Chronic cold stress increases excitatory effects of norepinephrine on spontaneous and evoked activity of basolateral amygdala neurons

Deanne M. Buffalari and Anthony A. Grace
Departments of Neuroscience, Psychiatry and Psychology, University of Pittsburgh, Pittsburgh PA, USA

Abstract
Neurons of the amygdala respond to a variety of stressors. The basolateral amygdala (BLA) receives dense norepinephrine (NE) innervation from the locus coeruleus, and stressful and conditioned stimuli cause increases in NE levels within the BLA. Furthermore, chronic stress exposure leads to sensitization of the stress response. The actions of NE in different structures involved in the stress circuit have been shown to play a role in this sensitization response. Here, we examine how chronic cold stress alters NE modulation of spontaneous and evoked activity in the BLA. In controls, NE inhibited spontaneous firing in the majority of BLA neurons, with some neurons showing excitation at lower doses and inhibition at higher doses of NE. NE also decreased the responsiveness of these neurons to electrical stimulation of the entorhinal and sensory association cortices. After chronic cold exposure, NE caused increases in spontaneous activity in a larger proportion of BLA neurons than in controls, and now produced a facilitation of responses evoked by stimulation of entorhinal and sensory association cortical inputs. These studies show that chronic cold exposure leads to an increase in the excitatory effects of NE on BLA neuronal activity, and suggest a mechanism by which organisms may display an enhancement of hormonal, autonomic, and behavioural responses to acute stressful stimuli after chronic stress exposure.

Key words: Amygdala, cold, locus coeruleus, norepinephrine, sensitization, stress.

Introduction
The amygdala is activated by a large variety of acute stressors (Akirav et al., 2001; Dayas et al., 1999; Rosen et al., 1998), and plays a facilitatory role in components of the stress response (Feldman et al., 1995; Herman et al., 2003; Van de Kar and Blair, 1999). The basolateral complex of the amygdala (BLA) receives dense noradrenergic (NE) projections from the locus coeruleus (LC) (Asan, 1998), which causes an α-adrenergic inhibition or a β-adrenergic excitation of BLA neurons (Buffalari and Grace, 2007). The LC has an excitatory influence on stress-related circuits as well (Al-Damluji, 1988; Plotsky et al., 1987; Sved et al., 2002; Ziegler et al., 1999). Levels of NE increase in the BLA during stress exposure (Galvez et al., 1996; Hatfield et al., 1999; Williams et al., 1998), and neurons of the BLA respond to stressful stimuli that also activate LC neurons (Aston-Jones et al., 1991; Buffalari and Grace, 2007; Correll et al., 2005; Rasmussen et al., 1986). In addition to their roles in acute stress responses, the LC and BLA may play roles in chronic stress.

Chronic or repeated exposure to stressful stimuli leads to a sensitization of many components of the stress response. Prior exposure to chronic cold enhances activation of LC neurons to footshock (Mara and Grace, 1997) and corticotrophin-releasing hormone (Jedema et al., 2001). Stress-induced NE efflux in target regions is enhanced after chronic cold or repeated immobilization (Gresch et al., 1994; Jedema et al., 1999; Nisenbaum et al., 1991; Pacak et al., 1992), which may cause alterations in LC targets. Stress-induced release of NE in the bed nucleus (Cecchi et al., 2002), paraventricular nucleus of the hypothalamus (Ma and Morilak, 2005a) and amygdala...
(Ma and Morilak, 2005b) facilitates activation of the HPA axis to acute immobilization stress. Therefore, in addition to alterations in the NE system that occur with chronic stress, alterations in NE post-synaptic actions play a role in the behavioural effects of chronic stress.

Chronic stress is also known to alter BLA activity. Chronic immobilization increases spines density on BLA pyramidal neurons (Mitra et al., 2005), and stress enhances plasticity in BLA circuits (Vouimba et al., 2004, 2006). Furthermore, chronic cold exposure alters responses of BLA neurons to footshock (Correll et al., 2005). This present study investigates how exposure to chronic cold alters NE modulation of BLA neurons. The effects of NE on BLA spontaneous and afferent-evoked activity was examined. Entorhinal cortex (EC) or sensory association cortex (Te3) were stimulated. Chronic cold exposure was used as a model of chronic stress, as cold-exposed animals reliably exhibit sensitization of the NE system (Gresch et al., 1994; Jedema et al., 1999; Nisenbaum et al., 1991; Pacak et al., 1992). To investigate the time-course of the development of alterations in LC targets during chronic cold exposure, two experimental groups were used: rats exposed to 7 d cold (which is subthreshold for stress-induced sensitization; Finlay et al., 1997) or 14 d cold. These studies examine the role of the amygdala as a contributor to the sensitization of the stress response.

