

**ROLE OF LOCUS COERULEUS AND AMYGDALA PROJECTIONS TO VENTRAL  
SUBICULUM IN STRESS REGULATION**

by

**Witold J. Lipski**

Physics B.A., Colby College, 2000

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This dissertation was presented

by

Witold J. Lipski

It was defended on

November 28, 2011

and approved by

German Barrionuevo, M.D., Department of Neuroscience

Judy Cameron, Ph.D., Department of Psychiatry

Linda Rinaman, Ph.D., Department of Neuroscience

Susan Sesack, Ph.D., Department of Neuroscience

Barry Waterhouse, Ph.D., Departments of Neuroscience and Anatomy, Drexel University

College of Medicine

Dissertation Advisor: Anthony A. Grace, Ph.D., Departments of Neuroscience, Psychiatry and

Psychology

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Witold J. Lipski, PhD

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The hippocampal formation, and the ventral subiculum (vSub), constitute a central node in mediating the forebrain's stress response, particularly to psychogenic stressors. The vSub regulates midbrain dopamine neuron population activity via its output to the nucleus accumbens (NAc)-pallidal-ventral tegmental area circuit, providing a potential functional link between stress and dopaminergic system. However, it is not known how appropriate vSub activity is generated in response to stress. The vSub receives projections from two stress-related inputs, locus coeruleus (LC) and basolateral nucleus of the amygdala (BLA). In this study, direct LC innervation of the vSub and indirect input through the BLA was investigated as potential mediators of stress responses in the vSub.

The effect of norepinephrine (NE) on single vSub neurons was examined using microiontophoresis combined with electrophysiological recording in anesthetized rats. The response to NE in the vSub was compared to the effect of electrical stimulation of the LC. Iontophoretic NE inhibited vSub neurons, while LC stimulation inhibited 16%, and activated 38% of neurons. Inhibition was mediated by alpha-2, whereas activation by beta-adrenergic

receptors. Moreover, vSub neurons were activated by BLA stimulation, and LC pre-stimulation both potentiated and suppressed these responses in neuronal subpopulations.

To examine the activation by stress, the effect of footshock on vSub neurons was tested. Footshock inhibited 13%, and activated 48% of neurons. Responses to footshock were correlated to LC stimulation-evoked responses in single neurons. Prazosin, an alpha-1 antagonist, reversed footshock-evoked inhibition, revealing an underlying activation. Inactivation of the BLA did not block LC stimulation- or footshock- evoked activation, while LC inactivation blocked both.

To examine stress effects on vSub neurons involved in modulation of DA activity, a subpopulation of vSub neurons projecting to the NAc was identified using retrograde tracing. Restraint stress activated these neurons as measured by Fos expression.

These results suggest that the LC NE system plays an important role in mediating responses to stress in the vSub. Stress-induced activation of the LC NE system evokes inhibition and activation in the vSub, both of which may contribute to stress adaptation. An imbalance of this system may lead to pathological stress responses in mental disorders.

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*In times of stress, be bold and valiant.*

-Horace (65-8 BC)

## **1.0 GENERAL INTRODUCTION**

### **1.1 STRESS, STRESS RESPONSES AND BRAIN REGIONS INVOLVED IN STRESS**

#### **1.1.1 Definition of stress and stress responses**

Stress can refer to a multitude of scenarios vastly different in their characteristics and consequences. Indeed, the use of the terms “stress” and “stress response” is often ambiguous and indiscriminate in the literature. The most common definition of stress can be distilled to some variation of the following: any actual or perceived threat to the maintenance of homeostasis (B S McEwen 1998; T. a Day 2005). Homeostasis – or a stable equilibrium – can be taken to imply that a single ideal steady state exists for living organisms. While this definition is oversimplified, efforts to reinterpret the term homeostasis with terms such as allostasis and allostatic load (David S Goldstein & B. McEwen 2002) contribute to the confusion. Thus, the concept of allostasis posits a dynamic state in which an organism strives to maintain a balance between multiple stressors. Correspondingly, the consequences of the continuous activation of allostatic mediators are referred to as the allostatic load. While such distinctions are largely semantic, it is important to recognize that living organisms are not static, and that stressors continually change the trajectory of their internal state. With this in mind, 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). It is also important to point out that 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 (Pacák 2000; Dayas et al. 2001; Pacák & Palkovits 2001).

