of LC is able to activate the amygdala subnuclei as measured by *c-fos* expression.

Key words: noradrenergic system, locus coeruleus, amygdala subnuclei, c-Fos

The noradrenergic nucleus locus coeruleus (LC) is a brainstem nucleus that provides the major source of norepinephrine (NE) to the forebrain (Counts and Mufson 2012, Sara 2009). The LC is involved in a broad number of physiological and psychological functions, including arousal, memory, cognition, pain processing, behavioral flexibility, and stress reactivity (McCall et al. 2015). Additionally, the role of LC in modulating emotional memories (Berridge 2005, Clewett et al. 2014, Counts and Mufson 2012, Tanaka et al. 2000), probably through coordinated action with the amygdala (Sterpenich et al. 2006), is well-known. The amygdala plays a key role in fear conditioning (LeDoux 2012), a paradigm widely use to understand the mechanism underlying associative learning and memory formation (Ehrlich et al. 2009). We can distinguish three groups of extensively interconnected subnuclei in the amygdala: the lateral nucleus (LA), basal (BA) and accessory basal nuclei (AB) and central nucleus (CeA) (LeDoux 2000). According to Pitkänen et al. (1997), the LA, specifically the lateral dorsal subnucleus (LAd), is the main point of entry for sensory inputs into the amygdala, given that it receives different polymodal inputs from the primary sensory cortex, hypothalamus and thalamus (Sah et al. 2003). Then, LAd projects to ventrolateral (LAvl) and ventromedial (LAvm) subnuclei, which connect with other amygdala areas (Pitkänen et al. 1997). On the other hand, CeA can be divided into its lateral (CeL), capsular (CeC) and medial (CeM) subdivisions (Pitkänen et al. 1997). The CeM is considered the principal output station from the amygdala, and it sends projections to different areas that mediate the expression of fear responses, such as the periaqueductal gray and brainstem (Sah et al. 2003). A subpopulation of CeL neurons also projects to brainstem targets important for fear conditioning (Veening et al. 1984). Moreover, CeL sustains a unidirectional connection with CeM and a reciprocal connection with CeC (Duvarci and Pare 2014).

In the past decade, several studies have shown the relationship between the noradrenergic system and the amygdala. Thus, it has been demonstrated that neuronal activity in the basolateral amygdala after footshock are mediated by LC input (Chen and Sara 2007). There are extensive projections from CeA to LC that include both excitatory and inhibitory connections (Retson and Van Bockstaele 2013, Reyes et al. 2011, Van Bockstaele et al. 1998, 1999), forming a complex circuit involved in stress, fear and anxiety (Fast and McGann 2017). In fact, it has been demonstrated that the LC may modulate the acquisition of fear conditioning (Sears et al. 2013), a paradigm involving the amygdala. Additionally, pharmacological studies have revealed that α-1 and β adrenergic receptors in the LA are related with fear learning and memory consolidation, respectively (Bush et al. 2010, Lazzaro et al. 2010, Schiff et al. 2017). Using optogenetic tech-

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Correspondence should be addressed to: Diana Cardona
Email: dcardona@ual.es

niques, Johansen and colleagues (Johansen et al. 2014) have provided strong evidence that LA neural plasticity underlying fear learning recruits noradrenergic neuromodulatory processes in addition to Hebbian mechanisms in LA pyramidal neurons. Thus, administration of β adrenergic agonists and antagonists directly into the LA facilitated and blocked associative learning, respectively (Johansen et al. 2014, Sears et al. 2013). However, it is important to determine through which LA subnuclei such a neuromodulatory effect is exerted.Although it has been reported that the LC densely innervates the amygdala, there are aspects about these connections that remain unclear, especially regarding the LC-CeA involvement in fear-conditioning processes. The aim of this study was to increase knowledge surrounding the functional connectivity between the LC and the amygdala subnuclei. To the best of our knowledge, this study is the first to assess the effects of LC electrical stimulation *in vivo* on the pattern of *c-fos* activity in several amygdala subnuclei. The analysis of *c-fos* immunoreactivity could inform us about whether there are differences in the activity of the amygdala subnuclei related to the LC electrical stimulation in conscious animals. The immediate early gene protein *c-fos* has been widely used as a marker of neuronal activity during the formation of conditioned fear (Chen and Sara 2007, Lanuza et al. 2008).