Materials and methods

Surgical preparation

All studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the USPHS, and all experimental procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (150–175 g at arrival) were anaesthetized with 8% chloral hydrate (400 mg/kg i.p.) and implanted with jugular catheters for administration of supplemental anaesthetic. Rats were then placed in a stereotaxic apparatus, with supplemental doses of chloral hydrate (i.v.) to maintain a constant level of anaesthesia. Temperature was monitored with a rectal thermometer probe and maintained at 37 °C. An incision was made along the scalp and the underlying skull was exposed. Burr holes were drilled in the skull and the dura under the burr holes was removed. The locations (from Bregma) of the BLA (−5.0 L, −3.0 C), Te3 (−6.1 L, −6.8 C, −4.7 V), and the EC (−5.0 L, −6.8 C, −8.2 V) were calculated using a stereotaxic atlas (Paxinos and Watson, 1997) for implantation of stimulating electrodes.

Electrophysiology

Multibarrel microiontophoretic electrodes (five barrels; Activational Systems, Warren, MI, USA) were constructed using a vertical microelectrode puller, broken back under microscopic control, and filled with 2% Pontamine Sky Blue in 2 m NaCl to yield electrodes with a central recording barrel impedance of 4–8 MΩ. These electrodes were lowered through the BLA in successive vertical tracks. Neurons within the BLA were isolated; those with a signal-to-noise ratio >3:1 were used for data analysis. Spontaneous activity was recorded for a minimum of 5 min before further manipulations.

Electrical stimulation

Bipolar, concentric stimulating electrodes were lowered into either the Te3 or the EC at the conclusion of surgical preparation. Recordings did not begin until a minimum of 45 min after lowering of the stimulating electrodes. Neurons responsive to either Te3 or EC stimulation were identified using a search-stimulate protocol. Single-pulse stimuli (300–900 μA, 0.25 ms, 0.5 Hz) were delivered to the Te3 or EC while the recording electrode was lowered through the BLA. Responsive neurons were identified and isolated. Neurons were characterized as having presumed orthodromic monosynaptic, orthodromic polysynaptic, or antidromic responses to EC stimulation based on the following criteria: Responses were operationally defined as orthodromic if they had an onset latency of <20 ms, showed a failure to show substantial changes in latency in response to increases in current intensity, their onset latency remained fairly consistent with ~1–5 ms of variability in evoked spike
latency, and evoked spikes follow paired pulse simulation at 50 Hz but not 400 Hz. Antidromic responses were characterized by their constant onset latency with virtually no variability, and their ability to followed paired pulses at 400 Hz. Monosynaptic responses were differentiated from polysynaptic spikes by an examination latency onset and variability, as well as polysynaptic failure to follow paired pulses at 50 Hz.

**Microiontophoresis**

The central barrel of the five-barrel microelectrode was filled with 2% Pontamine Sky Blue in 2 M NaCl for electrophysiological recordings. One of the outer barrels was filled with 3 M NaCl for automatic current balancing, and the remaining barrels were filled with 200 µM NE dissolved in 0.1 M NaCl. NE was retained with (−) current and ejected with (+) iontophoretic current (E104B; Fintronics, Orange, CT, USA). Retaining currents ranged between 12 nA and 18 nA while drug ejection currents ranged between 5 nA and 40 nA. Repeated, increasing doses from 5–40 nA were used with at least 2 min spontaneous activity measured between each period of drug ejection.

Neurons were tested for response to NE in a dose–response fashion (5–40 nA). Those neurons that responded to lower NE doses (5–10 nA) did so in a moderate, variable manner, often showing non-significant changes in firing rate. Significant and consistent responses emerged in BLA neurons exposed to higher NE doses (40 nA). Therefore, this dose was used for analysis of NE effects on spontaneous activity. However, evoked activity was significantly altered by lower NE doses, therefore the effects of NE on evoked activity focused on the 10 nA dose; effects are also reported for higher doses where tested.

