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NEUROSYSTEMS

Altered locus coeruleus–norepinephrine function following single prolonged stress

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Abstract

Data from preclinical and clinical studies have implicated the norepinephrine system in the development and maintenance of post-traumatic stress disorder. The primary source of norepinephrine in the forebrain is the locus coeruleus (LC); however, LC activity cannot be directly measured in humans, and previous research has often relied upon peripheral measures of norepinephrine to infer changes in central LC–norepinephrine function. To directly assess LC–norepinephrine function, we measured single-unit activity of LC neurons in a validated rat model of post-traumatic stress disorder – single prolonged stress (SPS). We also examined tyrosine hydroxylase mRNA levels in the LC of SPS and control rats as an index of norepinephrine utilisation. For electrophysiological recordings, 92 LC neurons were identified from 19 rats (SPS, 12; control, 7), and spontaneous and evoked responses to a noxious event (paw compression) were recorded. Baseline and restraint stress-evoked tyrosine hydroxylase mRNA expression levels were measured in SPS and control rats (n = 16 per group) in a separate experiment. SPS rats showed lower spontaneous activity but higher evoked responses, leading to an enhanced signal-to-noise ratio of LC neurons, accompanied by impaired recovery from post-stimulus inhibition. In concert, tyrosine hydroxylase mRNA expression in the LC of SPS rats tended to be lower at baseline, but was exaggerated following restraint stress. These data demonstrate persistent changes in LC function following stress/trauma in a rat model of post-traumatic stress, as measured by differences in both the electrophysiological properties of LC neurons and tyrosine hydroxylase mRNA transcription.

Introduction

Post-traumatic stress disorder (PTSD) is a severe psychiatric disorder that can occur following trauma (American Psychiatric Association, 1994). Although the neurobiology of PTSD is not fully understood, preclinical and clinical studies have implicated altered norepinephrine (NE) activity in the disorder. NE is important for mediating arousal (Foote *et al.*, 1980) and memory (McGaugh & Roozendaal, 2002), and altered NE activity may contribute to hyperarousal and re-experiencing symptoms associated with PTSD (Southwick *et al.*, 1997).

Although studies examining baseline NE levels in PTSD patients have yielded inconsistent results, challenge studies have suggested exaggerated responsivity to trauma-related stimuli (Southwick *et al.*, 1993; Murburg *et al.*, 1995; Liberzon *et al.*, 1999a). Similar findings in animal models of PTSD further support NE system involvement (Adamec *et al.*, 2007; Olson *et al.*, 2011). Thus, the empirical data suggest exaggerated stress-related NE release in PTSD, but inferences regarding central NE are limited, as these studies rely on peripheral measures of NE, and are dependent upon the assumption that peripheral measures reflect central activity.

The primary source of NE in the forebrain is the locus coeruleus (LC). LC neurons coordinate multiple components of the stress response (Foote *et al.*, 1983). The LC is the only source of NE for the hippocampus and neocortex, implicating it in fear and memory responses to trauma (Berridge & Waterhouse, 2003). LC neurons are activated by a number of acute stressors (Abercrombie & Jacobs, 1987; Simson & Weiss, 1988; Chiang & Aston-Jones, 1993; Curtis *et al.*, 2012), and chronic stress also increases LC neuron firing 24 h after stress terminates (Pavcovich *et al.*, 1990; Jedema & Grace, 2003).

As LC activity cannot be readily measured in humans, evidence for altered LC function in PTSD patients is extremely limited. A single postmortem study of three PTSD veterans found a 30% reduction in LC neurons as compared with trauma-exposed controls (Bracha *et al.*, 2005). However, neuronal counts provide little information regarding functionality, and it is impossible to determine from these data whether LC integrity was altered by trauma, or by other pre-deployment factors. The development of animal models affords the opportunity to directly examine the nature of LC activity changes following trauma.

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We measured single-unit activity of LC neurons following single prolonged stress (SPS). SPS produces multiple PTSD-like physiological changes, including enhanced negative feedback of the hypothalamic–pituitary–adrenal (HPA) axis (Liberzon *et al.*, 1997, 1999b) [for a detailed review, see Yamamoto *et al.* (2009)], the neuroendocrinological hallmark of PTSD (Yehuda *et al.*, 1993). Behaviorally, SPS animals show enhanced startle (Khan & Liberzon, 2004; Kohda *et al.*, 2007), indicating hyperarousal, and disrupted retention of extinction memories (Yamamoto *et al.*, 2008; Knox *et al.*, 2012a), a deficit shown by PTSD patients (Milad *et al.*, 2007). Thus, SPS results in physiological and behavioral changes reminiscent of PTSD symptoms linked to the LC–NE system.

Locus coeruleus discharge characteristics were quantified at baseline and following noxious stimulation. In addition, we measured baseline and stress-enhanced tyrosine hydroxylase (TH) mRNA in the LC, as increased NE utilisation is typically accompanied by TH mRNA upregulation (Smith *et al.*, 1991). We combined these methodologies to characterise changes in the LC–NE system following SPS.

Materials and methods

Animals

Fifty-one male Sprague Dawley rats (Charles River, Wilmington, MA, USA), aged 42–45 days and weighing 150–200 g upon arrival, were used in this study. Rats were housed at the Veterinary Medical Unit of the Ann Arbor Veterans Affairs Medical Center, and had 3 days to acclimate prior to the start of any procedure. Rats had *ad libitum* access to water and laboratory chow, and were handled at least three times prior to the start of experimental procedures. Experimental procedures were approved by the Veteran Affairs Institutional Animal Care Usage Committee, and were in accordance with National Institutes of Health guidelines for the treatment of animals.

