NORADRENERGIC MODULATION OF THE BASOLATERAL AMYGDALA: ALTERATIONS BY STRESS EXPOSURE

by

Deanne M. Buffalari

Neuroscience, BS, Allegheny College, 1999

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This dissertation was presented

by

Deanne M. Buffalari

It was defended on

September 21, 2006

and approved by

Dr. Alan Sved

Dr. Bita Moghaddam

Dr. Linda Rinaman

Dr. Edda Thiels

Dr. Stephen Maren

Dr. Susan Sesack

Dissertation Advisor: Dr. Anthony A. Grace

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The behavioral consequences of norepinephrine (NE) in the basolateral amygdala (BLA) have long been well established. NE increases in the amygdala in response to the presentation of aversive stimuli, presumably due to an activation of locus coeruleus (LC) neurons that send NE efferents to the BLA. The following studies examine the electrophysiological consequences of alterations of the NE system on neuronal activity within the BLA.

Single unit recordings of neurons of the BLA were performed, and responses to systemic administration of the anxiogenic agent yohimbine were examined. Yohimbine had both excitatory and inhibitory effects on spontaneous and afferent-evoked neuronal activity of BLA neurons. This was accompanied by a yohimbine-induced increase in NE levels within the BLA, confirmed with microdialysis.

To more precisely examine the effects of NE within the BLA on neuronal activity, we used iontophoresis combined with single unit recordings of BLA neurons. NE directly applied to BLA neurons causes predominantly inhibitory effects. Spontaneous activity was inhibited, presumably via alpha-2 receptor mechanisms, while a smaller subset of neurons were excited via beta receptor actions. NE also inhibited afferent-evoked activity of BLA neurons. Footshock and LC stimulation each caused both excitatory and inhibitory effects on BLA neuronal activity;

those effects could be mimicked by NE iontophoresis. Therefore, NE effects are representative of those caused by aversive stimulus presentation (footshock), or by activation of LC neurons.

Chronic stress alters the activity of the NE system, the responsivity of BLA neurons, and behavioral consequences of NE on targets. Our final studies addressed whether chronic cold exposure (7 or 14 days, 5C) alters NE modulation of BLA neuronal activity. After 14 days of cold exposure, NE caused more excitation of spontaneous and afferent BLA neuron activity, in contrast to the NE-induced inhibition seen in control rats. Seven days of cold stress caused only moderate changes in NE modulation of evoked activity. These data demonstrate that prolonged stress alters the way in which NE affects neuronal activity in target regions. We suggest BLA neurons become hyperexcitable, and this pathology may underlie some of the behavioral deficits and symptoms associated with exposure to chronic stress.

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PREFACE

The completion of this body of work involved the support of many people in several areas of my life over the past years. Without those people, seeing my graduate career reach its completion would not have been possible.

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1.0 GENERAL INTRODUCTION

1.1 STRESS, STRESS RESPONSES AND BRAIN REGIONS INVOLVED IN STRESS

1.1.1 The clinical implications of stress

The severely detrimental effects of stress exposure on biological and neurological processes have been revealed through numerous clinical and pre-clinical studies. Many different types of exposure to stressful stimuli or situations influence the onset, development, and progression of many diseases. These include post-traumatic stress disorder, panic disorder, generalized anxiety disorder (Sullivan et al. 1999), phobias, depression, and schizophrenia (Bremner et al. 1996b, Southwick et al. 1999). In addition, stress plays a role in peripheral diseases such as hypertension (Pickering 1990) and asthma (Oh et al. 2004), as well as contributing to neurodegenerative diseases like Alzheimer's disease (Landfield and Eldridge 1991). Stress increases the likelihood of relapse to drug taking and drug seeking behaviors (Shaham and Stewart 1995). Whereas the body's response to stress is often adaptive and enables a quick response to threatening or harmful stimuli, the situations above describe the maladaptive consequences of stress exposure. Having a greater understanding of the neurophysiological, autonomic, and behavioral consequences of the stress response, including alterations caused by

chronic stress exposure, may enable prevention or treatment of the negative consequences of stress exposure.

1.1.2 A clarification of terms: stress and stress responses

The terms “stress” and “stress response” are used in an increasingly indiscriminate and ambiguous manner to refer to a multitude of scenarios vastly different in their characteristics and consequences. The most common definition of stress is some version of the following: any actual or perceived threat to the maintenance of homeostasis (Pacak and Palkovits 2001, Day et al. 2006). This is, at best, oversimplified, and growing controversy over the usefulness of the reinterpretation of the term homeostasis with terms such as allostasis and allostatic load (Goldstein and McEwen 2002) contribute to the confusion. Correspondingly, the term stress response is used to refer to any one or combination of the exceedingly complex set of neuroendocrine, cardiovascular, immune and autonomic changes seen upon exposure to stressful stimuli or contexts (Kopin 1995). The imprecise use of the term stress is also evident in scientific referral to “stress circuitry” as a general combination of brain regions that are all activated in response to any stressful stimulus, when in fact the brain regions activated in response to stressful stimuli depends on the type of stress, the severity of the stress, the stress duration, and the state of the animal upon stress exposure (Pacak 2000, Dayas et al. 2001, Morrison 2001, Pacak and Palkovits 2001).

1.1.3 Stress and the involvement of two regions of interest: the locus coeruleus and the amygdala

In order to focus the broad scope of the data and literature available regarding stress, this document will consider stress with regard to two main CNS nuclei: the locus coeruleus (LC) and the amygdala. Both regions have been heavily implicated in response to certain types of stressful stimuli and certain components of the stress response. The stressful stimuli most commonly shown to activate the LC neurons are exteroceptive stimuli, or more specifically, psychogenic stressors (Rasmussen and Jacobs 1986, Abercrombie and Jacobs 1987, Grant et al. 1988, Ceccatelli et al. 1989, Aston-Jones et al. 1991, Pezzone et al. 1993, Passerin et al. 2000). Examples of such stimuli include as footshock, immobilization or restraint, and pain. The amygdala shows activation to these types of stimuli as well (Cullinan et al. 1995, Rosen et al. 1998, Dayas and Day 1999, Chowdhury et al. 2000, Akirav et al. 2001, Dayas et al. 2001). Interoceptive stimuli (such as hypertension) fail to reliably activate the LC or the amygdala (Graham et al. 1995), and therefore are not relevant to the focus of these studies or this discussion.

One component of the stress response reliably activated by psychogenic stressors is the hypothalamic-pituitary-adrenal axis. The LC plays a strong role in modulation of HPA axis activation in response to stress, as does the amygdala (Bhatnagar et al. 2004). A second focus of this document is the behavioral response to stress, namely behaviors indicative of fear and/or anxiety. The amygdala and the LC are implicated in these types of behaviors as well (Walker et al. 2003).

These behavioral and neuroendocrine portions of the stress response are thought to be driven by catecholaminergic neurons of the brain stem, including the LC (Cunningham and Sawchenko 1988, Cunningham et al. 1990, Habib et al. 2001). The role of the LC in the stress response has been well established, and is thought to be primarily facilitatory (Plotsky 1987, Al-Damluki 1988, Ziegler et al. 1999, Sved et al. 2002). However, despite having a prominent role in stress-related behaviors, the LC has limited direct projections to the paraventricular nucleus of the hypothalamus (PVN), which is often considered one of the main output nuclei responsible for portions of the stress response (Sawchenko and Swanson 1981, 1982, Cunningham 1988). Therefore, the LC must exert its effects through the innervation of another stress-related structure. A strong candidate is the amygdala, which also heavily influences many aspects of the stress response in a facilitatory manner (Feldman et al. 1995, Van de Kar and Blair 1999, Herman et al. 2003). Presentation of different stressful stimuli activates the amygdala (Rosen et al. 1998, Akirav et al. 2001, Dayas et al. 2001), and stimulation of the amygdala causes stress-related responses and behaviors (Saldivar-Gonzalez et al. 2003). Inactivation or lesions of the amygdala eliminate many aspects of the stress response (Feldman et al. 1981, Galeno et al. 1984, Van de Kar et al. 1991, Roozendaal et al. 1992, Sanders et al. 1994, Marcilhac and Siaud 1996, Strauss et al. 2003, Bhatnagar et al. 2004).

The LC sends a large afferent input to the amygdala (Asan 1998). Despite the widespread involvement of the central nuclei of the amygdala (CeA) in autonomic and behavioral aspects of the stress response, it is the basolateral complex (BLA) that receives the majority of LC input. Therefore, the majority of LC actions on the amygdala must be at the level of the BLA. The BLA could easily exert its effects through two main sets of projections: to the CeA, or to the bed nucleus of the stria terminalis (BNST, Figure 1). The BLA has dense

efferents to the CeA (Pitkanen et al. 1997), which projects to many downstream structures including the PVN (Gray et al. 1989), the bed nucleus of the stria terminalis (BNST, Walker et al. 2003), and the LC (Van Bockstaele et al. 2001). The role of the LC in the stress response is expanded upon below. The CeA projection to the LC mediates LC activation to certain types of stressful stimuli (Curtis et al. 2002). The BLA also has direct projections to the BNST which could subserve stress and anxiety responses (Walker et al. 2003).

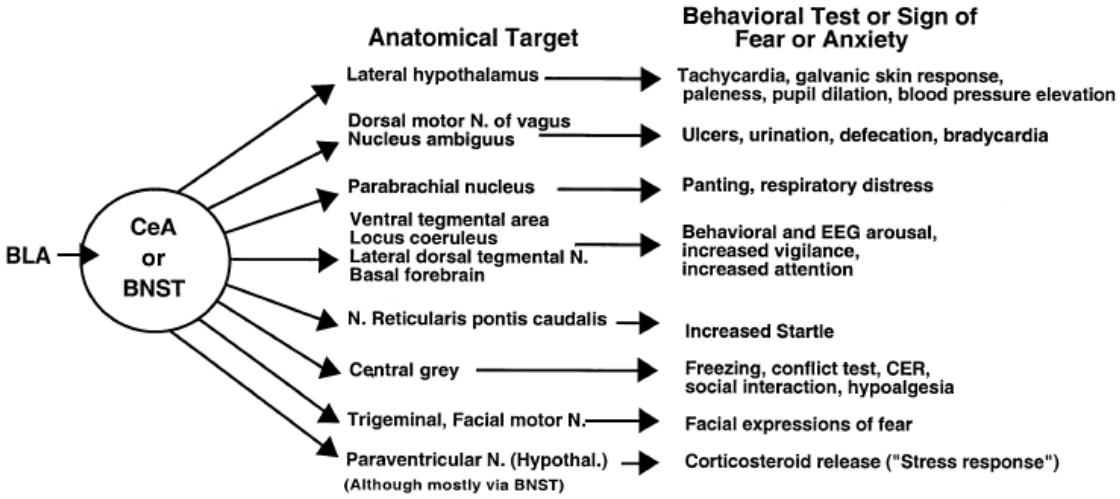


Figure 1 BLA projections to CeA and BNST influence fear and anxiety behavior

Despite the mutual importance of the BLA and the LC in the stress response, the influence of the NE projection arising from the LC that targets the BLA has been virtually ignored in stress-related research. The studies described in this thesis examine the effects of alterations of the NE system on the physiology of the BLA. The investigation will include how the effects of NE on the BLA are altered after chronic cold exposure.

1.2 THE LOCUS COERULEUS, NOREPINEPHRINE AND STRESS

1.2.1 The locus coeruleus: anatomy and physiology

The LC has been extensively examined as the largest source of NE in the brain, and the largest NE-containing cell group in the central nervous system (Moore and Bloom 1979, Pfister and Danner 1980, Foote et al. 1983, Moore and Card 1984). The LC is a bilateral structure located in the brainstem next to the fourth ventricle, and in the rat contains a mere 1600 neurons per side (Swanson 1976). However small, this group of neurons sends projections to the entire neuraxis, with each axon dividing into many collaterals targeting regions such as the hypothalamus, the amygdala, the thalamus, and the cortex (Swanson et al. 1976, Moore and Bloom 1979, Foote et al. 1983, Moore and Card 1984). These axons terminate in target regions and form small synaptic contacts (Olschowka et al. 1981, Papadopoulous et al. 1989, Parnavelas and Papadopoulous 1989). This extensive collateralization creates a system whereby activation of small numbers of LC neurons leads to nonspecific, coincident NE increases in many terminal areas.

The neurons of the LC contain tyrosine hydroxylase (TH) and dopamine-beta-hydroxylase (DBH), the enzymes necessary to synthesize NE, and therefore are all considered to be noradrenergic (Swanson et al. 1976, Grzanna and Molliver 1980). However, there are also neuropeptides colocalized in these neurons, including galanin and neuropeptide Y (Melander et al. 1986, Holets et al. 1988, Austin et al. 1990), which may also be released with LC activation. Upon release, the actions of NE are terminated by the norepinephrine transporter (NET), which

clears NE from the extracellular space (Barker and Blakely 1995, Blakely and Bauman 2000). Activation of presynaptic alpha-2a or alpha-2c autoreceptors also has an inhibitory influence on NE release. These autoreceptors cause a guanine-D-phosphate-binding-protein (Gprotein)-mediated hyperpolarization of LC neurons via opening of potassium (K+) channels, leading to decreased NE release (Grigg et al. 1996, Arima et al. 1998).

1.2.2 Effects of norepinephrine on neuronal activity

The effects of NE on neuronal activity are diverse and complex. The majority of studies document NE having inhibitory effects on spontaneous activity in various regions. These areas include the hippocampus (Curet and de Montigny 1988, Madison and Nicoll 1988, Bergles et al. 1996), the cerebellum (Freedman et al. 1977), the PVN (Chong et al. 2004), the red nucleus (Ciranna et al. 2000), and the medial septal nucleus (Alreja et al. 1996). Neurons of sensorimotor cortex (Bennett et al. 1998), superficial layers of cerebral cortex (Bevan et al. 1977), auditory cortex (Foote et al. 1975), and prelimbic prefrontal cortex (Kovacs and Hernandi 2002) are all also inhibited by NE. Electrical stimulation of the LC (which presumably increases NE in the hippocampus and cerebellum) has similar inhibitory effects on cellular activity in these regions (Hoffer et al. 1973, Segal and Bloom 1974, Loy et al. 1980), while destruction of these ascending NE projections causes excitation.

NE also has excitatory effects on spontaneous neuronal activity, though to a lesser degree than its inhibitory actions. NE depolarizes brainstem-projecting cortical neurons (Wang and McCormick 1993), hippocampal interneurons (Bergles et al. 1996), and lateral dorsal tegmental neurons (Kolheimer et al. 1999). Neurons of the subthalamic nucleus are excited by NE (Arcos

et al. 2003), as are interneurons of the piriform cortex (Marek and Aghajanian 1996). NE decreases GABAergic IPSPs in histaminergic neurons (Stevens et al. 2004), as well as in the PVN (Han et al. 2002, Chong et al. 2004).

Beyond simple inhibitory or excitatory effects on spontaneous activity, NE plays a large role in the modulation of neuronal evoked activity. Many studies document the function of NE as decreasing spontaneous activity, while simultaneously either increasing or decreasing evoked activity in the same neurons (Foote et al. 1975, Segal and Bloom 1976, Waterhouse et al. 1980, 1984, 1998, 2000, Waterhouse and Woodward 1980, Doze et al. 1991, Devilbiss and Waterhouse 2000), effectively altering the signal-to-noise ratio in target brain regions. As a result, NE has been described as a "gating mechanism" (Devilbiss and Waterhouse 2000), or "gain modulator", (Aston-Jones and Cohen 2005), focusing on the functional importance of electrophysiological actions. Therefore, our studies will examine the responses of BLA spontaneous neuronal activity to NE, but will also examine how NE affects BLA neuronal activity evoked by stimulation of afferents from entorhinal cortex (EC) and sensory association cortex (Te3).

1.2.3 Noradrenergic receptors

The variability of NE's electrophysiological effects is explained, in part, by the diversity of the receptor subtypes that it targets and their downstream effector systems. NE has its effects via nine different receptor subtypes that have been identified in the nervous system. These receptors are divided into three main classes: alpha-1 receptors including the alpha-1a, alpha-1b, and alpha-1d subtypes, alpha-2 receptors including the alpha-2a, alpha-2b, and alpha-2c subtypes, and beta receptors including the beta-1, beta-2, and beta-3 subtypes (Bylund et al. 1994,

Wozniak et al. 1995). All of these receptors are metabotropic, linked to a different Gprotein and consequently different second messenger systems. Alpha-1 receptors activate Gq proteins and stimulate phospholipase C, leading to intracellular calcium mobilization. Alpha-2 receptors are primarily inhibitory, activating inhibitory Gi proteins. This leads to a decrease in adenylyl cyclase and increases the opening of K+ channels (Beitner 1989, Grigg 1996, Arima 1998), an inhibitory result. Conversely, beta-receptors are typically excitatory and positively linked to adenylyl cyclase and cyclic-AMP via activation of Gs proteins (Pfeuffer 1977, Ross et al. 1978).

Evidence for the presence of all of these receptor classes has been found in the BLA. Different subtypes are more heavily localized to some nuclei over others. Of the alpha-1 receptors, 1a receptors are present in low to moderate levels in the entire BLA (Day et al. 1997). Alpha-1b receptors are low in all nuclei except for the lateral nucleus (LAT), where they are present in high quantities. This pattern is similar for alpha 1d receptors which are high in the lateral nucleus but present in only moderate levels in the basolateral (BL) and basomedial (BM) nuclei. Alpha-2 receptors have a somewhat reversed distribution; alpha-2a are moderate in the BL and BM but very low in the LAT (Nicholas et al. 1993, Talley et al. 1996,), while alpha-2c receptors are also very low in the LAT but even lower in BM, with moderate levels in BL (Rosin et al. 1996). Of the beta-receptors, the localization of beta-3 receptors has yet to be examined. Beta-1 and beta-2 are present in all three subnuclei of the BLA; each contains slightly more beta-1 receptors than beta-2 (Rainbow et al. 1984). Important to note is that the majority of studies examining receptors in the BLA are binding studies, which specify only that these types of receptors are present in the BLA complex. Receptors could be present on afferent noradrenergic terminals (autoreceptors), other axons invading the BLA (presynaptic heteroceptors), or on BLA neurons themselves. Only alpha-2a and alpha-2c have been examined

with mRNA studies, which indicates those types of receptors are certainly made by BLA neurons themselves, and not just present on afferent terminals.

In the BLA, the actions of NE at these receptors are complex. NE has been found to enhance excitatory neurotransmission via beta-receptors (Gean et al. 1992, Huang et al. 1996, Ferry et al. 1997), while producing inhibitory effects via alpha-2 receptors (Ferry et al. 1997). NE also reduces synaptic transmission in the BLA via actions at alpha-2 receptors (DeBock et al. 2003). Inhibitory, GABAergic transmission is facilitated by NE acting on alpha-1a receptors (Braga et al. 2004). NE also increases baseline firing rates of BLA neurons in vitro (Huang et al. 1994), and selectively enhances NMDA currents via actions at beta receptors (Huang et al. 1998a). Furthermore, it has been suggested that beta-receptors may function presynaptically in the BLA, causing excitatory effects and enhancing presynaptic calcium influx and transmitter release (Huang et al. 1996). However, the above studies examining the effects of NE on BLA neuronal activity have been performed exclusively in vitro. Therefore, the experiments outlined in this proposal will focus on the effects of NE on BLA neuronal activity measured in an in vivo preparation, allowing for an examination in the intact animal.

1.2.4 The locus coeruleus: role in acute stress

The excitatory effects of acute stress exposure on the noradrenergic system of the brain have been studied extensively (for review see Stanford 1995, Zigmond et al. 1995, Bremner et al. 1996a, 1996b, Sved et al. 2002, Morilak et al. 2005). Acute stress exposure causes increases in cfos in the LC (Ceccatelli et al. 1989, Pezzone et al. 1993, Passerin et al. 2000), and increased firing of LC neurons (Rasmussen and Jacobs 1986, Abercrombie and Jacobs 1987, Grant et al.

1988, Aston-Jones et al. 1991). Stress also increases NE efflux in terminal regions (Abercrombie 1988, Rossetti et al. 1990, Cenci et al. 1992, Nakane et al. 1994), and causes an upregulation of NE enzymes leading to the increased potential for NE synthesis (Serova et al. 1999, Chang et al. 2000). The behavioral effects of LC activation seem to mimic the effects of acute stress exposure, while lesions of the LC lead to loss or dampening of stress-induced neuroendocrine and behavioral responses (Redmond 1987, Ziegler et al. 1999).

1.2.5 The locus coeruleus: role in chronic stress

Chronic or repeated stress exposure causes a more complex, less well understood effect on the NE system of the brain than the more obvious excitatory effects of acute stress exposure (Abercrombie et al. 1992, Stanford et al. 1993, 1995, Zigmond et al. 1995, Morilak et al. 2005). Repeated footshock, tailshock, cold exposure or immobilization stress cause increased tissue levels of NE (Ingenito and Bonnycastle 1967, Thiery et. al 1968, Bhagat 1969, Adell et al. 1988). The basal activity of TH and DBH increases after chronic or repeated stress exposure (Thoenen et al. 1970, Zigmond and Harvey 1970, Melia and Duman 1991, Melia et al. 1992, Nisenbaum and Abercrombie 1992, Gordon et al. 1996, Serova et al. 1999). While some studies report an increased basal firing rate in LC neurons (Pavcovich et al. 1990, Mana and Grace 1997), other studies report no change after chronic stress (Simson and Weiss 1988, Curtis et al. 1995). However, chronic cold exposure does cause alterations in the electrophysiological characteristics of LC neurons (Jedema and Grace 2003).

Two interesting phenomena have been described after repeated or chronic stress exposure: habituation and sensitization of many components of the stress response. While

habituation implies a dampening of the autonomic, neuroendocrine and behavioral responses caused by stress with repeated exposure, sensitization describes an exaggeration of such responses after repeated exposure. Evidence exists for both scenarios. Hippocampal levels of NE and serotonin habituate quickly to repeated footshock; however, plasma cortisol levels sensitize (Hajos-Korcsok et al. 2003). While baseline levels of NE in terminal regions are unaltered after chronic stress exposure (Nisenbaum et al. 1991, Gresch et al. 1994, Terrazzino et al. 1995), stress-induced NE efflux is enhanced following chronic stress (Nisenbaum et al. 1991, Pacak et al. 1992, Gresch et al. 1994, Jedema et al. 1999), as is the stress-induced increase in TH or TH mRNA (Nisenbaum et al. 1991, Nisenbaum and Abercrombie 1992, Rusnak et al. 2001). In addition, prior chronic exposure to tailshock, footshock, and cold cause an enhanced response of LC neurons to excitatory input, footshock or hypotensive challenge (Simson and Weiss 1988, Curtis et al. 1995, Mana and Grace 1997). LC neurons also display a sensitized excitatory response to CRH after chronic cold exposure (Jedema and Grace 2001) and repeated footshock (Curtis et al. 1995).

1.2.6 The role of norepinephrine in target regions and sensitization of the stress response

Despite extensive knowledge of NE and LC responsivity to both acute and chronic stress, far less is known about alterations that may occur as a result of stress exposure in NE targets. As acute stressors cause increases in NE in terminal regions, and chronic stress sensitizes that response, it is likely that this may cause alterations in basal and stress-evoked activity in targets of NE projections. Stress-induced release of NE has been shown to facilitate activation of the HPA axis to acute immobilization, an effect that is specific to the BST (Cecchi et al. 2002) and the medial

amygdala (Ma and Morilak 2005a). More recent work has extended these findings to include the PVN as a site where NE has a facilitatory effect on HPA sensitization after chronic immobilization (Ma and Morilak 2005b). Therefore, in addition to alterations in the NE neurons themselves, alterations in the actions of NE at postsynaptic targets play a role in the behavioral effects seen after chronic stress. It has been suggested that the BLA may also play a role in the circuit responsible for the sensitization of the stress response after chronic stress as well (Bhatnagar and Dallman 1998). We will investigate the role of the BLA as a possible site modulating sensitization of the stress response by examining whether NE modulation of BLA neuronal activity is altered after rats have been exposed to chronic cold exposure.

One way in which NE effects on postsynaptic targets could be altered is through changes in NE receptors at terminal sites, which readily occurs during exposure to stress and manipulations of the NE system. This could include changes in receptor number, changes in the affinity of receptors for NE, or changes in the way in which receptors produce alterations in second messenger cascades. Evidence for all of these changes exists (Stone et al. 1983).

Acute presentation of many stressful stimuli causes changes in receptor number. These include a single footshock (Cohen et al. 1986) or a single exposure to a novel environment (Stanford and Waugh 1986) which lead to increased alpha-2 receptors in the cortex, while a single immobilization period causes an increase in alpha-2 receptors in the midbrain (U Prichard and Kvetnansky 1980). Single footshock also leads to a decrease in beta-receptors in the hypothalamus (Glavin et al. 1983); single immobilization stress causes the same in the cerebellum (U Prichard and Kvetnansky 1980). Acute novelty exposure leads to an increase in beta-receptors in the cortex (Stanford and Waugh 1986).

More variable effects on adrenergic receptor number have been reported with repeated or chronic stressors. Repeated immobilization, handling, variable stress, and tailshock all lead to a decrease in cortical beta-receptors (U Prichard and Kvetnansky 1980, Nomura et al. 1981, Stone and Platt 1982, Stanford et al. 1984, Yamanaka et al. 1987, Basso et al. 1993), as does food deprivation (Stone 1983). Beta-receptors in the hypothalamus are also decreased after repeated immobilization (Torda et al. 1981, Stone and Platt 1982), but increased after repeated footshock (Cohen et al. 1986). Repeated immobilization also decreases beta-receptors in the brainstem (Torda et al. 1981, Stone and Platt 1982), the cerebellum (UPrichard and Kvetnansky 1980), and the pineal body (Yocca and Friedman 1984). Chronic mild unpredictable stress causes decreased cortical beta-receptors as well (Papp et al. 1994). Alpha-1 receptors seem to be much more resilient in terms of their response to stress, showing no change after repeated immobilization or footshock (Stone 1981, Lynch et al. 1983, Cohen et al. 1986). However, alpha-2 receptors show variable effects, with increases in the brainstem reported in response to repeated immobilization (Torda et al. 1981) and repeated footshock (Cohen et al. 1986), while other groups report decreases in alpha-2 receptors in the cortex in response to very similar stressors (Stone 1981, Lynch et al. 1983, but see Cohen et al. 1986). Repeated handling also caused a decrease of alpha-2 receptors in the cortex (Stanford et al. 1984). This indicates the response of the NE is complex in terms of not only specificity of individual receptor response, but also location of those responses. Little is known about whether or not such changes occur in the BLA.

Receptor sensitivity and receptor signaling of intracellular cascades are altered after stress exposure. Repeated handling stress decreases the cyclic-AMP response to beta-receptor stimulation (Baamonde et al. 2002), while repeated tail pinch causes desensitization of alpha-receptors (Gomez et al. 1998). Repeated immobilization stress results in desensitized beta and

alpha2 receptors (Bellavia and Gallara 1998); restraint stress that is repeated daily leads to alpha-1 receptor desensitization and reduced cyclic-AMP response to receptor stimulation (Stone 1987). One conflicting study reports alpha-2 receptor hypersensitivity after chronic variable stress (Garcia-Vallejo et al. 1998). This difference may be due to the nature of the stressor. Any combination of the above effects could lead to alterations in the way target neurons respond to NE.

1.3 THE LOCUS COERULEUS SENDS AFFERENTS TO THE AMYGDALA: IMPORTANCE IN STRESS

1.3.1 The amygdala: anatomy and physiology

The amygdala is comprised of several distinct nuclei, with separate yet highly integrated functions. The different nuclei have distinct cytoarchitectonic and neurochemical features, as well as segregated anatomical connections. The focus here is on the BLA, which includes the lateral, basolateral, and basomedial nuclei, as these nuclei get the majority of the NE input from the LC (Asan et al. 1998). The BLA is said to be more cortical in nature than other nuclei of the amygdala. Its neurons and structure are more similar to cortical regions, than the CeA, for example, which is considered to be more striatal-like (Carlsen and Heimer 1988, McDonald and Pearson 1989, McDonald 1992). Two primary neuron types have been characterized in the BLA anatomically: large, glutamatergic pyramidal projection neurons, and different subgroups of

smaller, GABAergic, local circuit interneurons (McDonald 1982, 1985, 1992a, 1992b, 1996, McDonald and Pearson 1989, McDonald and Betette 2001). These GABAergic interneurons also label for acetylcholine, somatostatin, neuropeptide Y, vasoactive intestinal peptide, and cholecystokinin (McDonald 1989, McDonald and Pearson 1989), and are divided into at least four subclasses based on their neuropeptide-containing characteristics (McDonald and Betette 2001, McDonald 2001, Mascagni and McDonald 2003, Muller et al. 2003). Furthermore, these interneurons can be subdivided via an examination of their anatomical connectivity (McDonald and Bette 2001, McDonald and Mascagni 2002, McDonald et al. 2002, Muller et al. 2003a, b). These anatomical studies delineating neuronal subtypes in the BLA have been confirmed by electrophysiological studies (Millhouse and DeOlmos 1983, Washburn and Moises 1992, Rainnie et al. 1993, Pare and Gaudreau 1996, Rosenkranz and Grace 1999).

The intra-amygdala circuitry is extensive. Therefore, we will limit discussion to that of the BLA. Extensive interconnections link the BLA nuclei to one another, as well as to other nuclei within the amygdala (Pitkanen et al. 1997, Pitkanen 2000). Strong projections exist from the lateral nucleus to the basolateral and basomedial nuclei (Aggleton 1985, Pitkanen et al. 1995, 1997, Savander et al. 1995, 1996, 1997, Pitkanen 2000), and these projections are reciprocal (Pitkanen et al. 1997, Savander et al. 1997, Pitkanen 2000). These regions send projections on to the CeA (Pitkanen et al. 1997, Pitkanen 2000), which is typically considered the output region of the amygdala, supplying diverse efferent projections discussed in the following section (Gray and Magnuson 1987, Petrovich and Swanson 1997, Van de Kar and Blair 1999, Zahm et al. 1999). The CeA also sends input back to the BLA, though to a lesser degree (Pitkanen et al. 1997, Pitkanen 2000), and targeting only the basolateral nucleus (Jolkkenen and Pitkanen 1998).

1.3.2 The amygdala: afferent and efferent connections

The BLA receives a large amount of input from sensory regions, sensory association cortex, and thalamic nuclei. This input is thought to drive activity of the BLA and therefore amygdala-mediated behaviors in response to sensory stimuli (Cassell and Wright 1986, Van Vulpen et al. 1989, Arnault and Roger 1990, Turner et al. 1991, Rosen et al. 1992, Edeline et al. 1993, Mascagni 1993, Romanski et al. 1993, Campeau and Davis 1995, Li et al. 1996, Armony et al. 1997, Shi and Cassell 1997, 1998a, 1998b, 1999, McDonald 1998, Farb and Ledoux 1999, Poremba and Gabriel 1999). The BLA also receives dense innervation from dopaminergic neurons (Asan 1998, Brinley-Reed and McDonald 1999), serotonergic neurons (Moore et al. 1978), and NE neurons (Asan 1998). These catecholaminergic inputs may provide information about the valence of the sensory stimulus. For these experiments we will examine afferent inputs from the EC, Te3, and LC. The EC sends dense projections to the BLA (McDonald and Mascagni 1997) which are excitatory in nature (Brothers and Finch 1985). The BLA also receives afferents from Te3 (McDonald 1998) which cause excitatory responses in BLA neurons when activated (Rosenkranz and Grace 1999). Finally, the NE input from the LC to the BLA (Asan 1998) has been discussed in detail in previous sections.

The BLA has extensive efferent projections and can influence activity in regions such as the thalamus and prefrontal cortex (Holland 1993, Gallagher and Holland 1994, Kapp 1994, Shinonaga et al. 1994, Davis and Whalen 2001), sensory association cortex (Petrovich et al.. 1996, Poremba and Gabriel 1997, Pikkarainen and Pitkanen 2001), the hippocampus (Pikkarainen et al. 1999) and the nucleus accumbens (McDonald 1991, Mogenson and Yang

1991, Brog 1993, Johnson 1994, Shinonaga et al. 1994, Kirouac and Ganguly 1995, Alheid and Heimer 1996, Petrovich et al. 1996, Wright and Groenewegen 1996, Zahm et al. 1999). The BLA can also easily influence autonomic and brainstem targets through its projections to the CeA and the BNST (as discussed previously). In this way, the amygdala is positioned to collect a variety of sensory and processed cortical information, and influence many target regions that influence autonomic, motor, emotional, learning, and other behavioral responses.

1.3.3 Presentation of stressful stimuli or contexts activates the amygdala

The amygdala, like the LC, plays a prominent role in the behavioral, physiological, and autonomic responses to stress. The presentation of stressful stimuli or situations causes activation of the amygdala. In humans, the amygdala is activated during passive viewing or reading of emotionally evocative stimuli. It has been suggested the amygdala contributes to the pathology of post-traumatic stress disorder (PTSD, Rauch et al. 2000). Patients show an exaggerated response to emotional stimuli, and the amygdala might have an abnormal influence over downstream structures in PTSD (Gilboa et al. 2004). Increased amygdala activity is also associated with feelings of anxiety (Adolphs et al. 2001, Baxter and Murray 2002, Anand and Shekhar 2003). In rats, the amygdala shows cfos activation after noise, hemorrhage, immune challenge, restraint, swim, predator, social, cold water, and footshock stress (Cullinan et al. 1995, Rosen et al. 1998, Dayas and Day 1999, Chowdhury et al. 2000, Akirav et al. 2001, Dayas et al. 2001). Retrieval of aversive fear memories causes cyclic-adenosine-monophosphate-response-element-binding protein activation and cfos expression in the BLA (Hall et al. 2001). Extracellular signal-regulated protein kinase (ERK) phosphorylation is increased in the BLA

during performance of the cold water version of the spatial water maze, which is correlated with increased cortisol release (Akirav et al. 2001).

1.3.4 Stimulation of the amygdala elicits signs of stress, lesions disrupt aspects of the stress response

Stimulation of the amygdala leads to increases in autonomic responses like heart rate and blood pressure (Stock et al. 1978, Galeno and Brody 1983, Iwata et al. 1987, Sanders and Shekhar 1991, 1995b, Aslan et al. 1997, Sajdyk and Shekhar 1997, Tellioglu et al. 1997), increases in plasma corticosterone (Mason 1959, Setekleiv et al. 1961, Feldman et al. 1982, 1990, Weidenfeld et al. 1997), and behavioral responses such as immobility (Kaada 1951, Roozendaal et al. 1992b). However, other stimulation data is less clear, as studies show decreases in blood pressure and heart rate after amygdala stimulation as well (Kapp et al. 1982, Galeno and Brody 1983, Gelsema et al. 1987, 1989, Roder et al. 1999).