**Histology**

At the conclusion of electrophysiological experiments, Pontamine Sky Blue dye was ejected by passing constant current through the recording electrode (10 µA, 15 min) to mark the location of the recording site. Anodal current was passed through the stimulating electrode to create a small lesion to identify its location. Rats were killed by an overdose of anaesthetic, followed by decapitation and brain removal. Brains were fixed in 10% formalin for a minimum of 24 h, and then cryoprotected with 25% sucrose solution in 0.1 M phosphate buffer. Subsequently, coronal slices were cut on a cryostat into 60 µm sections, mounted on glass slides, and stained with Neutral Red and Cresyl Violet. Recording sites were identified by the presence of the Pontamine Sky Blue dye spot, and the location of stimulating electrodes was identified by the presence of a small lesion at the end of the electrode track.

**Chronic cold exposure**

Rats were randomly assigned to cold-exposed and control groups. After 5 d acclimation to the housing room during which all animals were pair-housed, cage-mates were assigned as matched pairs to either the control group or a cold-exposed group (7 d or 14 d). Rats that were to undergo chronic cold exposure were shaved caudally from their forelimbs and housed singly in hanging wire mesh cages in a cold room at 5 °C for 7 d or 14 d (Jedema et al., 1999, 2001; Mana and Grace, 1997). Controls were kept in the control housing room. Both control and cold-exposed rats were housed singly during the exposure phase prior to experimental use. Food and water were available ad libitum. Any animals showing signs of cold-induced injury or tissue damage were immediately removed from the protocol. In order to maintain consistency with protocols demonstrating sensitization of aspects of the stress response (Gresch et al., 1994; Jedema et al., 1999, 2001; Nisenbaum et al., 1991), chronic cold-exposed animals were removed from the cold room and housed singly in the colony room for 20–24 h before recordings.

**Data analysis**

Firing rate is expressed as spikes/s. Spontaneous firing rate was evaluated between groups (one- way ANOVA) to evaluate whether potential baseline differences might have influenced measurement of subsequent responses. An effect was labelled as a significant excitatory or inhibitory effect if, during NE iontophoresis, the average firing rate changed by ≥25% from baseline. This was based on conservative estimates of change necessary to yield significance in a t test, given the observed firing rate variability. Pre- and post-NE iontophoresis firing rates were compared using paired t tests. Percent neurons responding in an inhibitory manner vs. an excitatory manner was compared between control and cold-stress groups (χ²). The authors chose this level of analysis over others for several reasons. Measurements of absolute firing rate would not account for the washout that might occur if some neurons increase firing rate while others decrease it. It was difficult to measure differences in magnitude of excitation or inhibition, as across all groups of rats there was significant variation in the magnitude of inhibitory and excitatory effects. Furthermore, the authors often reached near maximal
effects during NE application, leading to potential ceiling effects when trying to examine NE iontophoresis in rats from both cold groups. Effects of NE iontophoresis on responses evoked by afferent stimulation were evaluated in the following manner. Current applied to EC or Te3 was adjusted to achieve between 50–60% probability of evoked spike responses. Fifty single pulses at this current intensity were applied and the number of stimuli resulting in evoked spikes was measured. This was compared to evoked spike firing during NE iontophoresis. These spike probabilities were compared using paired t tests. Changes in evoked responses were expressed as percent increase or decrease in the probability of evoking spike discharge in BLA neurons during NE iontophoresis. A minimum of 3 min of baseline spontaneous firing was recorded before delivery of a second stimulation period of 50 sweeps concomitant with NE iontophoresis. The proportions of neurons inhibited or excited in control rats, rats exposed to 7 d chronic cold, and rats exposed to 14 d chronic cold were compared using χ² tests. The magnitude of percent inhibition by NE on evoked activity in cold-exposed rats was compared to the NE-induced inhibition of evoked activity in control rats using t tests. Interactions between cold-stress groups and neuronal location with respect to baseline firing rates were examined using a two-way ANOVA.

Neurons examined for spontaneous activity displayed a wide range of firing rates and probably included both projection neurons and interneurons. In order to more confidently identify neurons as projection cells (as firing rate alone is not an entirely reliable predictor of projection neuron vs. interneuron; Likhtik et al., 2006; Rosenkranz and Grace, 2001), a subclass of neurons was confirmed as projection cells if they could be antidromically activated by EC stimulation. Histological verification also revealed subclasses of neurons in the lateral and basolateral nuclei of the BLA. These neurons were examined separately for their response to NE iontophoresis; pre- and post-NE firing rates were compared using paired t tests. Proportions of neurons in the lateral nucleus displaying excitatory vs. inhibitory responses were compared to proportions of neurons in the basolateral nucleus displaying excitatory vs. inhibitory responses using χ² analyses.