Single prolonged stress

Single prolonged stress refers to the application of three stressors (restraint, forced swim, and ether exposure) followed by a quiescent period of 7 days, which is required for the development of the SPS-related changes (Liberzon *et al.*, 1999b; Knox *et al.*, 2012a). Rats were restrained for 2 h, and this was followed immediately by 20 min of forced swimming in water (20–24 °C) in a container (68.13 L, 55.6 cm in diameter, 45.4 cm in height). Following 15 min of recuperation, during which rats were towel-dried and placed under a heat lamp, rats were exposed to ether in a desiccator until they were anesthetised. Rats were then left undisturbed in their home cage for a minimum of 7 days. The full SPS procedure refers to the application of the three stressors (restraint, forced swim, and ether exposure) plus a 7-day quiescent period. Control rats remained in their home cages.

Electrophysiological recording

Single-unit electrophysiological activity of LC neurons was recorded 0–9 days following the full 7-day SPS procedure (i.e. 7–16 days after the application of the three stressors) (n = 12) or control procedure (n = 7). In order to ensure that there was no effect related to the number of days between completion of SPS and testing of LC firing (created by the logistical constraints of SPS performed in batches and the time-intensive task of probing LC firing individually), we split our data into short-delay and long-delay groups – those with LC recordings conducted within 3 days, and those with LC recordings conducted 6–9 days after the completion of SPS. The groups did not differ significantly on any of our dependent measures, so all of the rats were analysed as a single group in further analyses. The methods used for recording LC discharge are similar to those previously reported (Curtis *et al.*, 1995). Micropipette glass electrodes were pulled from 2-mm capillary tubing, and filled with 2% pontamine sky blue in 0.5 M sodium acetate solution. Electrode tips were broken to 3–5 μm, and only electrodes with an impedance of 2–10 MΩ measured at 1000 Hz (glass electrode R/C meter, model 2700; AM Systems, Carlsborg, WA, USA) were used. Rats weighing 240–360 g were anesthetised with 5% isoflurane in air, mounted in a stereotaxic frame, and maintained at a surgical plane with 1–2% isoflurane. The level of anesthesia was monitored throughout the study to ensure that rats remained at this plane. Rats were positioned with the head oriented at 15° to the horizontal plane (nose down), the skull was opened, the dura was retracted, and an electrode was placed 1.1 mm lateral to the midline and 3.6 mm caudal to lambda. The electrode was advanced with a hydraulic probe drum drive (FHC, Bowdoin, ME, USA). Electrical signals were passed through an amplifier (Model 1800; AM Systems) and a noise eliminator (Hum Bug; Quest Scientific, North Vancouver, Canada), and fed into an oscilloscope, audio monitor and Cambridge Electronics Design 1401 data acquisition system with SPIKE 2 software (Cambridge Electronics Design, Cambridge, UK) for online visualisation and storage for offline analysis. LC neurons were tentatively identified according to the following criteria: (i) 2–3-ms-duration action potential with positive–negative waveform; (ii) notched waveform on the ascending phase of the action potential; (iii) spontaneous firing rates of 0.1–5.0 Hz; and (iv) biphasic response to tail pinch.

When a single unit of stable amplitude was isolated, an amplitude trigger converted each action potential into a digital pulse. Spontaneous firing was recorded for 180 s, and then LC evoked activity was measured following paw compression. Compressions were produced by placing the contralateral hind limb between the tips of a pair of 13-cm surgical forceps. The surgical forceps were equipped with a physical stopper to ensure that compression occurred to a fixed width between the forcep tips during all instances of paw compression. For each paw compression, pressure was applied to this fixed point for 1 s. This type of paw compression elicits a burst of spikes, and shows neither sensitisation nor habituation with repeated trials (West *et al.*, 2010). To determine the magnitude of the evoked response for a single unit, 10 paw compressions were applied, spaced at least 10 s apart. Each paw compression was event-stamped by use of the keyboard marker function in the data acquisition system, and SPIKE 2 software was used offline to generate peristimulus time histograms (PSTHs) time-locked to this event. Once a single-unit recording was complete, the micromanipulator was progressed until another unit was identified, and the procedure was repeated. One to 10 neurons were recorded from a single rat. At the end of the experiment, the recording site was marked by iontophoresis of the pontamine sky blue solution. Brains were harvested, frozen in isopentane on dry ice, and stored in a −80 °C freezer. They were later thawed in a cryostat (Leica, Bannockburn, IL, USA) at −20 °C, and 30-μm sections through the LC were taken and Nissl-stained. The data presented are only from those recordings for which the electrode was histologically verified as being within the LC. Data analysis was performed by an experimenter who was blind to condition.

TH mRNA expression

For determination of baseline TH mRNA levels, SPS (n = 8) and control (n = 8) rats were taken from the housing colony, and killed

by decapitation following the 7-day quiescent period of SPS. Stress-evoked TH mRNA expression was examined in SPS ($n = 8$) and control ($n = 8$) rats exposed to 90 min of restraint. Following the 7-day period of SPS, rats were restrained by placing them into individual cylindrical plastic containers, which were narrowed and fastened to ensure extremely limited movement. They were immediately killed after release from restraint. Sections (10 μm) through the LC were cut and thaw-mounted onto superfrost slides. To verify that the LC was present, adjacent sections were Nissl-stained.