Amygdala lesions also cause alterations in aspects of the stress response. Lesions of the amygdala cause a decrease in many of the hormonal measures of the stress response, including decreased ACTH secretion in response to stress (Feldman and Conforti 1981, Beaulieu et al. 1986, 1987, Feldman et al. 1994, Marcilhac and Siaud 1996), and decreased stress-induced corticosterone secretion (Feldman and Conforti 1981, Van de Kar et al. 1991, Roozendall et al. 1992). Such lesions also decrease behavioral measures of fear and anxiety. Freezing to cues or contexts paired with shock is reduced or eliminated in animals with amygdala damage (Roozendaal et al. 1991, Helmstetter et al. 1992a, Helmstetter 1992b, Phillips and Ledoux 1992, Helmstetter and Bellgowan 1994, Goldstein et al. 1996, Muller et al. 1997, Maren 1998).

Amygdala lesions also eliminate avoidance of cues or contexts naturally aversive or conditioned to be aversive (Pellegrino 1968, Grossman et al. 1975, Cahill and McGaugh 1990, Killcross et al. 1997, Bueno et al. 2005). Decreases in the facilitation of startle amplitude normally seen after exposure to stressors are eliminated in amygdala-lesioned rats (Hitchcock et al. 1989, Sananes and David 1992, Lee et al. 1996, Schanbacher et al. 1996, McNish et al. 1997). Excitotoxic lesions of the BLA also inhibit the HPA response to acute and chronic stressors (Bhatnagar et al. 2004). The BLA has been implicated in hypoalgesia and defensive freezing responses, and responses caused by exposure to aversive heat stimuli (Helmstetter and Bellgowan 1993). NMDA antagonism within the BLA blocks stress-induced enhancements in eye blink conditioning (Shors and Mathew 1998).

Stimulation of the amygdala via increased hormones and releasing factors associated with the stress response can affect behavioral measures of stress as well. Systemic administration of dexamethasone enhances performance on tasks involving footshock stress, an effect blocked by lesions of the BLA (Roozendaal et al. 1996). Glucocorticoid agonist administration directly into the BLA enhances performance on these same tasks involving aversive stimuli (Roozendaal and McGaugh 1997). Intra-amygdala corticosterone increases baseline plasma corticosterone levels, and also enhances the corticosterone response to stressful stimuli (Shepard et al. 2003), while CRH antagonists in the BLA decrease anxiety related behaviors (Robison et al. 2004). Administration of urocortin-1, a CRH agonist, into the amygdala causes anxiety-like behaviors as well (Spiga et al. 2006). Acute restraint stress increases CRH binding protein expression in the BLA (Lombardo 2001), and CRH blockade in the BLA impairs performance on inhibitory avoidance learning (Roozendaal et al. 2002). The BLA contains a high density of postsynaptic glucocorticoid receptors on both pyramidal neurons and interneurons (Johnson et al. 2005).

1.3.5 The amygdala is important for learning that involves stressful stimuli

There is extensive evidence for an involvement of the BLA in the modulation of memory by stress and stressful conditions (McGaugh and Roozendaal 2002). The BLA is important for many types of learning that involve aversive conditioning and learning (Maren 2003). Fear-potentiated startle relies heavily on the amygdala (Davis et al. 1993), as does Pavlovian fear conditioning (Maren 2001, Schafe et al. 2001), and memory for inhibitory avoidance (Liang et al. 1994). The encoding of fear memories is thought to rely on neurons in the lateral amygdala (Maren and Quirk 2004, Schafe et al. 2005). Several reports indicate that fear conditioning leads to a facilitation of synaptic transmission in the amygdala (Rogan et al. 1997, Blair et al. 2001, Goosens and Maren 2002, Tsvetkov et al. 2002, Dityatev and Bolshakov 2005, Rodriguez Manzares et al. 2005, Schroeder and Shinnick-Gallagher 2005). As NE rises in the amygdala during exposure to aversive stimuli, it may play a role in the plasticity that occurs in these circuits, and these circuits may be susceptible to exacerbated or unusual levels of stress exposure.

1.3.6 Stress-induced alterations in amygdala plasticity and morphology

Plasticity in the BLA is altered upon exposure to stress as well. Exposure to predators (Vouimba et al. 2006), placement on an elevated platform (Adamec et al. 2001, Vouimba et al. 2004), uncontrollable water stress (Kavashansky et al. 2006), and periods of restraint (Rodriguez-Manzanares et al. 2005) all facilitate plasticity within the BLA. However, some decreases in BLA plasticity after stress exposure have also been shown (Adamec et al. 2001, Kavashansky et al. 2006), demonstrating that the effect may depend on the nature or the severity of the stressful

stimulus. Neurons of the BLA are also sensitive to stressful stimuli. Acute foot-shock causes both short (Correll et al. 2005) and long latency (Pelletier et al. 2005) increases in the activity of BLA neurons, and inhibitory responses as well (Shors 1999, Correll et al. 2005). Repeated stress exposure dampens these inhibitory responses (Shors 1999), while chronic cold exposure eliminates inhibitory, but preserves excitatory responses to footshock (Correll et al. 2005). These alterations in firing rate may be due in part to increases in levels of NE within the BLA upon acute stressor exposure, and sensitization of the NE response with repeated or chronic stress administration.

Stress also alters dendritic morphology of BLA neurons. Chronic unpredictable stress causes an atrophy of dendrites in bipolar neurons of the BLA (Vyas et al. 2002). Chronic immobilization stress causes a large increase in spine density on BLA pyramidal neurons and stellate neurons; however, acute immobilization stress leads to a delayed development of new BLA neuronal spines (Vyas et al. 2002, Mitra et al. 2005). Such studies suggest that neurons of the amygdala that undergo hypertrophy may be hyperresponsive to afferent inputs. This may result in a disruption of the ability to further enhance transmission through these circuits, which could translate into difficulties in learning behaviors in two potential ways: undesirable associations made as a result of the potentiation that occurs in these circuits (such as in PTSD), or a decreased ability for the forming of new associations (learning impairments).

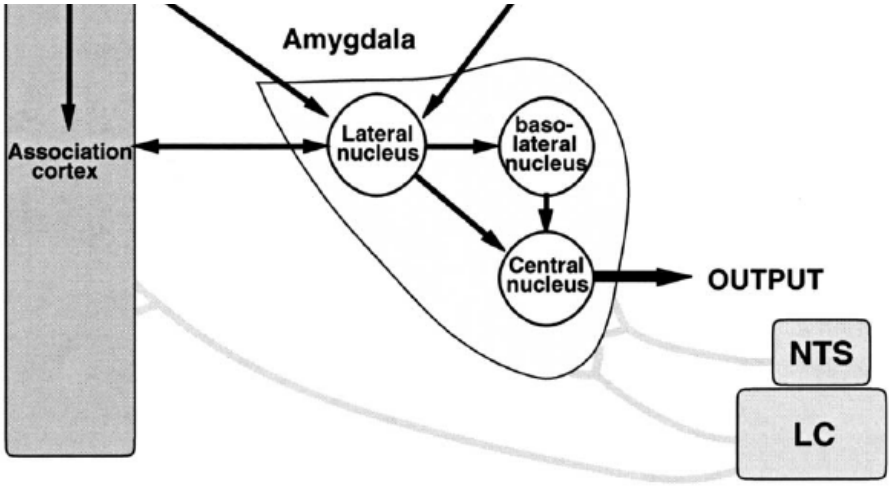
1.4 PUTTING IT ALL TOGETHER: THE AMYGDALA, NOREPINEPHRINE, AND STRESS

NE levels within the BLA are highly responsive to manipulations perceived as stressful by the animal. Exposure to footshock causes increased NE in the amygdala, as does exposure to other stressful stimuli (Tanaka 1991, Galvez et al. 1996, Hatfield et al. 1999,). Administration of yohimbine, an anxiogenic agent, also causes increases in amygdala NE (Khoshbouei et al. 2002). NE levels are also increased with exposure to contexts conditioned to be aversive (McIntyre et al. 2002). It is thought that during stress the amygdala nuclei are activated primarily by ascending catecholaminergic neurons (Habib 2001).

One of the most heavily studied functions of NE within the BLA is modulation of aversive memory. Noradrenergic transmission in the BLA has been shown to be important for tasks such as inhibitory avoidance (Ferry 1999a, 1999b, Nielson et al. 1999, Roozendaal 1999, Schneider et al. 2000, McIntyre et al. 2002) and contextual fear conditioning (LaLumiere 2003), which both involve footshock. Conditioned taste aversion, which involves aversive taste stimuli (Miranda 2003), is affected by NE manipulations within the BLA. Memory for fear-potentiated startle, which involves footshock and light stimuli, can also be impaired through blocking of NE receptors in the BLA (Schulz 2002). Humans tend to remember emotionally-evocative information better than information lacking an affective component. This effect is dependent on beta receptors (Cahill et al. 1994), and beta receptor blockade disrupts this facilitation of memory for emotional items and also blocks activation of the amygdala (van Stegeren et al. 2005), suggesting the beta-adrenergic action takes place within the amygdala. In rats, NE within the amygdala is also important in regulating the effects of stress on memory in other structures such as the hippocampus (Roozendaal 1999). Administration of NE alpha-2 agonists causes a

decrease in the expression of phosphorylated CREB and c-Fos normally seen during fear conditioning within the amygdala (Davies et al. 2004).

Learning and memory for tasks that involve some type of stressful or emotional stimulus is thought to rely on plastic mechanisms within the amygdala (as discussed previously, Rogan et al. 1997, Blair et al. 2001, Tsvetkov et al. 2002, Goosens and Maren 2002, Dityatev and Bolshakov 2005, Rodriguez Manzares et al. 2005). Activation of alpha-2 receptors within the BLA has been shown to inhibit long-term potentiation (LTP) and long-term depression (LTD) (DeBock et al. 2003). However, footshock stress can induce LTP of auditory-evoked potentials in the BLA (Garcia et al. 1998), and footshock stress increases NE in the BLA (Galvez et al. 1996, Williams et al. 1998, Hatfield et al. 1999). Footshock is often the stimulus used in such learning and memory conditioning paradigms. Therefore, footshock-induced increases in BLA NE may lead to a facilitation of evoked potentials that underlies learning of such tasks. We will model this scenario in these experiments by examining the effect of NE on BLA neuronal activity evoked by stimulation of two afferents, EC and Te3.



modified from Kobayashi and Kobayashi 2001

Figure 2 Summary of the regions of interest for the present experiments

1.5 RATIONALE FOR PROPOSED EXPERIMENTS

Neurons of the amygdala are sensitive to stressor administration (Shors 1999, Correll 2005) and NE (Gear et al. 1992, Huang et al. 1994, 1996, 1998, Ferry et al. 1997, DeBock et al. 2003, Braga et al. 2004). LC neurons, which send NE input to the BLA, are sensitive to chronic stress exposure, as are stress-induced effluxes of NE released in target regions, and NE receptors. Sensitization of behavioral and neuroendocrine aspects of the stress response has been shown to rely on the actions of NE in certain regions of the stress circuits responsible for such responses. However, studies to date have failed to demonstrate an alteration in the neurophysiological actions of NE on target neurons after chronic stress exposure. A series of in vitro studies demonstrated that NE modulation of BLA neuronal activity was altered in slices taken from animals that had been exposed to stress prior to sacrifice (Braga et al. 2004). This suggests the BLA as a strong candidate for alterations in NE modulation after chronic stress exposure. These in vitro studies demonstrate the need for further investigation into how NE may be modulating cellular activity within the BLA both before and after stress exposure. Here we will use chronic cold exposure as a model of chronic stress, as it has been shown repeatedly to cause sensitization of many aspects of the stress response (Nisenbaum et al. 1991, Pacak et al. 1992, Gresch et al. 1994, Jedema et al. 1999, Jedema and Grace 2001). An examination of these phenomena in vivo will allow for recordings in BLA in a preparation where all the connections between BLA and other structures remain intact. As there are several brain areas involved in the stress response, the way in which these structures interact is of importance. The proposed studies will provide

valuable physiologically relevant information as to how NE affects cellular activity of the BLA, and how chronic stress modifies these interactions.

1.5 HYPOTHESES AND RESEARCH OBJECTIVES

The following experiments examine the effects of alterations of the NE system on neuronal activity of the BLA. These studies aim to begin to elucidate the mechanisms by which NE levels within the BLA alter stress responses and aversive learning and behavior, and how those mechanisms may be altered after exposure to chronic stress. This will be accomplished through performance of the following aims:

1. Examine the effects of administration of yohimbine, an anxiogenic agent, on NE levels within the BLA, and on spontaneous and afferent-evoked activity of BLA neurons.

Neurons of the BLA are responsive to footshock stress (Shors 1999 and Correll et al. 2005), and to NE as demonstrated in vitro (Huang et al. 1994, Ferry et al. 1997). These studies examine BLA responses to administration of a pharmacological stressor known to increase NE levels in the BLA (Khoshbouei et al. 2002). Microdialysis will be used to confirm changes in extracellular NE levels in the BLA, and extracellular recordings will be used to monitor BLA neuronal activity to ask the following questions:

1a. Does systemic yohimbine administration alter NE levels within the BLA?

We hypothesize that yohimbine administration will increase BLA NE levels.

1b. Does systemic yohimbine administration affect the spontaneous activity of BLA neurons?

We hypothesize that yohimbine administration will have excitatory and inhibitory effects on BLA neuronal activity.

1c. Does systemic yohimbine administration affect BLA neuron activity evoked by stimulation of entorhinal cortex?

We hypothesize that yohimbine administration will facilitate the response of BLA neurons to EC stimulation.

1. Characterize how iontophoresis of NE within the BLA alters spontaneous and afferent-evoked activity of BLA neurons.

NE levels increase in the BLA in response to stress (Galvez et al. 1996, Williams et al. 1998, Hatfield et al. 1999). NE in the BLA is critical for the modulation of memory involving stressful stimuli such as footshock (McGaugh et al. 2002). However, little is known about how NE affects BLA neuronal activity in vivo. These studies will use extracellular recordings of BLA neurons concomitant with iontophoresis to ask the following questions:

2a. Does NE iontophoresis alter BLA neuron spontaneous activity?

We hypothesize that NE iontophoresis will have primarily inhibitory effects on BLA neuron spontaneous activity.

2b. Does clonidine iontophoresis alter BLA neuron spontaneous activity?

We hypothesize that clonidine iontophoresis will inhibit BLA neuronal spontaneous activity.

2c. Does systemic administration of propranolol alter BLA neuron spontaneous activity?

We hypothesize that propranolol will have no effect on BLA neuron spontaneous activity.

2d. Does systemic administration of propranolol alter NE-induced alterations of BLA neuron spontaneous activity?

We hypothesize that propranolol will enhance the inhibitory response of BLA neurons to NE iontophoresis.

2e. Do BLA neurons display similar responses to NE iontophoresis and footshock administration?

We hypothesize inhibitory responses to footshock administration will be mimicked by NE iontophoresis.

2f. Do BLA neurons display similar responses to NE iontophoresis and LC stimulation?

We hypothesize inhibitory responses to LC stimulation will be mimicked by NE iontophoresis.

2g. Does NE iontophoresis alter BLA neuron activity evoked by stimulation of EC and sensory association cortex (Te3).

We hypothesize that NE iontophoresis will facilitate the responses of BLA neurons evoked by EC and Te3 stimulation.

3. Investigate how exposure to chronic cold stress alters NE modulation of spontaneous and afferent-evoked activity of BLA neurons.

NE actions in regions like the PVN or BNST play a role in the sensitization of certain components of the stress response after repeated chronic exposure to stressful stimuli (Ma and Morilak 2005a, b). Neurons of the BLA show increased excitatory responses to footshock after chronic cold exposure (Correll et al. 2005). NE levels within the BLA increase during footshock (Galvez et al. 1996). These studies examine potential alterations of NE modulation of BLA neuronal activity after chronic cold exposure. Extracellular recordings from BLA neurons will be used in combination with iontophoresis of NE to investigate the following questions:

3a. Does exposure to 7 or 14 days of chronic cold stress alter baseline spontaneous and evoked activity in BLA neurons?

We hypothesize that 7 or 14 days of chronic cold stress will not alter baseline spontaneous or evoked activity in BLA neurons.

3b. Does chronic cold stress alter NE modulation of BLA spontaneous neuronal activity?

We hypothesize that NE iontophoresis will cause greater excitation in neurons of the BLA in rats exposed to 7 or 14 days of chronic cold stress.

3c. Does chronic cold stress alter NE modulation of BLA neuronal activity evoked by EC and Te3 stimulation?

We hypothesize that NE iontophoresis will have even greater facilitatory effects on BLA neuronal activity evoked by stimulation of EC and Te3.

2.0 ANXIOGENIC MODULATION OF SPONTANEOUS AND EVOKED NEURONAL ACTIVITY IN THE BASOLATERAL AMYGDALA

2.1 INTRODUCTION

The amygdala is known to play a central role in aversive learning, responses to stress, and modulation of memory by stressful experiences (LeDoux 2000, McGaugh 2004, Berretta 2005) as well as in Pavlovian fear conditioning (Maren 2003). Furthermore, this region is critical in mediating responses to anxiogenic stimuli or stress-related behaviors including elevated-plus maze, shock probe burying, and social anxiety tests (Sajdyk and Shekhar 1997, Treit and Menard 1997; Saldivar-Gonzalez et al. 2003). In human imaging studies, exposure to fearful faces causes marked activation of the amygdala, and fearful stimuli are remembered better than stimuli lacking an affective component (Rauch et al. 2000). Footshock, tail pinch, and other stressful stimuli cause amygdala activation as measured with cfos expression (Smith et al. 1997; Rosen et al. 1998). Neuronal activity within the amygdala is strongly affected by acute stressors, chronic stress exposure, and stimuli conditioned to be aversive (Shors 1999; Rosenkranz and Grace 2002; Correll et al. 2005). Plasticity within the amygdala is also affected by stress exposure (Vouimba et al. 2004). Given that the amygdala receives a large noradrenergic (NE) input from the locus coeruleus (LC; Asan 1998), an area known to be involved in stress responses (Sved et al. 2002), the role of the NE system of the amygdala in responses to stress and modulation of stress-related behaviors has been a topic of investigation.

The NE system of the LC is also known to play a central role in the stress response and reactivity to stressful stimuli. Neurons of the LC are activated by stressful stimuli (Rasmussen et al. 1986; Abercrombine and Jacbos 1987; Jacobs et al. 1991), leading to increased levels of NE in terminal regions including the amygdala (Galvez et al. 1996; Williams et al. 1998; Hatfield et al. 1999). During stress, the amygdaloid nuclei are believed to be activated primarily by ascending catecholaminergic neurons, and recently, the mechanism by which limbic areas may regulate responsivity to stress and anxiety has become a topic of interest (Habib et al. 2001, Singewald et al. 2003). The LC has been proposed to promote activation of forebrain areas such as the amygdala that feed back to the paraventricular nucleus of the hypothalamus, which is a primary output nucleus of the stress response (Passerin et al. 2000). The amygdala is well-situated anatomically to convey this feedback. The basolateral nuclei of the amygdala (BLA) receive NE input from the LC (Asan 1998) and have a strong influence over the central nucleus of the amygdala (CeA). The CeA (Rosenkranz et al. 2005), sends a corticotropin-releasing-hormone (CRH)-containing projection to the PVN (Gray et al. 1989) as well as a CRH-containing projection to the LC (Van Boeckstaele et al. 2001). Therefore, the amygdala is positioned to provide feedback not only to the PVN but also to the LC directly (Curtis et al. 2002).

Extensive evidence supports an involvement of the BLA in the modulation of memory by stress and stressful conditions (McGaugh and Roozendaal 2002). Manipulations of the levels of NE or NE receptors within the BLA after learning of tasks involving an aversive component have been shown to affect subsequent task performance (Gallagher et al. 1977; Introini-Collision et al.. 1991; Ferry et al. 1999). Moreover, levels of NE have been shown to correlate with performance on tasks involving an aversive stimulus (McIntyre et al. 2002). Thus, the BLA and

its afferent and efferent projections are believed to provide the means by which a stressful or emotionally evocative experience is remembered better than an experience lacking an emotional component (McGaugh 2002).

Yohimbine causes many of the neuroendocrine and sympathetic effects caused by other stressors. These include increased heart rate and blood pressure (Swann et al. 2005), a rise in plasma cortisol levels (Suemara et al. 1989) and plasma NE levels (Ambrisko and Hikasa 2002). It also causes reports of anxiety in humans (Cameron et al. 2000), and supports potentiation of the acoustic startle response in humans as well (Morgan et al. 1993). In rats, yohimbine supports reinstatement of food-seeking (Ghitza et al. 2005), cocaine seeking (Lee et al. 2004), and methamphetamine reinstatement (Shephard et al. 2004). Yohimbine is aversive to a degree sufficient to support contextual conditioning (Schroeder et al. 2003) and conditioned taste aversion (Myers et al. 2005). Fear-related behaviors (Pellow et al. 1985), anxiety, and drug craving (Stine et al. 2002) can all be elicited by yohimbine. Yohimbine causes an increase in the firing of neurons of the LC (Ivanov and Aston-Jones 1995), cfos activation in the amygdala (Singewald et al. 2003, Myers et al. 2005), and increased NE efflux in the amygdala (Khoshbouieu et al. 2002).

While there is clear behavioral and clinical evidence for the involvement of the noradrenergic system of the BLA in stress and anxiety, little has been done to examine the way in which the neurons of the BLA respond to stress-causing or stress-relieving pharmacological agents, or agents that affect the NE system of the BLA. In this study, we examine the responses of neurons of the BLA to yohimbine, an anxiogenic agent known to increase firing of the neurons of the LC (Ivanov and Aston-Jones 1995), elicit anxiety in humans (Cameron et al. 2000), and trigger fear-related behaviors in animals (Pellow et al. 1985). Furthermore, given that

the entorhinal cortex (EC) sends a dense projection to the BLA (McDonald 1997) and is involved in learning and performance of many behaviors that also involve the BLA (Bonini 2003; Dolcos 2005, Schenberg 2005), we examined how neurons of the BLA respond to stimulation of the EC. Studies suggest that the EC may modulate the learning of behaviors through an interaction with the BLA (Ferry 1999). Therefore, it is likely that increased levels of NE in the BLA during task performance may not only influence spontaneous BLA neuronal activity, but also affect the way in which afferent input is processed.

2.2 MATERIALS AND METHODS

2.2.1 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 (250-400g), housed in pairs and kept on a 12:12 light/dark cycle, were maintained at constant temperature and given food and water ad libitum. Rats were anesthetized with 8% chloral hydrate (400mg/kg, i.p.) and then implanted with jugular vein catheters. Rats were then placed in a stereotaxic apparatus, with supplemental doses of chloral hydrate administered intraperitonealy or intravenously to maintain a constant level of anesthesia. 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. Areas targeted were the BLA (-5.0L, -3.0C), and the EC (-5.0L, -6.8C, -8.2V). The locations of structures were calculated using a stereotaxic atlas (Paxinos and Watson 1986). Stimulating electrodes were lowered into the appropriate brain regions. Recordings did not begin until at least 45 minutes after placement of the stimulating electrodes.

2.2.2 **Electrophysiology**:

Single barrel electrodes were constructed from 2 mm Omegadot glass tubing (WPI) using a vertical microelectrode puller (Narishige; Japan), broken back under microscopic control and filled with 2% Pontamine sky blue in 2M NaCl to yield electrodes with an impedance of 6-12 MOhms. These electrodes were lowered through the BLA in successive vertical tracks. Neurons within the BLA were isolated, and only those with a signal-to-noise ratio greater than 3:1 were used for data analysis. Spontaneous activity was recorded for a minimum of 5 minutes and expressed as spikes/second. After recording of spontaneous activity, the neuron was examined for responsiveness to EC stimulation. If responsive, the nature of the response was characterized and recorded. Subsequently, yohimbine (500ug/kg) or 0.9%NaCl was delivered intravenously via the jugular vein catheter. This dose of yohimbine was chosen because it reliably and significantly increased NE levels in the BLA. One single dose of yohimbine was given per animal. Data collection continued for 5-30 minutes subsequent to drug infusion. For EC-responsive neurons, stimulation protocols were also run after drug infusion to examine the effects of drug on responses to EC stimulation.

2.2.3 **Electrical Stimulation**:

Bipolar, concentric stimulating electrodes were lowered into the EC at the conclusion of the surgical preparation. Recordings did not begin until a minimum of 30 minutes after lowering of the stimulating electrodes. BLA neurons responsive to EC stimulation were isolated using a search-stimulate protocol. Single-pulse stimuli (300-900uA, 0.25ms, 0.5Hz) were delivered to the EC while the recording electrode was lowered through the BLA to identify responsive neurons. Neurons were characterized as having presumed orthodromic monosynaptic, orthodromic polysynaptic, or antidromic responses to EC stimulation. Responses were operationally defined as orthodromic if they had an onset latency of <20ms, showed a failure to substantially change latency in response to increases in current intensity, their onset latency remained fairly consistent with approximately 1-5ms of variability in evoked spike latency, and they follow paired pulses at 50Hz but not 400Hz. Antidromic responses were characterized by their constant onset latency with virtually no variability in onset latency, and their ability to followed paired pulses at 400Hz. Monosynaptic responses were differentiated from polysynaptic spikes by an examination of onset latency and variability of latencies, as well as failure to followed paired pulses at 50Hz. Stimulation current was adjusted to a level where a spike was evoked approximately 50% of the time in the responsive neuron. The number of spikes evoked was recorded, as well as a minimum of 5 minutes of spontaneous activity, both before and after yohimbine administration**.**

2.2.4 Microdialysis:

Microdialysis probes (CMA 12, 2mm exposed tip) were lowered bilaterally into the BLA (-4.8L, -3.0L, -8.6V) of anesthetized, stereotaxically restrained rats. After lowering of probes, no samples were collected for a minimum of 90 minutes. Subsequently, baseline levels of NE within the BLA were measured in vivo for 60 minutes. Yohimbine (500ug/kg) was then administered via the jugular vein catheter and BLA NE levels measured for another 120 minutes.

2.2.5 Histology:

Recording, stimulation, and microdialysis probe placements were verified by histological analyses. At the conclusion of electrophysiological experiments, pontamine sky blue was ejected by passing constant current through the recording electrode (10nA, 15 minutes) to mark the recording site. Anodal current was passed through the stimulating electrode to create a small lesion for identification of the placement site of the stimulating electrode. Rats were euthanized by an overdose of anesthetic, followed by decapitation and brain removal. Brains were fixed in 10% formalin for a minimum of 24 hours, and then cryoprotected with 25% sucrose solution in 0.1M phosphate buffer. Subsequently, coronal slices were cut on a cryostat into 40μm sections, mounted on slides, and stained with cresyl violet. Recording sites were identified by the presence of the Pontamine sky blue dye spot, and the location of the stimulating electrodes was identified by the presence of a small lesion at the end of the electrode track. For microdialysis experiments, placement was verified by the location of the probe track within the tissue.

2.2.6 Data Analysis:

Electrophysiological characteristics were measured from neurons of the BLA, including firing rate (spikes/second), waveform shape, and action potential duration. For spontaneously active neurons that were not driven by EC stimulation, yohimbine was administered and the firing rate was measured for a minimum of 15 minutes after drug administration. For neurons monosynaptically activated by EC stimulation, the average onset latency was measured across 50 stimulation trials. One trial consisted of a single pulse stimulation applied to the EC (100-900uA) every 5 seconds. Spontaneous activity was also measured during the stimulation trials to determine if there was an effect of EC stimulation on overall firing rate. After each stimulation protocol, spontaneous activity was recorded for a minimum of 5 minutes before subsequent presentation of the next electrical stimulation. Predrug and postdrug administration or pre-stimulation and post-stimulation epochs were compared using paired t-tests. Overall increases or decreases in firing rate were reported as percent changes in firing rate from baseline. An individual neuron was considered to display a significant increase or decrease in firing rate if the difference between baseline firing and firing after drug administration was at least 30%. This is based on the conservative estimate of the degree of change necessary to yield a significant difference in a *t* test, given the observed level of variability in baseline firing rates.

The effects of location on baseline firing rates were examined using a one-way ANOVA. Examination of proportions of neurons excited or inhibited with regard to type of neuron or neuron location were examined using Chi Square tests.

Peri-stimulus time histograms were constructed from the EC stimulation data. Sweeps of 5 seconds were collected with the stimulus pulse occurring at time 0. Total firing rate was

summed across trials and plotted against time for each 5 second sweep. Effects of yohimbine on responses evoked by afferent stimulation were evaluated in the following manner: Current applied to EC was adjusted to achieve between 50-60% probability of evoked spike responses. One hundred single pulses at this current intensity were applied to the EC and the number of stimuli that resulted in evoked spikes within the BLA was measured. This was compared to the number of afferent stimuli that evoked spike firing after systemic yohimbine administration. Changes in evoked spikes were expressed as percent increase or decrease in the probability of evoked responses of BLA neurons resulting from yohimbine administration. Differences in probabilities before and after yohimbine administration were tested for significance using paired t-tests.

Previous reports classified BLA neurons into two major groups (Rosenkranz and Grace 1999). This classification system was modified and applied to our neuronal population. Due to concerns about using extracellular waveforms to measure action potential durations, we antidromically activated a group of BLA neurons with EC stimulation (n=8) to ensure that they were representative of projection neurons. These neurons all displayed a firing rate of less than 0.2Hz and action potential duration of greater than 3.4 msec. We then examined our population of spontaneously active neurons and classified all those with similar firing rates of less than 0.2Hz and action potential duration greater than 3.4 msec as projection neurons. In contrast, all neurons with firing rates of greater than 1.0Hz exhibited action potential durations of less than 2.2msec and were classified as putative interneurons. Neurons that did not fall within these classification criteria were not included in any analyses based on neuron type. Firing rates of projection neurons (both those that were antidromically activated and those classified as such based on criterion) and interneurons were compared using t-tests.

2.3 RESULTS

2.3.1 Spontaneous activity:

A total of 240 neurons within the BLA complex were isolated and recorded. Of these neurons, 61 were examined under control conditions (not during use of the search-stim protocol); these neurons exhibited an average firing rate of 1.24±0.28Hz. The other neurons (n=179) were examined during tonic stimulation of the EC; these neurons exhibited an average baseline firing rate of 1.68±0.35Hz. There was no significant difference between the firing rates of these two cell groups (p=0.34, t-test), and therefore for further analyses they were grouped together. The neurons recorded were located within the lateral (Lat) nucleus (n=63), the basolateral (BL) nucleus (n=105), and the basomedial (BM) nucleus (n=11 (See Figure 3). The average baseline firing rates did not differ across neuron location (Lat FR=1.6±0.03Hz, BL FR=1.5±0.27Hz, BM FR=1.9±0.064Hz, p=0.45, one-way ANOVA).

Neurons were classified as projection neurons or interneurons based on either antidromic activation (projection neurons, Figures 5, 6) or firing rate and action potential duration (Figure 4). Those neurons classified as projection neurons (n=16, Avg FR= 0.09±0.003Hz) had significantly lower spontaneous firing rates than those neurons classified as interneurons (n=16, Avg FR= 2.6±0.3Hz, p=0.02, t-test, Figure 4).

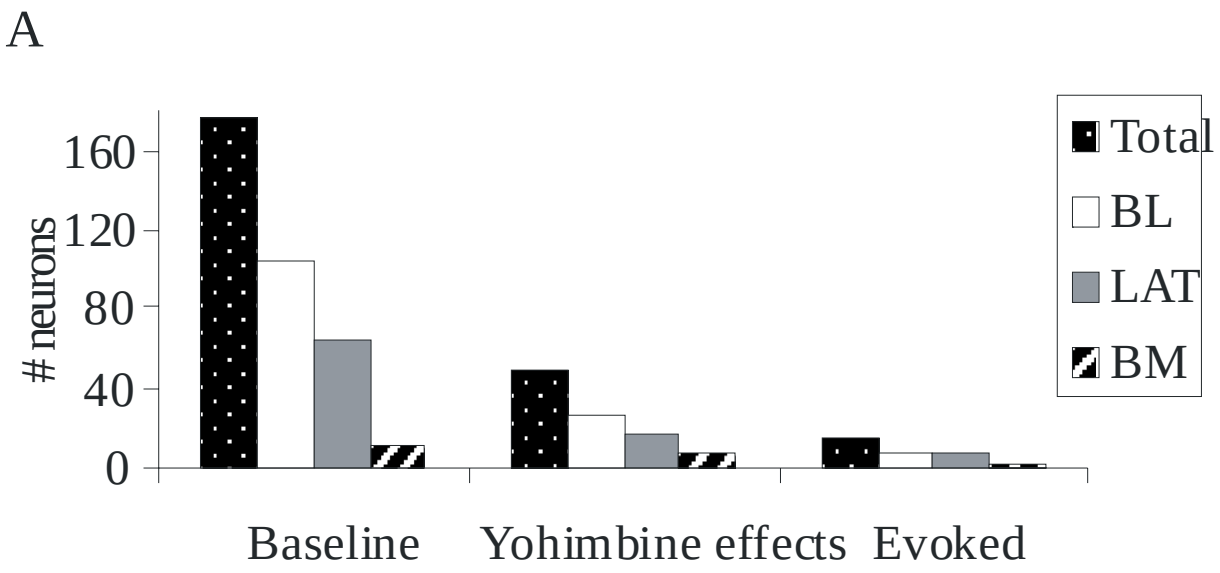


Figure 3 Distribution of BLA neurons examined for yohimbine responsivity. Distribution of amygdala neurons examined in this study. Neurons that were tested for response to yohimbine were located in the lateral, basolateral and basomedial nuclei.