Sixteen adult male Sprague-Dawley rats (weighing 250–274 g at the beginning of the procedures) were obtained from Janvier Laboratories (France). The animals were housed individually in plastic cages and maintained at a constant temperature of 22±2°C, a relative humidity of 50±10%, and a 12-hour light/dark automatic cycle (lights off from 08:00 to 20:00 h). All animals had *ad libitum* access to food (Panlab rodent chow, Barcelona, Spain) and water. All behavioral protocols and surgery techniques were approved by the University of Almeria Bioethics Committee and were in accordance and compliance with the guidelines for the use and care of experimental animals (Spanish Royal Decree 53/2013 and European Communities Council Directive (2010/63/UE).

Rats were anesthetized with equithesin (2.4 ml/kg) and atropine (0.05 mg/kg) to prevent respiratory distress. A bipolar stainless electrode (Plastic One) insulated with polyamide (tip diameter, 0.125 mm; impedance, 15–30 kΩ) except for the tip was used to deliver the electrical stimulation in the LC. The tip of the electrode (0.3 mm) was targeted according to the coordinates obtained from the Atlas by Paxinos and Watson (Paxinos and Watson 1998) (AP: -9.8; ML: +1.2; DV: -7.2 from bregma). The head angle was set to 0°. As the implantation was unilateral, operations were performed counterbalancing between left and right hemispheres so that in half of the rats, the coordinates in ML were -1.2.

After a minimum of 7 days of recovery, LC electrical stimulation was administered in a stimulation session in chambers constructed of aluminum and Plexiglas walls (MED-Associates, Inc., St. Albans, VT). After one and a half hours of acclimation, three pulses of 1 s total duration, 600 μA intensity, were delivered at a frequency of 0.1 Hz (Chen and Sara 2007). The sham group did not receive electrical stimulation, although electrodes were implanted identical to the LC-stimulation group. One and a half hours after electrical stimulation, the animals were anesthetized using sodium pentobarbital (40 mg/kg) and received an electrical lesion in the LC in order to evaluate the correct electrode placement (a 600 μA constant current was delivered through the electrode tip for 10s at a frequency of 0.1 Hz). This delay of one and a half hours following stimulation corresponds to the maximal *c-fos* expression (Martinez et al. 2013).

After LC lesion, rats were transcardially perfused with isotonic phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and postfixed for 24 h and then processed for c-Fos-like immunoreactivity. Sections of 50 μm thickness were cut on a vibratome in the coronal plane. In accordance with Carvajal et al. (2007), the adjacent series around the amygdala were incubated for 20 minutes in 0.3% H_2O_2 in absolute methanol, rinsed (3X, PBS) and incubated for one hour in 3% normal goat serum in PBS. Slices were then transferred without rinsing to the primary antibody solution, which consisted of 1:15000 *c-fos* polyclonal rabbit IgG (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA). After 48h incubation at 4°C, slices were rinsed (10X, PBS, 2 h) and processed using the ABC method (Vector laboratories). Slice were incubated for one hour in a solution of biotinylated goat anti-rabbit IgG and rinsed again for one hour (10X, PBS). Slices were then transferred to avidin-biotin peroxidase for one hour, rinsed (3x in PBS, 30 min, then 3x