Prior to *in situ* hybridisation, slides were fixed in a 4% paraformaldehyde solution for 2 h and rinsed in standard saline citrate (SSC) buffer. Slides were acetylated in 0.1 M triethanolamine containing 0.25% acetic anhydride for 10 min, and dehydrated in a progressive series of alcohols. ^{35}S-labeled riboprobes were generated from TH cDNA inserted into the bssk plasmid (Kabbaj *et al.*, 2000; Flagel *et al.*, 2007). TH cDNA was provided by S. Watson (MBNI, Ann Arbor, MI, USA). Plasmids were cut with *Bam*H1, and ^{35}S-labeled riboprobes were generated in a reaction mixture consisting of ~1 μg of linearised plasmid, 4 μL of transcription buffer (Promega, Madison, WI, USA), 4 μL of [^{35}S]UTP (800 mCi/mL) (PerkinElmer, Waltham, MA, USA), 4 mM NTPs (CTP, ATP, and GTP), 10 mM dithiothreitol, 20 U of RNase inhibitor, and 28 U of RNA polymerase (T7). The reaction proceeded for 1 h at 37 °C. Twenty units of RNase-free DNAse was added to the reaction mixture for 15 min at room temperature. Riboprobes were separated from free nucleotides and proteins on a Sephadex G50–50 column (Biorad, Hercules, CA, USA). One microliter of the ^{35}S-labeled riboprobe was counted in a scintillation counter. Only probes that were labeled with $> 1 \times 10^6$ d.p.m. of radioactivity/μL were used.

Riboprobes were diluted in hybridisation buffer to yield approximately 1×10^6 d.p.m./100 μL of 50% formamide hybridisation buffer (Amnesco, Solon, OH, USA). Hybridisation buffer (100 μL) was applied, and sections were coverslipped. Slides were placed in sealed plastic boxes lined with filter paper moistened with 50% formamide, and incubated overnight at 55 °C. Coverslips were removed, and slides were rinsed several times in 2 × SSC and incubated in RNase A solution (Sigma-Aldrich, St Louis, MO, USA) (60 μg/mL) at 37 °C for 30 min. Slides were washed in decreasing concentrations of SSC, incubated in 0.1 × SSC for 60 min at 65 °C, dehydrated in a series of alcohols, and exposed to Kodak MR X-ray film for 24 h along with a C-14 standard (American Radiolabeled Chemical, St Louis, MO, USA).

Data and statistical analysis

Electrophysiological data

Baseline activity was recorded for 180 s, and the mean spontaneous firing rate for each cell was calculated. LC activity during stimulation (paw compression) trials was recorded as PSTHs. Discharge activity was analysed from 2 s before to 4 s after stimulation, and the cumulative number of spikes was plotted in 20-ms bins (300 bins in total). The 2 s before each paw compression represented tonic discharge. Evoked discharge was defined as that which occurred after stimulation and exceeded the mean tonic rate by two standard deviations, and was defined as ending after five consecutive bins fell below the mean tonic discharge rate. The duration of evoked response, peak firing rate and latency to peak were also measured. Signal-to-noise ratio was calculated as the ratio of evoked-to-tonic discharge rate. Spontaneous, tonic and evoked discharge rates, evoked response duration, peak, latency to peak and signal-to-noise ratio for SPS and control rats were compared by use of Student's *t*-test for independent samples. For an analysis of evoked response over time, a mean PSTH was generated for each experimental group by averaging the number of spikes in each 20-ms bin for every cell. A Kolmogorov–Smirnov test was conducted to determine whether the overall distribution of LC discharge activity was different for SPS and control rats. Components of the mean PSTHs were compared between groups by the use of repeated measures ANOVA with the factors stress and time. The post-stimulus inhibitory component was defined as the period commencing immediately following the evoked component and lasting for 2 s.

TH mRNA data

Locus coeruleus sections on film were digitised with a SCION 10-bit Crystal Clear Display camera (SCION, Frederick, MA, USA) and a 50-mm Mega Pixel fixed C-mount lens. Background noise was subtracted by use of a two-dimensional rolling ball procedure (radius: 50 pixels). Gray values were taken from four to eight LCs for each rat (two to four sections), and averaged to obtain a mean gray value. These scores were then averaged to obtain group means. The mean gray value of signal pixels from the LC was subtracted from baseline, derived from the mean gray value of an activity-poor area (in the brainstem ventral to the LC). Mean gray values were converted into mCi/mg by the use of C-14 standards. Group means were analysed with a two-factor ANOVA (SPS vs. control, and baseline vs. restraint). *Post hoc* comparisons were conducted where appropriate. TH mRNA levels after restraint stress were expressed as percentage increase from mean baseline TH mRNA levels of SPS and control rats, and subjected to a *t*-test (Sands *et al.*, 2000). Data from one rat identified as an outlier were discarded (> 2 standard deviations from the group mean).