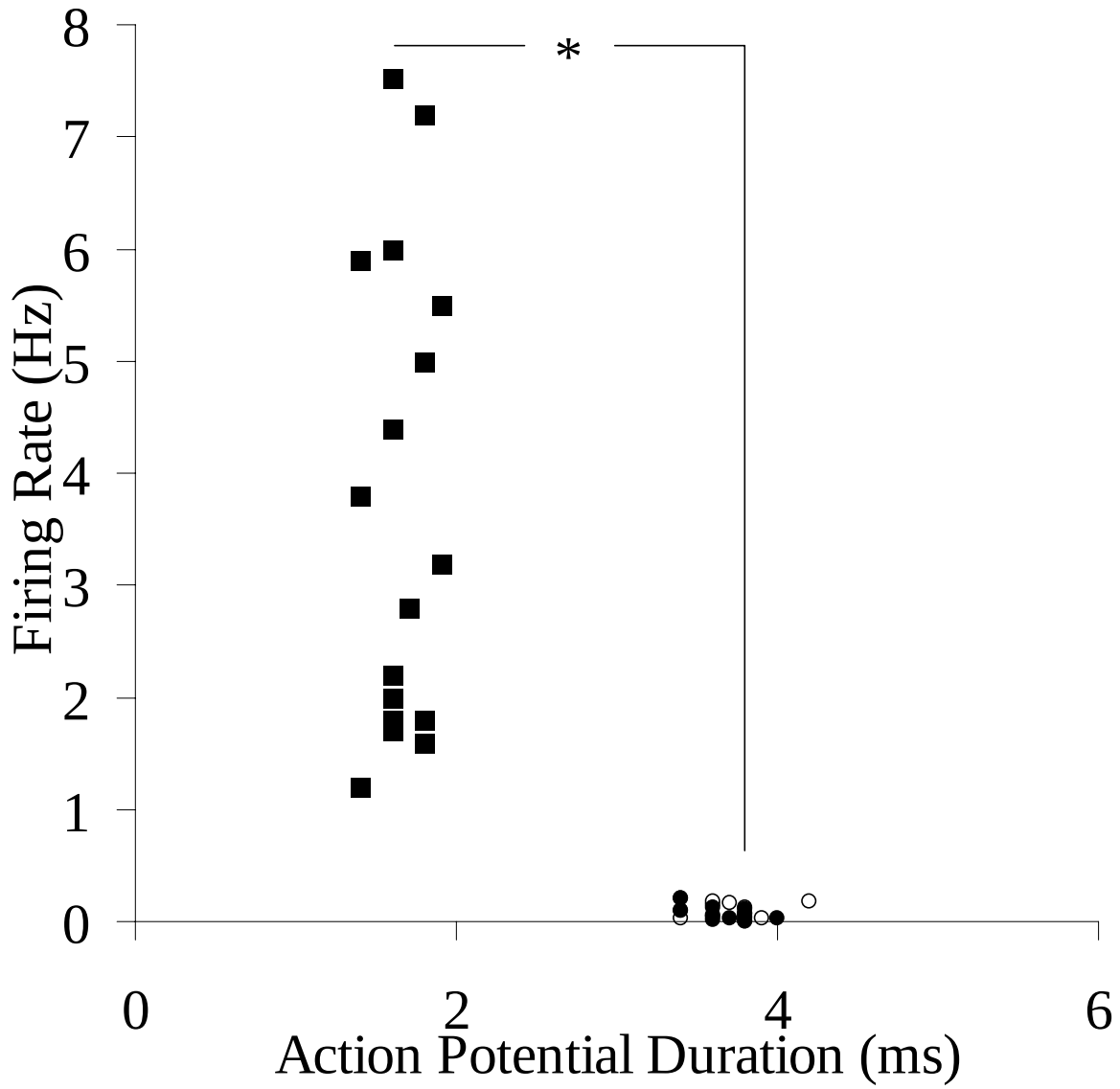


Figure 4 Projection neuron and interneuron characteristics. Neurons were also identified as projection neurons via antidromic activation (n=8), and classified as projection neurons (n=8), interneurons (n=16), or "unclassified" (n=35) based on firing rate and action potential duration. Neurons with longer action potential waveforms had significantly lower firing rates (p=0.02, t-test) and were operationally defined as projection neurons (open circles), some of which demonstrated antidromic activation from terminal sites (filled circles). Neurons with shorter duration action potential waveforms had significantly higher firing rates and were operationally defined as interneurons.

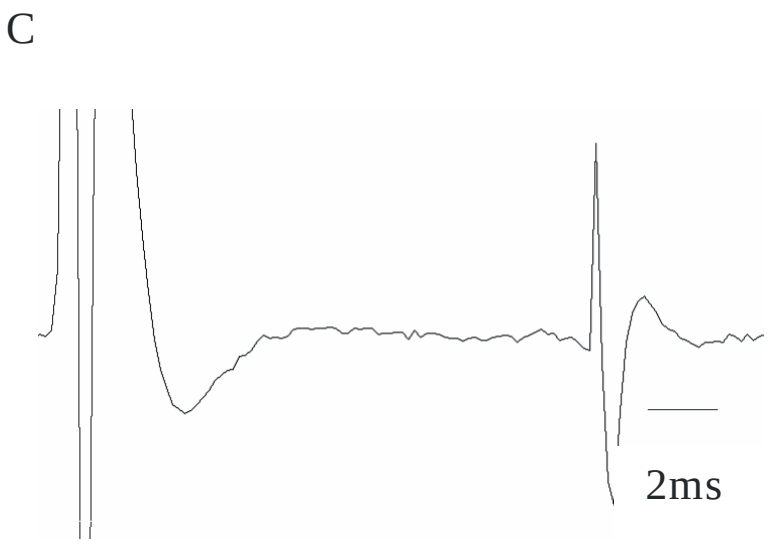


Figure 5 Electrophysiological trace of a BLA neuron antidromically activated by EC. Electrophysiological recording of a trace of a BLA neuron antidromically activated by stimulation of entorhinal cortex.

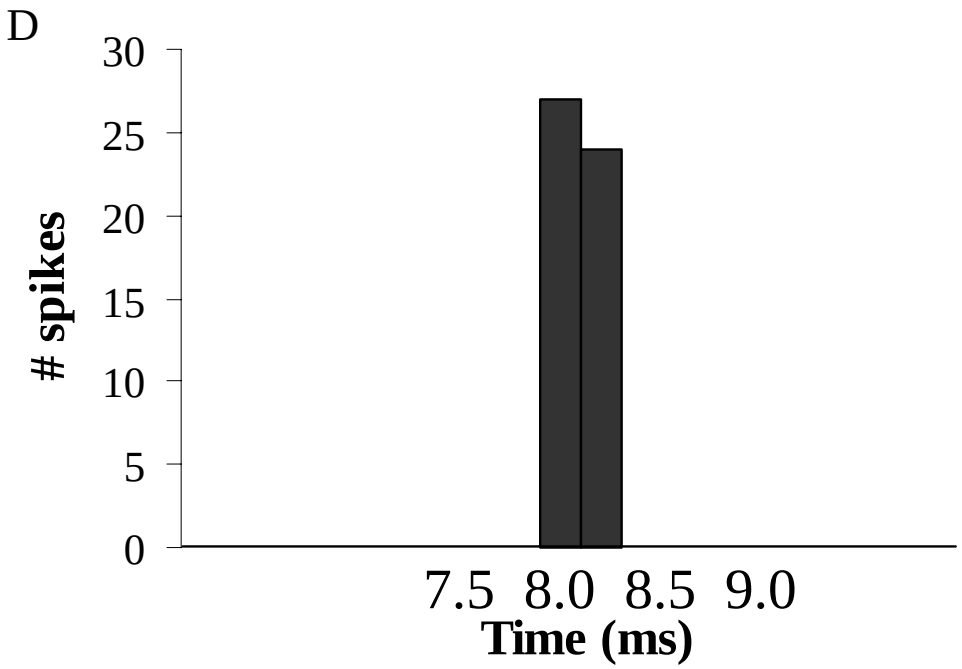


Figure 6 Latency distribution from an antidromically activated BLA neuron. Latency distribution for the neuron displayed in Figure 5. Note the constant spike latency evoked by EC stimulation.

2.3.2 NE Levels within the BLA

Yohimbine administration caused a significant increase in NE levels above baseline within the BLA. Systemic yohimbine (500ug/kg) caused a 100% increase in NE levels above baseline; this significant change (p=0.05) was observed at 15 minutes following yohimbine administration, and returned to baseline levels by 60 minutes post-administration (n=4, p=0.05, t-test, Figure 7). Administration of anesthetic (8% chloral hydrate) or saline (0.9M NaCL) did not alter NE levels measured within the BLA (data not shown).

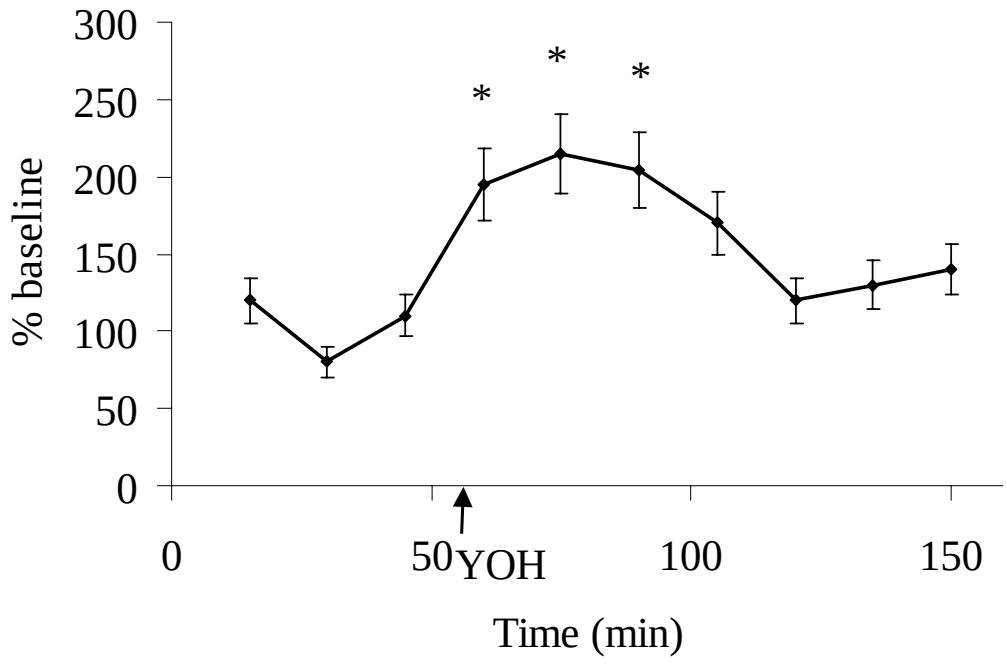


Figure 7 Systemic yohimbine causes increases in NE levels in the BLA. Systemic yohimbine administration (500ug/kg) caused a two-fold increase in NE levels within the BLA as measured by in vivo microdialysis (n=4, p=0.05, t-test). Increase in NE was significant within 15 minutes and returned to baseline after 60 minutes.

2.3.3 Yohimbine excites projection neurons of the BLA.

Systemic administration of yohimbine caused a substantial increase (by 68 ± 4.5%) in the spontaneous activity of BLA neurons (n=29, p=0.03, t-test) with an onset of approximately 30s after administration and lasting for up to 30 minutes. A significant proportion of neurons showed the opposite effect, with inhibitory responses of a similar magnitude and duration (72± 5.1%; n=27, p=0.02, t-test). Few neurons were unaffected by yohimbine administration (n=3; Figure 8). Systemic administration of saline (0.9M NaCl) caused no significant change in firing rate of BLA neurons from baseline (data not shown).

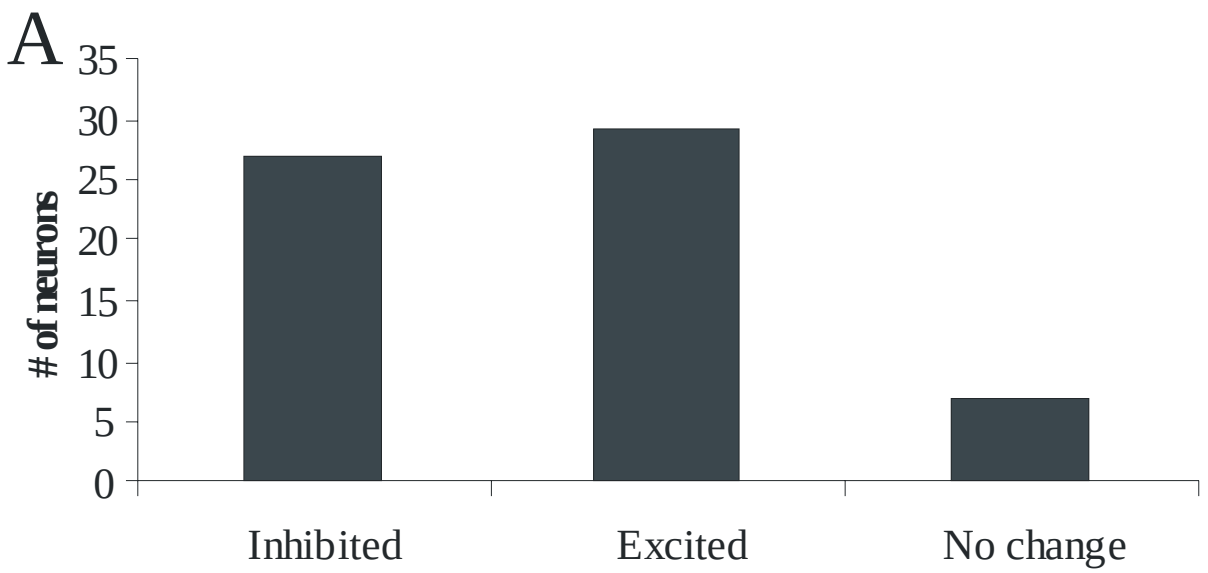


Figure 8 Yohimbine causes excitation and inhibition of spontaneous firing of BLA neurons. Systemic yohimbine administration causes both excitation (n=29, p=0.03, t-test) and inhibition (n=27, p=0.02, t-test) of spontaneous neuronal firing in the BLA.

In examining projection neurons versus interneurons, yohimbine was found to significantly excite (by 73 ± 8.3%) the majority of projection neurons within BLA (n=16, 8 confirmed by antidromic activation, p=0.03, t-test, Figures 5, 6), with fewer projection neurons displaying significant inhibition (57 ± 3.3% decrease in firing rate; n=8, p=0.04, t-test, Figure 9). Interneurons displayed equal proportions of neurons that were significantly excited (by 66 ± 4.0%; n=8, p=0.05, t-test) and significantly inhibited (by 59 ± 4.7%; n=8; p=0.05, t-test, Figure 9). However, the proportions of neurons displaying each effect were not significantly different between projection neurons and interneurons (p=0.3, Chi Square).

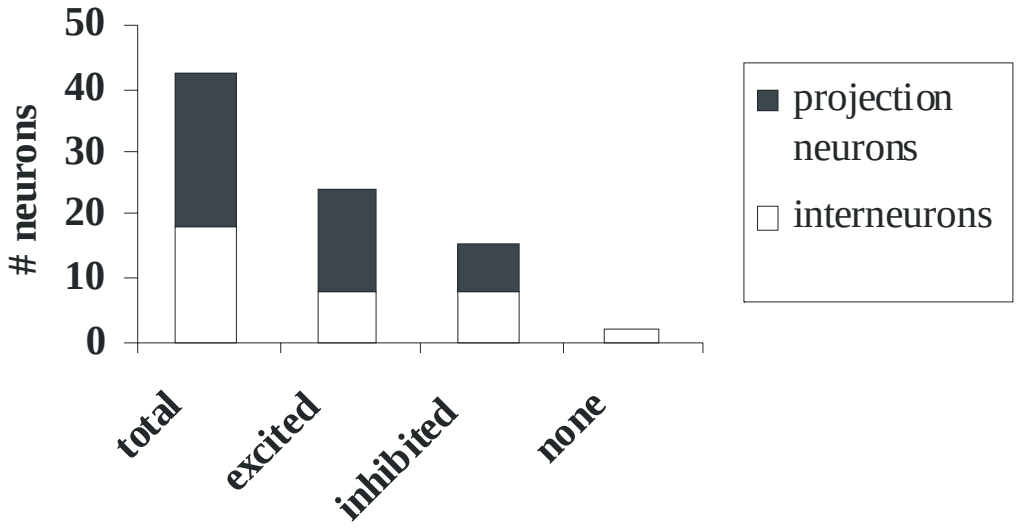


Figure 9 Responses of projection neurons and interneurons to yohimbine. Projection neurons were more frequently excited following systemic yohimbine administration (n=16/24), whereas equal numbers of interneurons exhibited excitation (n=8/16) and inhibition (n=8/16). However, the differences in this distribution were not statistically significant (p=0.3, Chi Square).

In examining neurons within different subnuclei of the BLA, we found that a similar proportion of neurons in the basolateral nucleus showed inhibition (48%) and excitation (41%). A greater proportion of neurons in the lateral nucleus, however, showed excitation (63%) than inhibition (37%), as did neurons in the basomedial nucleus (80% excitation, 20% inhibition; p=0.025, Chi-Square test, Figure 10).

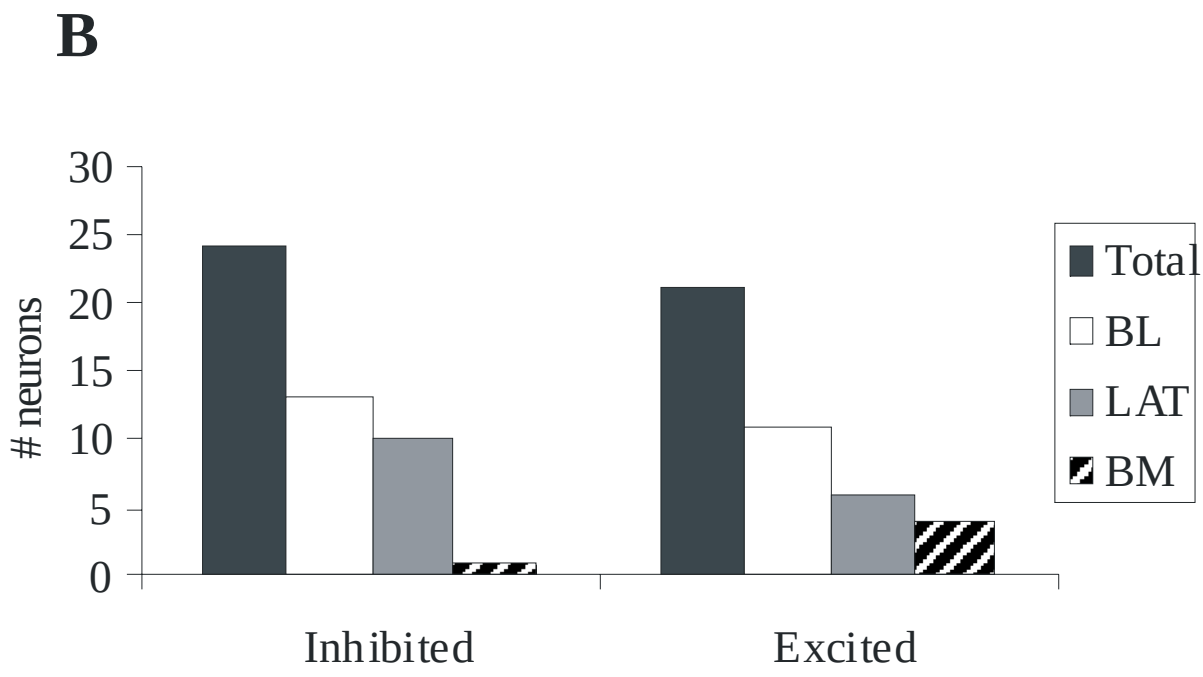


Figure 10 Responses of neurons in different BLA subnuclei to yohimbine. When examined based on neuron location, neurons in the basolateral nucleus show approximately equal numbers of neurons that were excited (n=12/25)and inhibited (n=13/25) by yohimbine, whereas a greater proportion of neurons in the lateral (n=10/16) and basomedial nuclei (n=4/5) showed excitation in response to yohimbine administration (Chi Square, p=0.05).

2.3.4 Yohimbine modulates excitatory responses to entorhinal cortex stimulation in a regionally specific manner.

Electrical stimulation of the entorhinal cortex caused predominantly orthodromic, presumably monosynaptic excitation of BLA neurons (n=71, avg. latency=11.2± 2.3 msec; Figures 11, 12). Yohimbine administration was found to predominantly facilitate (by 48 ± 7.1%; n=11, p=0.01, t-test), but also significantly inhibit (by 70 ± 6.4%; n=7, p=0.01, t-test) responsiveness of BLA neurons to EC stimulation (Figure 13). When neurons were examined with respect to location within specific BLA nuclei, some differences became apparent. A greater proportion of neurons in the lateral nucleus displayed yohimbine-induced facilitation of EC-evoked activity (n=5/7 neurons recorded) whereas there were an equal number of neurons in the basolateral nucleus that displayed facilitation and inhibition (n=3/6 facilitated, 3/6 inhibited), although these effects were not significant (p=0.25, Chi Square test). Neurons in the basomedial nucleus showed only facilitation of evoked activity (n=2; Figure 14). EC also caused other responses in BLA neurons including antidromic activation (n=42, avg. latency=9.1± 1.4msec, Figure 3D), polysynaptic excitation (n=3, avg. latency=29.2± 5.3msec), or short-duration inhibition (n=18, duration=280± 42msec).

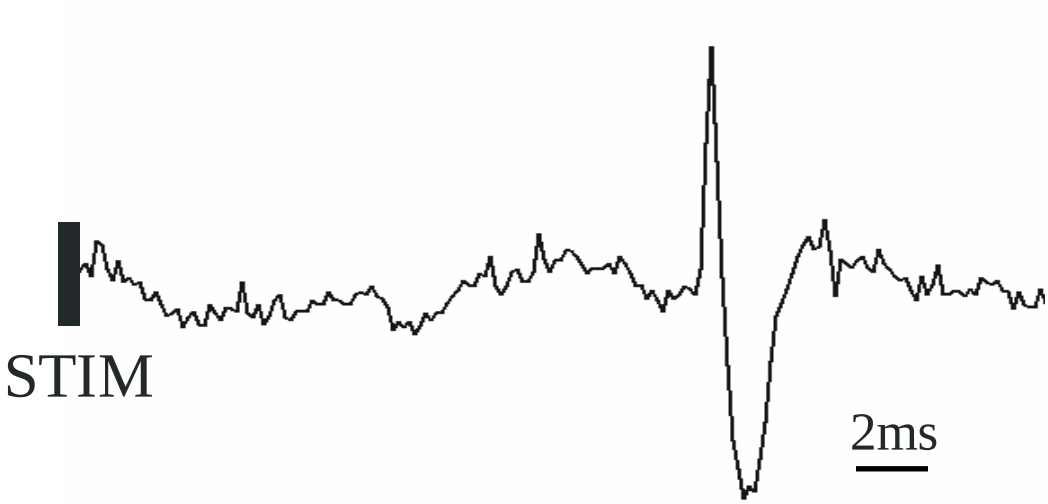


Figure 11 Electrophysiological trace of a neuron orthodromically activated by EC. Electrophysiological trace recording from a neuron that is orthodromically activated by stimulation of entorhinal cortex.

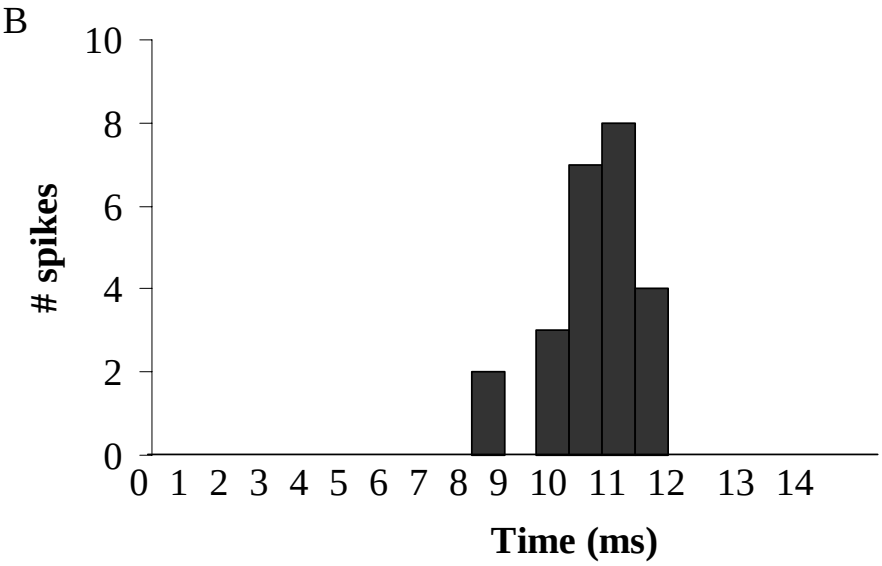


Figure 12 Latency distribution from orthodromically activated BLA neuron. Latency distribution for the neuron displayed in Figure 10. Note the variable onset latency evoked by EC stimulation.

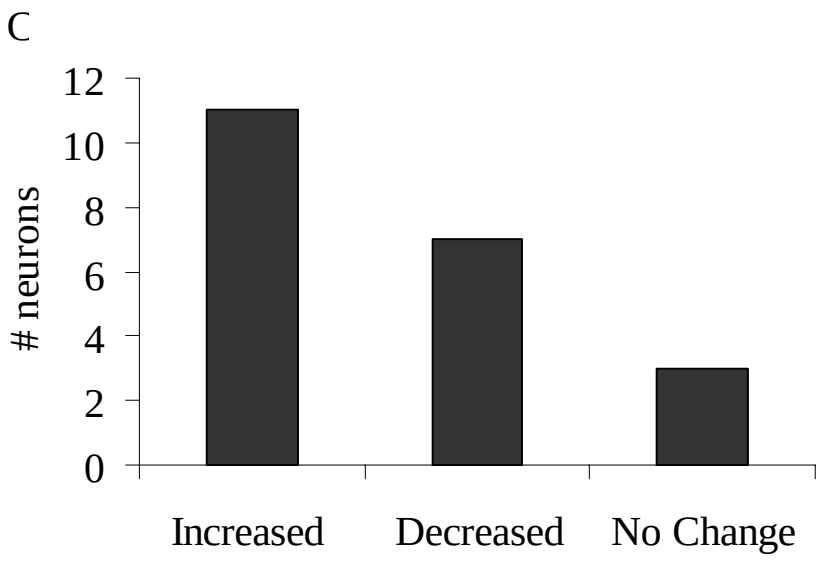


Figure 13 Yohimbine alters BLA neuronal activity evoked by EC. Yohimbine primarily increased EC-evoked activity of neurons in the BLA (n=11, p=0.01, t-test), but a subset of neurons exhibited significantly decreased EC-evoked activity (n=7, p=0.01, t-test).

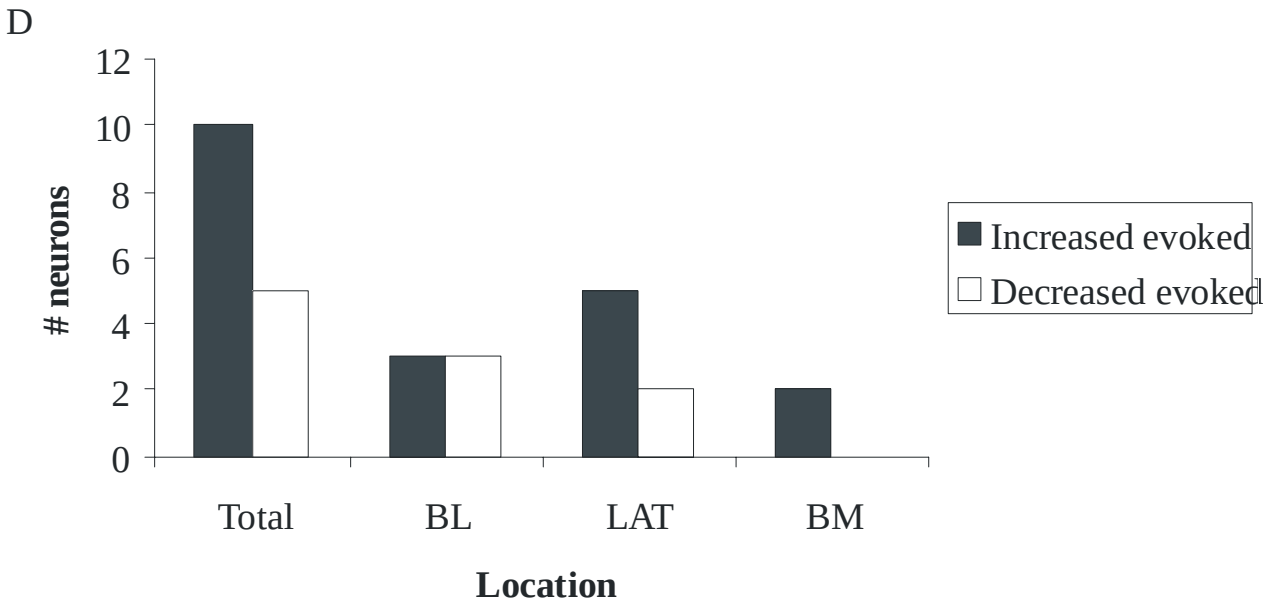


Figure 14 Yohimbine effects on EC-evoked activity in neurons in different BLA nuclei. An equal number of neurons in the basolateral nucleus showed facilitation (n=3/6) and inhibition (n=3/6) of EC-evoked activity, whereas more neurons in lateral (n=5/7) and basomedial (n=2/2) nuclei showed facilitation of EC-evoked activity, though these differences were not significant (p=0.25, Chi Square test).

2.3.5 Yohimbine affects spontaneous and evoked activity in a similar manner in individual BLA neurons

There was no clear relationship between the effects of yohimbine on spontaneous and evoked activity in BLA neurons. Although slightly more neurons showed spontaneous and evoked activity that was affected in a similar manner by yohimbine (n=10), with both factors showing either excitation/facilitation (n=6), or inhibition/attenuation (n=4), this was not significant (p=0.2, Chi Square). Remaining neurons showed facilitation of evoked activity with inhibition of spontaneous activity (n=3), or the opposite pattern of increased spontaneous activity with attenuation of evoked activity (n=2; Figure 15). All of these neurons were equally distributed among the different nuclei of the amygdala.

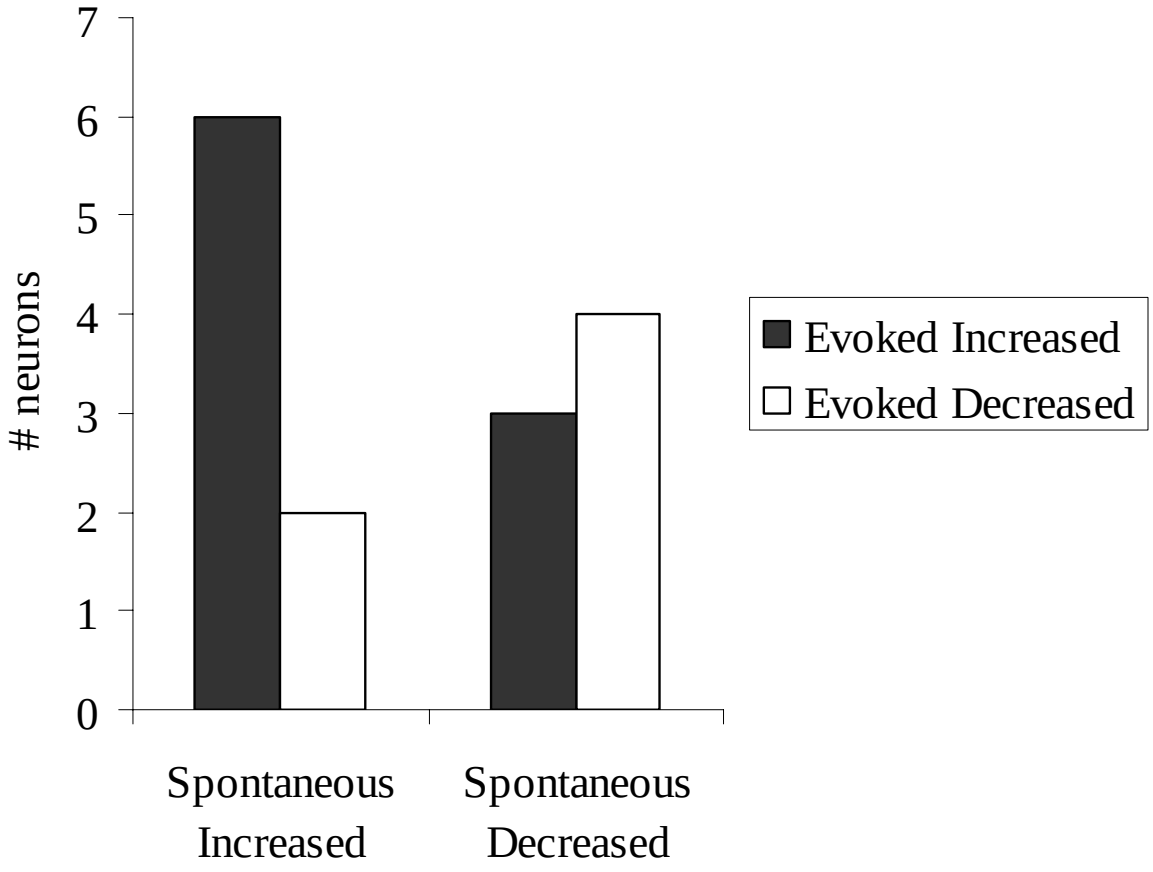


Figure 15 Yohimbine effects on spontaneous and eevoked activity of BLA neurons. Effects of yohimbine on spontaneous and evoked BLA neuronal activity. There is no clear correlation between the effects of yohimbine on baseline firing and evoked activity. While a greater proportion of neurons displayed changes in evoked and spontaneous activity in the same direction (increase in both, or decrease in both), this was not significant (p=0.2, Chi Square).

2.4 DISCUSSION

These studies confirm that administration of the anxiogenic drug yohimbine exerts a potent effect on neuronal activity within the BLA. Yohimbine administered systemically caused a two-fold increase in extracellular levels of NE within the BLA, which is similar to that reported to occur during exposure to a physical stressor (Galvez et .al. 1996). Electrophysiologically, yohimbine causes excitation in the majority of projection neurons within the BLA, whereas a smaller number of neurons exhibited inhibition. Yohimbine administration also alters responsivity of neurons to EC input, causing primarily an enhancement of responsivity, with fewer neurons showing decreased responsiveness. In addition, more neurons in the lateral nucleus of the BLA exhibited inhibition in response to yohimbine, both in terms of spontaneous activity and evoked activity, than in the basolateral nucleus of the BLA.

This is the first study to report the effects of anxiogenic drug administration on BLA neuronal activity in vivo. Amygdala activity responds to physical stressors (Shors 1999), stressful contexts (Schroeder et al. 2003), and stimuli conditioned to be stressful (Saddoris et al. 2005); here we show that amygdala activity is also affected by a pharmacological stressor. Furthermore, this is the first report to examine the effects of noradrenergic agents on neuronal activity in the BLA in vivo. There is a wealth of behavioral data confirming that NE signaling within the BLA is important for aversive learning, and here we reveal how increasing the activity of the NE system affects neuronal activity in the BLA.

The amygdala has long been implicated in portions of the stress response and stress-related behaviors. The BLA receives a large NE afferent input from the LC, and sends efferents to the CeA (Pitkanen et al. 1997), whose neurons are also responsive to stressful stimuli (Correll

et al. 2005). The CeA sends projections to downstream brain regions involved in many aspects of the stress response, including the periaqueductal gray (Rizvi et al. 1991), the dorsal vagal complex (Danielsen et al. 1989), and the paraventricular nucleus of the hypothalamus (Gray et al. 1989). Furthermore, the CeA has a direct projection to the LC (Van Boeckstaele et al. 2001) and may modulate these neurons directly (Curtis et al. 2002; Bouret et al. 2003; Ramsooksingh et al. 2003). We show here that pharmacological activation of LC efferents inhibits a portion of the neurons of the BLA. Inhibition of BLA neurons would cause disinhibition of neurons in the CeA, as BLA neurons have primarily an inhibitory influence over CeA neurons (Rosenkranz et al. 2005). This activation of CeA neurons would lead to activation of the paraventricular nucleus of the hypothalamus, a main output nucleus of the stress response, as well as provide feedback to the LC.