### **1.1.2 The clinical implications of stress**

The detrimental effects of stress exposure on neurological processes have been demonstrated through numerous clinical and pre-clinical studies. Whereas stress often triggers adaptive responses and enables a rapid reaction to threatening or harmful stimuli, it can also precipitate maladaptive changes (B S McEwen 1998). Various types of stressful stimuli influence the onset, development, and progression of a number of diseases including post-traumatic stress disorder, panic disorder, generalized anxiety disorder (Sullivan et al. 1999), phobias, depression, and schizophrenia (Southwick et al. 1999; Bremner, Krystal, Southwick & Charney 1996b; C. McDonald & Murray 2000). Stress also increases the likelihood of relapse to drug-taking and drug-seeking behaviors (Shaham et al. 2000; Shaham & J. Stewart 1995). Thus, understanding neural mechanisms underlying stress responsiveness may have important implications in the treatment of a variety of neuropsychiatric disorders and help optimize therapeutic strategies to prevent or treat the negative consequences of stress exposure. The studies conducted here were focused on the neurophysiological response to stress in the vSub, a brain region known to play an important role in the stress response (J P Herman & N K Mueller 2006), but whose mechanisms of stress responsiveness remain largely unexplored.

### **1.1.3 Stress and dopamine dysfunction**

The focus of this manuscript will be on the potential mechanisms of how stress plays a role in the development of disorders that involve dopamine dysfunction, in particular, drug abuse and schizophrenia. While the dopamine system plays a critical role in reward-related behaviors, it is also activated by stressful stimuli (Imperato et al. 1993; Piazza & Le Moal 1998). In rats, restraint stress has been shown to increase the population activity (i.e. the number of dopamine neurons firing spontaneously) of dopamine neurons in the ventral tegmental area (VTA) (Valenti et al. 2011).

Many clinical studies have documented that individuals undergoing stressful challenges show an increased vulnerability to drug addiction (G. Koob & Kreek 2007; Sinha 2008), and that this association is dependent on dopamine. Behavioral sensitization in rats, which is thought to reflect some of the neuroadaptive processes associated with drug addiction (Covington & Miczek 2001; T. E. Robinson & K. C. Berridge 1993), is also critically dependent on mesolimbic dopamine (Giorgi et al. 2005; R. C. Pierce & Kalivas 1997). It is a phenomenon whereby repeated administration of psychostimulants results in a gradual increase in the motor stimulant response after each subsequent dose. Importantly, stress during adolescence has been reported to alter behavioral sensitization to amphetamine (Kabbaj et al. 2002), and in rats, stress and psychostimulants cross-sensitize (Antelman et al. 1980; Piazza & Le Moal 1998). This cross-sensitization itself is thought to involve changes in mesolimbic dopamine (Cruz et al. 2011).

The association between the dopamine system, stress, and psychiatric disorders, including schizophrenia, has also been well-documented. In patients with psychotic disorders, stress may exacerbate preexisting deficits, promote the emergence of psychiatric aberrations and precipitate psychotic episodes (Pani et al. 2000, Moghaddam, 2002). Stress acutely activates

many CNS neurotransmitter and neuroendocrine circuits, including nigrostriatal (Keefe et al. 1993), mesocortical (Finlay et al. 1995; Gresch et al. 1994) and mesolimbic dopamine systems (Abercrombie et al. 1989). Substantial evidence points to a dysfunction in dopamine regulation as a central element in the pathophysiology of schizophrenia (Anthony A Grace 2010). Foremost, dopamine-mimetic drugs can induce psychosis (Angrist et al. 1975), whereas dopamine antagonists alleviate psychotic symptoms (CARLSSON & LINDQVIST 1963; Creese et al. 1976; Seeman et al. 1976; Kapur & Remington 2001). These findings are classic evidence pointing to dopamine involvement in schizophrenia. There is also a strong connection between stress and schizophrenia susceptibility. Thus, stress has been reported to precipitate relapse in schizophrenia patients that are in remission (Benes 1997). Early adolescent stress is one of the risk factors in the development of schizophrenia (Benes 1997; Tsuang 2000), and in children who have genetic risk for developing the disease, those that are hyper-responsive to stressors tend to be more vulnerable to developing psychosis (Johnstone et al. 2002).

In addition to dopamine, stress response in the CNS also involves other monoaminergic systems including norepinephrine (NE) (Rita J Valentino & E. Van Bockstaele 2008). Importantly, the NE system has also been implicated in the pathophysiology of drug abuse (Weinshenker & J. P. Schroeder 2007; G F Koob 1999; Gary Aston-Jones & Harris 2004), and in stress-induced reinstatement of drug seeking (Erb et al. 2000). The following section will review the forebrain NE system and its role in stress.