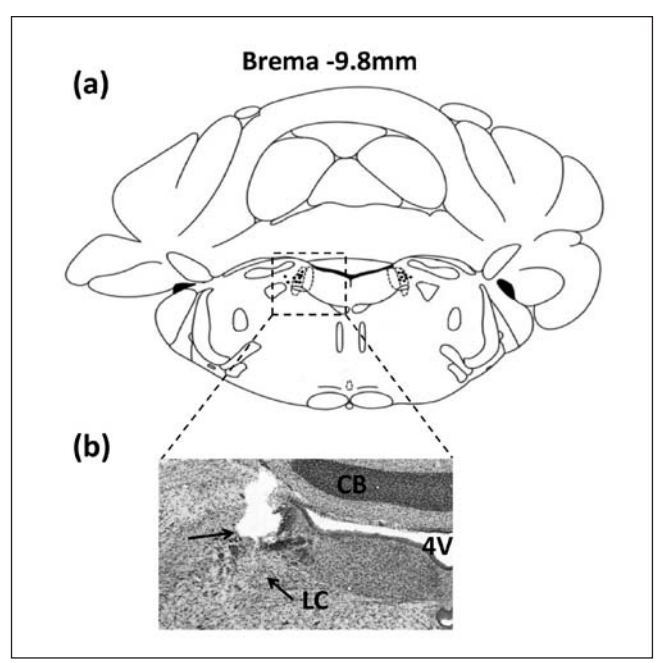

Fig. 1. Schematic representation showing electric stimulation sites (black dots) in the LC (a) and photomicrograph of a coronal brain section showing the tip of the electrode (black arrow head) at LC (b). Adapted from Paxinos & Watson stereotaxic atlas for rat brain.

in phosphate buffer, 30 min) and developed with diaminobenzidine tetrahydrochloride (3–4 minutes, DAB, Sigma). Slices were rinsed (10X, PBS), mounted on gelatin-coated slides and coverslipped with DPX.

Previous pilot studies revealed that c-Fos immunoreactivity was present in amygdala after the protocol of LC-stimulation was used. Stained sections were examined through a microscope (Nikon ECLIPSE E800) with 4× magnification; c-fos positive cells were scored through an RS Photometrics CoolSNAP model digital camera in selected brain regions (LA: LAd, LAvl, LAvm and CeA: CeL, CeC and CeM) by an observer blind to the experimental conditions. Based on Paxinos and Watson Rat Atlas, different antero-posterior levels of amygdala (eight sections from -1.8 mm to -3.6 mm) were chosen for the analysis (Paxinos and Watson 1998).

Border delineation, cell counting and amygdala subnuclei measurements were completed with ImageJ software (version 1.48a) as outlined above (Martinez et al. 2013). To verify the localization of the lesion, a subset of slices was stained using the Nissel protocol (Fig. 1). Only data from animals with an electrode tip in the LC were included in the statistical analysis. Data from three animals were discarded from the analysis.