Norepinephrine and other noradrenergic agents have been found to alter the signal–to-noise ratio of evoked responses by differentially affecting spontaneous and evoked activity of neurons in many regions (Foote et al. 1975, Segal and Bloom 1976, Waterhouse et al. 1980, 1984, 1998, 2000, Waterhouse and Woodward 1980, Doze et al. 1991, Devilbiss and Waterhouse 2000). Therefore, in addition to examining the effects of yohimbine on spontaneous activity, we also investigated how yohimbine affects the evoked activity of neurons of the BLA. The EC has an excitatory projection to the BLA (McDonald and Mascagni 1997). It is also involved in amygdala-dependent behaviors that are associated with aversive stimuli, such as inhibitory avoidance (Bonini et al. 2003), fear conditioning (Schenberg et al. 2005), and emotional recall (Dolcos et al. 2005). Given these data, the EC may be providing context-relevant information to the BLA, which we would predict would be affected by yohimbine. We found that yohimbine exerted a regionally-selective effect on neurons within distinct subnuclei of the BLA. Neurons

in lateral nucleus and basomedial nuclei of the BLA showed predominantly enhanced responsivity to stimulation of EC afferents following yohimbine. It is not surprising that the neurons within the basolateral nucleus exhibited distinct response patterns compared to the lateral regions. Recent studies suggest a dissociation of function for these nuclei in fear conditioning (Calandrea et al. 2005), as well as the responses to conditioned and unconditioned fear stimuli (Wallace and Rosen 2001), making it likely that the manner in which they respond to anxiogenics would also differ. Furthermore, closer examination of a circuit involving the BLA that underlies potentiated eating reveals activation of basomedial and lateral neurons, but few basolateral neurons. This activation depends not only on the input these neurons receive, but the neurons to which they project (Petrovich et al. 2005). This reveals a potential explanation for our observation that afferent input from EC is facilitated in some neurons and suppressed in others, which is elaborated upon below.

We hypothesize that the EC may be providing input to the BLA regarding conditioned stimuli that is important for the performance of behavioral tasks. During administration of a stressful stimulus, the lateral nucleus of the amygdala is positioned to receive convergent information about the stimulus features (e.g., context or other discriminative stimulus) from the EC and information about the aversive nature of the stimulus (e.g., stressful) from the locus coeruleus. Neurons within the EC develop differential firing to conditioned stimuli during discriminative avoidance (Freeman 1997). Moreover, neurons of the BLA develop similar responses which can be attenuated by lesions of the EC. Furthermore, levels of NE in the BLA increase during the performance of such tasks (McIntyre et al. 2002). Thus, NE may be modulating information entering the BLA in order to facilitate the propagation of information relevant to task performance onto downstream areas. We confirm that yohimbine administration

does indeed cause changes in responsivitiy of neurons of the BLA to afferent input from the EC, and that this occurs concomitant to an increase in NE levels in this area. The majority of BLA neurons become more responsive to EC stimulation; however, there is a population of neurons that display less responsivity. The neurons of the BLA have many target sites including the prefrontal cortex (Bacon et al. 1996), hippocampus (Petrovich et al. 2001), CeA (Pitkanen et al. 1997), and others. Therefore, one possibility is that NE modulates neuronal responses not only based on the afferent input that the BLA neurons are receiving, but also based on where these neurons are projecting, as demonstrated in the aforementioned study. During pathological or extreme anxiety-provoking situations, this modulation may be disrupted. Initial results suggest that after exposure to chronic stress, neurons of the BLA display much more excitation to both afferent stimulation and noradrenergic manipulations, which may be expressed as maladaptive behaviors (Buffalari and Grace 2005, Correll et al.. 2005;). Animals exposed to repeated or chronic stress show enhanced responsivity to moderate stressors (Bhatnagar and Dallman 1998), which may be caused in part by the enhanced responsivity of BLA neurons described above.

Through the use of microdialysis, we confirmed that yohimbine does cause an increase in extracellular norepinephrine within the BLA. However, as many of the same NE neurons originating in the LC send axon collaterals to several target regions, it is likely that yohimbine causes increases in NE in those terminal regions as well, many of which provide afferent input to the BLA, including the prefrontal cortex (Gabbot et al. 2005) and the hippocampus (Kishi et al. 2006). Therefore, while a subset of the responses seen in the BLA are likely due to direct actions of NE on BLA neurons, we cannot rule out that a portion of these responses may have arisen from the effects of increased NE in other terminal regions providing afferent input to the BLA. Furthermore, there is some evidence for post-synaptic alpha-2 receptors within the BLA

(U'Prichard et al. 1980), and therefore some of the effects observed here could be due to blockade of post-synaptic alpha-2 receptors.

There is an extensive literature confirming that the levels of NE within the BLA are related to levels of stress and anxiety, and that this directly influences performance on tasks involving aversive or stressful stimuli. This is the first study examining how manipulations of this NE input affect neuronal activity within the BLA in an intact, *in vivo* preparation. During anxiety-provoking situations, BLA neurons may be more active (modeled here as increased spontaneous activity) and more responsive to certain types of inputs (modeled here as increased evoked activity). This may be involved in the mechanism by which an organism in a stressful environment shows an exaggerated response to a non-threatening or moderately threatening stimulus. Furthermore, other input to BLA neurons not involved in the successful performance of avoidance or other behavior (such as that coming from prefrontal cortex involved in cognitive control over behaviors) may be inhibited. The noradrenergic system of the BLA plays a crucial role in the circuit subserving stress responses, and how stressful events are remembered better than those lacking an emotional component. These data provide information on the function of BLA neurons in relation to the NE system and anxiety. Both the amygdala and the central NE system display abnormalities in disorders such as PTSD, anxiety disorders, and depression. Therefore, gaining a greater understanding into how these systems and processes operate, in either an adaptive or maladaptive manner, in an intact organism lends insight into a potential pathophysiological underpinning of these symptoms and disorders, and direct future investigations as to appropriate approaches to their treatment.

3.0 NORADRENERGIC MODULATION OF BASOLATERAL AMYGDALA NEURONAL ACTIVITY: OPPOSING INFLUENCES OF ALPHA-2 AND BETA RECEPTORS

3.1 INTRODUCTION

The amygdala is a critical component of the systems mediating many aspects of the stress response, fear-motivated learning, and memory for emotionally evocative events (LeDoux 2000, McGaugh 2004, Berretta 2005). Moreover, the role of the amygdala in coordinating behavioral and neuroendocrine responses to stressful stimuli is well-established (Berretta 2005). The basolateral (BLA) subdivision of the amygdala receives input from catecholaminergic nuclei that modify its activity (Fallon et al. 1978, Asan 1998). Dopamine (Inglis and Moghaddam 1999), norepineprine (NE) (Galvez et al. 1996, Williams et al. 1998, and Hatfield et al.1999), and serotonin (Kawahara et al. 1993) all exhibit increases in the BLA in response to stress. Although the actions of dopamine (Rosenkranz and Grace 1999, Rosenkranz and Grace 2001) and serotonin (Stutzmann and LeDoux 1999) in the BLA in vivo have been examined in detail, little is known about the effects of NE on neuronal activity in the BLA in the intact animal.

NE within the amygdala is critical for memory of aversive events (McGaugh 2002). Thus, NE has been shown to modulate the consolidation of aversive learning within the BLA

(Gallagher et al. 1977), and levels of NE within the BLA correlate with subsequent performance on tasks involving stressful stimuli (McIntyre et al. 2002). The BLA receives a dense NE innervation originating primarily in the locus coeruleus (LC, Asan 1998). LC neurons are activated by stressful stimuli (Rasmussen et. al 1986, Abercrombie and Jacobs 1987, Grant et al. 1988, Aston-Jones et al. 1991), and presumably mediate the stress-induced increase in NE in the BLA. Neurons of the BLA are responsive to stressful stimuli as well (Shors 1999, Correll et al. 2005). Therefore, the pathway from LC is thought to be critical in providing information about stressful stimuli to the BLA and allowing for certain types of appropriate behavioral and neuroendocrine responses to stressors.

Despite the wealth of behavioral data validating the importance of BLA NE and aversive learning, little is known about the effects of NE within the BLA in vivo. These studies examine the effects of microiontophoresis of NE on BLA neuron spontaneous activity as well as activity evoked by stimulation of entorhinal cortex (EC) or sensory association cortex (Te3). Furthermore, we examined the contribution of beta-receptors and alpha-2 receptors to these effects of NE. Finally, we investigated similarities in the responses of BLA neurons to footshock or LC stimulation and NE application in order to gain insight into the role that NE plays in BLA neuronal response to acute stressors, as well as the role of the LC-BLA pathway in mediating these responses.

3.2 MATERIALS AND METHODS

3.2.1 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 (250-400g), were initially housed in pairs and kept on a 12:12 light/dark cycle, and were maintained at constant temperature and given food and water ad libitum. After 5 days of pair housing, one cagemate was removed from the cage and control housing room and used for chronic cold exposure experiments (see Chapter 4). The rats for these control studies remained singly-caged in the control housing conditions until removal for use for experiments. Upon removal, rats were anesthetized with 8% chloral hydrate (400mg/kg, i.p.) and then implanted with jugular catheters for anesthetic administration throughout the duration of the experiment. Rats were then placed in a stereotaxic apparatus, with supplemental doses of chloral hydrate given intravenously to maintain a constant level of anesthesia, and 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 of the BLA (-5.0L, -3.0C from bregma), sensory association cortex (Te3, -6.1L,-6.8C, -4.7V from bregma), LC (-1.1L, -3.6C, -5.8V from lambda), and the EC (-5.0L, -6.8C, -8.2V from bregma) were calculated using a stereotaxic atlas (Paxinos and Watson 1986). The location of the LC was verified by lowering a recording electrode, confirming the presence of neurons that displayed characteristic LC action potential

waveforms and exhibited an excitatory response to foot-pinch, and replacing the recording electrode with a stimulating electrode in the identified location. Stimulating electrodes were lowered into the brain, and recordings did not begin until at least 45 minutes after placement of the stimulating electrodes.

3.2.2 Electrophysiology:

Neurons within the BLA were isolated using 5-barrel microiontophoretic recording electrodes, and only those with a signal-to-noise ratio greater than 3:1 were used for data analysis. In all experiments, spontaneous activity was recorded for a minimum of 5 minutes and expressed as spikes/second. After recording of spontaneous activity, the neuron was examined for responsiveness to stimulation of entorhinal cortex, sensory association cortex (Te3), locus coeruleus (LC), or footshock stimulation, depending on the experiment. If responsive, the nature of the response was characterized and recorded. EC and Te3 stimulation protocols were also run concomitant with NE application. If unresponsive, NE (200uM) or clonidine (50uM) was applied via iontophoresis in successive increasing doses (5-40nA), and the nature of the response recorded.

3.2.3 Electrical Stimulation:

Bipolar, concentric stimulating electrodes were lowered into either the LC, Te3, or the EC at the conclusion of surgical preparation. Neurons responsive to either Te3 or EC stimulation were found using a search-stimulate protocol. Single-pulse stimuli (300-900uA, 0.25ms, 0.5Hz) were

delivered to the Te3 or EC while the recording electrode was lowered through the BLA, and responsive neurons were identified and isolated. Neurons were characterized as having presumed orthodromic monosynaptic, orthodromic polysynaptic, or antidromic responses to EC stimulation. Responses were operationally defined as orthodromic if they had an onset latency of <20ms, showed a failure to substantially change latency in response to increases in current intensity, exhibited a consistent onset latency with approximately 1-5ms of variability in evoked spike latency, and followed paired pulse stimulation at 50Hz but not 400Hz. Antidromic responses were characterized by a constant onset latency with absence of variability in evoked spike latency, and their ability to followed paired pulse stimulation at 400Hz. Monosynaptic responses were differentiated from polysynaptic spikes based on onset latency and variability of evoked spike latencies, as well as the failure of presumed polysynaptic spikes to follow paired pulse stimulation at 50Hz. Propranolol (50 mg/kg) was given I.V. via the jugular catheter.

3.2.4 Iontophoresis:

Five barrel microiontophoretic electrodes (Activational Systems, Warren, MI) were constructed using a vertical microelectrode puller, broken back under microscopic control, and filled with 2% Pontamine sky blue in 2M NaCl to yield electrodes with a central recording barrel impedance of 4-8 MOhms. These electrodes were lowered through the BLA in successive vertical tracks. The central barrel of the 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 filled with 200mM NE, pH 4.0, or 50mM clonidine, pH 4.0. All drugs were dissolved in 0.1M NaCl. Drugs were retained

using currents that ranged between -12 and -18 nA, and were ejected at currents that ranged between 5 and 40nA. The ejection was done using repeated, increasing doses from 5-40nA with at least 2 minutes of spontaneous activity recorded between each period of drug ejection.

Neurons were tested for dose responsivity to NE (5-40nA). Those neurons that responded to lower doses of NE (5-10nA) did so in a moderate, variable, and often nonsignificant manner. More clear, significant, and consistent responses emerged in BLA neurons to higher doses of NE (40nA). Therefore, this dose was used for analysis of NE effects on spontaneous activity. However, evoked activity was significantly altered by lower doses of NE. Therefore the effects of NE on evoked activity focused on the 10nA dose, although, effects are also reported for higher doses where tested.

3.2.5 Footshock:

A single footshock (5 mA, 0.5ms) was administered via two 28-gauge pins inserted into the lateral side of the footpad, and single unit responses were recorded. The footshock was repeated up to five times per neuron.

3.2.6 Histology:

At the conclusion of electrophysiological experiments, pontamine sky blue dye was ejected by passing constant current through the recording electrode (10nA, 15 minutes) to mark the location of the recording site. Anodal current was passed through the stimulating electrode to create a

small lesion to localize placement of the stimulating electrode. Rats were sacrificed by an overdose of anesthetic, followed by decapitation and brain removal. Brains were fixed in 10% formalin for a minimum of 24 hours, and then cryoprotected with 25% sucrose solution in 0.1M phosphate buffer. Subsequently, coronal slices were cut on a cryostat into 40µm coronal sections, mounted on slides, and stained with cresyl violet. Recording sites were identified by the presence of the Pontamine sky blue spot, and the location of the stimulating electrodes was identified by the presence of a small lesion at the end of the electrode track.

3.2.7 Data Analysis

Firing rate was calculated as spikes/second. An effect was labeled as a significant excitatory or inhibitory effect if during or shortly after the experimental manipulation (NE iontophoresis, clonidine iontophoresis, footshock or LC stimulation) the average firing rate increased or decreased by 20% or more from baseline. This was based on conservative estimates of the change necessary to yield significance in a t-test given the degree of firing rate variability. Pre and post-manipulation firing rates were compared using paired t-tests. For neurons monosynaptically activated by EC or Te3 stimulation, the average onset latency was measured across 50 stimulation trials. 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 evoking a spike response. Fifty single pulses at this current intensity were applied to the EC or Te3 and the number of stimuli that resulted in evoked spikes within the BLA was measured. This was compared to the number of afferent stimuli that evoked spike firing during NE iontophoresis. These spike probabilities were compared using

paired t-tests. Changes in evoked spikes were expressed as percent increase or decrease in the probability of evoked responses during NE iontophoresis. A minimum of 3 minutes of spontaneous firing was allowed before delivery of a second stimulation period of 50 sweeps concomitant with NE iontophoresis.

Responses were defined based on identification of neuronal subtype. Neurons were confirmed as projection cells if they exhibited antidromic activation from EC stimulation (n=8). These neurons were examined separately for their response to NE iontophoresis, and pre and post-NE firing rates were compared with paired t-tests. Subclasses of neurons were also examined based on neuronal location. Histological verification revealed subgroups of neurons located in the lateral and basolateral subnuclei of the BLA complex. These two groups of neurons were analyzed separately for their responses to NE iontophoresis. Proportions of neurons in the lateral nucleus displaying excitatory versus inhibitory responses were compared to proportions of neurons in the basolateral nucleus displaying excitatory versus inhibitory responses using Chi Square analyses.

3.3 RESULTS

3.3.1 Spontaneous Activity

A total of 85 neurons were recorded from 26 rats. Of these, 35 neurons were tested for their responses to NE alone (8 of those were antidromically activated and confirmed as projection neurons), 15 were examined for responses to Te3 (n=8) or EC (n=7) stimulation before or after NE iontophoresis, 5 were examined for responses to NE before and after propranolol

administration, 5 were examined for responses to NE and clonidine, 12 were examined for responses to NE and LC stimulation, and 13 were examined for responses to NE and footshock.

Neurons from the lateral (LAT) and basolateral (BL) nuclei of the basolateral complex were examined for the above responses. There was no difference in firing rate among neurons located in different nuclei of the basolateral amygdala (LAT FR= 1.04±0.14Hz, BL FR=1.2±0.24Hz, p=0.88, t-test). The average firing rate across all neurons was 1.1±0.19Hz. The average firing rate of neurons confirmed to be projection neurons by antidromic activation (n=8) was 0.22 ±0.04Hz, significantly less than spontaneously active neurons (p=0.01, t-test).

3.3.2 Norepinephrine inhibits spontaneous activity of BLA neurons

Iontophoresis of NE (200um, 40nA) significantly decreased spontaneous activity (by 32±4.1% in the majority of neurons of the BLA (n=24/35, p=0.02, t-test, Figures 16-18). A smaller proportion of neurons showed a significant increase in spontaneous activity (by 136±11.4%, n=8/35, p=0.02, t-test, Figures 19,20) with few cells showing no response to NE iontophoresis (n=3/35, data not shown).

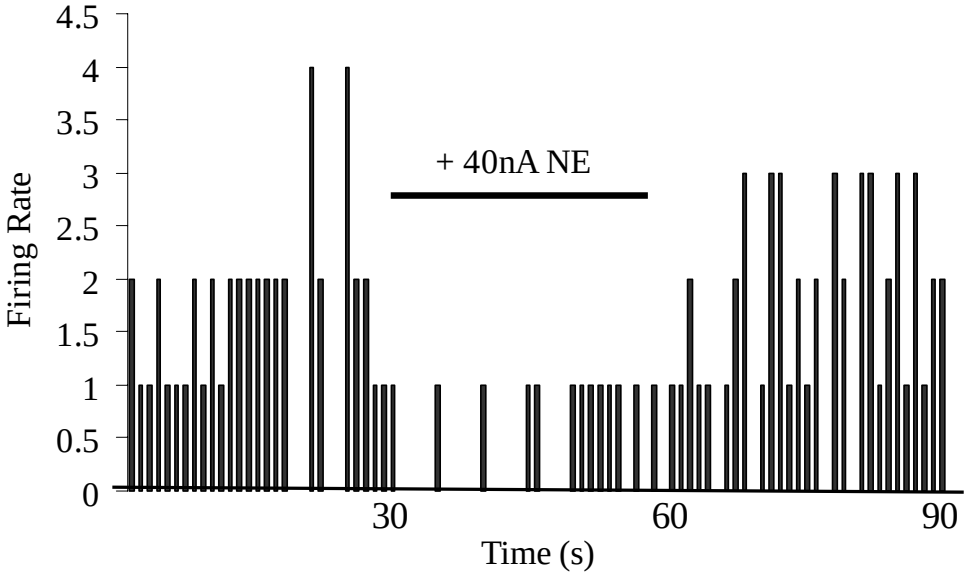


Figure 16 BLA neuron displaying an inhibition in response to NE. Iontophoresis of 40nA of NE onto a BLA neuron causes a decrease in spontaneous activity that returns to baseline after cessation of NE application.

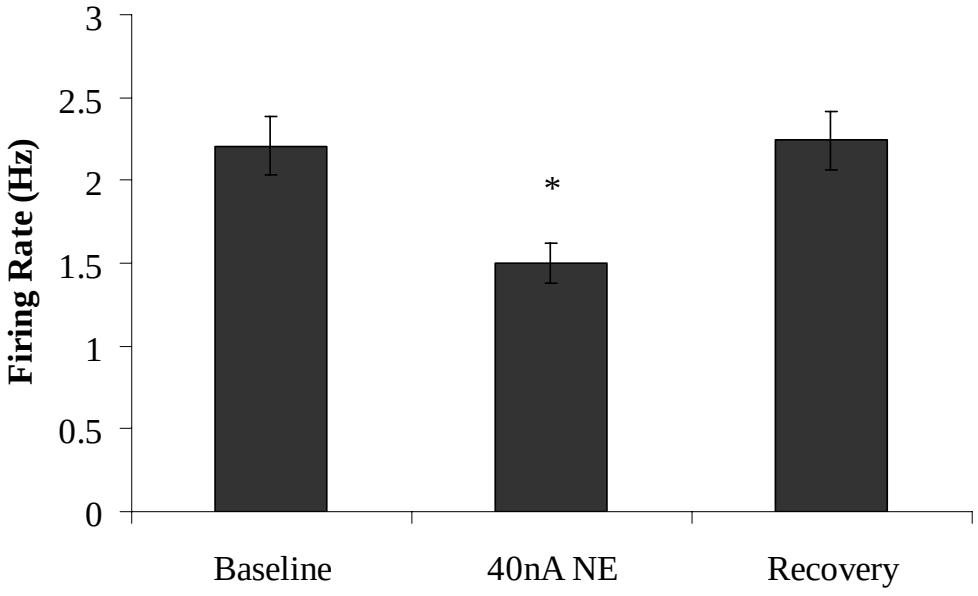


Figure 17 BLA neurons are significantly inhibited by iontophoresis of NE. The majority of neurons) displayed a significant inhibition of spontaneous activity with 40nA NE application (n=24/35, p=0.02, t-test).

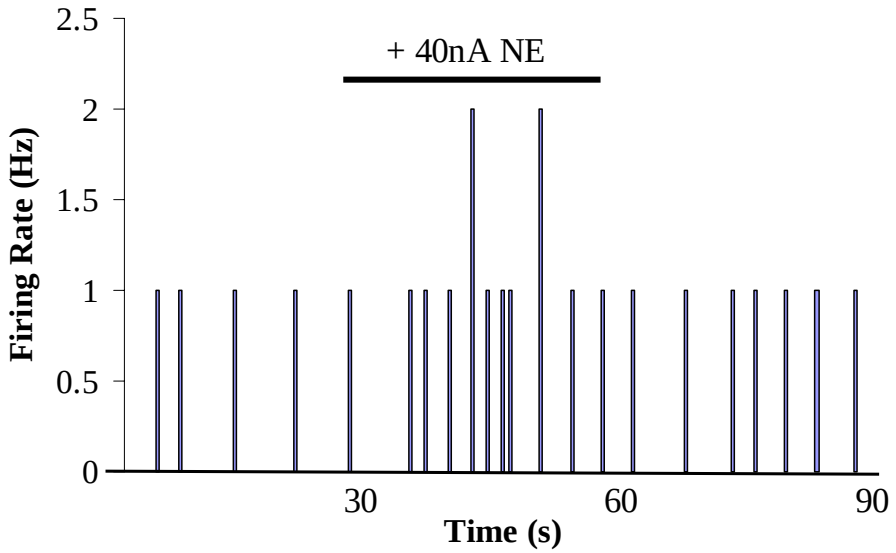


Figure 18 BLA neuron displaying an excitation in response to NE. Iontophoresis of 40nA of NE onto a BLA neuron causes an increase in spontaneous activity that returns to baseline after cessation of NE application.

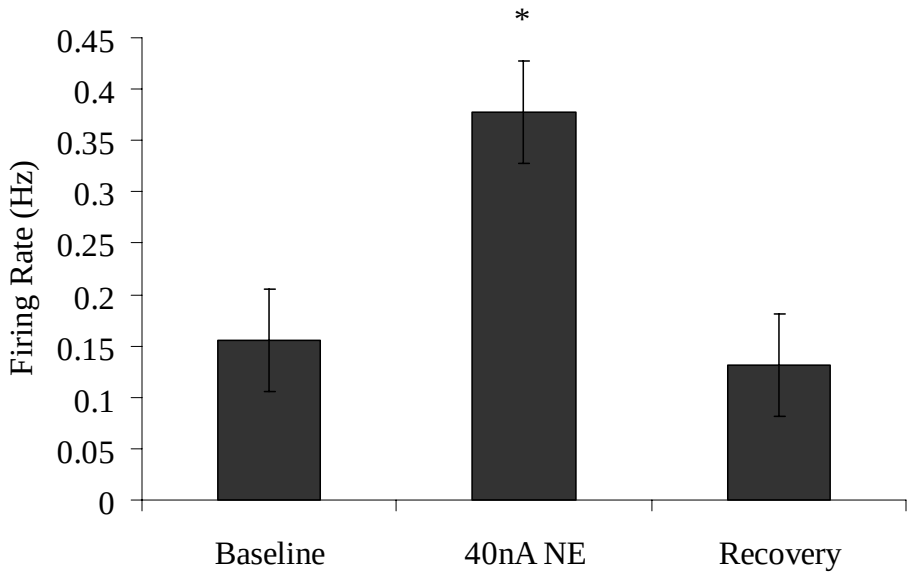


Figure 19 BLA neurons are significantly excited by iontophoresis of NE. A small number of neurons) showed a significant increase in spontaneous activity with 40nA NE application (n=8/35, p=0.02, t-test).

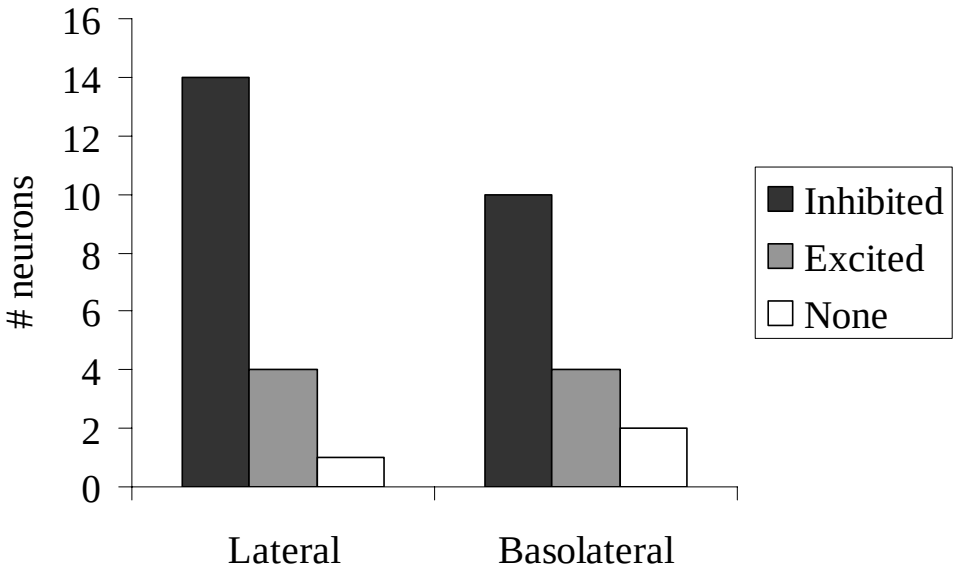


Figure 20 Neurons in different BLA subnuclei display similar responses to NE. Neurons in both the lateral and basolateral nuclei of the BLA displayed excitatory and inhibitory responses (no difference in proportions of neurons displaying inhibition/excitation, p=0.6, Chi Square).

Neurons from the lateral and basolateral nuclei were examined for their responses to NE. No significant differences were found in the proportions of neurons that were excited and inhibited in each of these nuclei (LAT: 74% inhibited, BL: 64% inhibited, p=0.6, Chi-Square). Therefore, there was no effect of location on NE effects (Figure 20). A subset of neurons was antidromically activated by electrical stimulation of EC, confirming them as projections cells. These neurons displayed only significant inhibitory responses to NE iontophoresis (by 58±9%, n=8, p=0.002, t-test, Figure 21).

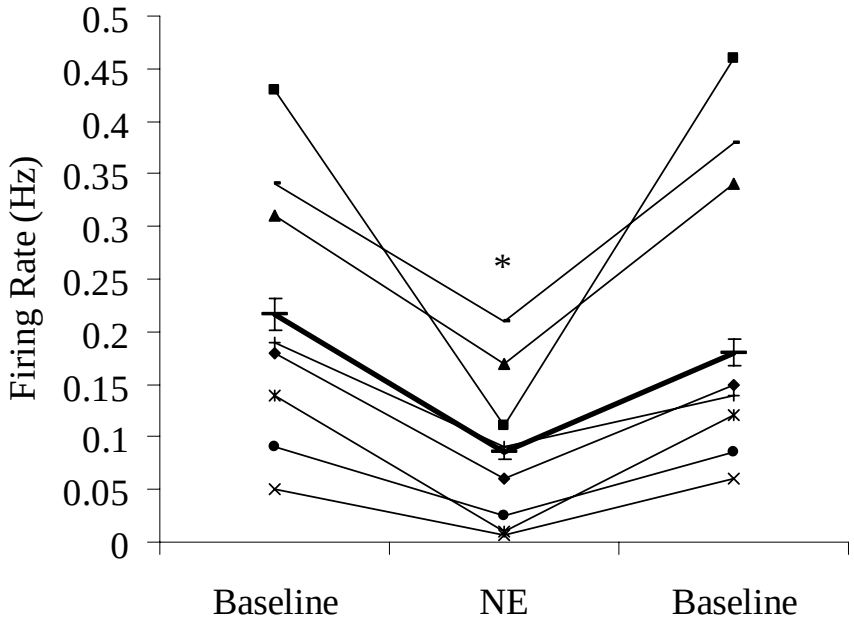


Figure 21 Projection neurons of the BLA are inhibited by NE. Neurons confirmed as projection cells via antidromic activation displayed significant inhibition to NE application (n=8, p=0.002, t-test).

3.3.3 Norepinephrine inhibits activity evoked by stimulation of entorhinal cortex (EC) and sensory association cortex (Te3).

Electrical stimulation of EC evoked orthodromic, excitatory responses in neurons of the BLA (n=7, Figure 22) with average latency to spike onset of 12.3±1.9ms. During iontophoresis of NE (10nA), the spike probability in response to entorhinal stimulation decreased significantly (by 62±8%, n=7/7, p=0.005, t-test, Figure 23). Electrical stimulation of Te3 caused orthodromic, excitatory responses in neurons of the BLA (n=8, Figure 24) with an average spike onset latency of 9.7±1.4ms. During iontophoresis of NE (10nA), the spike probability in response to Te3 stimulation decreased significantly (by 41±7%, n=8/8 neurons, p=0.002, t-test, Figure 25).

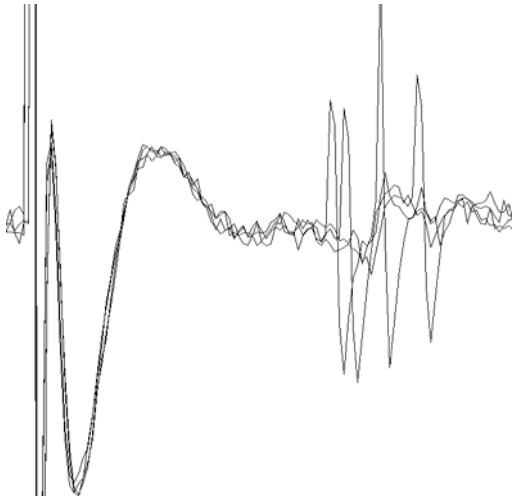


Figure 22 Electrophysiological trace of a neuron orthodromically activated by EC. Electrophysiological recording trace of a BLA neuron exhibiting orthodromic activation following EC stimulation (left).

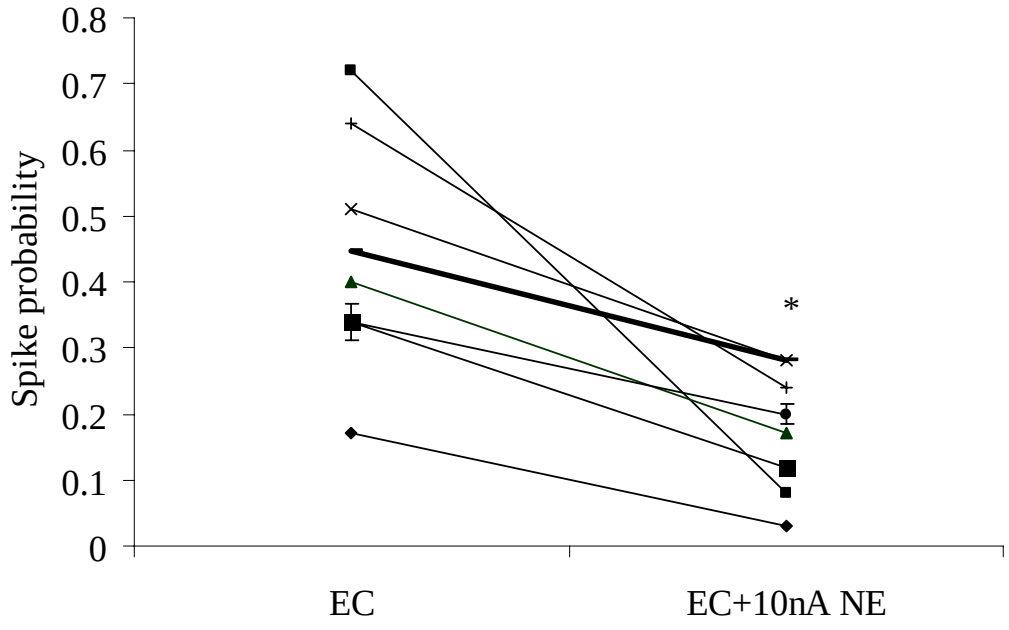


Figure 23 NE suppresses EC-evoked BLA neuron activity. Iontophoretic application of NE significantly suppresses responses to EC stimulation (n=7, p=0.005, t-test).

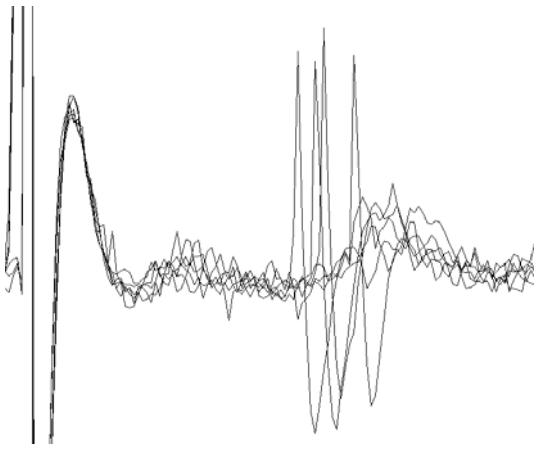


Figure 24 Electrophysiological trace of a neuron orthodromically activated by Te3. Electrophysiological recording trace showing orthodromic activation of a BLA neuron after Te3 stimulation.