Results

Electrophysiological data

Figure 1 shows representative PSTH and raster plots generated from a control and an SPS rat. Figure 2 shows an example set of overlaid action potentials from recorded LC neurons. This figure shows that the data presented were obtained from single units rather than from multiple LC units. Data from 92 neurons (SPS, 50; control, 42) were used in the analyses (for the number of cells recorded from each rat, see Table 1). SPS rats showed significantly lower baseline levels of spontaneous LC activity than controls ($t_{90} = 2.09$, $P = 0.039$) (Fig. 3A). Quantification of LC discharge during components of the PSTH revealed that the evoked LC activity of SPS rats was significantly higher than that of control rats ($t_{90} = 2.79$, $P = 0.006$) (Fig. 3B). In contrast, the tonic LC rate during the trial of noxious stimulation was not significantly different between groups ($t_{90} = 1.53$, $P = 0.13$) (Fig. 3C). The greater evoked discharge rate in the absence of a change in tonic rate resulted in a significantly higher signal-to-noise ratio in SPS than in control rats ($t_{90} = 3.02$, $P < 0.003$) (Fig. 3D). Between-group differences in peak LC response, latency to peak and evoked response duration were also analysed. Peak response tended to be higher in SPS rats ($P < 0.1$), but no other differences were found between SPS and control rats for any of these LC discharge characteristics. These data are presented in Table 2.

The averaged PSTHs generated from all LC neurons of SPS and control rats are shown in Fig. 4A. First, in order to determine whether the overall distribution of LC discharge activity was different for SPS and control rats, a Kolmogorov–Smirnov test was conducted. This analysis indicated that the distribution of LC discharge activity was significantly different between groups ($P = 0.0002$).

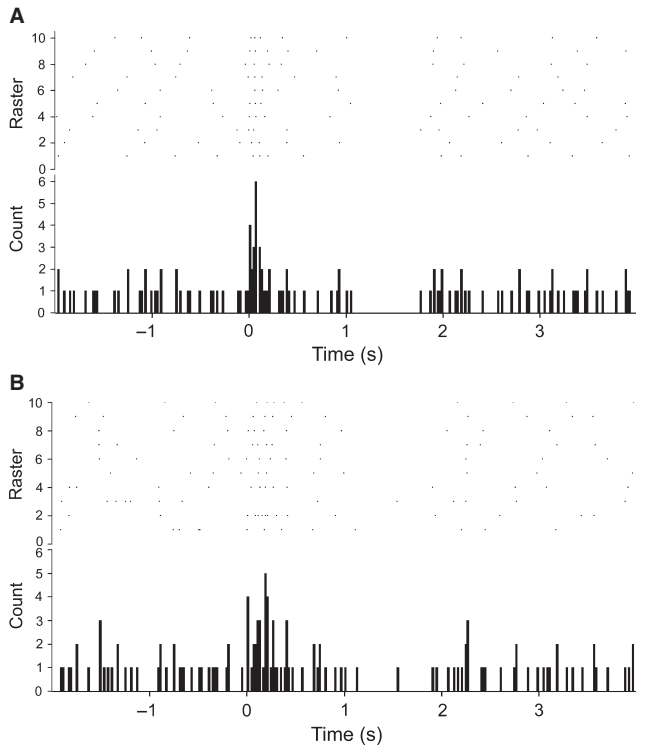

FIG. 1. Representative PSTHs of an LC single-unit response to paw compression from an SPS (A) and control (B) rat.

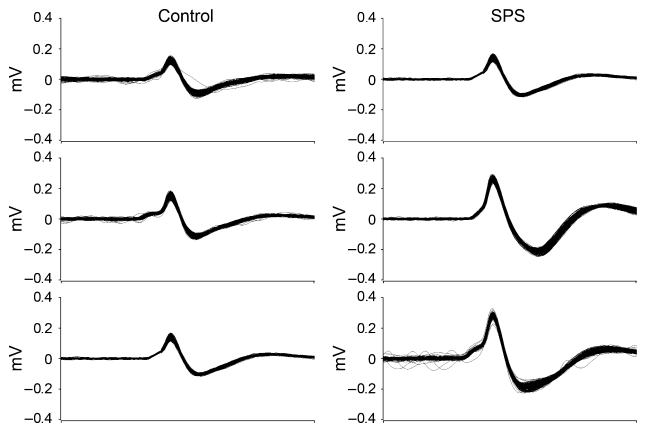

FIG. 2. Example set of overlaid action potentials of recorded LC neurons from SPS and control rats.

Second, components of the histograms containing the evoked response (Fig. 4B) and post-stimulus inhibition (Fig. 4C) were isolated and compared by the use of repeated measures ANOVAS. The excitatory component of the PSTH was exaggerated in SPS rats (time × stress interaction: $F_{18,1620} = 2.04$, $P = 0.025$). The data were re-binned for *post hoc* analyses, and these analyses revealed that the bin corresponding to 0–0.06 s after stimulation was significantly different in SPS rats ($t_{90} = 2.65$, $P = 0.009$). SPS rats also tended to show slower recovery from post-stimulus inhibition ($F_{49,4410} = 1.51$, $P = 0.064$). Bins corresponding to 1.40–1.58 s, 1.60–1.78 s and 1.80–2.0 s were significantly different between stress conditions ($t_{90} = 2.66$, $t_{90} = 2.85$, and $t_{90} = 2.57$, and $P = 0.009$, $P = 0.005$, and $P = 0.010$, respectively).

TABLE 1. Number of cells recorded from each rat

Rat	Stress condition	No. of cells
1	SPS 1	1
2	SPS 2	6
3	SPS 3	7
4	SPS 4	9
5	SPS 5	10
6	SPS 6	1
7	SPS 7	6
8	SPS 8	1
9	SPS 9	1
10	SPS 10	5
11	SPS 11	1
12	SPS 12	2
13	CON 1	3
14	CON 2	6
15	CON 3	3
16	CON 4	7
17	CON 5	8
18	CON 6	9
19	CON 7	6

CON, control.