## 1.2 THE LOCUS COERULEUS, NE, AND STRESS

### 1.2.1 The locus coeruleus: anatomy and physiology

The LC has attracted much interest in the literature, as the biggest source of NE in the brain, and the central nervous system's largest noradrenergic cell group despite containing only 1600 neurons per side (R. Y. Moore & Bloom 1979; Foote et al. 1983; R. Y. Moore & Card 1984). The LC is located bilaterally in the brainstem, next to the fourth ventricle (Swanson 1976). The axons of this small group of neurons innervate the entire neuraxis, and form small synaptic contacts in target regions (Olschowka et al. 1981, Papadopoulos et al. 1989, Parnavelas and Papadopoulos 1989). The highly collateralized projections of the LC extend to regions such as the hypothalamus, the amygdala, the thalamus, and the cortex (Swanson 1976; R. Y. Moore & Bloom 1979; Foote et al. 1983; R. Y. Moore & Card 1984). This creates a highly divergent system whereby activation of a few LC neurons leads to a relatively nonselective, coincident NE release in many terminal areas; however, some heterogeneity in the projections of distinct LC neuron subsets has also been described (D. J. Chandler & B.D. Waterhouse 2011)

The neurons that comprise the LC are considered to be noradrenergic, since they contain tyrosine hydroxylase (TH) and dopamine-beta- hydroxylase (DBH), the enzymes necessary to synthesize NE (Swanson 1976). However, in addition to NE, neuropeptides – including galanin and neuropeptide Y – are localized in these neurons (Melandier et al. 1986; Holets et al. 1988; Austin et al. 1990), and may be released along with NE, particularly with phasic activation of the LC (Bicknell & Leng 1981; Bicknell 1988). After NE is released from LC terminals, its actions are terminated by the norepinephrine transporter (NET), which clears NE from the extracellular space (Blakely & Bauman 2000). In addition, activation of presynaptic alpha-2a or alpha-2c

autoreceptors inhibits NE release, providing negative feedback regulation of NE signaling. Autoreceptor activation causes guanine-D-phosphate-binding-protein (G-protein)-mediated hyperpolarization of LC neurons through opening of potassium (K<sup>+</sup>) channels, which in turn suppresses NE release (Arima et al. 1998).

### **1.2.2 The effects of norepinephrine on neuronal activity**

NE has diverse and complex effects neuronal activity. Numerous studies have reported NE having primarily inhibitory effects on spontaneous activity in various brain areas. These regions include the hippocampus (Curet & de Montigny 1988a; Madison & Nicoll 1988; Bergles et al. 1996), the amygdala (D M Buffalari & A A Grace 2007), the cerebellum (R. Freedman et al. 1977), the PVN (Chong et al. 2004), the red nucleus (Ciranna et al. 2000), and the medial septal nucleus (Alreja & W. Liu 1996). In addition, NE has been found to inhibit neurons in neocortical areas, including the 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 (Kovács & Hernádi 2002).

Similar inhibitory effects on neuronal activity have been evoked by electrical stimulation of the LC, which presumably increases NE in target regions. Thus, LC stimulation inhibits neurons in the hippocampus, amygdala, and the cerebellum (Segal & Bloom 1974; D M Buffalari & A A Grace 2007; Hoffer et al. 1973; Loy et al. 1980), while destruction of ascending NE projections from the LC causes an increase in spontaneous activity.

While the inhibitory actions of NE appear to dominate in most targets of the LC, excitatory effects of this transmitter on spontaneous neuronal activity have also been documented. Thus, NE activates brainstem-projecting cortical neurons (Z. Wang & McCormick

1993), hippocampal interneurons (Bergles et al. 1996; Hillman et al. 2007; Hillman, Doze, et al. 2005), and lateral dorsal tegmental neurons (Kohlmeier & Reiner 1999). Neurons of the subthalamic nucleus are depolarized by NE (Arcos et al. 2003), as are interneurons of the piriform cortex (Marek & Aghajanian 1996). NE has also been found to decrease GABAergic IPSPs in histaminergic neurons (Stevens et al. 2004), as well as in the PVN (Chong et al. 2004).