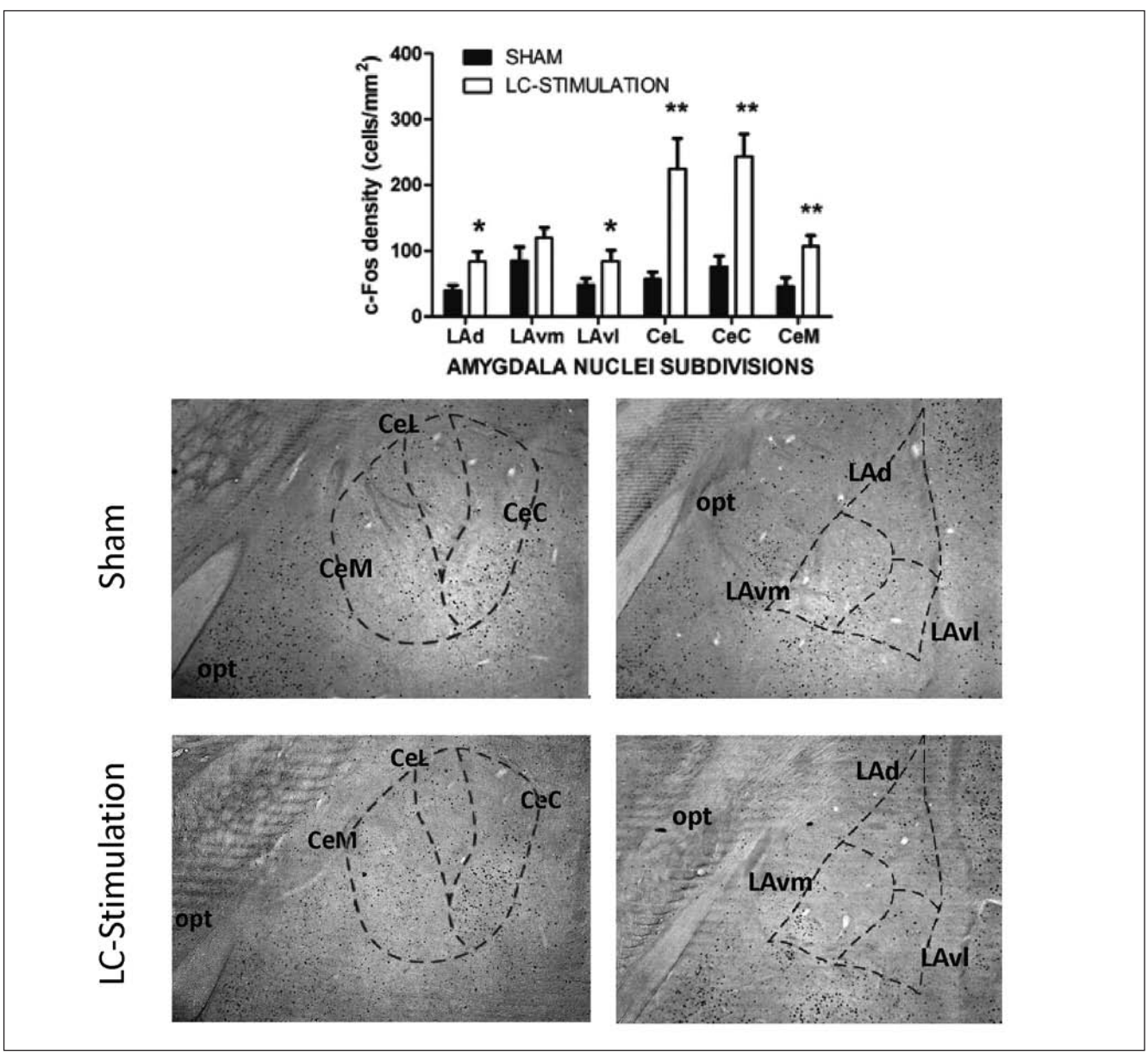

Fig. 2. (Top) Total *c-fos* average density in amygdala subnuclei after ipsilateral LC electrical stimulation. Data represent mean±SEM (* indicates significant statistical differences, p≤0.05; ** indicates significant statistical differences, p≤0.01). (Bottom) Representative microphotographs depicting *c-fos* expression in lateral amygdala subnuclei (left) or central amygdala subnuclei (right) both in sham and LC-stimulation subjects and ipsilateral to stimulation (LA and CeA images correspond to approximately -3.3 mm and -2.3 AP from bregma respectively).

C-fos density (*c-fos* positive cells by the area (1 mm^2) of the region of interest) was analyzed and compared with Mann-Whitney U analysis to determine whether significant differences ($p \leq 0.05$) existed between the sham and LC-stimulation groups.

Total average density per c-fos amygdala subnuclei was analyzed with Mann-Whitney U Test. Significant statistical differences in *c-fos* expression ipsilateral to LC electrical stimulation (Fig. 2) were found in LAd ($Z=2.14$; $p \leq 0.05$), LAvl ($Z=2$; $p \leq 0.05$), CeL ($Z=2.42$; $p \leq 0.01$), CeC ($Z=2.42$; $p \leq 0.01$) and CeM ($Z=2.42$; $p \leq 0.01$). Contralateral to LC electrical stimulation (Fig. 3), we found significant statistical differences in LAvl ($Z=2.42$; $p \leq 0.01$), CeL ($Z=2.85$; $p \leq 0.01$) and CeC ($Z=2.71$; $p \leq 0.01$). No other effects, nor their interactions, reached statistical significance ($p > 0.05$).