TABLE 2. Means (± standard deviation) of LC discharge characteristics

	Condition			
	Control	SPS	*t*	d.f.
Peak rate (Hz)	21.90 (6.53)	25.00 (8.69)	−1.90*	90
Latency to peak (s)	0.089 (0.047)	0.087 (0.072)	0.15	90
Evoked response duration (s)	0.49 (0.22)	0.44 (0.23)	0.99	90

d.f., degrees of freedom. *$P < 0.1$.

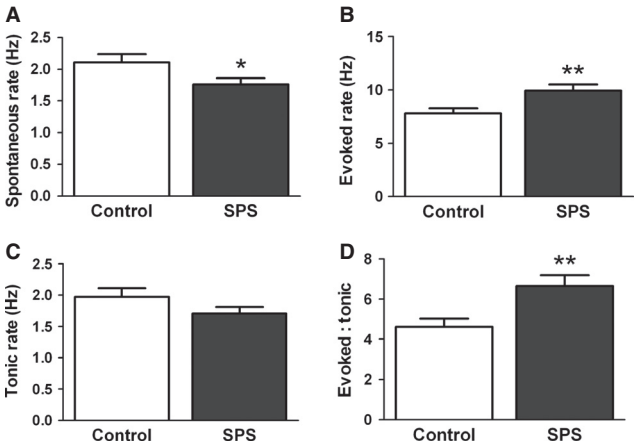

FIG. 3. SPS effects on LC discharge characteristics. Bars indicate the mean LC discharge for control (white) and SPS (black) rats. (A and B) SPS rats showed significantly lower baseline levels of spontaneous LC unit firing than control rats (A), but significantly higher evoked responses to paw compression than controls (B). (C and D) Although tonic activity was not significantly different (C), the signal-to-noise ratio was significantly higher in SPS rats than in controls (D). *$P < 0.05$, **$P < 0.01$.

TH mRNA data

Figure 5A shows a representative autoradiogram of TH mRNA in the LC. Restraint stress increased TH mRNA expression in the LC of all rats ($F_{1,27} = 30.05$, $P < 0.0001$). A two-factor ANOVA revealed

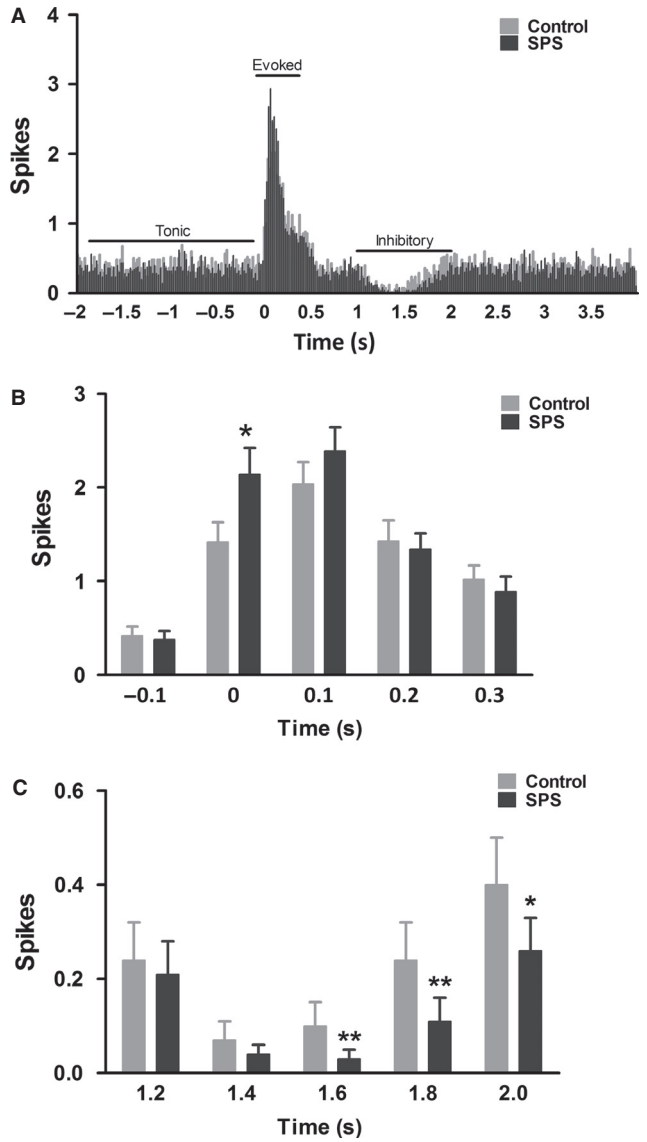

FIG. 4. Averaged PSTHs showing SPS effects on evoked LC discharge characteristics. (A) The entire average PSTH of all LC neurons from SPS and control rats. Bars indicate the frequency of spikes (Hz) in 20-ms bins for control (gray) and SPS (black) rats. (B and C) Components of the PSTH are broken down to show average evoked and inhibitory phases plus several bins for reference. $*P < 0.05$, $**P < 0.01$.