In addition to purely inhibitory or excitatory effects on spontaneous activity, NE has more complex effects neuronal evoked activity. Many studies have documented the modulatory role of NE in decreasing spontaneous activity, while simultaneously either increasing or decreasing evoked activity in the same neurons (Foote et al. 1975; Segal et al. 1991; B D Waterhouse & Woodward 1980; B D Waterhouse et al. 1984; B D Waterhouse, Moises & Woodward 1998b; B D Waterhouse et al. 2000; Doze et al. 1991; Devilbiss & B D Waterhouse 2000b) effectively altering the signal-to-noise ratio in target brain regions. Thus stressing the functional importance of its physiological actions, NE has been described as a “gating mechanism” (Devilbiss and Waterhouse 2000), or “gain modulator”, (Aston-Jones and Cohen 2005).

LC neurons are known to exhibit two modes of firing. The tonic mode is characterized by irregular slow firing of 0.1-5 Hz, while the phasic mode consists of short bursts of action potentials of 10–20 Hz (T. Akaike 1982; Florin-Lechner et al. 1996; Devilbiss & B D Waterhouse 2000b). Phasic activity is thought to signal salient stimuli (C. W. Berridge & Barry D Waterhouse 2003), while tonic activity reflects levels of arousal (C. W. Berridge & Barry D Waterhouse 2003). Importantly, phasic stimulation causes a higher increase in NE release in the prefrontal cortex than the same number of stimulation pulses delivered at a lower rate (Florin-

Lechner et al. 1996). In the experiments described in this document, burst stimulation of the LC will be used to mimic the phasic firing of LC neurons to acute stressful stimuli.

### **1.2.3 Noradrenergic receptors**

The multifarious physiological effects of NE can be attributed, in part, to the diversity of the receptor subtypes it activates in target regions, and downstream effector systems. Nine different receptor subtypes that bind NE 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 (Wozniak M et al. 2000; Bylund et al. 1994). NE receptors are metabotropic, and are linked to different G proteins, which in turn activate different second messenger systems. Alpha-1 receptors activate Gq proteins and stimulate phospholipase C, leading to mobilization of calcium from intracellular stores. Alpha-2 receptors have primarily inhibitory effects, activating inhibitory Gi proteins. This leads to a decrease in adenylyl cyclase activity, and the opening of K<sup>+</sup> channels (Arima et al. 1998), which generally hyperpolarizes the neuron. 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).

### **1.2.4 The locus coeruleus: role in stress**

The activation of the brain's LC noradrenergic system by exposure to acute stress has been extensively studied (Morilak et al. 2005; S. C. Stanford 1995; Zigmond Finlay, J.M., Sved,

A.F. 1995; Bremner, Krystal, Southwick & Charney 1996a; Bremner, Krystal, Southwick & Charney 1996b; Sved et al. 2002). Acute stress exposure results in increased c-fos expression in the LC (Ceccatelli et al. 1989; M. A. Pezzone et al. 1993; Passerin et al. 2000), and increased firing of LC neurons (Rasmussen & Jacobs 1986; Abercrombie & Jacobs 1987a; Grant et al. 1988; G Aston-Jones et al. 1991). Accordingly, acute stress increases NE efflux in terminal regions (Abercrombie et al. 1988), and results in an up-regulation of NE-synthesizing enzymes, which in turn increases the capacity of neurons to synthesize the neurotransmitter (Serova et al. 1999). In addition, the behavioral effects of LC activation mimic the effects of acute stress exposure, while lesions of the LC lead to loss or dampening of stress-induced neuroendocrine and behavioral responses (Ziegler et al. 1999).

Repeated or chronic exposure to stress causes a more complex, less well understood effects on the NE system, compared with the more straightforward activating effects of acute stress (Abercrombie Keller, R.W., Zigmond, M.J. 1992; S. C. Stanford 1995; Zigmond Finlay, J.M., Sved, A.F. 1995; Morilak et al. 2005). Repeated tailshock, footshock, immobilization stress, and cold exposure have been reported to increase tissue levels of NE (Ingenito & Bonnycastle 1967; Thierry et al. 1968; Bhagat 1969; Adell et al. 1988). Accordingly, chronic or repeated stress exposure has been found to increase the basal activity of TH and DBH (Serova et al. 1999; Gordon et al. 1966; Nisenbaum & Abercrombie 1992). While some studies report an increased basal firing rate in LC neurons after chronic stress (Pavcovich et al. 1990; Mana & A A Grace 1997), other studies report no change (Simson & Weiss 1988; Curtis et al. 1995). However, chronic cold exposure causes alterations in the electrophysiological characteristics of LC neurons (Jedema & A A Grace 2003).