LA and *CeA subnuclei* per level were analyzed with *Mann-Whitney U Test (*Table I). *LAd subnucleus* showed significant statistical differences in *c-fos* expression in ipsilateral -3.60 ($p \leq 0.05$) and contralateral side -2.80 mm and -3.30 mm from bregma ($p \leq 0.05$). *LAvm subnucleus* showed significant differences ipsilaterally at -3.30 mm

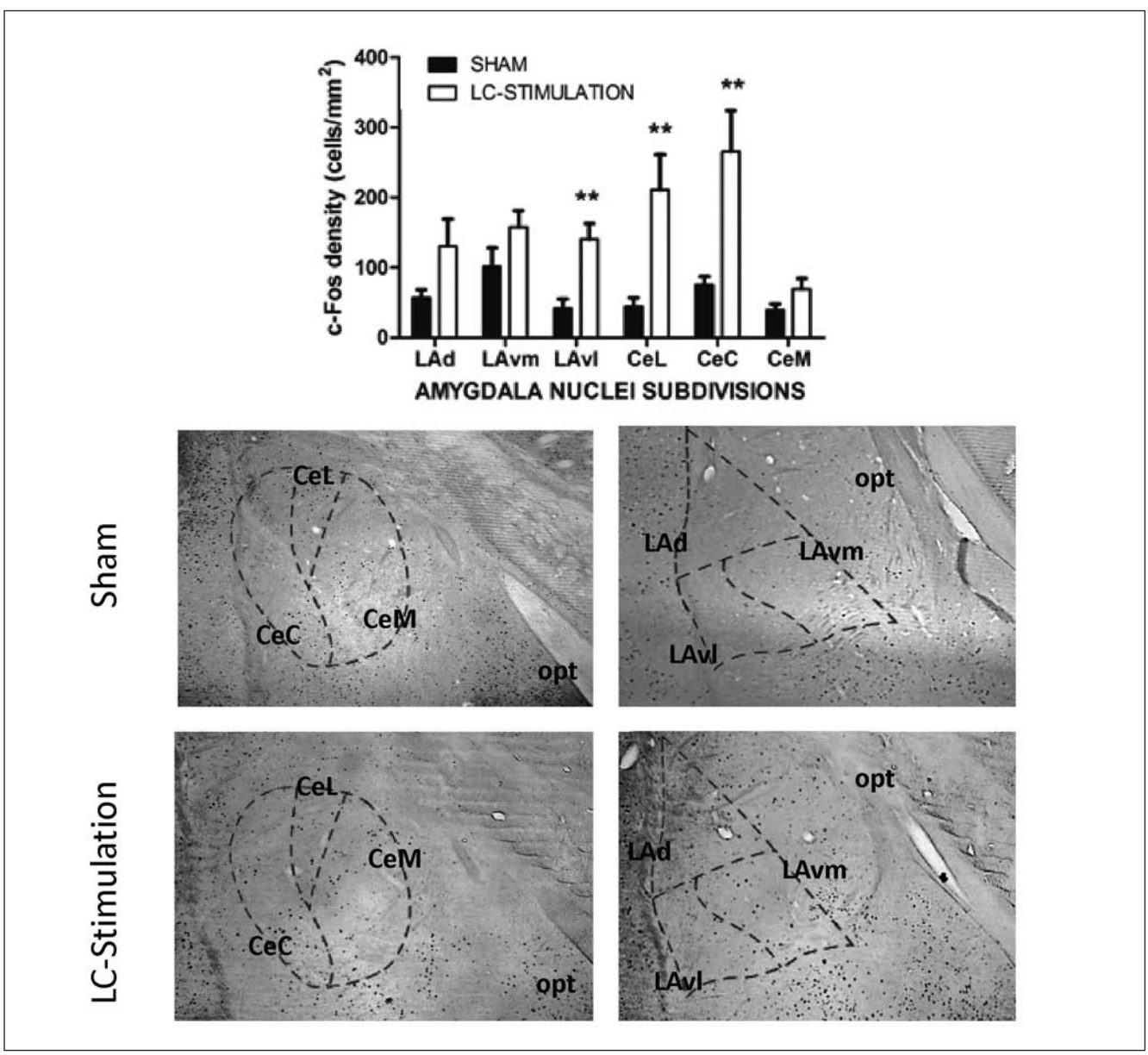

Fig. 3. (Top) Total *c-fos* average density in amygdala subnuclei after contralateral LC electrical stimulation. Data represent mean±SEM (* indicates significant statistical differences, $p \leq 0.05$; ** indicates significant statistical differences, $p \leq 0.01$). (Bottom) Representative microphotographs depicting *c-fos* expression in lateral amygdala subnuclei (left) or central amygdala subnuclei (right) both in sham and LC-stimulation subjects and contralateral to stimulation (LA and CeA images correspond to approximately -3.3 mm and -2.3 AP from bregma respectively).

from bregma (p≤0.05). In *LAvl subnucleus* we found significant statistical differences in -3.60 mm ipsilateral (p≤0.05) and in -2.56 mm contralateral side (p≤0.05). In the analysis carried out on the CeA subnuclei, we demonstrated that *in vivo* LC stimulation increased c-fos expression in *CeL subnuclei* [ipsilateral: Bregma -2.30 mm (p≤0.05), -2.56 mm (p≤0.01) and -2.80 mm (p≤0.01); contralateral: Bregma at -2.30 mm (p≤0.05), -2.56 mm (p≤0.05), -2.80 