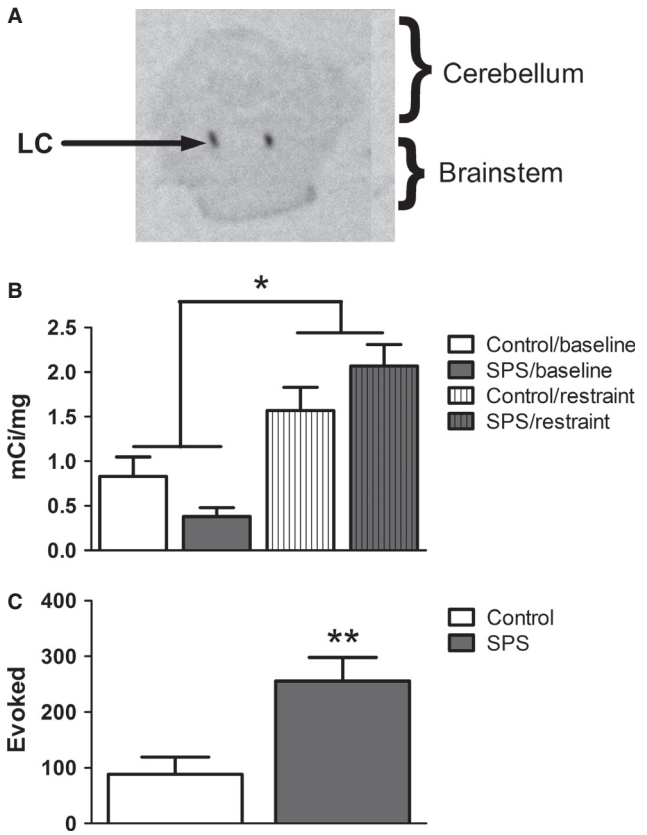

FIG. 5. SPS effects on TH mRNA expression in the LC. (A) Representative autoradiogram of TH mRNA in the LC detected by *in situ* hybridisation. (B) Bars indicate mean miC/mg detected. SPS treatment differentially affected TH mRNA expression in SPS and control rats at baseline as compared with after restraint stress. (C) The percentage increase in TH mRNA expression (from mean baseline score) following restraint was significantly greater for SPS than for control rats. $*P < 0.05$, $**P < 0.01$.

a significant interaction ($F_{1,27} = 4.63$, $P = 0.040$), indicating that SPS treatment differentially affected the TH mRNA response to restraint stress (Fig. 5B). *Post hoc* comparisons indicated that baseline levels of TH mRNA expression tended to be lower in SPS rats than in controls at trend level significance ($t_{9.71} = 1.89$, $P = 0.089$). To examine 'reactivity' of the system while controlling for baseline TH mRNA levels, we calculated TH mRNA expression as the percentage increase from mean baseline scores, and compared the SPS and control groups. SPS rats showed significantly greater increases in TH mRNA expression following restraint stress than controls ($t_{14} = 3.21$, $P = 0.006$) (Fig. 5C).

Discussion

Using a validated animal model for PTSD (SPS), we set out to directly examine hypothesised changes in LC–NE activity following stress/trauma exposure. SPS exposure decreased rates of spontaneous LC activity, exaggerated phasic responses to a noxious event, and decreased recovery from post-stimulus inhibition. At the molecular level, TH mRNA expression in the LC tended to be lower in SPS rats at baseline, but stress-enhanced TH mRNA expression was augmented by SPS, mirroring our electrophysiological findings.

It has long been suggested that NE activity is altered in PTSD (Kosten *et al.*, 1987). However, as LC function cannot be directly measured *in vivo*, these inferences rely on the assumption that peripheral and central systems are co-activated and peripheral NE levels reflect central NE activity. Studies examining baseline NE levels in PTSD have yielded inconsistent results, with reports of NE concentrations that are increased (De Bellis *et al.*, 1997; Liberzon *et al.*, 1999a), decreased (Murburg *et al.*, 1995), and unchanged (McFall *et al.*, 1990, 1992), although reduced numbers of α2-adrenergic binding sites suggest potential adaptation to higher circulating NE levels (Perry *et al.*, 1990), and the study of autonomic markers also points to an overactive NE system (Orr *et al.*, 2002). Challenge studies have provided more consistent findings, and suggest that NE alterations in PTSD may be better characterised as exaggerated responsivity to trauma-related stimuli (McFall *et al.*, 1990; Liberzon *et al.*, 1999a). The α2 antagonist yohimbine, which increases synaptic NE through blockade of presynaptic autoreceptors, causes anxiety, panic and flashbacks in patients with PTSD (Southwick *et al.*, 1993). Our new data, examining central nervous system NE function

in a PTSD rat model, support this characterisation, as we demonstrate, using two different and complementary methodologies, that SPS rats have decreased baseline LC–NE function. Furthermore, our data suggest that SPS induces exaggerated phasic responses to noxious stimulation and elevates TH mRNA synthesis in response to restraint stress. Together, these data indicate altered LC–NE function following SPS.