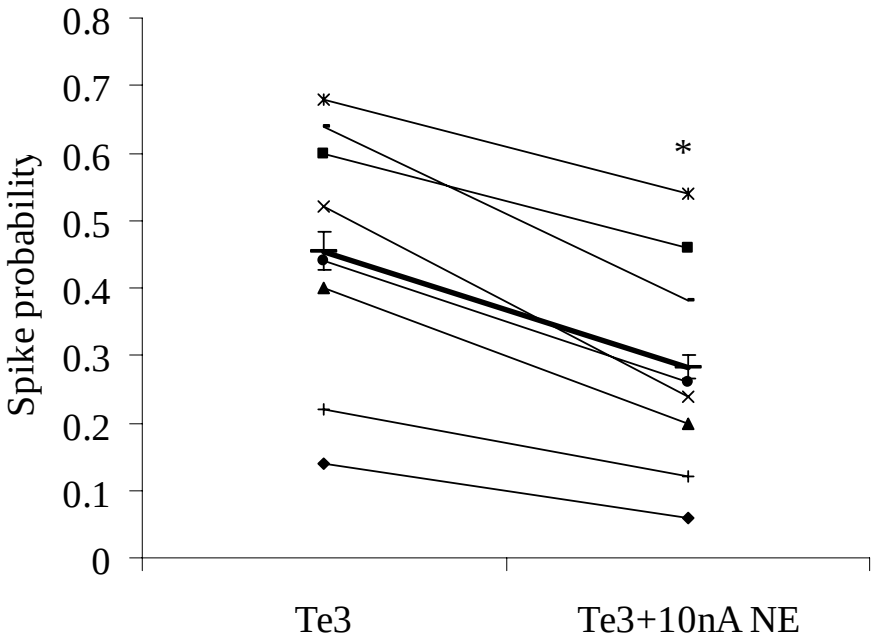


Figure 25 NE suppresses Te3-evoked BLA neuron activity. Iontophoretic application of NE significantly suppresses responses to Te3 stimulation (n=8, p=0.002, t-test).

A significant and consistent decrease in evoked spiking (both EC and Te3-evoked) with application of NE emerged at lower doses of NE (10nA) than did the decrease in spontaneous firing (40nA). A portion of neurons (n= 6) did not recover baseline evoked spiking probabilities to levels where it was sufficient to continue testing at higher doses of NE; however, in those that did exhibit recovery to baseline (n=9), NE iontophoresis (40nA) caused further inhibition of spiking probability (by 70±11%, p=0.00006, t-test).

3.3.4 Norepinephrine-induced inhibition is potentiated by systemic propranolol administration and mimicked by iontophoresis of clonidine.

Iontophoresis of clonidine (50um, 10nA) an alpha-2 agonist, caused a significant decrease in spontaneous activity of BLA neurons (by 65±7.0%, n=5, p=0.02, t-test, Figure 26). Iontophoresis of NE (40nA, 200um) inhibited spontaneous activity in these same neurons (by 61±6.8%, n=5, p=0.03, t-test, Figures 26, 27). Therefore, NE appears to act at alpha-2 receptors to cause inhibitory effects in BLA neurons.

Systemic administration of propranolol (I.V., 0.5mg/kg) caused a significant decrease in spontaneous activity of BLA neurons (by 46±5.2%, n=5, p=0.03, t-test, Figure 28). Furthermore, iontophoresis of NE (10nA, 200um) caused greater inhibition of BLA neuronal activity than before propranolol (at 10nA NE, controls: 35±6.2% inhibition, after propranolol: 99±11.2% inhibition, n=5, p=0.007, t-test, Figures 29, 30).

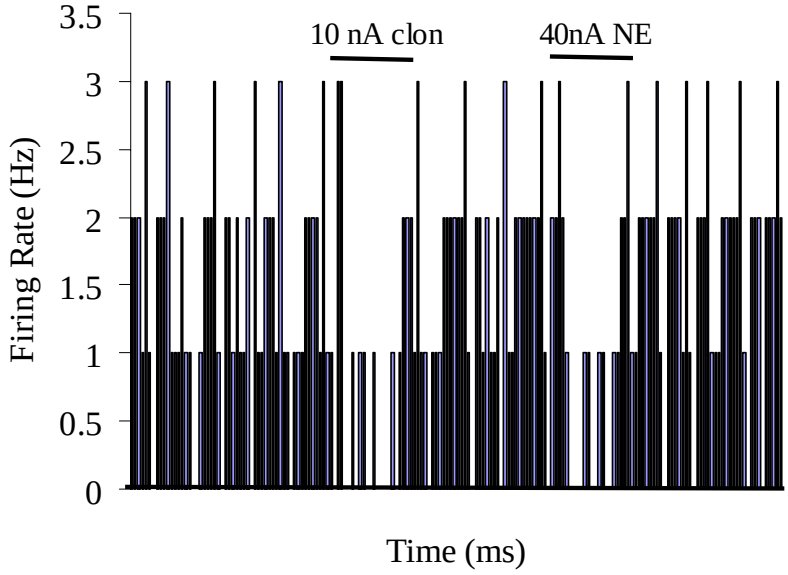


Figure 26 Clonidine and NE inhibit spontaneous activity of a BLA neuron. Firing rate histogram showing iontophoretic application of clonidine causing an inhibition of spontaneous activity of a BLA neuron. This same neuron exhibited inhibition of spontaneous activity in response to NE application.

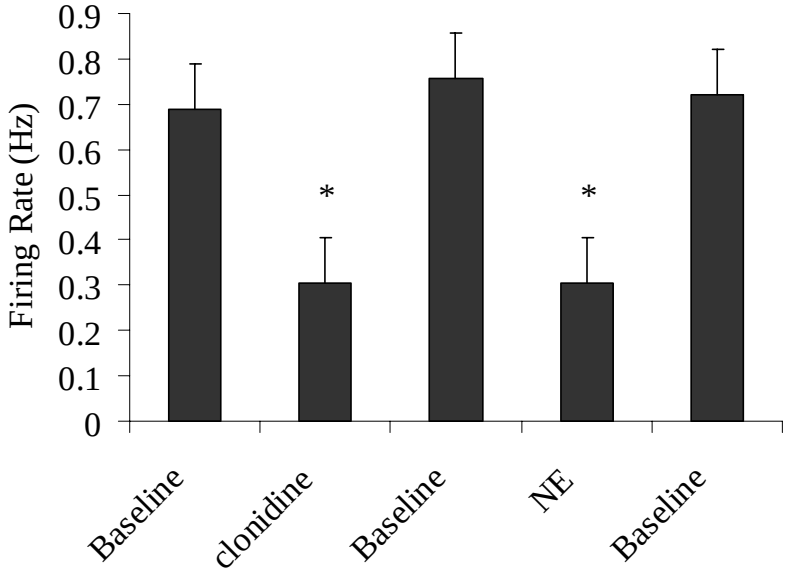


Figure 27 Population of neurons inhibited by NE and clonidine. All neurons that displayed significant inhibitory responses to clonidine iontophoresis (n=5, p=0.02, t-test) also displayed significant inhibitory responses to NE iontophoresis (n=5/5, p=0.03, t-test).

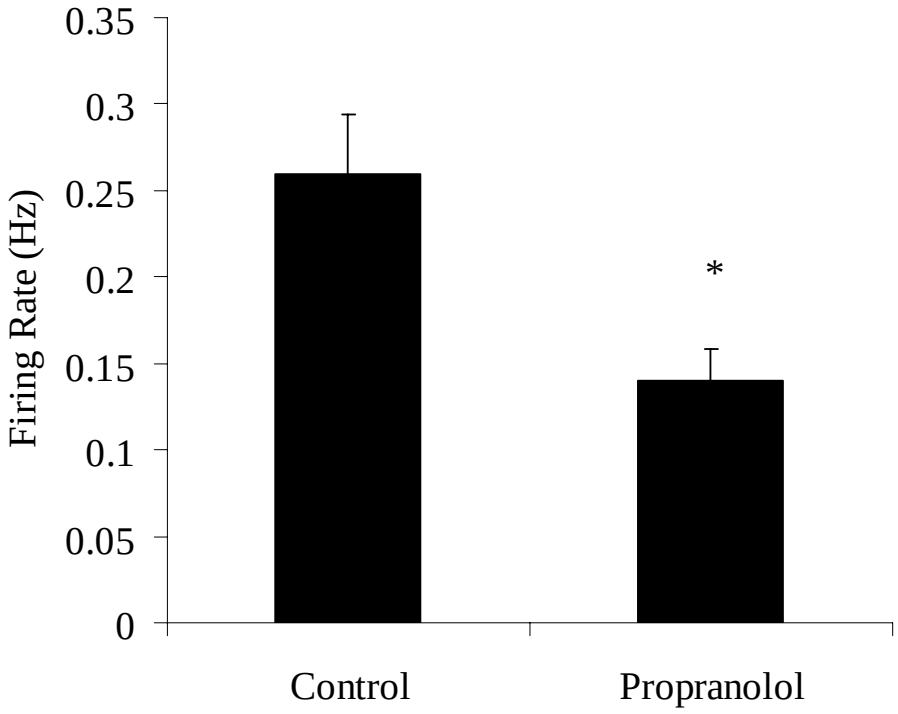


Figure 28 Propranolol decreases spontaneous BLA neuron activity. Excitatory effects of NE are mediated via beta receptors. Propranolol administration (500ug/kg, IV) caused a significant decrease in the spontaneous firing rate of BLA neurons (n=5, p=0.03, t-test).

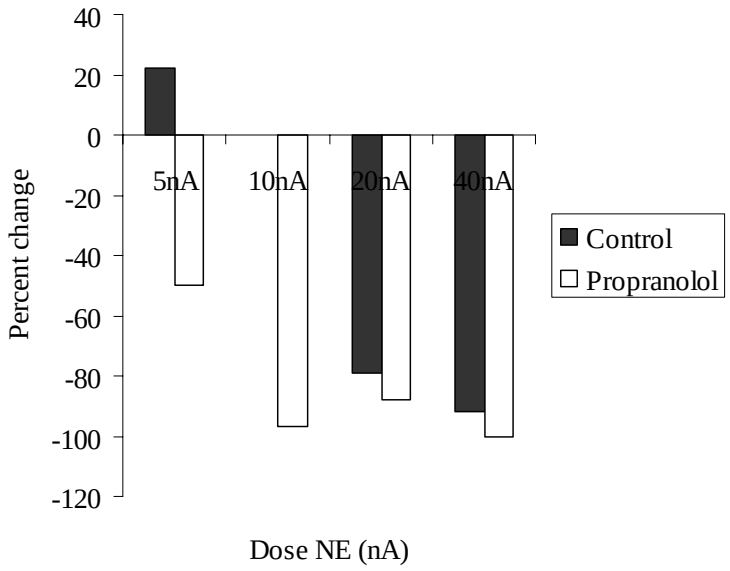


Figure 29 Propranolol potentiates NE-mediated inhibition of a BLA neuron. Propranolol administration significantly potentiates the dose-dependent inhibitory effects of NE iontophoresis in a BLA neuron.

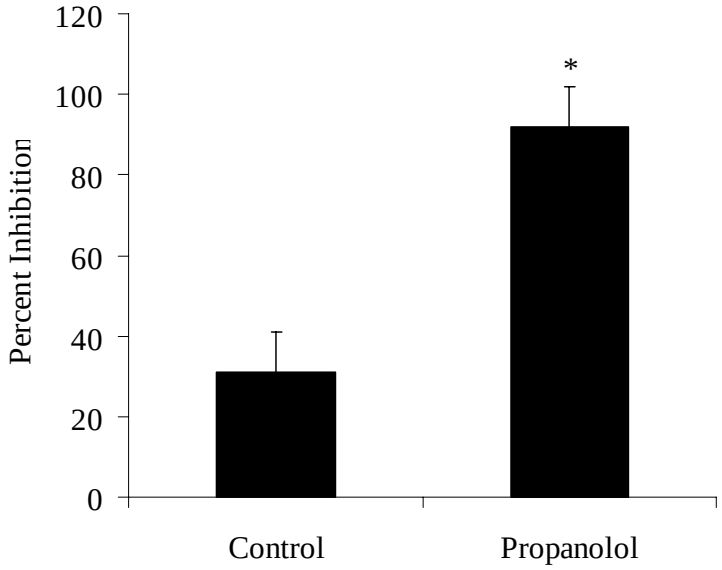


Figure 30 Population of BLA neurons where propranolol potentiated NE inhibition. All BLA neurons showed a significant potentiation of the inhibition displayed in response to NE iontophoresis after propranolol administration (n=5, p=0.007, t-test).

3.3.5 Footshock and LC stimulation cause similar responses as those produced by NE iontophoresis.

Electrical stimulation of the LC caused a significant inhibition in the spontaneous activity of neurons of the BLA (by 62+/-7.1%, n=8/12, p=0.01, t-test, Figure 31). Of these neurons, 6 were tested for their response to NE iontophoresis, and all 6 displayed NE-induced inhibition (by 51+/-12%, n=6/6, p=0.004, t-test, Figure 32, 33). LC stimulation also caused significant excitation of BLA neurons (by 146+/-13%, n=4/12, p=0.001, t-test, Figure 34). All of these neurons (n=4) were tested for responses to NE iontophoresis, and 3 were also excited by NE (by 104+/-18%, n=3/4, p=0.001, t-test, Figure 35, 36). One neuron tested did not significantly respond to NE iontophoresis.

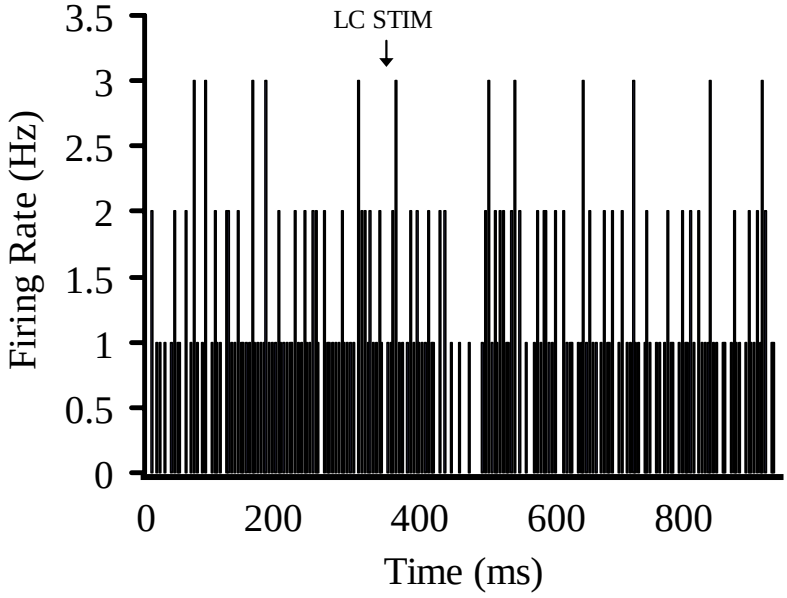


Figure 31 BLA neuron inhibited by LC stimulation. Firing rate histogram of a BLA neuron showing inhibition in response to LC stimulation.

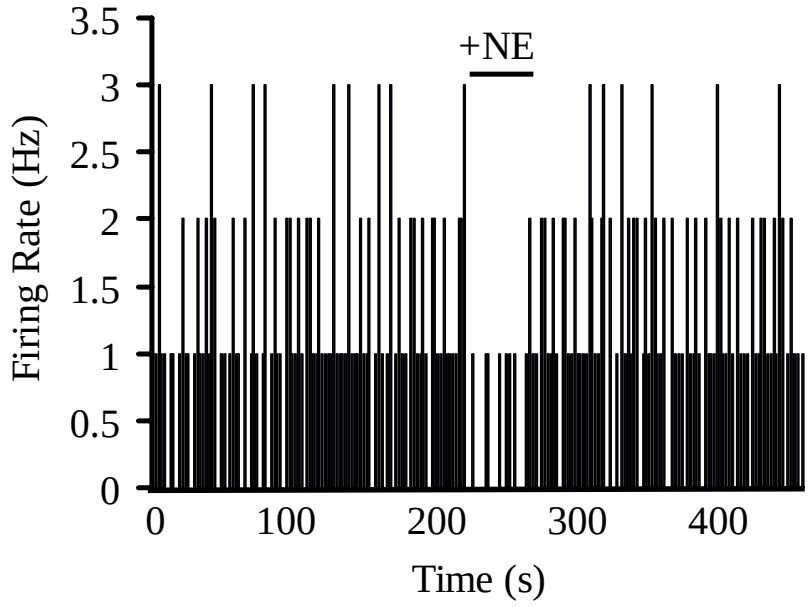


Figure 32 Same BLA neuron is inhibited by NE iontophoresis. This same BLA neuron showed inhibition in response to NE iontophoresis.

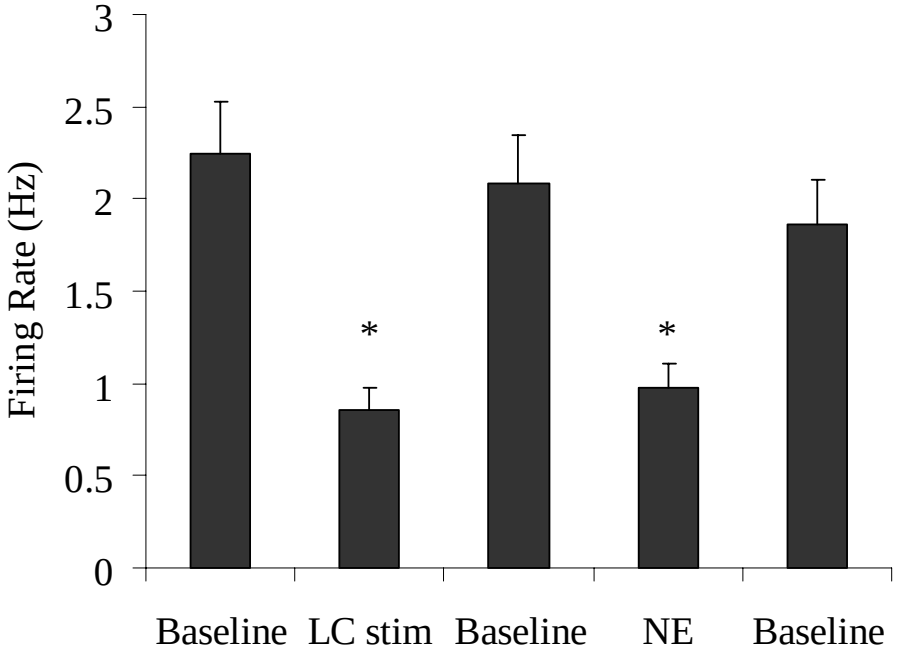


Figure 33 Population of BLA neurons showing inhibitory responses to LC stimulation. Of the BLA neurons that displayed significant inhibitory responses to LC stimulation (n=8, p=0.01, t-test) a subset (n=6) that were tested for responses to NE iontophoresis also displayed inhibition to NE iontophoresis (n=6/6, p=0.004, t-test).

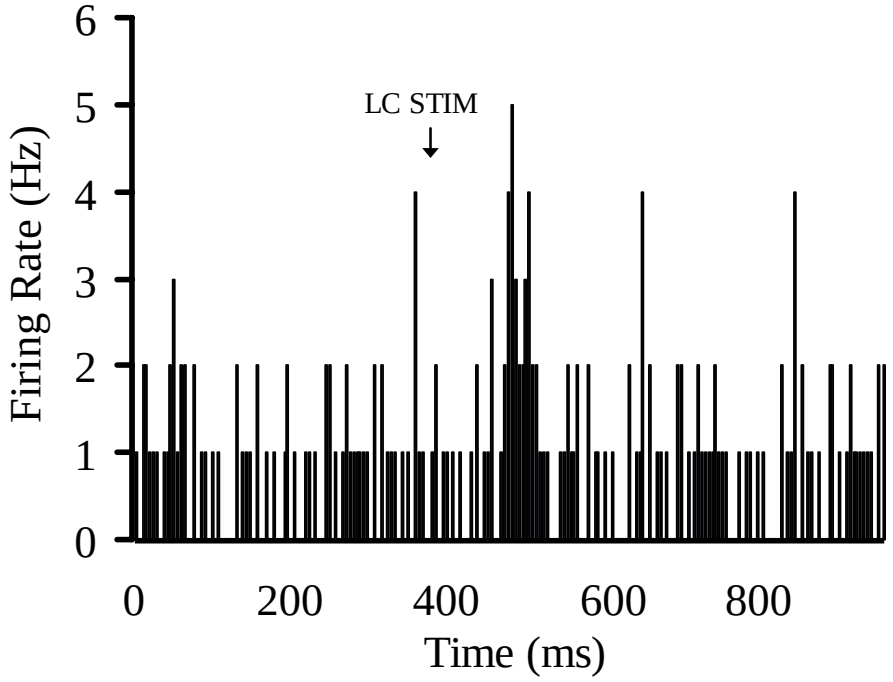


Figure 34 BLA neuron excited by LC stimulation. Example of a BLA neuron showing excitation in response to LC stimulation.

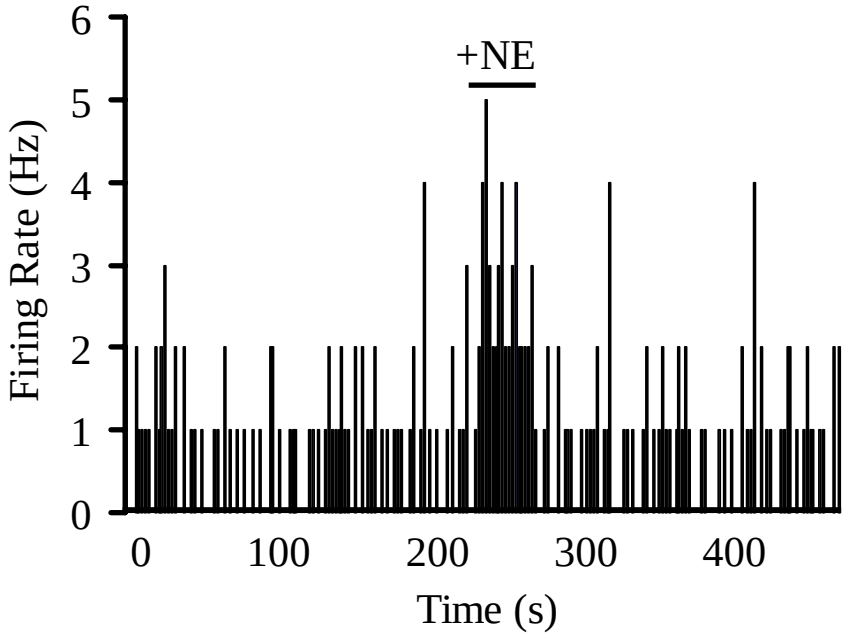


Figure 35 Same BLA neuron excited by NE iontophoresis. This same BLA neuron showed excitation in response to NE iontophoresis.

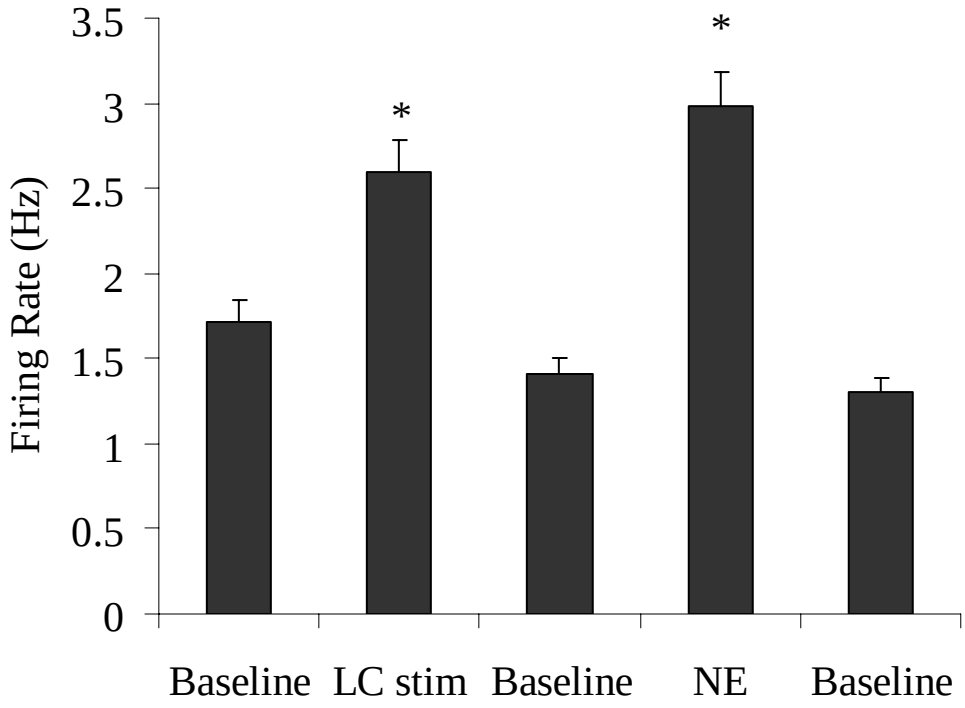


Figure 36 Population of BLA neurons showing excitatory responses to LC stimulation. The majority of BLA neurons that displayed significant excitatory responses to LC stimulation (n=4, p=0.001, t-test) also displayed excitatory responses to NE iontophoresis (n=3/4, p=0.01, t-test).

Footshock administration (5mA, 0.5ms) caused a significant inhibition in spontaneous firing of BLA neurons (by 75+/-11%, n=8/13, p=0.003, t-test, Figure 37). All neurons inhibited by footshock also displayed inhibitory responses to iontophoresis of NE (by 44+/-9%, n=8/8, p=0.004, t-test, Figure 38, 29). Footshock administration also caused excitation of BLA neurons (by 212+/-29%, n=5/13, p=0.003, t-test, Figure 41). Of these neurons, the majority also displayed excitatory responses to NE iontophoresis (by 118+/-23%, n=4/5 p=0.002, t-test, Figure 42, 43). One neuron tested did not significantly respond to NE iontophoresis.

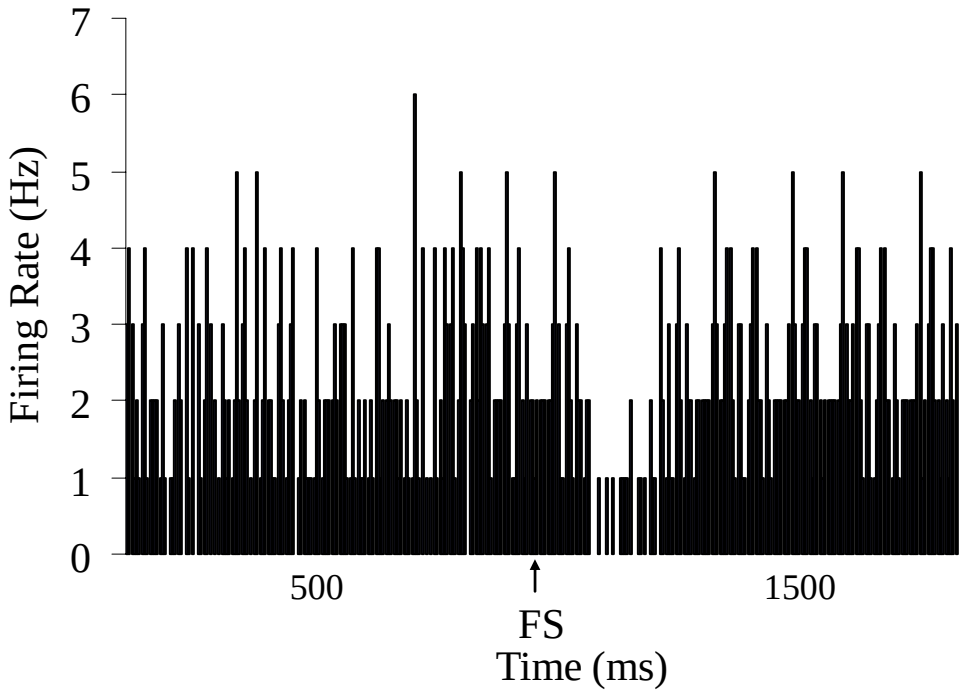


Figure 37 BLA neuron inhibited by footshock. Firing rate histogram of a BLA neuron showing inhibition in response to footshock .

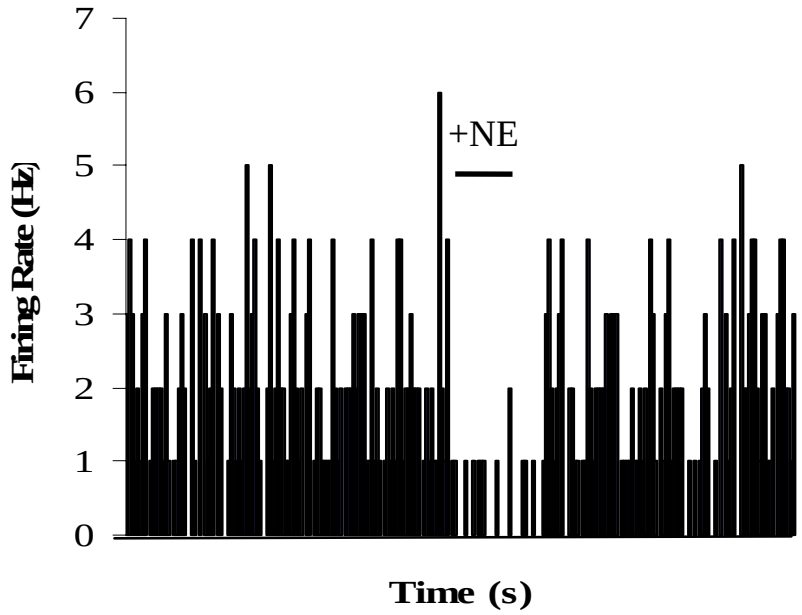


Figure 38 Same BLA neuron inhibited by NE iontophoresis. This same BLA neuron showed inhibition in response to NE iontophoresis.

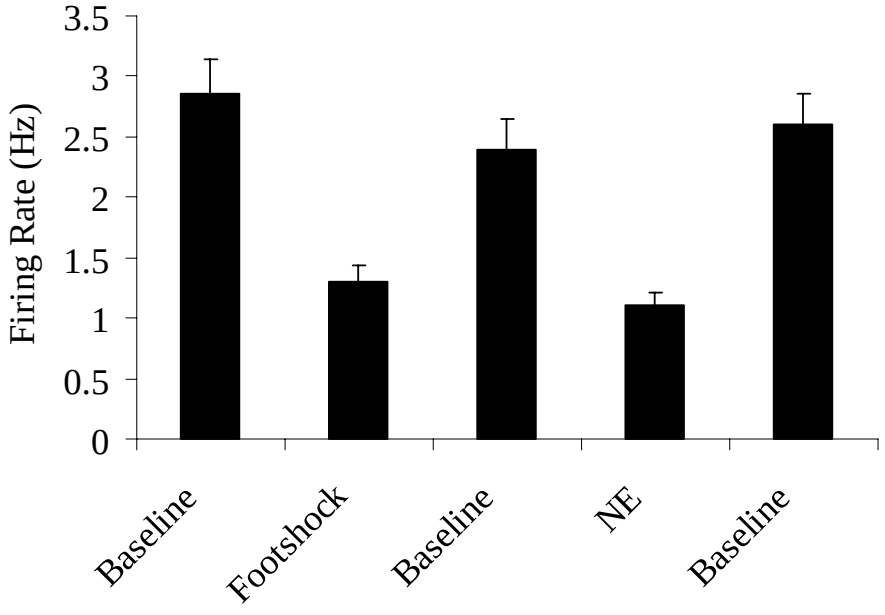


Figure 39 Population of BLA neurons showing inhibitory responses to footshock. All neurons that displayed significant inhibitory responses to footshock (n=8/13, p=0.003, t-test) also displayed significant inhibitory responses to NE iontophoresis (n=8/8, p=0.004, t-test).

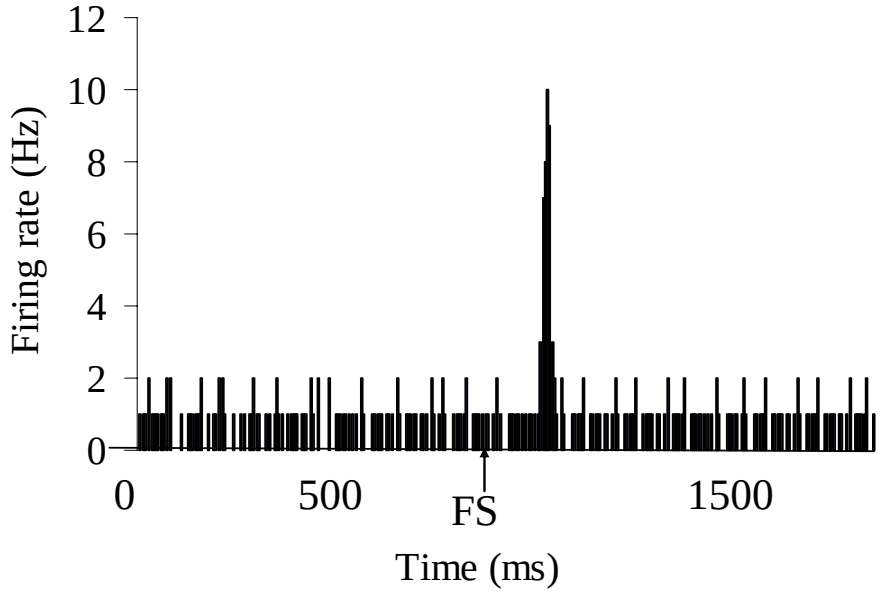


Figure 40 BLA neuron excited by footshock. Example of a BLA neuron showing excitation in response to footshock.

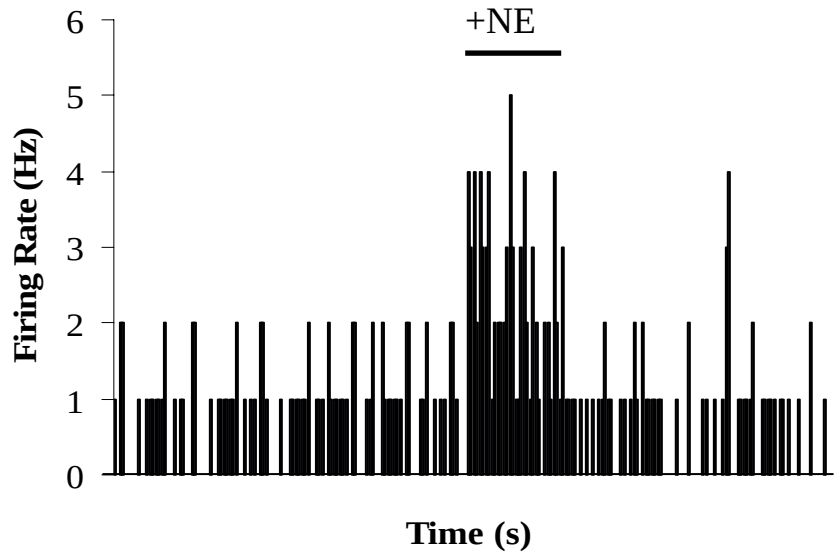


Figure 41 Same BLA neuron excited by NE iontophoresis. This same BLA neuron showed excitation in response to NE iontophoresis.