mm (p≤0.01) and -3.14 mm (p≤0.01)]; *CeC subnucleus* [ipsilateral: Bregma -1.80 mm (p≤0.05), -2.12 mm (p≤0.05), -2.30 mm (p≤0.01), -2.56 mm (p≤0.05) and -3.14 mm (p≤0.01); contralateral: Bregma -1.80 mm (p≤0.05), -2.30 mm (p≤0.01), -2.56 mm (p≤0.05), -3.14 mm (p≤0.05) and -3.30 mm (p≤0.01)] and *CeM subnucleus* [ipsilateral: Bregma -2.30 mm and -2.80 mm (p≤0.05); contralateral: Bregma -2.12 mm and -2.56 mm (p≤0.05)]. No other effects, nor their interactions, reached statistical significance (p>0.05).

The present research provides for the first time, to our knowledge, evidence surrounding the effects of *in vivo* electrical stimulation of the LC on amygdala subnuclei activity. Specifically, our results show that *in vivo* electrical stimulation of the noradrenergic system through its major nucleus is able to induce *c-fos* expression. We found that these differences occur both ipsilateral, in LAd, LAvl, CeL, CeC and CeM, and contralateral, LAvl, CeL, and CeM, to the stimulation side when the total average density was analyzed. Ad-

Table I. (*Top*) *C-fos* average density in LA and CeA subnuclei at different AP coordinates from bregma ipsilateral to LC electrical stimulation. (*Bottom*) *C-fos* average density in LA and CeA subnuclei at different AP coordinates from bregma contralateral to LC electrical stimulation. Data represent Mean ± S.E.M. (* represent significant statistical differences, p≤0.05)