The fact that stress can alter LC–NE system function is well established. Increased LC discharge and NE turnover has been demonstrated following acute stress regimens (Korf *et al.*, 1973; Abercrombie & Jacobs, 1987; Abercrombie *et al.*, 1988), and chronic stress has been also shown to sensitise LC neuronal responses to subsequent stress exposure. For example, Simson & Weiss (1988) contributed an early report that prolonged uncontrollable tail shock stress exaggerates LC evoked responses, as do chronic cold, repeated restraint, or electric shock exposure (Simson & Weiss, 1988; Curtis *et al.*, 1995; Conti & Foote, 1996; Mana & Grace, 1997; Jedema *et al.*, 2001; Jedema & Grace, 2003). NE release and TH mRNA activity are also enhanced by these treatments (Thierry *et al.*, 1968; Stone *et al.*, 1978; Irwin *et al.*, 1986; Adell *et al.*, 1988; Anisman & Zacharko, 1990; Angulo *et al.*, 1991; Nisenbaum *et al.*, 1991; Watanabe *et al.*, 1995; Rusnak *et al.*, 1998). These effects are mediated by adaptation in LC neurons (rather than alterations in afferent input), as they are reproduced *in vitro* (Jedema & Grace, 2003), and probably involve α2-receptor-activated hyperpolarisation of LC neurons via the opening of potassium channels (Aghajanian & VanderMaelen, 1982; Williams & Clarke, 1995; Arima *et al.*, 1998). These findings, while providing important general information about stress-induced plasticity in the LC, typically describe processes that immediately follow stress exposure or occur up to 24 h following the cessation of stress. They do not, however, address the long-term, sustained alteration in the LC–NE system seen in some psychiatric disorders, and thus offer limited PTSD-relevant information. The SPS model, however, interrogates sustained changes that are present or develop at least 7 days after stress exposure. This model also describes specific systemic changes in the other regions of the brain that might have direct effects on LC function. We have demonstrated in previous publications that SPS-specific changes develop over the 7-day sensitisation period, and are not present immediately or soon after (24 h) the stress exposure. This suggests that the sustained effect of SPS on LC function may involve different mechanisms than those described in general stress studies. Furthermore, SPS is one of only two models of PTSD (SPS and predator exposure) (Kozlovsky *et al.*, 2009) that reproduce the signature neuroendocrine abnormality associated with PTSD of enhanced glucocorticoid negative feedback (Pitman *et al.*, 2012). This is consistent with the glucocorticoid hypersensitivity found in PTSD patients (Yehuda *et al.*, 1993). Critically, glucocorticoid receptor (GR) upregulation is observed in SPS only after the 7-day quiescent period has elapsed (Liberzon *et al.*, 1997, 1999a), and our laboratory has recently demonstrated that this upregulation is necessary for the expression of SPS-specific extinction retention deficits (Knox *et al.*, 2012a,b), which, in turn, are critical to PTSD symptomology (Milad *et al.*, 2007). Thus, our study is the first to demonstrate sustained LC–NE system abnormalities after SPS, and provides a unique opportunity to study the interaction between LC–NE system changes, HPA axis changes, and memory abnormalities, all of which are known to be central to PTSD.

In the present article, we also report lowered baseline rates of LC discharge in SPS rats. These effects differ from what Simson & Weiss (1988), and others, have observed following exposure to acute stressors [in fact, it was reported as elevated (see Cedarbaum & Aghajanian (1978) and Abercrombie & Jacobs (1987)]. Similarly, Abercrombie and colleagues reported no effect of chronic cold stress on basal NE levels in the hippocampus or basal rates of NE synthesis (Abercrombie *et al.*, 1988). These differences further highlight the unique nature of the SPS model, and may be accounted for by either the number and type of multiple stressors involved in SPS, or the 'quiescent' component of SPS, which increases the time interval between stress and electrophysiological recording. Given the relatively small number of animals in the present study, however, and the modest effect size, independent replication of this finding will strengthen confidence in this result. Overall, the findings reported here demonstrate that SPS leads to enduring changes in LC–NE system function, characterised by reduced basal activity in conjunction with elevated phasic activity, and suggest a threat detection system that is hyper-responsive to stimulation, offering a plausible mechanism for the development of long-term post-trauma changes leading to persistent psychopathology.

In addition to observing changes in LC neuronal activity following SPS, we found differences in TH mRNA expression levels between SPS and control rats. We have used restraint – a single acute mild stressor-that is known to induce an elevation in TH mRNA in the LC (Smith *et al.*, 1991), as a probe of LC 'reactivity'. SPS rats tended to have lower TH mRNA expression at baseline, but showed exaggerated expression following stress. These results are intriguing, as many studies show increases in TH mRNA level shortly after the cessation of acute or chronic stress (Zigmond *et al.*, 1974; Stone *et al.*, 1978; Richard *et al.*, 1988; Watanabe *et al.*, 1995), but our data demonstrate sustained changes in transcription reactivity in LC neurons that match electrophysiological changes in these neurons. These data suggest that, in addition to causing persistent changes in the rate of firing of LC neurons, SPS results in corresponding long-term changes in the synthesis of NE. Future studies could include analyses of protein levels, enzyme activity or TH phosphorylation to provide additional evidence of functional changes in NE synthesis.

How might these LC–NE system changes contribute to PTSD symptomatology? As discussed previously, the LC is implicated in mediating arousal, and changes in noradrenergic activity may contribute to the hyperarousal associated with PTSD. In a study by Southwick and colleagues, an observed correspondence between NE metabolite levels and intrusive memory symptoms in response to yohimbine challenge led to the suggestion that NE system hyper-responsiveness may alter the threshold for activation in the amygdala and hippocampus, and facilitate the retrieval of intrusive memories (Southwick *et al.*, 1993). Other functional impairments commonly found in people with PTSD include decreased attention and sleep disruption, which have also been attributed to LC–NE system disturbances (Gil *et al.*, 1990; Raskind *et al.*, 2007). A number of studies have demonstrated physiological and behavioral changes reminiscent of these symptoms by using SPS, including enhanced acoustic startle (modeling hyperarousal) (Khan & Liberzon, 2004) and impaired fear memory regulation (modeling intrusive memories) (Yamamoto *et al.*, 2008; Knox *et al.*, 2012a). Given the role of the LC–NE system in mediating arousal and memory processes (Foote *et al.*, 1980; McGaugh & Roozendaal, 2002), it is plausible that the changes in the LC reported here contribute to these effects of SPS. No research to date has investigated performance on attentional tasks or sleep disturbances in SPS animals. Assessment of these behaviors and determining whether a direct link exists between LC activity alterations and the established behavioral changes associated with SPS are important questions for future research.