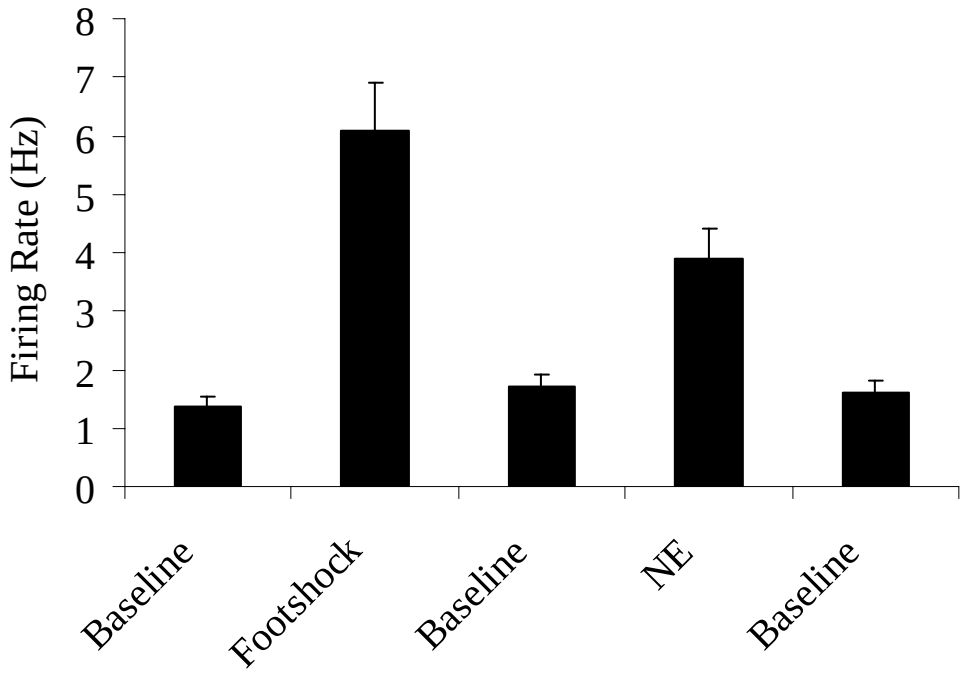


Figure 42 Population of BLA neurons showing excitatory responses to footshock. The majority of BLA neurons that displayed significant excitatory responses to footshock (n=5/13, p=0.003, t-test) also displayed excitatory responses to NE iontophoresis (n=4/5, p=0.002, t-test).

3.4 DISCUSSION

This study demonstrates that the NE input from the LC exerts potent alpha-2-mediated inhibition of spontaneous and evoked activity of neurons in the BLA. NE delivered locally via iontophoresis has potent inhibitory effects on spontaneous activity in the BLA, and this inhibition is mimicked by an alpha-2 receptor agonist. NE also has smaller excitatory effects mediated by beta receptors. The effects of both footshock and LC stimulation on BLA neurons can be reproduced by NE iontophoresis. NE also potently inhibits activity evoked by stimulation of EC and Te3, which is consistent with an overall inhibitory effect of NE in the BLA. This study provides the first *in vivo* electrophysiological data to complement substantial behavioral studies examining the role of NE in the BLA.

These data are also consistent with evidence from electrophysiological studies performed in vitro in the BLA. NE in the BLA is reported to enhance excitatory neurotransmission via beta-adrenergic receptors (Gean et al. 1992, Huang et al. 1996, Ferry et al. 1997) while producing inhibitory effects via alpha-2 receptors (Ferry 1997). NE also reduces synaptic transmission in the BLA and disrupts plasticity via actions at alpha-2 receptors (DeBock et al. 2003). We demonstrate here that the inhibitory actions of NE were mimicked by administration of the alpha-2 agonist clonidine, suggesting NE has its inhibitory effects at least in part via alpha-2 receptors. Furthermore, NE in vivo produces a moderate tonic excitatory effect on BLA neurons via beta receptors which is lost with beta-receptor blockade, which causes a decrease in spontaneous firing rate. In fact, the excitation caused by NE, though present in a fewer number

of neurons (n= 8/35) than the inhibition (n=24/35), was much greater in magnitude (136%) than the inhibition (32%). Furthermore, blockade of beta receptors leads to a stronger inhibition of neuronal activity by NE iontophoresis, presumably due to blockade of the excitatory actions of NE mediated via beta-receptors, thereby unveiling a more potent inhibition via alpha-2 receptors.

The LC sends a large NE projection to the BLA. However, little is known about the specific targets of these NE afferents. Previous studies reveal that NE terminals make asymmetric synapses onto GABAergic neurons in the BLA (Li et al. 2001). Therefore, the inhibition of projection neurons caused by NE may potentially be mediated via excitation of GABAergic inhibitory interneurons which synapse onto glutamatergic projection neurons within the BLA (Muller et al. 2003, 2006). In neurons that exhibited properties consistent with BLA interneurons (i.e., high firing rate and tendency toward brief action potential duration; Rosenkranz & Grace, 1999), we saw both excitatory and inhibitory effects. However, in addition to their synapses onto projection neurons (Muller et al. 2003, 2006), interneurons are known to synapse onto other interneurons within the BLA (Li et al. 2002, Muller et al. 2005). Therefore, excitation of an interneuron that in turn inhibits another interneuron could potentially explain these two types of effects. There is evidence for alpha-2 and beta receptors within the BLA (Alburges et al. 1993). Alpha-2 receptors function as autoreceptors for the NE system (Grigg et al. 1996, Arima et al. 1998), and therefore presynaptic inhibitory alpha-2 receptors could also account for the inhibitory effects of NE and clonidine. However, there is some evidence of post-synaptic alpha-2 receptors in the BLA as well (U'Prichard et al. 1980). These potential mechanisms have not been distinguished within the BLA to date. Although not conclusive, the observation that LC stimulation produces the same inhibitory responses as NE iontophoresis is

consistent with a predominantly postsynaptic alpha-2 adrenergic inhibition of BLA neuronal activity.

NE in the BLA enhances learning and consolidation of tasks involving aversive stimuli, most often footshock (McGaugh 2002). One might interpret our data showing a predominantly inhibitory effect of NE in the BLA as being contradictory to the facilitative effect exerted by NE on behavior. However, these behavioral effects of NE are mediated via beta receptors (McGaugh et al. 1988, Introini-Collision et al. 1991, Ferry et al. 1999, Strange and Dolan 2004). Our data demonstrate that the actions of NE in the BLA via beta-receptors are, in fact, excitatory. Furthermore, these behavioral effects have never demonstrated that facilitation of behavior involves an activation of the BLA. Norepinephrine having mainly inhibitory effects in the BLA could serve two functions. First, having an overall inhibitory effect on the majority of neurons of the BLA could serve to enhance the signal-to-noise ratio of those select units that are excited. This would increase transmission of those excited units onto downstream BLA targets to subserve behavioral responding. Alternatively, an inhibition of the BLA could lead to a disinhibition of the central nucleus of the amygdala (CeA), as the BLA has a predominantly inhibitory influence over the CeA (Rosenkranz et al. 2006). Disinhibition of the CeA could lead to activation of downstream brain targets involved in the behavioral response. The CeA sends excitatory, CRH-containing projections to the LC (Van Boeckstale et. al 2001) and paraventricular nucleus of the hypothalamus (Gray et al. 1989), two regions heavily involved in the production of portions of the stress response and stress-related behaviors.

In addition to its inhibitory effects on spontaneous BLA neuronal activity, NE also caused a decrease in activity evoked by stimulation of EC and Te3. This contrasts with the observation in other brain regions that NE alters the signal-to-noise ratio by decreasing baseline

firing but increasing evoked responses (Foote et al. 1975, Segal and Bloom 1976, Waterhouse et al. 1980, 1984, 1998, 2000, Doze et al. 1991, Waterhouse and Woodward 1980, Devilbiss and Waterhouse 2000). At face value this would appear to be inconsistent with stress-related learning, in which the EC is thought to modulate the learning of certain behaviors through an interaction with the BLA (Ferry et al. 1999). However, it is possible that NE modulation of these inputs is altered during learned associations. Therefore, in the absence of learned associations, the NE system may function to suppress activity within circuits in which fear-conditioned behavior has not taken place.

The stress response often functions as an adaptive mechanism by which organisms can react to potentially threatening or harmful stimuli with appropriate behavioral responses. Noradrenergic modulation of structures involved in these stress circuitries plays a critical role in many of these responses. However, extensive exposure to stress is a common factor associated with the development and/or enhancement of pathology and disease. Activation of the HPA axis by novel stressors is enhanced after exposure to chronic immobilization stress, an effect that has been shown to rely on NE modulation of the BNST and the PVN (Cecchi et al. 2002, Ma and Morilak 2005a, b) and may result in maladaptive behavioral responses to stress. Here, we examine the NE modulation of the BLA. Lesions of the BLA inhibit HPA responses to acute stress (Bhatnagar et al. 1998), and the BLA has been suggested as a potential site involved in sensitization (Bhatnagar et al. 2004). These and other data combined with the data found here suggest the BLA as another important nucleus that may be susceptible to stress-induced changes that may lead to sensitization. A more complete understanding of how NE alters transmission in regions such as the BLA that serve as important modulators of the stress response can lead to insights into how transmission within these circuits modulates stress-related behaviors.

4.0 CHRONIC COLD STRESS INCREASES EXCITATORY EFFECTS OF NOREPINEPHRINE ON SPONTANEOUS AND EVOKED ACTIVITY OF NEURONS OF THE BASOLATERAL AMYGDALA

4.1 INTRODUCTION

The amygdala is activated by a large variety of acute stressors (Rosen et al. 1998, Akirav et al. 2001, Dayas et al. 2001), and plays a facilitatory role in components of the stress response (Feldman et al. 1995, Van de Kar and Blair 1999, Herman et al. 2003). The basolateral complex of the amygdala (BLA) receives dense noradrenergic (NE) projections from the locus coeruleus (LC, Asan et al. 1998), which also has an excitatory influence on some of the stress circuitries (Plotsky 1987, Al-Damluki 1988, Ziegler et al. 1999, Sved et al. 2002). Levels of NE increase in the BLA during stress exposure (Galvez et al. 1996, Hatfield et al. 1997, Williams et al. 1998), and neurons of the BLA are sensitive to stressful stimuli that also activate LC neurons (Rasmussen and Jacobs 1986, Abercrombie and Jacobs 1987, Grant et al. 1988, Aston-Jones et al. 1991, Shors et al. 1999, Correll et al. 2005). In addition to their roles in acute stress responses, the LC and BLA may play a role in chronic stress.

Chronic or repeated exposure to stressful stimuli leads to a sensitization of many components of the stress response, with organisms displaying exaggerated or hyperactive

responses to stressful stimuli. Prior exposure to chronic cold and repeated footshock enhances activation of LC neurons to acute stress, footshock (Simson and Weiss 1988, Curtis et al. 1995, Mana and Grace 1997), and to corticotrophin-releasing hormone (Jedema and Grace 2001). Stress causes the release of NE in brain regions innervated by the LC (Abercrombie 1988, Rossetti et al. 1990, Cenci et al. 1992, Nakane et al. 1994). This NE efflux is enhanced after chronic cold exposure or repeated immobilization (Nisenbaum et al. 1991, Pacak et al. 1992, Gresch et al. 1994, Jedema et al. 1999). This enhanced NE may lead to alterations in stress-induced activity in targets of the LC. Stress-induced release of NE has been shown to facilitate activation of the HPA axis to acute immobilization stress. This effect is specific to the BNST (Cecchi et al. 2002), the PVN (Ma and Morilak 2005a) and the amygdala (Ma and Morilak 2005b). Therefore, in addition to alterations in the NE neurons and NE efflux that occur with chronic or repeated exposure to stressful stimuli, alterations in the actions of NE at postsynaptic targets play a role in the behavioral effects of chronic stress.

The basolateral amygdala is sensitive to the effects of chronic stress. Chronic immobilization stress causes a large increase in spine density on BLA pyramidal neurons (Mitra et al. 2005), and stress enhances plasticity in BLA circuits (Vouimba et al. 2004, 2006). Chronic cold exposure eliminates inhibitory responses of BLA neurons to footshock, leaving only excitatory responses intact (Correll et al 2005). In this study, we investigate how exposure to chronic cold alters spontaneous activity and NE modulation of neurons of the BLA. This is examined in terms of NE impact on spontaneous activity, as well as activity evoked via stimulation of entorhinal cortex (EC) and sensory association cortex (Te3), which both send excitatory projections to the BLA. We use chronic cold exposure as a model of chronic stress. Animals exposed to chronic cold reliably exhibit signs of sensitization of the NE system to

stressful stimuli such as LC neurons more sensitive to CRH or enhanced NE release in terminal regions (Nisenbaum et al. 1991, Pacak et al. 1992, Gresch et al. 1994, Jedema et al. 1999, Jedema et al. 2001). In order to investigate the time course of the development of alterations in LC targets during chronic cold exposure we ran our experiments in two experimental groups: rats exposed to 7 days of cold and rats exposed to 14 days of cold. Seven days of cold exposure does not lead to sensitized NE release in terminal regions, while 14 days of exposure does (Finlay et al 1997). Therefore, an examination of the development of changes in the BLA may lend insight into whether or not potential changes in BLA neurons are all necessarily due to sensitized NE efflux, or whether alterations may develop earlier in regions of the brain other than the LC. These studies may demonstrate an important role for the amygdala as a contributor to the sensitization of portions of the stress response.

4.2 METHODS

4.2.1 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 (250-400g), were initially housed in pairs upon entrance to the colony and kept on a 12:12 light/dark cycle, and were maintained at constant temperature and given food and water ad libitum. Rats were pair-housed for 5 days. Each pair was then separated, with one of the cage

mates now singly-housed and remaining in the control housing room until removal for experimental use (used as a control rat, see previous data, Chapter 3). The other cage mate was shaved and placed in the cold room, also singly-housed for 7 or 14 days, depending on experimental group. Upon removal, each cold-exposed animal was singly-housed in the control room for 24 hours before final removal and experimental use (see Section 4.2.7 for further details on cold exposure paradigm). Upon removal, rats were anesthetized with 8% chloral hydrate (400mg/kg, i.p.) and then implanted with jugular catheters for continued anesthetic administration. Rats were then placed in a stereotaxic apparatus, with supplemental doses of chloral hydrate given intravenously to maintain a constant level of anesthesia, and 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 of the BLA (-5.0L, -3.0C from bregma), sensory association cortex (Te3, -6.1L,-6.8C, -4.7V from bregma), and the EC (-5.0L, -6.8C, -8.2V from bregma) were calculated using a stereotaxic atlas (Paxinos and Watson 1986). Stimulating electrodes were lowered into the brain, and recordings did not begin until at least 45 minutes after placement of the stimulating electrodes.

4.2.2 **Electrophysiology**:

Multibarrel electrodes (five barrels; Activational Systems, Warren, MI) were constructed using a vertical microelectrode puller, broken back under microscopic control, and filled with 2% Pontamine sky blue in 2M NaCl to yield electrodes with a central recording barrel impedance of 4-8MOhms. These electrodes were lowered through the BLA in successive vertical tracks.

Neurons within the BLA were isolated, and only those with a signal-to-noise ratio greater than 3:1 were used for data analysis. In all experiments, spontaneous activity was recorded for a minimum of 5 minutes and expressed as spikes/second.

4.2.3 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 minutes after lowering of the stimulating electrodes. Neurons responsive to either Te3 or EC stimulation were found using a search-stimulate protocol. Single-pulse stimuli (300-900uA, 0.25ms, 0.5Hz) 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. Responses were operationally defined as orthodromic if they had an onset latency of <20ms, showed a failure to substantially change latency in response to increases in current intensity, their onset latency remained fairly consistent with approximately 1-5ms of variability in evoked spike latency, and they follow paired pulses at 50Hz but not 400Hz. Antidromic responses were characterized by their constant onset latency with virtually no variability, and their ability to followed paired pulses at 400Hz. Monosynaptic responses were differentiated from polysynaptic spikes by an examination of onset latency and variability of latencies, as well as polysynaptic failure to follow paired pulses at 50Hz.

4.2.4 Microiontophoresis:

The central barrel of the 5-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 NE was used in the remaining barrels. Drug barrels of the multibarrel pipette were filled with 200mM NE, pH 4.0, dissolved in 0.1M NaCl. NE was retained with (-) current and ejected with (+) iontophoretic current (E104B; Fintronics). Retaining currents ranged between 12 and 18 nA while drug ejection currents ranged between 5 and 40nA. The ejection was done using repeated, increasing doses from 5-40nA with at least 2 minutes of spontaneous activity measured between each period of drug ejection.

Neurons were tested for dose responsivity to NE (5-40nA). Those neurons that responded to lower doses of NE (5-10nA) did so in a moderate, variable, and often nonsignificant manner. More clear, significant, and consistent responses emerged in BLA neurons to higher doses of NE (40nA). Therefore, this dose was used for analysis of NE effects on spontaneous activity. However, evoked activity was significantly altered by lower doses of NE, therefore the effects of NE on evoked activity focused on the 10nA dose, however, effects are also reported for higher doses where tested.

4.2.5 Histology:

At the conclusion of electrophysiological experiments, Pontamine sky blue was ejected by passing constant current through the recording electrode (10nA, 15 minutes) to mark the location of the recording site. Anodal current was passed through the stimulating electrode to create a

small lesion for identification of placement of the stimulating electrode. Rats were sacrificed by an overdose of anesthetic, followed by decapitation and brain removal. Brains were fixed in 10% formalin for a minimum of 24 hours, and then cryoprotected with 25% sucrose solution in 0.1M phosphate buffer. Subsequently, coronal slices were cut on a cryostat into 40µm coronal sections, mounted on slides, and stained with cresyl violet. Recording sites were identified by the presence of the Pontamine sky blue spot, and the location of the stimulating electrodes was identified by the presence of a small lesion at the end of the electrode track.

4.2.6 Data Analysis

Firing rate was calculated as spikes/second. An effect was labeled as a significant excitatory or inhibitory effect if during the NE iontophoresis the average firing rate increased or decreased by 25% or more from baseline. This was based on conservative estimates of the 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. For neurons monosynaptically activated by EC or Te3 stimulation, the average onset latency was measured across 50 stimulation trials. 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 to the EC or Te3 and the number of stimuli that resulted in evoked spikes within the BLA was measured. This was compared to the number of afferent stimuli that evoked spike firing during NE iontophoresis. These spike probabilities were compared using paired t-tests. Changes in evoked spikes were expressed as percent increase or decrease in the probability

of evoked responses of BLA neurons resulting from NE iontophoresis. A minimum of 3 minutes of spontaneous firing was allowed 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 days of chronic cold, and rats exposed to 14 days of chronic cold were compared using Chi Square tests. The magnitude of percent inhibition of NE on evoked activity in cold-exposed rats was compared to the NE-induced inhibition of evoked activity in control rats using t-tests. The effects of 7 or 14 days of chronic cold on baseline firing rates were compared using a one way ANOVA. Interactions between cold stress groups and neuronal location with regard to baseline firing rates were examined using a two-way ANOVA.

Projection neurons were examined and analyzed separately as a subclass of neurons. Neurons were confirmed as projection cells if they could be antidromically activated by EC stimulation. These neurons were examined separately for their response to NE iontophoresis, and pre and post-NE firing rates were compared using paired t-tests.

Subclasses of neurons were also examined based on neuronal location. Histological verification revealed subgroups of neurons located in the lateral and basolateral nuclei of the BLA complex. These neurons were analyzed separately for their responses to NE iontophoresis, and proportions of neurons in the lateral nucleus displaying excitatory versus inhibitory responses were compared to proportions of neurons in the basolateral nucleus displaying excitatory versus inhibitory responses using Chi Square analyses.

Data concerning control animals were previously reported in this manuscript (Chapter 3). To ensure against unnecessary use of more animals, data collected regarding NE effects on spontaneous and evoked activity reported in Chapter 3 were re-examined and compared to data collected from rats in each cold exposed group (7 day and 14 day). To minimize differences

between these groups (as described previously), animals were initially housed as pairs, and then cage mates were separated with one placed in the control group and one placed in the experimental group. All rats had similar housing conditions (except for the temperature variable), and food and water availability. All example neurons depicted in this chapter (Figures 45, 46, 49, 51, and 53) were recorded from rats exposed to chronic cold.

4.2.7 Chronic Cold Exposure:

Rats were randomly assigned to cold-exposed and control groups. After five days of 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 or 14 days). 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 or 14 days (Jedema et al. 1999, Jedema et al. 2001, Mana and Grace 1997). Rats used as controls were kept in the control housing room (data reported in Chapter 3 and used as control data in Chapter 4). Both control and cold exposed rats were singly housed for the duration of their time in housing rooms until 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 (Nisenbaum et al. 1991, Gresch et al. 1992, Jedema et al. 1999, Jedema and Grace 2001), chronic cold-exposed animals were removed from the cold room and housed singly in the colony room for 20–24 hours before recordings.

4.3 RESULTS

4.3.1 Spontaneous Activity

A total of 128 neurons were examined from 32 rats (n=13 control rats, n=7 rats cold-exposed for 7 days, n=12 rats cold-exposed for 14 days). In control rats, 35 neurons were examined for NE-induced changes in spontaneous activity (8 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 7 days of cold stress, 15 neurons were examined for NE-induced changes in spontaneous activity (6 of those were antidromically activated and confirmed as projection neurons), and 15 were examined for responses to Te3 (n=7) or EC (n=8) stimulation before or after NE iontophoresis. In rats exposed to 14 days of cold stress, 32 neurons were examined for NE-induced changes in spontaneous activity (7 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.

4.3.2 Chronic cold does not alter spontaneous activity of BLA neurons after 7 or 14 days.

There were no significant differences in spontaneous firing rates of neurons of the BLA in control rats (avg FR= 1.1±0.45Hz), rats exposed to 7 days of cold (avg FR= 0.75±0.11Hz), or rats exposed to 14 days of cold (avg FR= 1.4±0.13Hz, Figure 43). Although there was a trend

for decreased spontaneous firing rates after 7 days and increased spontaneous firing rates after 14 days, these were not significant ($p=0.35$, one way ANOVA).

The spontaneous firing rates of neurons in control rats, 7 day cold rats and 14 day 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 1.19±0.19 Hz.

The average firing rate of neurons confirmed by antidromic activation to be projection neurons (n=21, all groups) was 0.18±0.09Hz, significantly lower than the firing rate of spontaneously active neurons ($p=0.01$, t-test). The spontaneous activity of projection neurons was no different ($p=0.7$, one way ANOVA) in control rats (avg FR= 0.22±0.04Hz, n=8), rats exposed to 7 days of cold (avg FR= 0.13±0.04Hz, n=6), or rats exposed to 14 days of cold (avg FR= 0.17±0.03Hz, n=7). As the majority of projection neurons were located in the basolateral nucleus (n=6 in controls, n=4 in 7 day cold rats, n=5 in 14 day cold rats), projection neurons were not further divided based on neuronal location for analysis of interaction between experimental group and neuron location.

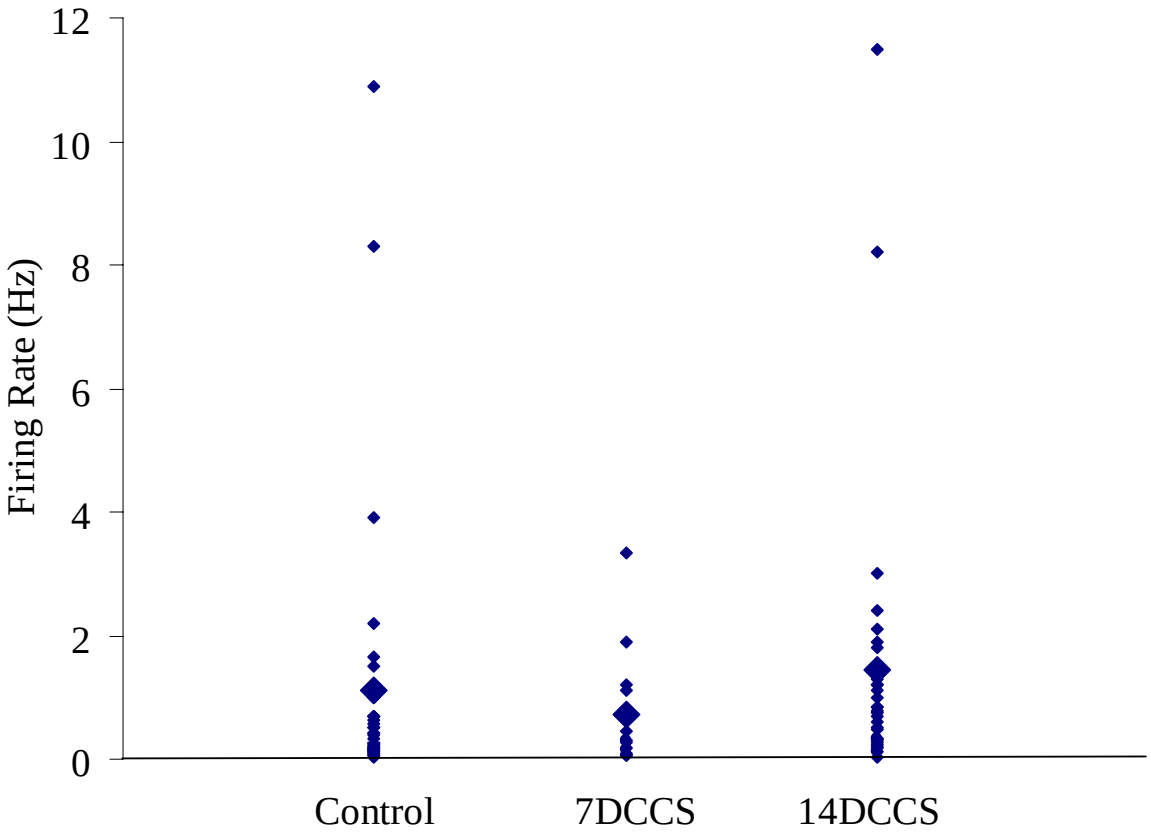


Figure 43 Chronic cold exposure does not alter BLA neuron activity. Chronic cold exposure did not alter average spontaneous baseline firing of BLA neurons (p=0.35, one way ANOVA, larger symbols represent average firing rates).

4.3.3 Chronic cold alters NE modulation of spontaneous activity after 14 but not 7 days

Iontophoresis of NE (200um, 40nA) caused a significant decrease in spontaneous activity (by 32±4.1%, p=0.01, t-test) of the majority of neurons (n= 24/35) of the BLA in control rats (Figure 46). A smaller proportion of neurons (n=8/35) showed a significant increase in spontaneous activity (by 136±11.4%, p=0.02, t-test, Figure 46). Neurons from rats exposed to 7 days of chronic cold stress displayed responses to NE that were not significantly different from those observed in control rats (p=0.35, Chi Square, Figure 46). However, in rats exposed to 14 days of 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 Square, Figures 44- 46). No differences in the magnitude of NE-induced inhibition or excitation was seen (data not shown).

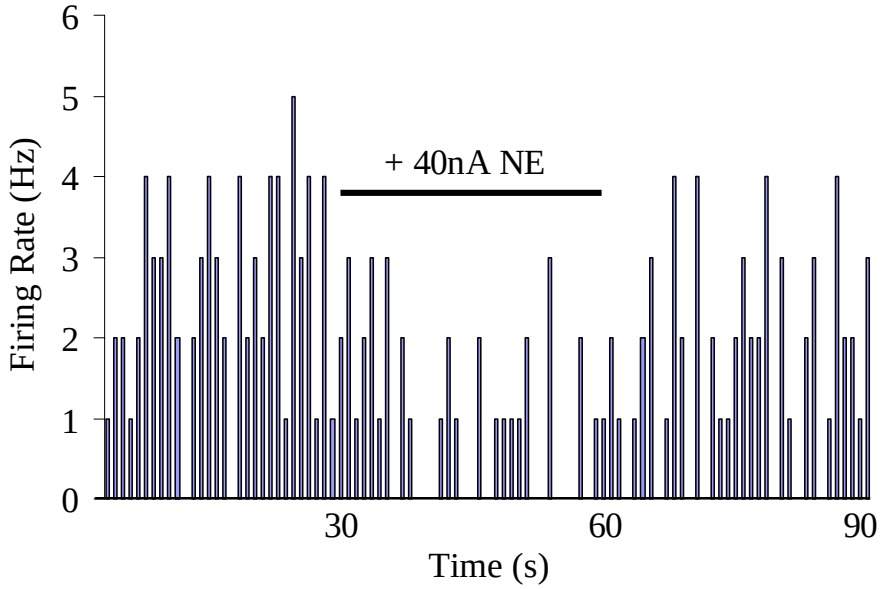


Figure 44 BLA neuron displaying an inhibition in response to NE. Iontophoresis of 40nA of NE onto a BLA neuron causes a decrease in spontaneous activity that returns to baseline after cessation of NE application (14 day cold stress rat).

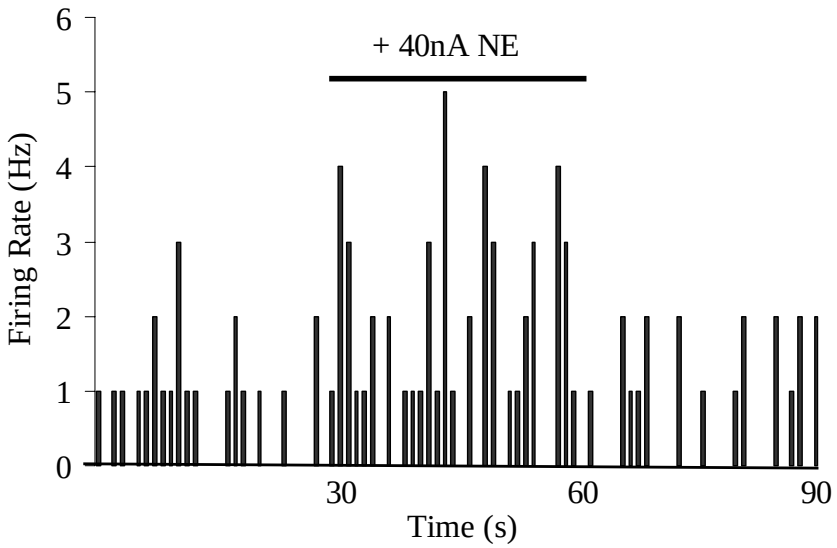


Figure 45 BLA neuron displaying an excitation to NE. Iontophoresis of 40nA of NE onto a BLA neuron causes an increase in spontaneous activity that returns to baseline after cessation of NE application (14 day cold stress rat).

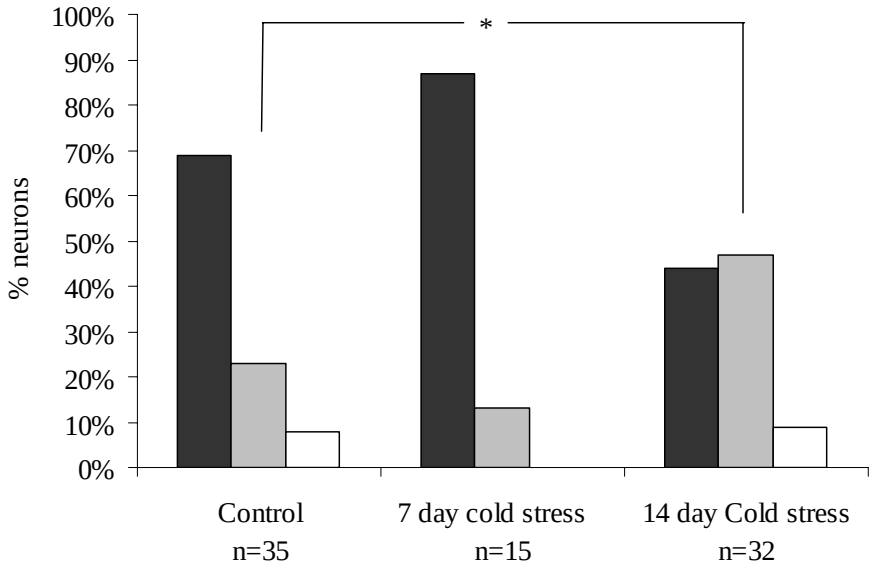


Figure 46 Chronic cold exposure alters proportion of neurons excited by NE. In control rats and rats exposed to 7 days of cold stress, the majority of neurons displayed inhibitory responses to NE iontophoresis (no differences, p=0.35, Chi Square). After 14 days of cold stress, significantly different proportions of BLA neurons displayed excitatory responses to NE iontophoresis (p=0.02, Chi Square, black bars= inhibited neurons, grey bars= excited, white bars = not affected).

A subset of neurons were antidromically activated and confirmed as projection neurons in control, 7 day cold exposed, and 14 day cold exposed rats (Figure 47). In controls, NE significantly inhibited all projection neurons (n=8/8, p=0.002, t-test, Figure 48). In rats exposed to 14 days of cold stress significantly different proportions of responses were seen (p=0.05, Chi Square). A portion of neurons were still inhibited by NE (n=4/7, p=0.01, t-test), however, other projection neurons displayed excitatory responses to NE iontophoresis (n=3/7, p=0.03, Figure 48). There were no significant differences between control rats and rats exposed to 7 days of cold stress (p=0.48, Chi Square), in that all projection neurons were inhibited in rats exposed to 7 days of cold stress (n=6/6, p=0.02, t-test, Figure 48).

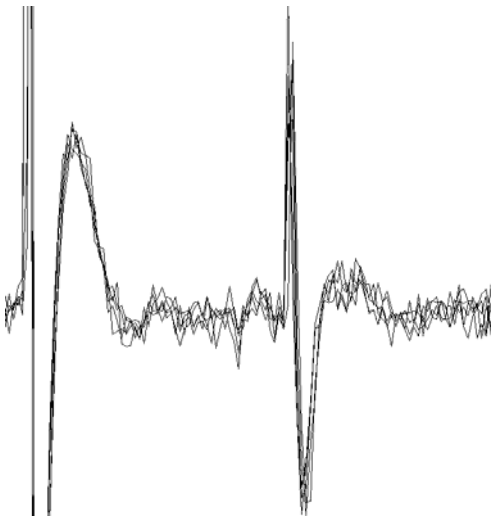


Figure 47 Electrophysiological trace of a neuron antidromically activated by EC. Electrophysiological recording trace of a BLA neuron exhibiting antidromic activation following EC stimulation (14 day cold exposed rat).