IPSILATERAL

	LA SUBNUCLEI						CeA SUBNUCLEI					
	LAd		LAvm		LAvl		CeL		CeC		CeM	
	Sham	*LC-Stimul.*	*Sham*	*LC-Stimul.*	*Sham*	*LC-Stimul.*	*Sham*	*LC-Stimul.*	*Sham*	*LC-Stimul.*	*Sham*	*LC-Stimul.*
-1.80	16,93±16,93	36,81±23,34					28,07±12,58	99,83±34,03	63,04±21,20	161,42±39,41*	54,04±18,64	74,35±10,94
-2.12	22,34±9,92	63,32±15,95					27,31±12,20	191,53±61,03	84,70±23,41	293,97±88,52*	26,42±6,61	91,28±30,27
-2.30	59,08±15,07	104,13±19,54					59,41±29,34	187,32±57,22*	70,62±33,50	250,48±47,21**	32,89±8,09	78,69±15,49*
-2.56	30,57±12,64	118,51±54,58	93,63±39,73	109,14±42,37	44,85±44,85	73,40±48,25	82,55±23,08	279,66±56,08**	56,46±22,93	255,01±58,45*	55,15±28,25	116,48±38,56
-2.80	47,99±12,62	130,62±43,67	81,27±29,89	151,65±28,64	81,74±40,14	122,43±70,45	81,54±19,57	387,16±103,91**	79,35±32,08	153,60±41,92	63,23±37,97	157,83±36,19*
-3.14	64,55±21,56	69,65±26,19	155,19±37,41	160,22±30,46	63,18±27,51	111,91±31,47	79,27±32,35	246,46±88,73	76,35±34,06	351,91±73,39**		
-3.30	79,73±28,59	67,88±25,22	58,37±12,91	119,97±16,19*	28,35±9,70	71,67±32,16	43,85±19,98	259,44±84,88	103,10±68,59	249,03±56,09		
-3.60	44,43±16,43	94,59±13,16*	41,52±7,14	76,13±30,45	18,85±10,00	61,83±11,67*						

CONTRALATERAL

	LA SUBNUCLEI						CeA SUBNUCLEI					
	LAd		LAvm		LAvl		CeL		CeC		CeM	
	Sham	*LC-Stimul.*	*Sham*	*LC-Stimul.*	*Sham*	*LC-Stimul.*	*Sham*	*LC-Stimul.*	*Sham*	*LC-Stimul.*	*Sham*	*LC-Stimul.*
-1.80	49,52±20,30	110,45±39,04					65,81±35,16	128,89±36,39	65,09±10,30	162,15±39,11*	66,77±30,79	85,35±19,46
-2.12	60,77±19,43	103,70±23,80					23,23±8,47	113,81±60,21	124,73±27,01	216,50±31,92	16,38±5,98	54,98±20,94*
-2.30	75,27±21,09	161,14±64,98					10,28±6,29	100,24±29,12*	42,29±22,21	230,62±62,96**	51,82±13,76	37,21±8,88
-2.56	70,08±32,09	147,88±59,84	107,99±35,10	200,89±40,29	61,18±39,01	292,84±72,28*	84,34±35,90	239,82±54,40*	107,00±14,96	280,24±59,61*	18,98±7,87	68,44±17,60*
-2.80	56,83±21,64	210,51±41,34*	89,80±27,71	205,63±42,12	13,13±13,13	91,28±52,20	84,76±17,91	527,46±197,62**	90,91±43,79	139,95±33,92	35,15±14,31	115,94±37,40
-3.14	71,34±20,97	107,54±38,84	178,50±61,87	136,23±41,41	42,19±26,05	80,38±33,66	29,83±7,49	237,86±65,40*	49,74±25,17	392,75±184,04**		
-3.30	28,83±10,55	139,60±38,99*	85,07±35,16	100,05±17,98	43,85±11,88	86,39±23,66	15,10±15,10	217,78±82,03	43,46±28,97	505,86±185,19*		
-3.60	41,66±12,77	134,81±69,76	43,84±24,58	184,53±86,74	48,19±9,30	100,55±41,88						

ditionally, in our analysis of *c-fos* expression by levels, in most subnuclei, we found bilateral results. That is, we found an increase of c-fos expression in LAd (-3.60 mm), LAvl (-3.60 mm), and LAvm (-3.30 mm) following ipsilateral LC electrical stimulation. Our data are in agreement with a recent study by Sears et al. showing that unilateral connection between the LC and ipsilateral LA would be enough to enhance memory formation (Sears et al. 2013). However, we also found unexpected contralateral effects on LAd (-2.80 and -3.30 mm) and LAvl (-2.56 mm). Although most of the projections of the LC are ipsilateral (Sara 2009), it has been demonstrated through tracing studies that LC projections to somatosensory areas (that are directly connected with amygdala subnuclei) could be bilateral (Simpson et al. 1997). In fact, an *in vivo* tracing study using manganese-enhanced MRI demonstrated that LC projections reach amygdala bilaterally (Eschenko et al. 2012). Thus, these findings demonstrate that even though the projections from LC to LA and CeA subnuclei are mainly ipsilateral, the contralateral structures also receive afferences from LC. In accordance with these findings, our research also provides evidence of bilateral LC-noradrenergic activity in the amygdala subnuclei. However, this does not rule out that observed *c-fos* expression in the amygdala could be the results of secondary effects of LC stimulation, considering that LC projections are ubiquitously present throughout the CNS (Aston-Jones and Waterhouse 2016; Samuels and Szabadi 2008). *C-fos* does not have the temporal specificity required to differentiate direct versus indirect activation of the amygdala after LC stimulation; therefore, additional studies are needed.