Recent theories of LC function propose specific contributions of LC neural activity to behavior, such that different modes of LC

discharge facilitate distinct cognitive-behavioral processes. Spontaneous LC firing is suggested to determine the state of arousal and vigilance, and mediate environmental scanning and attention to irrelevant stimuli, thereby facilitating task disengagement. Conversely, phasic activity is suggested to mediate focused attention, and occurs in response to salient or task-relevant stimuli (Aston-Jones & Cohen, 2005). Although the application of these findings to those reported here must be approached with caution, as these data are derived from awake animals and the present study measured LC activity in anesthetised rats, they may still have significance for interpreting the present results. SPS rats showed spontaneous activity of LC neurons that was lower than that of controls, and a greater phasic response to a stimulus. These findings may indicate that appropriate task disengagement and flexible performance would be inhibited in SPS animals. The elevated phasic response to a noxious event and signal-to-noise ratio following noxious stimulation seen in SPS rats may indicate greater reactivity to salient events. To the extent that these LC activity changes are preserved in awake animals, SPS rats may have LC activity that promotes hyper-responsiveness to specific environmental events and reduced behavioral flexibility. Experiments designed to test these hypotheses represent exciting avenues for future research.

The present findings provide evidence that LC–NE activity is disrupted by SPS, but the mechanism by which this occurs is not yet clear. It has been suggested that trauma could directly alter LC activity via excitotoxic effects of glutamate release in the LC (Aston-Jones *et al.*, 1994). SPS exposure has been linked to structural abnormalities at the cellular level. For example, it was recently reported that SPS rats show signs of apoptosis in the amygdala (Ding *et al.*, 2010). However, we found no evidence of neurotoxicity following SPS in the hippocampus, amygdala or medial prefrontal cortex by using magnetic resonance spectroscopy (Knox *et al.*, 2010). An alternative possibility is that LC activity changes are caused by more indirect mechanisms; convergent evidence suggests that, during stress, corticotropin-releasing factor and NE may cross-sensitise each other, creating a feed-forward loop in the presence of inappropriate negative feedback from GRs (Sawchenko & Swanson, 1982; Dunn & Berridge, 1987; Valentino & Foote, 1988; Koob, 1999). Via upregulation of GRs in the hippocampus, SPS is known to lead to sensitisation of inhibitory components of the HPA axis (Liberzon *et al.*, 1997, 1999b), and upregulated corticotropin-releasing factor has been reported in the hypothalamus of SPS animals (Wang *et al.*, 2009). Therefore, it may be that a combined change in glucocorticoid feedback and stress-related NE release alters regulation of the stress response in SPS animals. Studies designed to address the precise nature of LC–NE system and HPA axis interactions in SPS animals are needed to address this possibility; the results of these will be important in advancing our understanding of how stress/trauma can lead to significant and persistent psychopathology.

There are several methodological limitations of the present work that should be noted. First, a limitation of using paw compression to evoke LC responses is that, owing to the manual application of the compression, it is possible that the force applied may vary across trials. Such variation would introduce noise into the dataset, and may also be potentially subject to experimenter bias. Owing to experimenter constraints, the experimenter was not blind to stress condition; however, the paw compression procedure has features that ensure that the compressions are less likely to be subject to experimenter bias, so as to eliminate the possibility that the experimenter may unintentionally influence the result (Simson *et al.*, 1988; Simson & Weiss, 1989; Grant & Weiss, 2001; West *et al.*, 2010). A physical instrument was employed (described in detail in Materials and methods) to ensure that equal force was employed in all instances of paw compression. In addition, the research group that introduced this methodology have reported the results of tests conducted with different intensities of paw compression that have shown that the response of LC neurons to paw compression is essentially 'all or none in nature', and that the magnitude of the LC response is insensitive to variations in the magnitude of the paw compression (Simson & Weiss, 1988). These findings indicate that, even if unintentional experimenter effects had influenced the pressure of paw compressions administered, this would have been unlikely to have affected the results obtained. Furthermore, we would like to note that our finding of reduced spontaneous LC activity in SPS animals involves a 3-min baseline recording of LC cell activity, prior to any experimenter manipulation. Therefore, this finding is highly unlikely to have been influenced by any experimenter bias. Future studies that address these limitations are needed to replicate the reported findings, delineate the mechanism by which LC activity is altered following SPS, and explore the potential for rescue of LC function following stress/trauma.

In summary, the data presented provide evidence of altered LC–NE activity in an animal model of PTSD. SPS rats showed lower spontaneous activity but higher evoked responses, leading to an enhanced signal-to-noise ratio of LC neurons, accompanied by impaired recovery from post-stimulus inhibition. In concert, baseline TH mRNA expression in the LC tended to be lower, and stress-evoked TH mRNA upregulation was exaggerated in SPS rats. These data provide convergent evidence that SPS lowers basal activity and enhances the reactivity of the LC-NE neurons. Understanding the neuropathological sequelae of stress/trauma is crucial for deepening our understanding of PTSD and for the development of novel targeted treatment strategies.

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Abbreviations

GR, glucocorticoid receptor; HPA, hypothalamic–pituitary–adrenal; LC, locus coeruleus; NE, norepinephrine; PSTH, peristimulus time histogram; PTSD, post-traumatic stress disorder; SPS, single prolonged stress; SSC, standard saline citrate; TH, tyrosine hydroxylase.

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