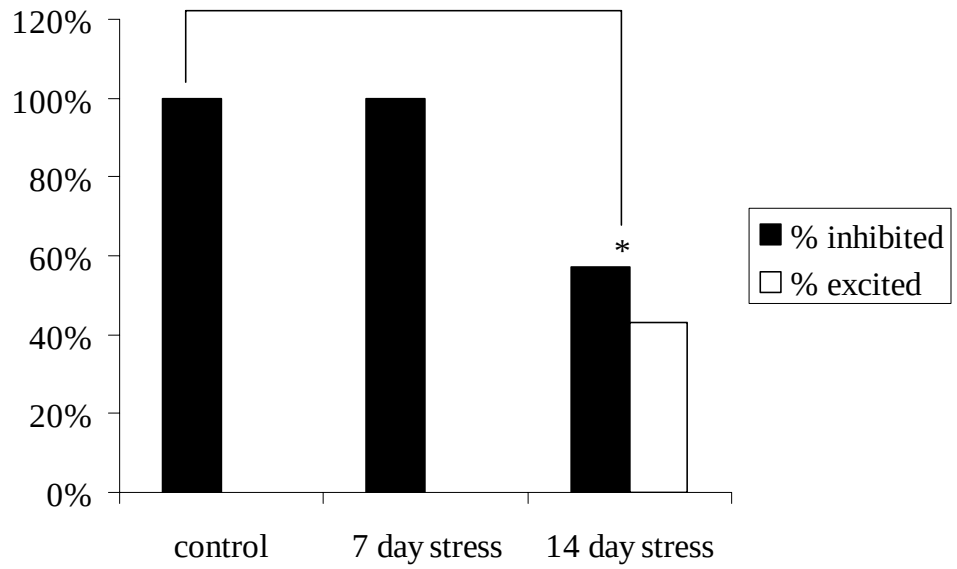


Figure 48 Chronic cold alters NE inhibition of projection neurons. Confirmed projection neurons (100%) displayed inhibitory responses during NE iontophoresis in control rats and rats exposed to 7 days of cold (no differences, p=0.48, Chi Square). After 14 days of cold, significantly different proportions of neurons are inhibited and excited (p=0.05, Chi Square). Projection neurons displayed both inhibitory (57%) and excitatory (43%) responses to NE iontophoresis.

4.3.4 Chronic cold alters NE modulation of evoked activity after 7 and 14 days

Electrical stimulation of EC caused orthodromic, excitatory responses in neurons of the BLA in both control and stressed rats (Figure 49). Stress exposure did not alter baseline responses to EC stimulation (latency, spike probability, # neurons per track). In control rats iontophoresis of NE (10nA), significantly decreased the spike probability of neurons in response to EC stimulation (n=7/7, p=0.005, t-test, Figure 50). In rats exposed to 14 days of cold, iontophoresis of NE

(10nA) resulted in NE-induced effects on EC-evoked activity that were significantly different from controls. In 14 day cold rats, NE decreased the spike probability of some neurons in response to EC stimulation (n=5/9, p=0.05), however, in a subset of neurons NE increased the spike probability (n=4/9, p=0.03, Figure 50). These alterations in the proportions of neurons showing inhibitory versus excitatory effects were significantly different (p=0.04, Chi Square). In addition, after 14 days of 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±8%, which was significantly different from baseline EC-evoked activity (p=0.005 t-test, Figure 53). After 14 days of cold, this dropped to an average decrease of 8±3%, 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 53). After 7 days of 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 day cold exposed rats (p=0.01, Chi Square, Figure 50). However, while NE still inhibited EC-evoked activity in 7 day cold rats (by 25±4%, p=0.02, t-test), it was to a significantly lesser degree 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 53).

Electrical stimulation of Te3 caused orthodromic, excitatory responses in neurons of the BLA in both control and stressed rats (Figure 51). Stress exposure did not alter the nature of baseline responses to Te3 stimulation (latency, spike probability, # neurons per track). 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 52). In rats exposed to 14 days of cold, iontophoresis of NE (10nA) resulted in significant differences in the proportions of BLA

neurons displaying inhibitory/excitatory effects of NE on Te3-evoked activity (p=0.001, Chi Square). In 14 day cold exposed rats, iontophoresis of NE (10nA) significantly increased the spike probability in the majority of neurons in response to Te3 stimulation (n=5/7, p=0.02), whereas in a subset of neurons NE decreased the spike probability (n=2/7, p=0.01, Figure 52). Cold exposure also decreased the magnitude of NE-induced inhibition of Te3-evoked activity of BLA neurons (p=0.006, t-test, Figure 53). In control rats, NE significantly decreased spike probability by an average of 41±7% from baseline. After 14 days of cold exposure, this changed to an average increase of 2±8% (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 days of cold exposure, NE decreased spike probability in the majority of neurons (n=4/7), but not to a significant degree (by 13±6%, 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 day cold rats were compared to controls (p=0.04, Chi Square). There were no differences in the magnitude of NE-induced decrease of Te3-evoked activity in rats exposed to 7 days of cold versus controls (p=0.06, t-test), although this was close to statistical significance. One neuron did display increased spike probability (by 38%, n=1/7), with others displaying no significant change (n=2/7, Figure 53).

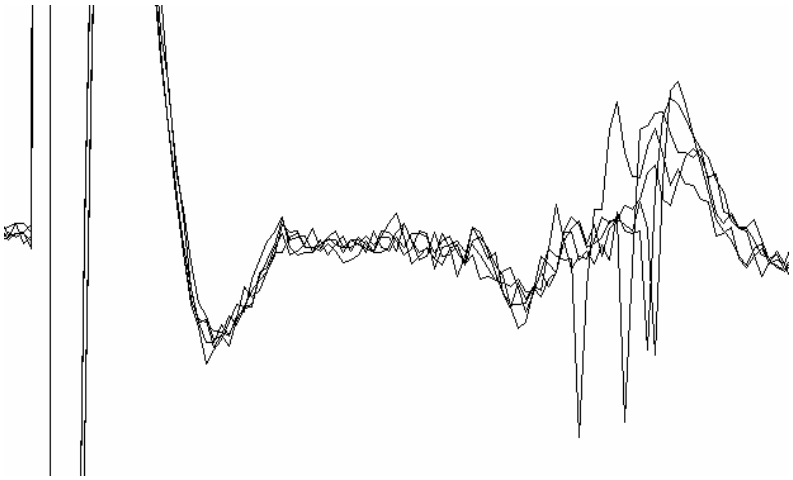


Figure 49 Electrophysiological trace of a BLA neuron orthodromically activated by EC. Electrophysiological recording trace of a BLA neuron exhibiting orthodromic activation following EC stimulation (14 day cold exposed rat).

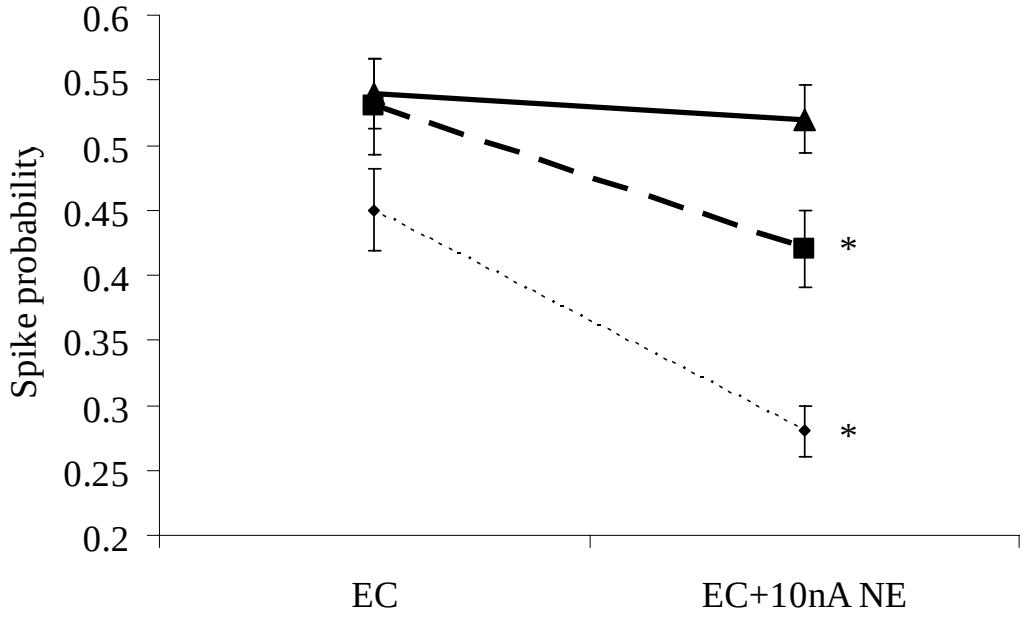


Figure 50 Chronic cold alters NE modulation of EC-evoked activity. NE iontophoresis inhibits EC-evoked activity in BLA neurons in control rats (dotted line). After 7 days of cold stress, NE still inhibits evoked activity, but to a lesser degree (dashed line). After 14 days of cold stress, NE loses its ability to inhibit evoked activity, and facilitates evoked activity in a portion of neurons (solid line).

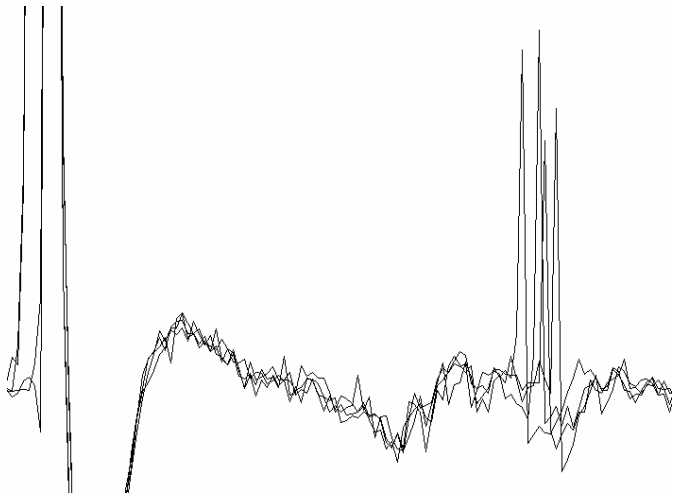


Figure 51 Electrophysiological trace of a neuron orthodromically activated by Te3. Electrophysiological recording trace of a BLA neuron exhibiting orthodromic activation following Te3 stimulation (14 day cold exposed rat).

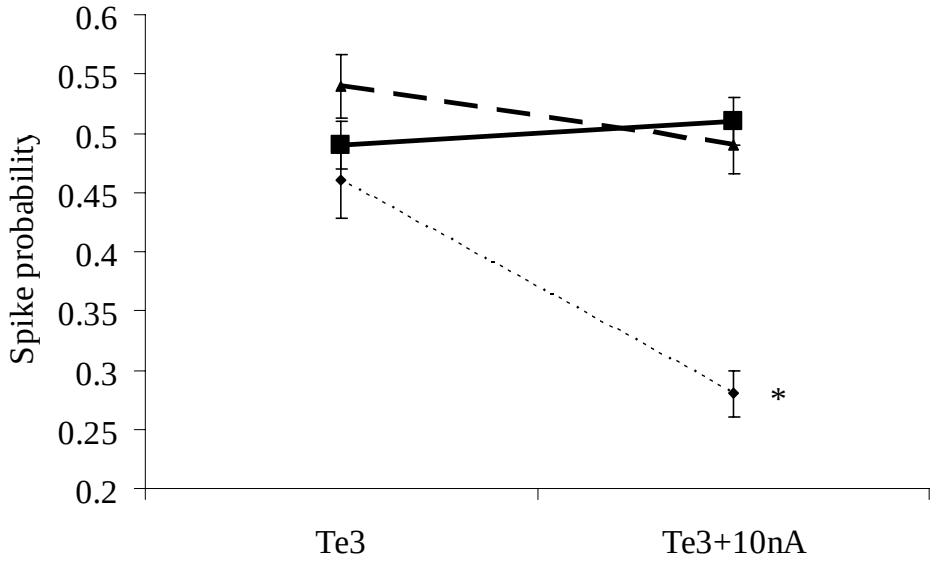


Figure 52 Chronic cold alters NE modulation of Te3-evoked activity. NE iontophoresis inhibits Te3-evoked activity in BLA neurons in control rats (dotted line). After 7 days of cold stress, NE still inhibits evoked activity, but to a lesser degree (dashed line). After 14 days of cold stress, NE facilitates evoked activity on average in BLA neurons (solid line).

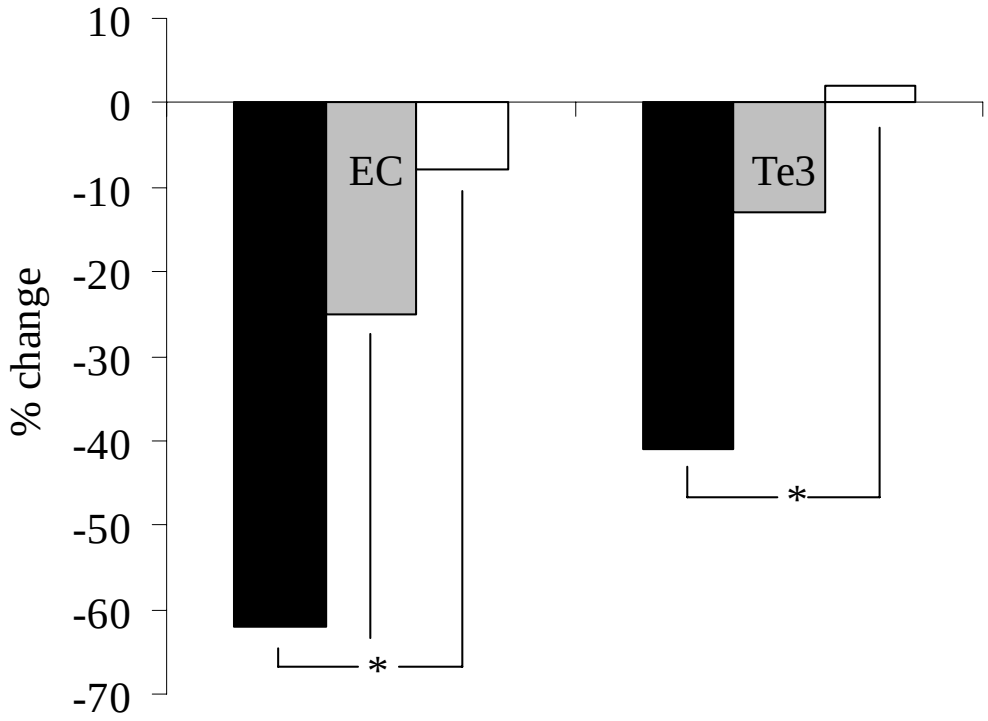


Figure 53 Chronic cold alters magnitude of NE modulation of BLA evoked activity. NE significantly inhibits activity evoked by stimulation of EC (62%) and Te3 (42%) in control rats (black boxes). After 7 days of cold stress, the degree to which NE inhibits evoked activity has significantly decreased (EC 25%, Te3 13%, gray bars). After 14 days of cold stress (white bars), NE inhibits EC-evoked activity by a marginal amount (8%) and actually moderately facilitates Te3-evoked activity (2%).

3.4 DISCUSSION

This study is the first to demonstrate chronic stress-induced alterations in the responses of neurons in the BLA to NE. Following chronic cold exposure, there was a significantly higher proportion of BLA neurons exhibiting excitatory responses to NE iontophoresis, but only after 14 days of exposure. Chronic cold exposure also decreases the inhibitory effects of NE on BLA neuronal activity evoked by stimulation of EC and Te 3. Stress potentiates NE-induced increases in spontaneous activity only after 14 days of exposure, but causes a facilitation of some afferent-evoked activity after 7 days exposure. This facilitatory effect of NE on evoked activity was more pronounced after 14 days cold exposure. These data are the first to provide evidence that chronic stress-induced alterations in the NE system (Nisenbaum et al. 1991, Pacak et al. 1992, Gresch et al. 1994, Mana and Grace et al. 1997, Jedema et al. 1999, Jedema and Grace 2001) also impact the electrophysiological activity in NE target regions. They are also consistent with a role for the amygdala as an important site influencing sensitization of the stress response, and extend previous work examining other regions that may underlie pathological changes that occur as a result of stress exposure.

Repeated administration of the same stressor leads to habituation of the stress response to that stressor. Neurons of the BLA 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 neurons of the BLA as well. 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),

demonstrating either a potentiation of the excitatory component of the response, or an elimination of the inhibitory component. Our present work suggests a mechanism by which these effects may be occurring. During footshock, NE is increased in the BLA (Galvez et al. 1996, Williams et al. 1998, Hatfield et al. 1999). In control rats, neurons of the BLA display largely inhibitory responses to NE, with few neurons displaying excitatory responses. After chronic cold exposure, NE causes primarily excitatory responses in neurons of the BLA, with many fewer displaying inhibition. This may explain the increase in excitatory responses of BLA neurons to footshock following chronic cold exposure.

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 (Nisenbaum et al. 1991, Pacak et al. 1992, Gresch et al. 1994, Jedema et al. 1999), which may in turn 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. Finally, there were no significant differences in dose-responsivity between groups.

A second mechanism by which NE's neuronal actions 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 et al. 1996, Meyer et al. 2000). However, chronic cold exposure leads to increased sensitivity of alpha-2 receptors in the hippocampus (Nisenbaum et al.. 1993). With regard to stress, the LC and the BLA may act in a coordinated manner to facilitate the stress response,

whereas the hippocampus plays an inhibitory role (Herman et al. 1995, 1998). Indeed, stress decreases plasticity in the hippocampus while increasing plasticity in the BLA (Vouimba et al. 2004). Furthermore, BLA neurons display dendritic hypertrophy after stress (Vyas et al. 2002, Mitra et al. 2005), while hippocampal neurons exhibit dendritic atrophy (Wantanabe et al. 1992, Margarinos et al. 1996). Alpha-2 receptor sensitization in the hippocampus would lead to an NE-mediated decrease in the inhibitory role of the hippocampus. In contrast, a decrease in inhibitory alpha-2 receptor effects in the LC would enhance LC activity, and a corresponding decrease in alpha-2 receptor function in the BLA would lead to a NE-induced facilitation of its excitatory role. Such a scenario corresponds well with a sensitized response.

In addition to alterations in receptor number, changes in receptor affinity, Gproteins mediating responses, or second messenger systems may all play a role in alterations of NE actions on neuronal activity. All NE receptors are metabotropic, therefore relying on Gproteins and second messenger cascades to achieve their effects. Studies have reported repeated tail pinch or immobilization causes decreased sensitivity of alpha-2 receptors (Gomez et al. 1998), and receptors that are less efficacious in producing intracellular cAMP responses (Bellavia and Gallara 1998). Either of these effects could contribute to the results seen in the present studies. However, one of these studies (Bellavia and Gallara 1998) also demonstrates subsensitive beta-receptors, and another found hypersensitive alpha-2 receptors (Garcai-Vallejo et al. 1998) after chronic variable stress. This evidence complicates interpretation of the current results.

Prior to chronic stress, NE inhibits BLA responses to EC and Te3 stimulation. However, after chronic stress exposure, some of these inputs are potentiated in the presence of NE. 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. As chronic stress seems to disrupt the delicate balance of excitatory and inhibitory mechanisms present within the BLA, this may lead to nonspecific potentiation of inputs, causing non-conditioned stimuli to elicit responses normally observed following conditioning. Indeed, chronic stress has been shown to increase dendritic spines within the BLA (Mitra et al. 2005), a phenomenon often associated with the type of plasticity observed following conditioning (Radley et al. 2006). Moreover, stress is known to enhance synaptic plasticity in BLA circuits (Vouimba et al. 2004). Therefore, after chronic stress exposure, inputs that were previously inhibited would now be potentiated. This may manifest itself behaviorally by producing rats that are hyperresponsive to stressful stimuli, as has been reported (Nisenbaum et al. 1991, Pacak et al. 1992, Gresch et al. 1994, Jedema et al. 1999). Furthermore, it suggests a mechanism by which these rats may be less easily conditioned or poorer learners if mechanisms of plasticity have been disrupted. Rats exposed to chronic restraint stress (Conrad et al. 2003) or chronic psychosocial stress (Park et al. 2001) demonstrate impairments in spatial memory. Chronic restraint stress also causes impairments in object recognition memory (Beck et al. 1999).

Stress is thought to be associated with a reduction in GABAergic transmission in the amygdala. In control animals, BLA pyramidal neurons are under tight inhibitory control by GABAergic synapses (Rainnie et al. 1991, Washburn and Moises 1992, Woodson et al. 2000). However, immobilization and cold lead to decreased levels of GAD and GABA in the brain (Otero Losado 1988), and swim stress decreases GABA concentrations as well (Briones-Aranda et al. 2005). Restraint stress decreases GABAergic inhibitory control of the stress response by the amygdala (Marineja et al. 2002). GABAergic blockade in the BLA leads to anxiety responses (Sanders and Shekhar 1991, Sajdyk and Shekhar 1997), 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 potentially account for the inhibitory effects seen in control rats; NE exciting GABAergic interneurons which synapse onto glutamatergic pyramidal cells within the BLA (Muller et al. 2006). Examined in vitro, stress disrupts normal NE-induced facilitation of GABAergic transmission normally seen in control rats (Braga et al. 2004). Similar processes may be induced following cold exposure. After chronic stress, decreased GABAergic transmission could result from a desensitization or decrease in the number of 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 current data.

Extensive exposure to stress is often associated with the development and/or enhancement of pathology and disease. Indeed, post-traumatic stress disorder has been modeled 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 PVN, BNST, 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 are the first to suggest 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 the

understanding of how such disorders develop, and aid in the design of potential treatments for such conditions.

5.0 GENERAL DISCUSSION

5.1 SUMMARY OF RESULTS AND CONCLUSIONS

These studies provide further support for a critical role of the BLA in the stress response, and more specifically, suggest a role in the behavioral consequences of sensitization of the stress response. An examination of the response of BLA neurons to a pharmacological stressor, to increased NE within the BLA known to occur during stress, and to chronic cold stress exposure reveals BLA neurons are sensitive to all of these manipulations. Yohimbine leads to increases and decreases in both spontaneous and afferent-evoked neuronal activity in the BLA, presumably in part due to its ability to increase NE levels in the BLA. A more precise examination of NE within the BLA specifically was accomplished through the use of iontophoresis of NE directly onto BLA neurons. This technique revealed the apparent alpha-2-receptor mediated inhibitory actions of NE on BLA spontaneous neuronal activity and activity evoked by stimulation of EC and Te3. Small numbers of neurons display beta receptor mediated excitatory responses to NE iontophoresis. Furthermore, effects of LC stimulation and footshock administration on BLA neurons are mimicked with iontophoresis of NE. Finally, these experiments demonstrate that neurons of the BLA are susceptible to alterations induced by chronic cold exposure. After chronic cold, there is a shift in NE's actions on BLA neurons from primarily inhibitory to primarily excitatory. Spontaneous activity now increases in response to NE in the majority of

BLA neurons. In addition, before chronic cold NE caused only inhibition of afferent-evoked activity, but after chronic cold NE loses the ability to selectively depress evoked activity in some neurons, and causes a facilitation of evoked activity in others.

NE-induced inhibition of spontaneous BLA neuronal activity is in concordance with many other studies citing NE as having primarily inhibitory effects in other regions (see Introduction). However, a portion of those studies also demonstrate that NE causes an increase in evoked activity concomitant with inhibition of spontaneous activity. We anticipated similar results here, with NE decreasing spontaneous activity but increasing evoked. We examined two regions that send excitatory afferent input to the BLA, Te3 and EC. In contrast to what was expected, NE inhibited evoked activity. Our suggestions explaining this difference are explained in subsequent sections of this document. After chronic cold stress, a portion of the evoked responses to afferent inputs are facilitated during NE iontophoresis. This suggests the possibility that during cold stress, there is a nonspecific potentiation of afferent inputs to the BLA (expanded upon below). This could result in hyperresponsivity to stimuli that activate those inputs, and an occlusion of further potentiation of these or other inputs, which may manifest itself in learning and memory impairments. The consequences of increased BLA activation to NE may be an enhanced excitatory influence over the stress response, perhaps to a maladaptive degree.

5.2 POTENTIAL MECHANISMS OF NE-INDUCED EFFECTS

In control rats, we hypothesize that BLA neurons contain a combination of alpha-2 and beta receptors. LC neurons have low tonic firing rates, therefore, there are low levels of NE present in the BLA under baseline conditions. These low levels of NE have minor excitatory actions on BLA neurons via beta receptors. This is supported by our data showing that blockade of beta receptors in the BLA decreases spontaneous firing rates of BLA neurons. With low levels of NE iontophoresis (5-10nA), a mixture of beta-mediated excitatory effects and alpha-2-mediated inhibitory effects result in no clear net effect of NE on BLA neuron activity (the inconsistency and moderate effects of low doses of NE on BLA neuron activity was the rationale for the use of the higher dose, see Methods Chapter 3). After iontophoresis of higher doses of NE (40nA), in the majority of neurons the alpha-2 mediated inhibition becomes the dominant effect. This may be due to a higher prevalence of alpha-2 receptors than beta receptors located postsynaptically on the majority of BLA neurons (or more or less sensitive receptors, respectively). In addition, at higher doses of NE, both presynaptic and postsynaptic alpha-2 receptors may be activated. This would lead to a combination of direct inhibition via postsynaptic alpha-2 receptor-mediated increases in K+ permeability, along with a decreased release of glutamate from afferent terminals due to presynaptic alpha-2 receptor mediated inhibition (see Figure 54). Previous in vitro work suggests that NE decreases EPSPs but does not affect IPSPs, eliminating NE actions at GABAergic terminals from our consideration of primary mechanism potentially mediating effects. Therefore, we consider the role of presynaptic alpha-2 receptors on glutamatergic terminals.

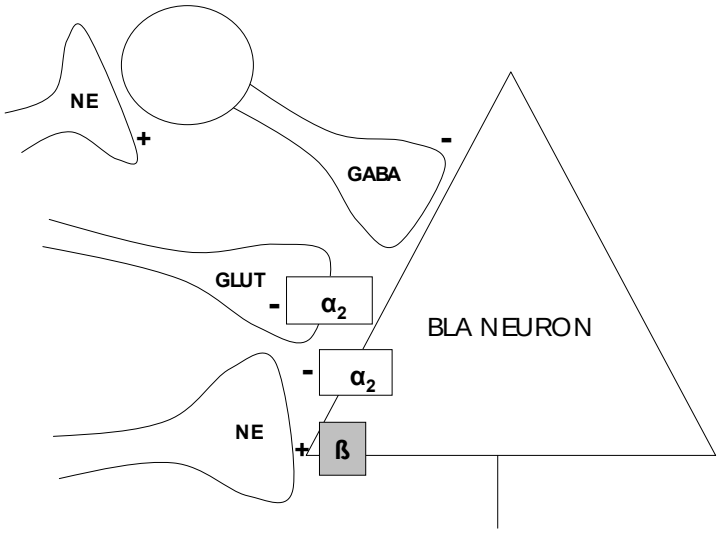


Figure 54 Diagram illustrating proposed actions of NE within the BLA.

While NE may indeed act on these receptors, we would argue that presynaptic alpha-2 receptor effects on glutamatergic terminals would be somewhat minimal during baseline conditions in the BLA, as BLA neurons generally have very low firing rates and therefore either do not receive much tonic glutamatergic input or this excitatory input is outweighed by inhibitory control (this is expanded upon later, as is the potential role of presynaptic alpha-2 receptors on GABAergic terminals). Decreasing minimal amounts of glutamate release via activation of presynaptic alpha-2 receptors would therefore have marginal effects. Therefore, we suggest there may be a greater distribution of alpha-2 receptors present postsynaptically on the BLA neurons which leads to an ability of alpha-2 receptor mediated inhibition to "outcompete" postsynaptic beta receptor-mediated excitation in the majority of BLA neurons.

NE-induced inhibition of BLA neurons was potentiated during beta-blockade, presumably due to activation of available alpha-2 receptors. This lends further support to our argument that individual BLA neurons have both alpha-2 and beta receptors. Blockade of beta-

receptors leads to an enhancement of inhibitory responses, explained here by the elimination of beta-receptor mediated excitation "competing" with alpha-2 receptor mediated inhibition. Finally, the replication of our NE-induced effects with stimulation of LC lends further weight to the argument that these NE effects on spontaneous activity are predominantly postsynaptic.

Perhaps the most beneficial future studies that would aid in the determination of the mechanisms by which NE has its effects in the BLA would investigate anatomy rather than physiology. The only studies confirming mRNA for adrenergic receptors in the BLA report on alpha-2 receptors. Therefore, this also lends support to our assertion that alpha-2 mediated effects are predominantly postsynaptic. However, the paucity of data on exact cellular location of receptors within the BLA makes interpretation challenging. Future studies looking at the colocalization of adrenergic receptors with neurons labeled for glutamate or GABA in the BLA would be beneficial. In addition, more studies looking at mRNA to determine whether receptors are probably located postsynaptically would aid in interpretation of our electrophysiological results.

However, our data clearly present the question: why do some BLA neurons display excitatory responses even at high doses of NE? We present two explanations: these neurons contain a higher proportion of beta receptors than alpha-2 receptors, or, alpha-2 receptor mediated effects target GABAergic terminals in addition to glutamatergic terminals. Alpha-2 actions on GABAergic terminals would lead to a decrease in GABA release, and therefore less inhibition of BLA neuron activity (perhaps to a degree that would cause excitation). Our data argue against this second option. BLA neurons are bombarded by GABAergic input (as described in the introduction), which contributes to keeping spontaneous firing rates low. Given the amount of GABAergic input BLA neurons receive, we would expect more excitation if

inhibition of GABAergic terminals is one of the alpha-2 receptor-mediated actions. If alpha-2 receptor mediated inhibition can decrease GABAergic neurotransmission, it is likely NE would have lead to excitatory responses in more than only ~25% of BLA neurons, which is what was reported here.

Therefore, differential distribution of alpha-2 and beta receptors on BLA neurons could account for the inhibitory and excitatory effects of NE on spontaneous activity. This suggests that there may be subsets of BLA neurons designated by the receptor distribution to inhibit or excite in response to NE. We feel this designation may be related to two other factors: the type of inputs these neurons receive, and the type of output these neurons send. This is expanded on below with regard to BLA afferents and efferents, and consideration of the circuitry related to responses to stressful stimuli.

5.3 POTENTIAL MECHANISMS OF NE-INDUCED EFFECTS: EVOKED ACTIVITY

Iontophoresis of NE inhibited EC and Te3-evoked BLA activity in all control rats (alterations due to cold effects are addressed later). This is most likely mediated via alpha-2 receptors as well, although there are two types to again consider: presynaptic and postsynaptic. Activation of presynaptic alpha-2 receptors present on glutamatergic terminals in the BLA from EC and Te3 would lead to increased K+ permeability and a resultant decrease in glutamate release from such terminals. Alternatively, a direct inhibitory effect via postsynaptic alpha-2 receptor activation would directly inhibit BLA neurons and render them less responsive to afferent input. The data that speak most directly to the mechanism NE uses to decrease evoked activity are the alterations in NE modulation of evoked activity in cold exposed animals. We demonstrated that cold-

induced alterations in NE modulation of evoked activity become apparent before those on spontaneous activity. This argues for a scenario whereby these two effects occur via different mechanisms. If NE inhibited spontaneous and evoked activity via the same postsynaptic alpha-2 receptors, we would expect alterations of this modulation to develop with a similar time course. However, this is not the case, suggesting that while NE effects on spontaneous activity likely occur due to activation of postsynaptic alpha-2 receptors, NE modulation of evoked activity likely occurs via activation of presynaptic alpha-2 receptors present on glutamatergic terminals (our data do not exclude some role of postsynaptic alpha-2 receptor activation in contributing to the effects on evoked activity, but suggest, at the very least, a combination of these two mechanisms would occur).

5.4 NE, THE LC, AND FOOTSHOCK: BLA NEURONAL RESPONSES

Footshock administration activates neurons of the LC and leads to NE efflux in the BLA (see Introduction). Our data demonstrate that BLA neurons display similar responses to footshock, LC stimulation, and NE iontophoresis. It is our contention that footshock-induced alterations in BLA neuronal activity are due, at least in part, to increases in LC neuron activity. We suggest that upon footshock administration, LC neurons become active, NE is released in the BLA, and this leads to increases or decreases in BLA neuron spontaneous activity. However, upon footshock administration, it is likely that other brain regions are activated, and that some of those may send input to the BLA. Therefore, it is unreasonable to assume that the responses of BLA neurons to footshock are solely due to NE increases in the BLA. In fact, one of our neurons that

displayed an excitatory response to footshock did not display any response to NE. This particular response to footshock was of much shorter latency than the others that were replicated with NE iontophoresis. This suggests it may have been due to activation of a glutamatergic afferent to the BLA from a region that is also responsive to footshock, rather than a consequence of NE.

5.5 POSSIBLE REASONS FOR DIFFERENTIAL RESPONSES OF BLA NEURONS TO NE: DEPENDENCE ON AFFERENT INPUT

BLA neurons receive a variety of inputs (as discussed in the introduction). Upon presentation of a stressful stimulus, it seems logical that BLA neurons would want to encode not only the valence of the stimulus, but other details regarding the stimulus, which involve differential inputs. In the case of auditory fear conditioning, a subset of BLA neurons will respond to input from the auditory cortex and/or thalamus, regions that are both activated by the auditory tone. Simultaneously, neurons of the LC are activated in response to presentation of the footshock (stressful stimulus). Perhaps these two inputs converge onto single neurons of the BLA. This has been argued for auditory fear conditioning (Schafe et al 2001). Furthermore, these neurons may display excitatory responses to sensory related input and NE input as well. The combined excitatory response of BLA neurons to sensory and NE input may provide sufficient BLA neuronal activation to provide efferent output that subserves appropriate behavioral responding to such a stimulus (i.e. escape, freezing, etc). Therefore, if BLA neurons that receive input regarding sensory stimuli or contextual information are the same neurons that are also excited by NE, we would argue these neurons have a higher distribution of beta receptors. Conversely, in a

scenario where it is beneficial to ignore a stressful stimulus, perhaps input from other regions, such as PFC, inhibits neurons of the BLA. Again, with regard to convergence of inputs, BLA neurons that are inhibited by PFC may also be inhibited by NE (and have a higher distribution of alpha-2 receptors). This would lead to a scenario whereby the effects of activation induced by one input (i.e. thalamus) may not be sufficient to significantly alter BLA neuron firing to a degree that would have substantial downstream effects and alter behavioral responding, while convergent activation (i.e. thalamus and LC) or inhibition by two inputs would suffice. However, this interpretation does not lend itself well to the role of the amygdala in learning and conditioning, as described below.

5.6 ACTIONS OF NE IN THE BLA: CONSIDERATION OF CONDITIONING AND BLA PLASTICITY

While it may be the case that BLA neurons have different levels of alpha-2 or beta receptors, and that this distribution may correspond to the differential inputs these BLA neurons receive, the above scenario seems too rigid and oversimplified. It leaves little room for learning and behavioral flexibility, and the data demonstrating an involvement of the amygdala in aversive learning are substantial. Therefore, we suggest that plasticity, or more specifically, for this discussion, long-term potentiation, plays an important role in modulation of the responses of BLA neurons to not only NE but afferent input. For our studies, in a naïve animal, it may be beneficial for the majority of neurons to respond to NE in an inhibitory manner via a more dominant alpha-2 mediated effect. As animals experience stressful or fear-related learning and conditioning, inputs to the amygdala representing the details of those situations (context, sensory

stimuli) may undergo potentiation. For example, if a certain context is conditioned as fearful, input from the hippocampus may be potentiated. However, simultaneous input regarding the valence of the context may be signaled by the LC. Some form of plasticity involving an upregulation of beta receptors would lead to excitatory BLA neuronal responses to NE in that subset of neurons. Plasticity and potentiation are known to involve changes in receptor distribution, the most common of these involves insertion of AMPA receptors into the postsynaptic membrane during potentiation (Lu et al 2001). The combination of enhanced excitatory responses to hippocampus and NE may alter BLA neuronal output in a manner consistent with appropriate behavioral responding (i.e. freezing). One would reason, then, that our animals display so little excitation in response to NE because they are behaviorally naïve. This could easily be tested by performing experiments looking at NE modulation of spontaneous activity in control rats and comparing them to rats that have undergone some conditioning paradigm.