Results

Spontaneous activity

A total of 140 neurons were examined from 35 rats [control rats (n = 16), 7-d cold-exposed rats (n = 7), 14-d cold-exposed rats (n = 12)]. In control rats, 47 neurons were examined for NE-induced changes in spontaneous activity (eight of those were antidromically activated and confirmed as projection neurons), and 15 were examined for responses to Te3 (n = 8) or EC (n = 7) stimulation before or during NE iontophoresis. In rats exposed to 7-d cold stress, 15 neurons were examined for NE-induced changes in spontaneous activity (six of those were antidromically activated and confirmed as projection neurons), and 15 were examined for responses to Te3 (n = 8) or EC (n = 7) stimulation before or after NE iontophoresis. In rats exposed to 14-d cold stress, 32 neurons were examined for NE-induced changes in spontaneous activity (seven of those were antidromically activated and confirmed as projection neurons), and 16 were examined for responses to Te3 (n = 7) or EC (n = 9) stimulation before or after NE iontophoresis.

Chronic cold does not alter spontaneous activity of BLA neurons after 7 d or 14 d

There were no significant differences in spontaneous firing rates of BLA neurons in control rats [average firing rate (avFR) = 1.3 ± 0.45 Hz], rats exposed to 7 d cold (avFR = 0.75 ± 0.11 Hz), or rats exposed to 14 d cold (avFR = 1.4 ± 0.13 Hz, Figure 1). Although there was a trend for decreased spontaneous firing rates after 7 d and increased spontaneous firing rates after 14 d, these were not significant (p = 0.35, one-way ANOVA).

The spontaneous firing rates of neurons in control rats, 7-d cold rats and 14-d cold rats did not differ...
when examined based on neuronal location. That is, there was no significant interaction between experimental group and neuron location (two-way ANOVA, \( p > 0.1 \)). The average firing rate across all groups and all locations was \( 1.2 \pm 0.17 \) Hz.

The average firing rate of neurons confirmed by antidromic activation to be projection neurons (\( n = 21 \), all groups) was \( 0.18 \pm 0.09 \) Hz, which was significantly lower than the firing rates of spontaneously active neurons (\( p = 0.01 \), \( t \) test). Spontaneously active neurons displayed a wide range of firing rates and probably included both projection neurons and interneurons. The spontaneous activity of projection neurons was no different (\( p = 0.7 \), one-way ANOVA) in control rats (avFR = \( 0.22 \pm 0.04 \) Hz, \( n = 8 \)), rats exposed to 7 d cold (avFR = \( 0.13 \pm 0.04 \) Hz, \( n = 6 \)), or rats exposed to 14 d cold (avFR = \( 0.17 \pm 0.03 \) Hz, \( n = 7 \)). As the majority of projection neurons were located in the basolateral nucleus [controls (\( n = 6 \)), 7-d cold rats (\( n = 4 \)), 14-d cold rats (\( n = 5 \))], projection neurons were not further divided based on neuronal location for analysis of interaction between experimental group and neuron location.

**Chronic cold alters NE modulation of spontaneous activity after 14 d but not 7 d**

NE iontophoresis (200 \( \mu M \), 40 nA) caused a significant decrease in spontaneous activity (by \( 30 \pm 5.1 \% \), \( p = 0.001 \), \( t \) test) of the majority of neurons (70\%, \( n = 33/47 \)) of the BLA in control rats (Figure 2a). A smaller proportion of neurons (21\%, \( n = 10/47 \)) showed a significant increase in spontaneous activity (by \( 107 \pm 9.1 \% \), \( p = 0.005 \), \( t \) test). Neurons from rats exposed to 7 d chronic cold stress displayed responses to NE that were not significantly different from those observed in control rats (\( p = 0.35 \), \( \chi^2 \), Figure 2c). However, in rats exposed to 14 d chronic cold stress, significantly more neurons displayed excitatory responses to NE than in control rats, and significantly fewer neurons displayed inhibitory responses to NE than in control rats (\( p = 0.02 \), \( \chi^2 \), Figure 2b example neuron, Figure 2c group data). No differences in the magnitude of NE-induced inhibition or excitation were observed (data not shown).