Repeated or chronic stress exposure has been associated with both habituation and sensitization of many components of the stress response. Habituation refers to a dampening of the autonomic, neuroendocrine and behavioral stress responses with repeated exposure, while sensitization describes an exaggeration of stress responses after repeated exposure. Both phenomena have been observed. Thus, hippocampal levels of NE and serotonin habituate quickly to repeated footshock; however, plasma cortisol levels sensitize (Hajós-Korcsok et al. 2003). Chronic stress exposure does not alter baseline levels of NE in terminal regions (Nisenbaum et al. 1991; Gresch et al. 1994; Terrazzino et al. 1995); however, stress-induced NE efflux is enhanced following chronic stress (Nisenbaum et al. 1991; Gresch et al. 1994; Pacak et al. 1992; Jedema et al. 1999), as is the stress-induced increase in TH or TH mRNA (Nisenbaum et al. 1991; Nisenbaum & Abercrombie 1992; Rusnák et al. 2001). Additionally, prior chronic exposure to stressors such as tailshock, footshock, and cold result in an enhanced response of LC neurons to excitatory input, footshock and hypotensive challenge (Simson & Weiss 1988; Curtis et al. 1995; Mana & A A Grace 1997). LC neurons also display a sensitized excitatory response to CRH after chronic cold exposure (Jedema et al. 2001) and repeated footshock (Curtis et al. 1995).

### **1.3 AMYGDALA AND STRESS**

#### **1.3.1 The amygdala: anatomy and physiology**

The amygdala comprises several nuclei, with distinct yet highly integrated functions. The nuclei of the amygdala have different cytoarchitectonic and neurochemical features, as well as

segregated anatomical connections. The focus here is on the basolateral amygdala (BLA), which includes the lateral, basolateral, and basomedial nuclei, as these nuclei receive the majority of the NE input from the LC (Asan 1998). Two primary neuron types have been characterized in the BLA anatomically: large, glutamatergic pyramidal projection neurons, and distinct subgroups of smaller, GABAergic, local circuit interneurons (A. J. McDonald 1982; A. J. McDonald 1985; A. J. McDonald & Betette 2001). The intra-amygdala circuitry is extensive; therefore, the discussion here will be limited to the BLA, which is the part of the amygdala projecting to the hippocampal formation (Pikkarainen et al. 1999). Extensive interconnections link the BLA nuclei to one another, as well as to other nuclei within the amygdala (Pitkänen et al. 1997). Strong reciprocal projections exist from the lateral nucleus to the basolateral and basomedial nuclei (Pitkänen et al. 1997; Savander et al. 1995; Savander et al. 1996; Pitkänen et al. 1995). These regions send projections on to the central nucleus of the amygdala (CeA) (Pitkänen et al. 1997), which is considered the output region of the amygdala.

### **1.3.2 The amygdala: afferent and efferent connections**

The BLA receives a large amount of input from sensory regions, including the 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 (van Vulpen & Verwer 1989; A. J. McDonald 1998). The BLA also receives dense innervation from dopaminergic neurons (Asan 1998), serotonergic neurons (R. Y. Moore et al. 1978), and NE neurons (Asan 1998). These catecholaminergic inputs may provide information about the valence of the sensory stimulus.

The BLA has extensive efferent projections and can influence activity in regions such as the thalamus and prefrontal cortex (Gallagher & Holland 1994; Laviolette et al. 2005), sensory association cortex (Petrovich et al. 1996; Pikkarainen & Pitkänen 2001), and the nucleus accumbens (Petrovich et al. 1996). The BLA is also reciprocally connected with the hippocampal formation (Pitkänen et al. 2000; Pikkarainen et al. 1999).

The BLA can influence autonomic and brainstem targets through its projections to the central nucleus of the amygdala (CeA) and the bed nucleus of stria terminalis (BNST; Pitkänen, Savander, and LeDoux 1997; T S Gray, Carney, and Magnuson 1989). In this way, the amygdala is positioned to collect a variety of sensory cortical information, and influence many target regions that influence autonomic, motor, emotional, learning, and other behavioral responses.