In addition to an altered response to NE that may occur during conditioning and suggested resultant potentiation, NE modulation of evoked activity may be altered as a result of conditioning. While in naïve rats, NE inhibits evoked activity, after conditioining NE may selectively facilitate input from afferents relevant to the learning experience. This could also be due to an upregulation of beta receptors postsynaptically on BLA neurons or to a downregulation of alpha-2 receptors (either pre or postsynaptic). Therefore, future experiments examining NE modulation of BLA activity should include evoked activity along with spontaneous activity. It may be useful to examine two separate paradigms, one using more sensory-related stimuli that may engage Te3 inputs to BLA, and one using more contextual stimuli that may engage EC inputs to BLA. Furthermore, an extension of such studies to include an examination of thalamic

input would be useful. Despite extensive data relating conditioning and behavior to plasticity within the amygdala, such studies have yet to examine the role of NE in such phenomena.

5.7 POSSIBLE REASONS FOR DIFFERENTIAL RESPONSES OF BLA NEURONS TO NE: DEPENDENCE ON EFFERENT OUTPUT

Our data speak to two viable options with regard to how LC/NE-induced inhibition of BLA neuron activity alters BLA output and downstream effects. The first was delineated above, whereby, through learning, an alteration of responses to NE and afferent inputs may lead to selective activation of BLA neurons with precise outputs to downstream targets mediating behavioral responding. The second is drastically different, suggesting that the behaviorally relevant effect of NE on BLA neuron activity is not the beta receptor-mediated excitation, but actually the alpha-2 receptor mediated inhibition.

While the first option is supported by a multitude of data examining BLA neuron excitation in response to conditioned stimuli, the second is supported by some anatomical studies looking at how the BLA connects with other regions involved in some stress circuitries. LC neuron activation in response to presentation of a stressful stimulus would lead to primarily inhibitory responses in BLA neurons. This inhibition of the BLA would lead to a disinhibition of the CeA, as the BLA has primarily inhibitory effects over BLA neurons (Rosenkranz et al 2006). Activation of the CeA could lead to effects on downstream targets involved in behavioral responding. For example, the CeA sends excitatory, CRH-containing projections to the PVN (Van Boeckstale et al 2001). This could be a pathway by which the BLA has an excitatory influence over HPA responses to stressful stimuli. Alternatively, the CeA also has an excitatory

influence on the LC (Ramsooksingh et al 2003, Bouret et al 2003). This also could lead to an excitatory effect of the BLA on portions of the stress response, facilitating LC activation and creating a positive feedback loop (see Figure 55). However, important to consider is the low spontaneous firing rates of BLA neurons. Would further inhibition of these slow firing neurons be enough to have a potent effect in terms of disinhibiting the CeA?

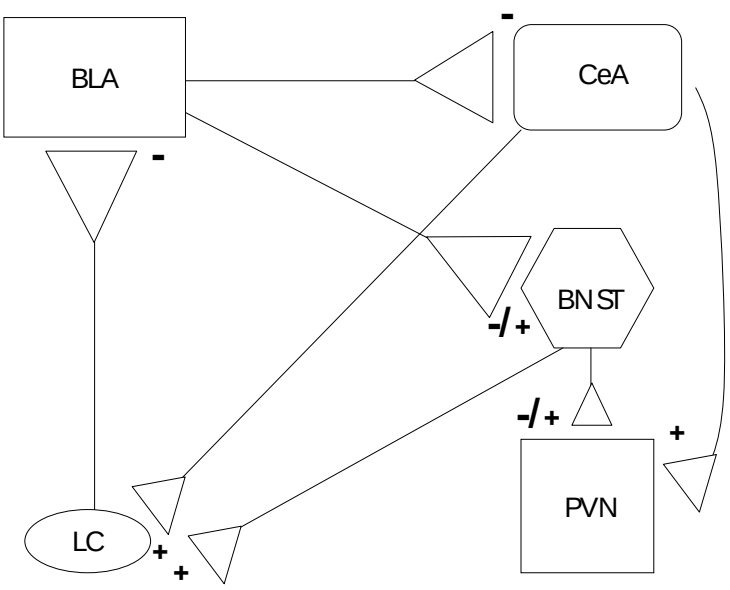


Figure 55. BLA and Stress Circuitry. Diagram examining how LC/NE induced inhibition of the BLA could alter activity in efferent targets (here, CeA and BNST), and potential downstream effects on regions involved in the stress response (here, LC and PVN).

Another important structure to consider is the BNST, whose role in the behavioral response to stress has been documented (Walker et al 2003). The BLA sends projections to the BNST (Dong et al 2001), both directly and indirectly via the CeA. Neurons of the BNST display primarily excitatory responses to BLA stimulation (Casada and Dafny 1982). Therefore, inhibition of BLA neurons would lead to no activation of BNST neurons (and perhaps some

inhibition, although due to the low tonic firing rates of BLA neurons, it is unlikely that BLA sends much tonic excitatory input to the BNST). Neurons of the LC display excitatory responses to stimulation of the BNST (Dr. Hank Jedema, personal communication). This provides an alternate pathway whereby inhibition of the BLA would NOT lead to further excitation of the LC and may even influence the LC in an inhibitory manner (see Figure 55). Data regarding the effects of BNST output on the PVN are less clear.

If inhibition of the BLA can lead, at least in part, to further activation of the LC, what, then, terminates the stress response? Other structures may then come on line to inhibit the BLA or other regions activated by stress to limit the effects of the stress response. The PFC is thought to have inhibitory control over the stress response, its projections to the BLA have a strong inhibitory component (Rosenkranz and Grace 2001). It may play a role in terminating the response of the BLA to stressful stimuli.

Consideration of these potential pathways, along with the overwhelming data suggesting that BLA neurons respond in an excitatory manner to stimuli conditioned to be aversive, lend weight to our first suggestion that an excitatory effect of NE on BLA neurons may be the behaviorally relevant effect. Inhibition of the BLA, whose neurons already fire at such low rates to allow for activation of downstream targets of the BLA seems like the less ideal scenario and would limit the degree to which the BLA could signal (a neuron can only inhibit to a level of no firing). Therefore, activation of the BLA seems the more reasonable goal of inputs encoding stress-related stimuli. With regard to our proposed circuit, this would still allow for an activation of LC or PVN neurons via the BNST and a facilitatory effect on the stress circuit. Furthermore, although the BLA effects on CeA activity are primarily inhibitory, there are excitatory components to this projection (Rosenkranz et al 2006). Therefore, activation of the BLA could

lead to some excitation of CeA neurons. And finally, while the projections we described above originating in the CeA were excitatory, CRH projections, the CeA also sends GABAergic efferents (Sun et. al 1994, Saha et al 2000, Jia et al 2005). Therefore, inhibition of the CeA by BLA activation could lead to disinhibition of some targets of the CeA.

An interesting future experiment would investigate neurons that are excited and inhibited by NE, and where those neurons project. Placement of stimulating electrodes in regions such as CeA, BNST, or others and attempts at antidromic activation may reveal whether or not there is any specificity in terms of neuronal response to NE and efferent target. This may further elucidate the circuitry by which the BLA affects downstream targets in response to NE.

5.8 POSSIBLE MEANS OF STRESS-INDUCED ALTERATIONS IN NE MODULATION

There are many ways in which the neurophysiological actions of NE on BLA neurons may be altered after chronic stress exposure. It is well documented that after chronic stress exposure, NE efflux in terminal regions caused by acute stress exposure is enhanced (Nisenbaum et al. 1991, Pacak et al. 1992, Gresch et al. 1994, Jedema et al. 1999). Other studies have reported that the effects of NE on neuronal activity may be dose-dependent. Higher levels of NE after chronic stress may exert different actions on BLA neurons. Our data, however, argue against this interpretation. Though our examination of dose response was very limited as the responses of BLA neurons to lower doses of NE were variable and often insignificant, increasing doses of iontophoretic NE caused greater levels of inhibition in almost all BLA neurons. Perhaps more important is the fact that we never saw a switch in BLA neurons from inhibitory responses at

lower doses of NE to excitatory responses at higher doses of NE. This refutes the idea that increased excitatory responses of BLA neurons are simply due to sensitized NE efflux.

The most obvious means by which altered responses may occur is at the level of receptor upregulation or downregulation (or sensitivity). As described above and based on our control data, we would expect a downregulation of alpha-2 receptors, an upregulation of beta-receptors, or some combination of these two responses. Studies do show decreases in alpha-2 receptor levels after repeated stress (Stone 1981, Lynch et al. 1983, Stanford et al. 1984), however, this data is not completely clear in that many studies report different effects in different regions to different stress paradigms. A specific examination in the amygdala has yet to occur.

In addition to alterations in receptor number, changes in receptor sensitivity could account for our effects. Findings that document less sensitive alpha-2 receptors (Gomez et al. 1998) that are less efficacious in producing cAMP responses (Bellavia and Gallara 1998) after repeated tail pinch or immobilization support our results. Again, this has not been examined in the amygdala.

Other reported alterations in the NE system caused by chronic stress should be mentioned as potential contributors to our effects. Recent evidence demonstrates that while chronic cold stress does not alter levels of NET, it alters NET localization (Miner et al. 2006). Evidence shows that the amount of NET present in the plasma membrane of axon terminals in the prefrontal cortex was doubled after exposure to chronic cold. However, the functional relevance of this is unclear, while increased NET on the membrane may be a response to increased levels of NE which have been reported after microdialysis, whether this increased NET can compensate for increased NE is unclear. An examination of NET levels in the BLA may reveal data more relevant to the interpretation of our studies.

Finally, regulators of G protein signaling (RGS) are susceptible to alterations by exposure to chronic stress (Ni et al. 1999). As all adrenergic receptors are metabotropic and work via Gprotein signaling, alterations in regulation of that signaling process could have potent effects on NE modulation of neuronal activity. Levels of mRNA for RGS4 decrease in the PVN after chronic unpredictable stress, but increase in the LC (Ni et al. 1999). It has been suggested that such alterations play a role in long-term adaptations of structures to stress. Either of these changes could contribute to the alterations of NE modulation of BLA neuronal activity seen in the present experiments, but our techniques cannot speak to these potential changes. In vitro examination of BLA neurons after chronic stress may speak to such effects.

Particularly challenging with regard to interpretation are the data demonstrating alterations of NE modulation of evoked activity after only 7 days of cold exposure. Other studies examining sensitization of the NE system due to cold exposure show effects emerging after 14 days only, not after 7 days of exposure. This suggests there may be alterations in the NE system after 7 days of exposure not yet detected experimentally. An example of this would be increases in NE levels at the synapse too subtle for detection with microdialysis. Such an effect could lead to alterations in BLA neurons such as changes in receptor sensitivity or number. Our data in particular argue that a downregulation or decrease in presynaptic alpha-2 receptors may be one of the first responses to cold exposure. This would explain why the effects of cold on NE modulation of evoked activity emerge before the effects on NE modulation of spontaneous activity.

5.9 STRESS AND DISRUPTION OF BLA GABAERGIC TRANSMISSION

Pyramidal neurons of the BLA are under tight inhibitory control from GABAergic local circuit neurons in the BLA. These neurons provide inhibitory input via three mechanisms. Baseline recordings of BLA pyramidal neurons indicate that they are under a tonic GABAergic inhibitory influence, as reflected by the presence of rhythmic spontaneous IPSPs (Rainnie et al. 1991). Feed forward inhibition is also thought to occur upon the stimulation of afferents: under normal conditions, stimulation of BLA afferents leads to two overlapping responses. First occurs an AMPA/kainate and NMDA dependent excitatory response, with a later, inhibitory response mediated by GABA-A receptors (Rainnie et al. 1991, Washburn and Moises 1992, Woodson et al. 2000). Underlying this proposed source of inhibition is the assumption that afferents synapse on both pyramidal neurons and also inhibitory interneurons, which contact the pyramidal neurons. Feedback mechanisms of inhibition also exist in the BLA. Antidromic activation of BLA efferent fibers causes inhibition of projection neurons, via activation of a collateral from the projection neuron onto an inhibitory interneuron which synapses back onto the projection neuron (Matsuda ad Fujimura 1996). The combination of these three inputs serves to keep BLA projection neurons firing at very low rates.

Due to the low spontaneous firing rates of these neurons, activation of a subset of neurons leads to a high signal-to-noise ratio. This could create a highly efficient signaling system. Such a system may be influenced by NE in the following manner: upon presentation of a salient stimulus, LC neurons would increase their firing and NE levels in the BLA would increase. As demonstrated through our studies, this inhibits the majority of neurons via alpha-2 receptor mechanisms, but a select few become activated via beta receptors. This reinforces the high

signal to noise ratio by not only activating a subset of neurons (via beta-receptor activation) in a region with low spontaneous firing rates, but also further dampening activity (via alpha-2 receptor activation) of those neurons not involved in the response. As explained above, this distribution of excited/inhibited neurons could be altered during learning.

Restraint causes decreases in GABAergic inhibitory control of the stress response in the amygdala (Martineja et al. 2002). Acute restraint and tail-shock stress lead to a disruption of GABAergic transmission within the BLA as reported in control rats (Braga et al. 2004). However, our data argue against any disruption in tonic GABAergic activity. Our studies revealed no differences in baseline firing rates or in baseline responses of BLA neurons to afferent stimulation. If disruptions in tonic GABAergic inhibition occurred, one would expect to see an increase in baseline firing rates, particularly in neurons identified as projection neurons, as such neurons are under such tight GABAergic inhibition. One of those sources of inhibition is feed-forward GABAergic activity (see above). The lack of differences in baseline evoked activity suggest this feed-forward inhibition also goes unaltered after chronic cold. Important to note, however, is that this interpretation may be limited by our use of extracellular techniques. If, for example, chronic stress leads to a decrease in the slow, GABAergic IPSP seen in BLA neurons after excitatory EPSPs, in order for us to see such an effect, the neuron would need to be firing at higher rate. In a slowly firing neuron, the IPSP is probably over before the next spike occurs, therefore the effect of decreasing that response cannot be seen at the level of recording extracellular spikes. More subtle effects such as these could be better revealed with the use of intracellular recordings. In fact, intracellular recordings would be particularly useful to examine BLA neurons and subthreshold responses, given their low baseline firing rates. Another important way in which intracellular recordings would aid in interpretation of NE effects is by

providing a means to identify whether a projection neuron or an interneuron is being recorded by cell filling after physiological recording. An alternative way to determine this would be to attempt to juxtacellularly label neurons recorded extracellularly.

One mechanism by which NE inhibits neuronal activity may be via activation of GABAergic interneurons. NE forms asymmetrical synapses onto GABAergic interneurons in the BLA (Li et al. 2001). Therefore, NE may rely on GABAergic neurotransmission to accomplish at least a portion of its inhibitory effects. After chronic stress, NE in the BLA causes much more excitation than it did prior to stress exposure. However, as our studies used direct application of NE onto BLA neurons, it is unlikely that NE ejected from the iontophoretic electrode reached and affected adjacent interneurons that project onto and potentially inhibit the neurons we were recording from. Therefore, our experiments did not account for potential alterations in this portion of the LC/NE-BLA circuitry.

5.9 CORTICOTROPIN-RELEASING HORMONE, THE AMYGDALA, AND STRESS

While our data focus on the modulation of the BLA by NE before and after chronic cold exposure, this certainly is not a sufficient examination of how stress exposure may affect the BLA. There are many other physiological changes that occur during stress that could influence the amygdala, sensitization, and the functioning of the stress response. CRH is briefly examined here as a candidate that could affect BLA neurons during prolonged stress. CRH plays a critical role in the initial hormonal cascade leading to the release of corticosterone from the adrenal cortex (Vale et al. 1981). Studies suggest CRH may also activate LC neurons in response to stress (Curtis et al. 1997), and some behavioral effects of stress can be replicated with repeated

injections of CRH (Servatius et al. 2005). Evidence suggests that poor regulation of CRH may contribute to the development of stress-related disorders including panic, anxiety, and PTSD (Arborelius et al. 1999, Boyer 2000).

During stress, CRH is released in the amygdala (Merlo 1995, Cratty et al. 1999, Koob and Heinrichs 1999), which has a high abundance of CRH receptors (Van Pett et al. 2000, Reul and Holsober 2002). Activation of these receptors may be a potential substrate for stress-induced alterations in affective behaviors (Dunn and Berridge 1990, Lee and Davis 1997, Sadjyk et al. 1999, Herringa et al 2004, Gehlert et al 2005). Repeated administration of CRH2 receptor agonists directly into the BLA leads to anxiety-like symptoms (Sajdyk et al. 1999), and sensitization of the stress response, or priming effects (Sadjyk et al. 1999).

The source of CRH in the BLA is unclear. Studies demonstrate CRH-containing neurons in the CeA and BNST, regions that send projections back to the BLA (Swanson et al. 1983, VanBoeckstale et al. 1999, VanPett et al. 2000), although these projections are not particularly robust (Jokkenen and Pitkanen 1998). Other potential sources include sparse amounts of CRH in the prefrontal cortex and the dentate (Palkovits et al. 1983), however, these regions are far less implicated in the stress response than the CeA and BNST. Acute footshock stress increases CRH levels in the CeA (Roozendaal et al. 2002) and the proximity of the CeA to the BLA, as well as the transport of peptides long distances in the brain (Bittencourt and Sawchenko 2000) suggest that the CRH present in the CeA may also be able to diffuse and affect the BLA as well.

Direct application of CRH into the amygdala increases the excitability of BLA neurons (Rainnie 1992). Chronic urocortin, a CRH agonist, decreases tonic inhibition of BLA projection neurons, mechanistically thought to occur via a decrease in the activity of GABAergic interneurons, which normally cause inhibition of pyramidal neurons (Rainnie et al. 2004). As

described above, under normal conditions, a balance of excitatory and inhibitory inputs maintains BLA neuronal activity within a reasonably low range and prevents nonspecific potentiation of inputs due to NMDA activation and postsynaptic calcium influx (Rainnie et al. 1991). CRH may potentially disrupt this balance of excitatory and inhibitory inputs, leading not only to hyperexcitability, but the potential for nonspecific potentiation of inputs and plasticity. However, again our data argue against changes in tonic GABAergic inhibition in the BLA, as well as changes in feed-forward inhibition upon stimulation of afferents. This suggests that NE may in fact be particularly critical for alterations in BLA circuits that occur during stress exposure, potentially including those responses currently attributed to CRH effects.

5.10 YOHIMBINE, NE AND ANXIETY

Yohimbine is a pharmacological agent whose primary mechanism of action is blockade of noradrenergic alpha-2 receptors (Elmslie et al. 1990, Arnsten et al. 1999). As alpha-2 receptors act as autoreceptors in the noradrenergic system, it is thought that yohimbine decreases feedback inhibition of NE and increases levels of NE in the synapse. Yohimbine administration causes increases in NE cell firing (Ivanov and Aston-Jones 1995) as well as increases in extracellular levels of NE in terminal regions including the hypothalamus (Pacak et al. 1992), the hippocampus (Abercrombie et al. 1988), the prefrontal cortex (Garcia et al. 2004), and the amygdala (Khoshbouei et al. 2002 and present studies). Yohimbine also increases cfos labeling in the hypothalamus, locus coeruleus, and the amygdala (Myers et al. 2005), and contexts associated with yohimbine lead to an activation of the basolateral amygdala, lateral septum, and hypothalamus (Schroeder et al. 2003).

Yohimbine clearly has actions in the amygdala. Increased NE within the amygdala could cause increases and decreases in BLA neuronal activity similar to those seen during NE iontophoresis. However, this interpretation becomes complicated when integrated with our hypotheses on NE actions in the BLA. We suggest NE-mediated inhibition acts primarily via postsynaptic alpha-2 receptors. This is eliminated as a possible source of inhibition in yohimbine studies. Despite yohimbine-induced NE increases in the BLA, yohimbine also blocks BLA postsynaptic alpha-2 receptors. Therefore, the inhibitory responses seen in BLA neurons must be due to some source other than postsynaptic alpha-2 receptor-mediated inhibition. In a similar manner, alpha-2-mediated inhibition of glutamate release is also eliminated as a possible source. While alpha-2 blockade may allow for NE to target beta receptors and excite BLA neurons specifically in the BLA, an alternative explanation is needed for the inhibitory effects. Clearly, the actions of systemic yohimbine are far less specific than NE iontophoresis, and lead to potential increases in NE in any region of the brain receiving NE afferents. Furthermore, alpha-2 receptors are blocked in any region containing those receptors as well, which could lead to changes in activity. Any one of these regions could project to the BLA and alter its neuronal activity. Therefore, the interpretation of these effects must include actions of yohimbine on alpha-2 receptors in other regions, as well as the consequences of increased NE on regions that may provide afferent input to the BLA.

Yohimbine has many pharmacological and behavioral applications (Tam et al. 2001). It has antinociceptive properties (Dessaint et al. 2004), and is used for the treatment of obesity and depression (Tam et al. 2001). Yohimbine administration in humans increases plasma cortisol and NE levels (Ambrisko and Hikasa 2002), and increases blood pressure (Swann et al. 2005), respiration, restlessness, irritability (Cameron et al. 2000), feelings of anxiety (Holmberg and

Gershon 1961, Charney et al. 1983), and impulsivity (Swann et al. 2005). Patients with PTSD or panic disorder display exacerbated anxiety responses to yohimbine compared to controls (Charney et al. 1992, Southwick et al. 1999), as do children with anxiety disorders (Sallee et al. 2000). In animals, yohimbine decreases social interaction (Pellow et al. 1985, Ghitza et al. 2005), causes motor activation (Schroeder et al. 2003), and feeding suppression (Myers et al. 2005). It impairs performance on memory tasks (McAllister 2001, Park et al. 2001), supports conditioned flavor avoidance (Myers et al. 2005) and conditioned place aversion (File 1986), and increases fear-potentiated startle (Davis et al. 1979). Furthermore, yohimbine causes reinstatement of drug-seeking behavior for methamphetamine (Shepard et al. 2004), and cocaine (Lee et al. 2004), and food-seeking behavior as well (Ghitza et al. 2005). Animals are more sensitive to yohimbine after chronic psychosocial stress (Park et al. 2001).

These studies demonstrate that yohimbine significantly alters activity in the BLA. The BLA is known to be involved in many of the above mentioned behavioral responses associated with yohimbine administration, including anxiety responses, fear-potentiated startle, and others. Therefore, it is likely that the behavioral effects of yohimbine are mediated, in part, by its actions in the BLA, although its actions at other receptors and in other brain regions cannot be discounted. Furthermore, amygdala pathology and hyperactivation has been documented in patients with PTSD, panic disorder, and anxiety disorders (DeBellis et al. 200, Deckersbach et al. 2006). The exaggerated response to yohimbine administration may be due to alterations in the actions of yohimbine or increased NE on a hyperactive amygdala.

5.11 POTENTIAL BEHAVIORAL IMPLICATIONS OF CHRONIC COLD-INDUCED ALTERATIONS IN NE MODULATION OF BLA NEURONAL ACTIVITY

The idea that specific BLA inputs may be potentiated during learning is well established (see Introduction). We have presented this idea in reference to the role that NE may play in affecting BLA neuronal activity, as well as modulating evoked activity. Extending this idea to our chronic cold data, one could argue that cold exposure is a stressful experience, made more severe by the chronic exposure paradigm used in these studies. During a severely stressful experience involving excessive NE release, this potentiation of inputs to BLA neurons may become less specific. Therefore, while our animals are learning about the cold environment, this potentiation of inputs may be maladaptive or even "saturating" to some degree, leaving decreased ability of BLA inputs for further potentiation. Behaviorally, this may result in animals displaying learning impairments. Indeed, after chronic stress exposure, many learning impairments have been documented. Chronic restraint (Wright and Conrad 2005), immobilization, cold water exposure (Nishimura et al 1999), and psychosocial stress (Park et al 2001) all impair learning on a variety of tasks. Furthermore, chronic stress may lead to difficulties in extinction, another form of new learning whereby animals learn that a stimulus is no longer threatening or aversive. In fact, PTSD could be attributed, in part, to such effects. Patients have learned, perhaps to a maladaptive degree, to fear a certain stimulus. However, that fear does not remain specific to that stimulus, and it is triggered by other, less specific stimuli or situations. Also, the unlearning, or extinction of that fear is exceedingly difficult. Other behaviors including anxiety could also be related to a version of maladaptive fear learning, i.e. a version of exaggerated mild fear of stimuli that in other people , does not evoked fear. Patients usually refer to this fear as "worry".

Many types of stress lead to animal behaviors indicative of anxiety such as decreased exploration, anhedonia, decreased weight gain and sexual behaviors (Willner 1997, Hata et al. 2001, Vyas and Chattarji 2004).

Future studies would further expand on the role of the amygdala in such effects. An extension of the examination of NE modulation of afferent inputs beyond EC and Te3 would be revealing. In the present studies, EC and Te3-evoked activity is facilitated by NE after cold exposure. One could argue that this facilitation is due to some potentiation that occurred as a result of learning about the cold environment. An examination of other inputs would indicate how widespread and perhaps nonspecific that potentiation of inputs is. In addition, if previously suggested studies reveal that Te3 or EC are potentiated during conditioning paradigms, an examination of whether or not these inputs can undergo further potentiation during the conditioning of cold-exposed animals may reveal contributions of the physiology of these circuits to behavior.

In complement to an examination of the physiology, behavioral studies would provide further information regarding the relevance of this alteration of NE modulation of the BLA to behavioral effects. Little data is available regarding behavioral performance of animals exposed to chronic cold. We would suggest these animals would display learning impairments, particularly with regard to tasks involving the amygdala (although other, more widespread disruptions may occur). This would be accompanied by a deficiency in the ability of BLA inputs to be specifically potentiated during such learning/conditioning experiences.

It must be emphasized that the effects of stress on behavior rely heavily on the severity and duration of stress exposure. Stress does facilitate some learning experiences (Conrad et al. 1999). While moderate levels of stress may lead to moderate increases in NE which help to

facilitate memory formation and consolidation, extreme levels of stress and exacerbated NE increases may disrupt it. This too is demonstrated in our electrophysiological data. Whereas after 14 days of chronic cold exposure the above alterations in NE modulation occur, after 7 days the effects are much less dramatic, if present at all. We see little disruption of the NE-induced inhibition of spontaneous activity, and less dramatic disruption of NE modulation of afferent-evoked activity, lending electrophysiological support to the idea that a stressor needs to be of a certain duration or severity in order to induce pathological effects.

This leaves us with the question: what are the consequences of increased excitatory responses in BLA neurons in terms of BLA output? For consideration of this point, we return to our proposed example circuit. We concluded that it is likely that NE-induced excitation of BLA neurons is the behaviorally relevant effect that allows for activation of downstream target regions of the BLA, primarily via projections to the BNST (although through the CeA via disinhibition). This allows for an excitatory influence of the BLA on the stress response, as has been suggested (see Introduction). We also suggested the NE excitation of BLA neurons is selective, and may be altered during learning. Therefore, a more widespread excitation of BLA neurons by NE would lead to a loss of that selectivity, and an increased output of BLA neurons. This may lead to maladaptive effects in terms of overexcitation of downstream targets. Consider, for example, the LC. If BLA outputs via the BNST feedback to further excite the LC, we suggested perhaps this response is terminated via inhibitory afferents to the BLA (though we suggested mPFC inhibition as a potential source, there are other options). If the BLA-excitatory circuit becomes hyperexcited, inhibitory inputs may no longer be sufficient to terminate the stress response. This would result in an exaggerated, prolonged, and potentially maladaptive response to stress, the

consequences of which have been previously discussed, and the clinical relevance of which is review at the conclusion of this document.

5.12 EXPERIMENTAL CAVEATS AND POSSIBLE CONFOUNDS

The use of anesthetic for all of these electrophysiological studies is a significant confounding variable that may alter neuronal activity levels and responses. This is particularly challenging in relation to the low basal firing rates of BLA neurons, which make it difficult to reliably detect inhibitory responses. Furthermore, the anesthesia eliminates cognitive appraisal of the aversive footshock stimulation and possibly also learning or habituation that may occur with repeated footshock presentations. To partially account for this, repetitions of footshock were limited.

As discussed in the introduction, neurons of the LC contain NE, but also a variety of different peptides. Electrical stimulation of the LC should activate all neurons and fibers. Therefore, we cannot ensure that the release of neurotransmitter from these terminals is limited to NE only, meaning we cannot ensure that the effects seen after LC stimulation are due to NE as well. Supporting the theory that NE is mediating these effects are the data demonstrating that the effects of LC stimulation can be replicated by applying NE via iontophoresis. However, this does not eliminate the possibility that a peptide released from LC neurons could have similar effects as NE. The peptides known to be colocalized in LC neurons are galanin and NPY. Both have been implicated in mood and anxiety disorders (Carvajal et al. 2006, Ogren et al. 2006). Galanin antagonists are being investigated as a possible treatment for depression (Ogren et al. 2006), due to their actions on the serotonin system. The actions of galanin in the CeA are anxiolytic (Khoshbouei et al. 2002), however, the source of galanin was shown to be local

galanin-containing neurons, as these effects were still present after lesions of the noradrenergic CeA afferents (Barrera et al. 2006). Limited electrophysiological data suggest that galanin has mainly inhibitory effects. Hippocampal LTP was reduced by galanin (Mazarati et al. 2000), and galanin inhibits spontaneous and glutamate-evoked activity in the arcuate nucleus (Dong et al. 2006). With regard to NPY, its actions also seem to be inhibitory, causing decreases in spontaneous activity and hyperpolarization in neurons of the hypothalamus, as well as decreases in excitatory postsynaptic potentials, an effect though to be mediated by pre-synaptic receptors (Fu et al. 2004). Excitatory neurotransmission is also decreased in the olfactory bulb by NPY (Blakemore et al. 2006), and a decreased release of norepinephrine via presynaptic actions of NPY has been found in cardiac tissues (Potter et al. 2006).

Consideration of possible peptidergic contributions to the effects of LC stimulation on BLA neuronal activity reveals that as galanin and NPY do both have inhibitory effects, and therefore could contribute to the inhibitory actions seen after LC activation. However, two main points argue against this interpretation. First, the effects of LC stimulation on BLA neurons were mimicked by NE iontophoresis, suggesting a similar mechanism of action. Also, both excitatory and inhibitory responses were seen after LC stimulation, and each type of response was replicated with NE iontophoresis. There have been no reported excitatory electrophysiological actions of galanin or NPY, therefore it is unlikely these peptides are contributing to these responses.

While iontophoresis was an excellent technique for examining, in a more precise manner, the response of BLA neurons to local NE manipulations, the limitations of this technique makes interpretation challenging. In using such a technique, it is difficult to determine whether one is hitting postsynaptic receptors present on BLA neuron dendrites or soma, or possibly receptors

present on presynaptic terminals. Furthermore, one cannot confidently ensure that small, local inhibitory interneurons adjacent to projection neurons are not simultaneously affected by NE iontophoresis. This adds the further complicating factor that NE could be causing inhibitory effects via activation of an inhibitory interneuron which synapses onto the projection neuron being recorded. A lack of anatomical data specifying localization of adrenergic receptors to BLA projection neurons, GABAergic/peptidergic interneurons, or postsynaptic terminals does not simplify the interpretation of our effects.

Interpretation of the yohimbine studies is also arduous. In addition to its effects on alpha-2 receptors, yohimbine has moderate binding affinities at serotonergic (Winter and Rabin 1992), dopaminergic (Scatton et al. 1980), and alpha-1 receptors (Doxey et al. 1984). While the confirmation that yohimbine administration increased NE within the BLA lends support to the idea that at least a portion of these effects were due to NE actions within the BLA, other possibilities cannot conclusively be ruled out.

Norepinephrine could also be having effects via alpha-1 receptors in the amygdala. Alpha-1 receptors have also been shown to play an important role in the modulation of stress and anxiety-related behaviors. Blockade of alpha-1 receptors with antagonists in the BNST attenuates the immobilization stress-induced increases in ACTH and anxiety behaviors on the elevated plus maze (Cecchi et al. 2002). Pharmacological stress-induced impairment of working memory can be alleviated by blockade of alpha-1 receptors (Birnbaum et al. 1999). Alpha-1 antagonists administered in the central amygdala attenuate the effects of immobilization stress on social interaction (Cecchi et al. 2002b). In the BLA, NE has been shown to enhance GABAergic transmission via alpha-1 receptors (Braga et al. 2004). However, other studies have demonstrated alpha-1 receptor potentiation of the excitatory effect of beta-receptor stimulation

(Ferry et al. 1999). Therefore, it is unclear exactly what the contribution of alpha-1 receptors would be to physiology of neurons of the BLA examined in these experiments. In order to have a complete understanding of how NE acts in the BLA in vivo, these studies should extend to include an examination of alpha-1 receptors.

5.14 CLINICAL RELEVANCE (PTSD, DEPRESSION, ETC)

Dysfunction of the noradrenergic system has been implicated in mood and anxiety disorders (Charney et al. 1995, Bremner et al. 1996), including PTSD (Southwick et al. 1999), panic disorder (Charney et al. 1995), depression (Schildkraut et al. 1992), and attention-deficit/hyperactivity-disorder (Berridge et al. 2001). Anxiogenic responses induced by yohimbine are enhanced in PTSD patients; with yohimbine causing increases in cortical activity as well (Bremner et al. 1997). Furthermore, some antidepressants used for treatment of depression target, at least in part, the noradrenergic system (Grant and Weiss 2001).

The implication of NE in many disorders overlaps well with disorders also thought to involve the amygdala. Alterations in amygdala volumes and amygdala activation have been demonstrated in anxiety and stress-related disorders. Females with depression display increased amygdala volumes (van Elst et al. 2001), as do subjects with generalized anxiety disorder (De Bellis et al. 2000). Depressed patients also have greater baseline levels of amygdala activation (Drevets et al. 2000), which returns to normal in patients who respond to antidepressant treatment (Drevets et al. 1999), and greater amygdala responses to masked emotional faces (Sheline et al. 2001). Patients with PTSD have a hyperresponsive amygdala, and display greater activations of the amygdala to fearful stimuli (Deckersbach et al. 2006). Therefore, perhaps the

pathology lies in part in the interactions of the locus coeruleus and the amygdala. Considerable previous work has confirmed with certainty that the NE system of the brain and the amygdala are both highly susceptible to stress-induced alterations. We suggest here how an altered response to NE inputs may result in a maladaptive response to stress. These studies elaborate on the pharmacology involved in how these regions interact in control situations, in relation to acute stress presentation (footshock stress), and after exposure to chronic stress, making them highly relevant to clinical investigation of mood, anxiety, and other disorders thought to involve stress.

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