A subset of neurons were antidromically activated and confirmed as projection neurons in control, 7-d cold-exposed, and 14-d cold-exposed rats (Figure 3a). In controls, NE significantly inhibited all projection neurons (\( n = 8/8 \), \( p = 0.002 \), \( t \) test, Figure 3b). In rats exposed to 14 d cold stress significantly different proportions of responses were seen (\( p = 0.05 \), \( \chi^2 \)). A portion of neurons were still inhibited by NE (57\%,
**Chronic cold alters NE modulation of evoked activity after 7 d and 14 d**

Electrical stimulation of EC caused orthodromic, excitatory responses in neurons of the BLA in both control and stressed rats (Figure 4a). Stress exposure did not alter baseline responses to EC stimulation (latency, spike probability, number of neurons per track, data not shown). In control rats iontophoresis of NE (10 nA), significantly decreased the spike probability of neurons in response to EC stimulation ($n=7/7$, $p=0.005$, t test, Figure 4b). In rats exposed to 14 d cold, NE iontophoresis (10 nA) resulted in NE-induced effects on EC-evoked activity that were significantly different from controls. In 14-d cold rats, NE decreased the spike probability of 56% of neurons in response to EC stimulation ($n=5/9$, $p=0.05$); however, in 44% of neurons NE increased the spike probability ($n=4/9$, $p=0.03$, Figure 4f). These alterations in the proportions of neurons showing inhibitory vs. excitatory effects were significantly different ($p=0.04$, $\chi^2$). In addition, after 14-d cold exposure, the magnitude of the overall NE-induced change was significantly decreased. In control rats, NE decreased spike probability by an average of $62\pm8\%$, which was significantly different from baseline EC-evoked activity ($p=0.005$, t test, Figure 4e). After 14 d cold, this dropped to an average decrease of $8\pm3\%$, which was not significantly different from baseline EC-evoked activity ($p=0.65$, t test), and was significantly less than the inhibition seen in controls ($p=0.001$, t test, Figure 4e). After 7-d cold stress, NE decreased spike probability in the majority of neurons ($n=6/8$), and therefore the proportions of neurons displaying excitation/inhibition were not significantly different between controls and 7-d cold-exposed rats ($p=0.03$, $\chi^2$, Figure 4f). However, whereas NE still inhibited EC-evoked activity in 7-d cold rats (by $25\pm4\%$, $p=0.02$, t test), the degree of inhibition was significantly less than in control rats ($p=0.03$, t test), and not in all neurons ($n=1/8$ neurons increased spike probability by $14\%$, $n=1/8$ neurons no change in evoked activity, Figure 4f).

Electrical stimulation of Te3 caused orthodromic, excitatory responses in BLA neurons in both control and stressed rats (Figure 4c). Stress exposure did not alter the nature of baseline responses to Te3 stimulation (latency, spike probability, number of neurons per track, data not shown). In control rats, NE iontophoresis significantly decreased the spike probability of neurons in response to Te3 stimulation ($n=8/8$, $p=0.002$, t test, Figure 4d). In rats exposed to 14 d
Figure 4. Noradrenergic modulation of evoked activity in basolateral amygdala (BLA) neurons is altered after 7 d and 14 d chronic cold exposure. (a) Electrophysiological trace of a BLA neuron displaying an excitatory, orthodromic spike in response to entorhinal cortical stimulation. (b) In control rats (–•–), BLA activity evoked by stimulation of entorhinal cortex (EC) was significantly decreased during microiontophoresis of norepinephrine (NE). In rats exposed to 7 d or 14 d cold, the inhibitory actions of NE were lessened (7 d, – –) and abolished (14 d, - -). (c) Electrophysiological trace of a BLA neuron displaying an excitatory, orthodromic spike in response to sensory association cortical stimulation. (d) In control rats (–•–), BLA activity evoked by stimulation of sensory association cortex (Te3) was significantly decreased during microiontophoresis of NE. In rats exposed to 7 d or 14 d cold, the inhibitory actions of NE on evoked activity were lessened (14 d, - - ) and abolished (7 d, – –). (e) Chronic cold exposure decreased the magnitude of NE-induced inhibition of Te3 and EC-evoked activity after 14 d exposure, but not 7 d. (f) Chronic cold exposure decreased the proportion of neurons that showed NE-induced inhibition of Te3 and EC-evoked activity after 7 d or 14 d cold exposure (■, control; □, 7 d; □, 14 d) (* p < 0.05).
cold, NE iontophoresis (10 nA) resulted in significant differences in the proportions of BLA neurons displaying inhibitory/excitatory effects of NE on Te3-evoked activity ($p=0.001$, $\chi^2$). In 14-d cold-exposed rats, NE iontophoresis (10 nA) significantly increased the spike probability in 71% of neurons in response to Te3 stimulation ($n=5/7$, $p=0.02$), whereas in 29% of neurons NE decreased the spike probability ($n=2/7$, $p=0.01$; Figure 4d, f). Cold exposure also decreased the magnitude of NE-induced inhibition of Te3-evoked activity of BLA neurons ($p=0.006$, $t$ test, Figure 4e). In control rats, NE significantly decreased spike probability by an average of $41\pm7\%$ from baseline. After 14 d cold exposure, this changed to an average increase of $2\pm8\%$ (not significant from baseline, $p=0.65$, $t$ test), which was significantly different than the NE-induced inhibition seen in controls ($p=0.006$, $t$ test). After 7-d cold exposure, NE decreased spike probability in 57% of neurons ($n=4/7$), but not to a significant degree (by $13\pm6\%$, $p=0.08$, $t$ test). There were significant differences in the proportions of neurons displaying inhibitory/excitatory effects on evoked activity to NE when 7-d cold rats were compared to controls ($p=0.04$, $\chi^2$, Figure 4f). There were no differences in the magnitude of NE-induced decrease of Te3-evoked activity in 7-d cold exposed rats vs. controls ($p=0.06$, $t$ test), although a trend was noted. One neuron did display increased spike probability (by 38%, $n=1/7$), with others displaying no significant change ($n=2/7$, Figure 4f).