The existence of a LA-CeL-CeM circuit that could be critical for the acquisition of fear memories has been suggested (Lee et al. 2013). Thus, LA is a critical structure in associative acquisition of threat learning (for a review, see Sears et al. 2013). Electrophysiological data have shown that LAd neurons respond to polymodal sensory information (Romanski et al. 1993). The activation that we found in LAd following LC stimulation might indicate that it is in this subnuclei where the noradrenergic system modulates the acquisition of threat conditioning. Previous pharmacological studies have shown that LA noradrenergic receptors are able to modulate associative learning both in conditioned fear learning (Dębiec et al. 2011, Johansen et al. 2014, Sears et al. 2013) and in avoidance tasks (Ferry et al. 1999). LAv neurons (specially the LAvl) respond to somatosensory (or nociceptive) information (Romanski et al. 1993). We have found that LC stimulation induces ipsilateral activation in LAvm (-3.30 mm) and LAvl (-3.60 mm) and contralateral activation in LAvl (-2.56). Thus, our data suggest that activation of the noradrenergic system would influence the LAvl processing of sensorial (nociceptive) information during the acquisition of fear conditioning. In addition to an increase in the activity of LA subnuclei, our results have shown an increase in activity in CeL, CeC and CeM ipsilateral and contralateral to the stimulation side. This demonstrates a bilateral noradrenergic modulation of this LA–CeA subnuclei axis with robust bilateral LC effects through the CeA subnuclei. Therefore, it is tempting to speculate that the noradrenergic system through the bilateral projections of LC to CeA could modulate the expression of emotional memories (LeDoux 2000). This bilateral activation could not be explained by inter-hemisphere CeA connections, given that the CeA subnuclei does not project to the contralateral amygdala (Jolkkonen and Pitkänen 1998). As noted above, it has been demonstrated that the projections from LC to LA and CeA subnuclei are mainly ipsilaterally but also contralaterally, which would explain these bilateral effects (Eschenko et al. 2012).

In summary, data from our study provide evidence that *in vivo* electrical stimulation of the LC is able to active the amygdala subnuclei as measured by *c-fos* expression. Our results show that the noradrenergic system could be a potent modulator of the activity of the amygdala subnuclei. It is important to note that the electrical stimulation method may stimulate not only LC neurons but also the fibers of passage, which may not be noradrenergic. However, there is strong evidence for an increase of norepinephrine release in brain areas, including the amygdala, innervated by the LC in response to stressful stimuli (Chen and Sara 2007, Johnson et al. 2011, Passerin et al. 2000). Taken together, these data suggest that LC, through the release of norepinephrine, could modulate the acquisition and expression of conditioned fear, which seems to be affected in many emotional disorders (Dębiec et al. 2011, Stoppel et al. 2006). However, to verify this hypothesis and rule out the influence of other neurotransmission systems, additional pharmacological or genetic studies directed to block the noradrenergic system in the amygdala following LC stimulation need to be performed. To determine the subtype of noradrenergic receptors implicated, selective inhibition of α1 or β-adrenergic receptors will we carried out. Specifically, the LC stimulation and presumably subsequent release of NE should be able to modulate the acquisition of fear memories through ipsilateral projection to LAd and bilateral projection to LAv as well as the expression of fear responses through the bilateral projections of the LC to the CeA subnuclei.

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