**Discussion**

This study demonstrates that chronic stress induces alterations in the responses of BLA neurons to NE. Following chronic cold exposure, a significantly higher proportion of BLA neurons exhibited excitatory responses to NE iontophoresis, but only after 14 d exposure. Chronic cold exposure also decreases the inhibitory effects of NE on BLA neuronal activity evoked by stimulation of EC and Te3. Stress causes a facilitation of some afferent-evoked activity after 7 d exposure. This facilitatory effect of NE on evoked activity was more pronounced after 14-d cold exposure. Thus, chronic stress-induced alterations in the NE system (Gresch et al., 1994; Jedema et al., 1999; Jedema et al., 2001; Mana and Grace, 1997; Nisenbaum et al., 1991; Pacak et al., 1992) also impact the electrophysiological activity in NE target regions. This characterizes the BLA as an important site influencing sensitization of the stress response, and extends previous work examining other regions that may underlie pathological changes that occur as a result of stress exposure.

**Effects of stress on BLA neurons and their responses to NE**

Repeated administration of the same stressor leads to habituation of the stress response to that stressor. BLA neurons display a decreased response to footshock with repeated presentations (Shors, 1999). However, after repeated or chronic stress, portions of the stress response display sensitization, or a potentiated response, to novel stressors. This phenomenon is also displayed by BLA neurons. In control rats, footshock causes both excitatory and inhibitory responses in BLA spontaneous activity. However, after exposure to chronic cold stress, BLA neurons display only excitatory responses to this stimulus (Correll et al., 2005). The present study suggests a mechanism by which these effects may be occurring. During footshock, NE is increased in the BLA (Galvez et al., 1996; Hatfield et al., 1999; Williams et al., 1998). In control rats, BLA neurons display largely inhibitory responses to NE, with few neurons displaying excitatory responses. After chronic cold exposure, NE causes more excitatory responses in BLA neurons, with few neurons displaying inhibition. This increase in excitatory responses to NE may explain the increase in excitatory responses of BLA neurons to footshock following chronic cold exposure. The finding that this only happens with lengths of stress exposure that lead to sensitization (i.e. 14 d) suggests that this is the behaviourally relevant alteration underlying the sensitized response. However, we did see changes in NE modulation of evoked activity after 7 d, despite evidence examining NE efflux showing the sensitization of the NE system does not occur at 7 d exposure (Finlay et al., 1997). We suggest that alterations may be occurring at the receptor level on presynaptic glutamatergic terminals before they occur on post-synaptic BLA neurons. Alternatively, those studies examining sensitized NE efflux used microdialysis, which examines extracellular levels of NE. Smaller, more subtle changes that occur at the level of the synapse after 7 d may not be detectable with this technique. Furthermore, use of the combined extracellular recording/iontophoresis technique may also be unable to detect more subtle changes occurring that may be revealed with intracellular or in-vitro recordings. However, we felt the importance of examining these phenomena in the intact rat was critical to the interpretation of the relevance of these changes to behavioural and physiological consequences of stress.
Several potential mechanisms are suggested for the alteration in the effects of NE on BLA neuronal activity by chronic stress. First, after chronic stress, stress-induced NE efflux in terminal regions is enhanced (Gresch et al., 1994; Jedema et al., 1999; Nisenbaum et al., 1991; Pacak et al., 1992), which may lead to differential effects on BLA neuronal activity. Our data argue against this possibility. Dose-dependent effects of NE demonstrate increasing levels of inhibition with increasing doses of NE in the majority of BLA neurons. Furthermore, in a subset of neurons, excitatory responses were seen at low doses of NE, with inhibitory responses emerging at higher doses.

A second mechanism by which the neuronal actions of NE could be modified is via an alteration in the levels of NE receptors in the BLA. Chronic social stress decreases $\alpha_2$-receptor binding in the LC, suggesting a loss of NE-induced feedback inhibition on LC neurons (Flugge 1996; Meyer et al., 2000). Furthermore, chronic stress decreases $\alpha_2$-mediated inhibition of neurons in the LC via an increase in regulator of G-protein signalling-7 (Jedema et al., 2008). Chronic cold exposure increases sensitivity of $\alpha_2$ receptors in the hippocampus (Nisenbaum and Abercrombie, 1993). The LC and BLA may act in a coordinated manner to facilitate the stress response, whereas the hippocampus and prefrontal cortex play an inhibitory role (Herman et al., 1995, 1998). While no one has examined the effect of stress or NE on neurons in these regions specifically, some authors have examined the effects of stress on plasticity. Stress decreases plasticity in the prefrontal cortex (Maroun, 2006) and hippocampus, and causes dendritic atrophy in the hippocampus as well (Magarinos et al., 1996; Watanabe et al., 1992), while increasing plasticity and causing dendritic hypertrophy in the BLA (Mitra et al., 2005; Vouimba et al., 2004; Vyas et al., 2002). $\alpha_2$-receptor sensitization in the hippocampus decrease the inhibitory role of the hippocampus on stress reactivity, but desensitization enhances the facilitatory LC and BLA influence on the stress response. Such a scenario corresponds well with a sensitized response to stress following chronic exposure.

Changes in receptor affinity, G protein-mediated responses, or second-messenger systems may all also play a role in alterations of NE actions on neuronal activity. Repeated tail pinch or immobilization causes receptors to be less efficacious in producing intracellular cAMP responses (Bellavia and Gallara, 1998), possibly secondary to changes in regulators of G-protein signalling proteins (Jedema et al., 2008). However, one of these studies (Bellavia and Gallara, 1998) also demonstrates subsensitive $\beta$-receptors, and another found hypersensitive $\alpha_2$-receptors (Garcia-Vallejo et al., 1998) after chronic variable stress. This evidence complicates interpretation of the present results.

Previous studies have used firing rate and spike duration together to differentiate presumed projection neurons (low) from putative interneurons (high; Rosenkranz and Grace, 2001). However, considered alone, firing rate does not appear to be adequate to identify neuronal subtypes (Likhtik et al., 2006; Rosenkranz and Grace, 2001). Furthermore, these studies suggest that neurons that can be reliably identified as interneurons make up a very small population of BLA neurons. Given the large variation in firing rates, we used antidromic activation to precisely confirm neurons as projection neurons. While these neurons were inhibited in control and 7-d cold-exposed animals, a portion of them were excited in 14-d cold-exposed animals. Of course, this study cannot distinguish between a direct excitatory effect on projection neurons vs. one mediated by decreased NE-induced inhibition of GABAergic interneurons that synapse onto BLA pyramidal neurons.

Effects of stress on evoked responses

Prior to chronic stress, NE was found to inhibit BLA responses to EC and Te3 stimulation. However, after chronic stress exposure, some of these inputs are potentiated in the presence of NE. We anticipated NE may differentially affect sensory input (Te3) vs. higher cortical input (EC). However, this was not the case. During conditioning to aversive stimuli, it is possible that one or both of these inputs undergoes learning-related alterations. However, in the absence of conditioning, such changes are not relevant in control rats. Chronic stress-induced alterations may lead to non-specific potentiation of inputs. Indeed, chronic stress has been shown to increase dendritic spines within the BLA (Mitra et al., 2005), and enhance synaptic plasticity in BLA circuits, phenomena often associated with conditioning (Radley et al., 2006). If, after chronic stress exposure, inputs that were previously inhibited are potentiated, this may manifest itself behaviourally by producing rats that are hyper-responsive to stressful stimuli, as has been reported (Gresch et al., 1994; Jedema et al., 1999; Nisenbaum et al., 1991; Pacak et al., 1992). Furthermore, these rats may be poorer learners if mechanisms of plasticity have been disrupted. Rats exposed to chronic restraint or psychosocial stress demonstrate impairments in spatial and object memory (Conrad et al., 2003; Park et al., 2001).
Stress is associated with a reduction in GABAergic transmission in the amygdala. BLA pyramidal neurons are under tight inhibitory control by GABAergic synapses (Rainnie et al., 1991; Rosenkranz and Grace, 2002; Washburn and Moises, 1992; Woodson et al., 2000). However, immobilization and cold lead to decreased levels of glutamic acid decarboxylase and GABA in the brain (Otero Losado, 1988), and swim stress decreases GABA concentrations as well (Briones-Aranda et al., 2005). Restrain stress decreases GABAergic inhibitory control of the stress response by the amygdala (Martijena et al., 2002). GABAergic blockade in the BLA leads to anxiety responses (Sajdyk and Shekhar, 1997; Sanders and Shekhar, 1991), an effect also seen after restraint stress (Rodriguez-Manzanares et al., 2005). These data are consistent with disruption of NE-GABAergic circuits in the BLA by chronic stress.

NE afferents form asymmetrical synapses onto GABAergic interneurons of the BLA (Li et al., 2001). This could contribute to the inhibitory effects seen in control rats; i.e. NE-induced excitation of GABAergic interneurons that synapse onto glutamatergic pyramidal cells within the BLA (Muller et al., 2006). Stress disrupts normal NE-induced facilitation of GABAergic transmission in the BLA seen in control rats (Braga et al., 2004). Similar processes may be induced following cold exposure. Decreased GABAergic transmission could result from a desensitization or decrease in NE receptors present on these GABAergic interneurons. This would decrease GABA neuron excitation by NE, leading to less inhibition in BLA pyramidal neurons, an effect demonstrated in these data.

The present studies cannot rule out whether the changes in evoked responses occurred due to changes in amygdala neurons vs. an alteration in the afferent neurons in the EC or Te3. However, the data presented here strongly favour an action of chronic cold that is mediated by a change in BLA neurons. First, cold exposure did not affect baseline responses of BLA neurons to afferent stimulation: the current amplitude required to evoke an orthodromic BLA neuron spike, the number of responsive neurons per track, and the average current amplitude required to reach 50% spike probability were not different between control and cold-exposed animals. Furthermore, these cold-exposed changes were limited to NE modulation, and NE was iontophoresed directly onto BLA neurons, which support an intra-amygdala mechanism. Finally, the finding that the change in the response of the BLA neuron to stimulation of either a primary sensory afferent (Te3) or a limbic afferent (EC) was essentially identical strongly supports a chronic cold-induced alteration within BLA neurons themselves, rather than a common effect on two very different afferent structures. Nonetheless, it is certainly possible that alterations of glutamatergic afferent terminals targeting BLA neurons may have also contributed to the chronic cold-induced changes observed.

It should be noted that the present studies do not definitively distinguish between effects caused by 14-d cold exposure vs. effects caused by 7-d cold exposure that may have a delayed onset (revealed in the present study at 14 d). However, previous studies examining NE efflux in response to a stressor after 7-d or 14-d cold exposure show conclusively that acute stress-evoked NE efflux, as measured with microdialysis, was enhanced in animals exposed to 14-d but not 7-d cold exposure even when the animals exposed to 7 d cold were not evaluated until 14 d following a return to a normal environment (Finlay et al., 1997). Therefore, it is unlikely that the present results can be explained by effects that occur with 7 d exposure that have a delayed onset, and that 14-d cold exposure are required to induce the changes observed.

**Implications**

Extensive exposure to stress is often associated with the development and/or enhancement of pathology and disease. Indeed, post-traumatic stress disorder has been modelled as a severe pathological form of the sensitization of the stress response (Rau et al., 2006). Previous studies have suggested a potential involvement of the BLA in the circuit mediating the sensitization of the stress response. The importance of NE in the paraventricular nucleus of the hypothalamus, bed nucleus of the stria terminalis, and medial amygdala has been established (Ma and Morilak, 2005a, b). The present studies lend further support for an important role of the BLA in sensitization, and provide an electrophysiological mechanism by which NE could act in target regions to mediate such a condition. Identifying the structures and circuits that may undergo pathological changes during abnormal levels of stress, and the mechanisms underlying such changes, will promote an understanding of how such disorders develop, and aid in the design of potential treatments for such conditions.

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Statement of Interest
None.

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