### **1.3.3 The amygdala: role in stress**

Like the LC, the amygdala plays a prominent role in the behavioral, physiological, and autonomic responses to stress. The presentation of stressful stimuli or situations causes engagement of the amygdala. In humans, the amygdala is activated during passive viewing or reading of emotionally evocative stimuli. Thus, it has been suggested the amygdala contributes to the pathology of post-traumatic stress disorder (PTSD) (Rauch et al. 2000). Increased amygdala activity is also associated with feelings of anxiety (Anand & Anantha Shekhar 2003). In rats, the amygdala shows c-fos expression following noise, hemorrhage, immune challenge, restraint, swim, predator, social, cold water, and footshock stress (Cullinan et al. 1995; Rosen et al. 1998; Dayas et al. 2001; Chowdhury et al. 2000; Akirav et al. 2001). Furthermore, retrieval of aversive fear memories causes cyclic-adenosine-monophosphate-response-element-binding (CREB) protein activation and c-fos 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).

Stimulation of the amygdala leads to increases in autonomic stress responses like heart rate and blood pressure (Galeno & Brody 1983; Iwata et al. 1987; Sajdyk & A Shekhar 1997; Aslan et al. 1997), increases in plasma corticosterone (Feldman et al. 1982; Weidenfeld et al. 1997), and behavioral responses such as immobility (KAADA 1951). In the experiments described in this manuscript, we will electrically stimulate the amygdala in order to model stressful stimuli, and to investigate the role of this structure in generating stress responses in the vSub.

## **1.4 VENTRAL SUBICULUM AND STRESS**

### **1.4.1 The ventral subiculum: anatomy and physiology**

The vSub is continuous with area CA1 of the hippocampus and shares some cytoarchitectonic features with this structure. It is composed of three cell layers: 1) a molecular layer, which is continuous with strata lacunosum-moleculare and radiatum of the CA1, 2) a pyramidal cell layer, which is larger and less dense than in the CA1 and 3) a polymorphic layer (O'Mara et al. 2001). The rat subiculum consists of glutamatergic pyramidal projection neurons and GABAergic local-circuit interneurons (Menendez de la Prida et al. 2003; Menendez de la Prida et al. 2002; Staff et al. 2000; Funahashi & M. Stewart 1997). The pyramidal neurons extend their apical dendrites into the molecular layer and their basal dendrites into the deep portions of the pyramidal cell

layer, while the smaller GABAergic interneurons are interspersed among the pyramidal cells (O'Mara et al. 2001). Interneurons in the CA1 have been classified based upon morphological features as well as expression of calcium binding proteins and peptides (Somogyi & Klausberger 2005). It is not known whether the same interneuron types exist in the subiculum (O'Mara et al. 2001).

It is noteworthy that somatostatin expressing interneurons in area CA1 that express alpha-1 receptors (Hillman, Knudson, Carr, Doze, & Porter, 2005) correspond to a population of GABAergic interneurons targeting both the basal and apical dendrites of pyramidal cells (Somogyi & Klausberger, 2005). NE may therefore suppress glutamatergic input to pyramidal neurons by activating GABAergic inhibition of pyramidal dendrites via alpha-1 receptors. Pyramidal neurons in the vSub send glutamatergic projections to the caudomedial portion of the NAc (Aylward & Totterdell, 1993; O'Mara et al., 2001; Witter & Groenewegen, 1990), which is connected to the VTA via the ventral pallidum (VP). In this way, activity of the ventral subiculum affects the population activity of VTA dopamine neurons (Floresco, Todd, & Grace, 2001; Floresco, West, Ash, Moore, & Grace, 2003).

#### **1.4.2 Ventral Subiculum: afferent and efferent connections**

The ventral subiculum (vSub) is a region of the hippocampal formation receiving major input from area CA1 (Amaral et al. 1991) within the hippocampus, as well as from the entorhinal, perirhinal and postrhinal cortices (M P Witter et al. 2000; Kloosterman et al. 2004). The inputs to the vSub originating from the other parts of the cortex are few and include the presubiculum (van Groen & Wyss 1990) and retrosplenial cortex (Menno P Witter 2006). Subcortically, afferent projections to the vSub originate from the medial septal nucleus (J. P. Chandler &

Crutcher 1983) and nucleus of the diagonal band (Nyakas et al. 1987); the amygdaloid complex (Pikkarainen et al. 1999) and the endopiriform nucleus (Behan & Haberly 1999). The subiculum receives thalamic projections that originate mainly in the nucleus reuniens, the paraventricular nucleus and the parataenial nucleus (Van der Werf et al. 2002). Furthermore, the ventral subiculum receives heavy projections from the supramammillary region (Haglund et al. 1984) and the nucleus incertus (Goto et al. 2001). Finally, the subiculum is innervated by several monoaminergic cell groups including the noradrenergic locus coeruleus, the dopaminergic ventral tegmental area, substantia nigra and retrorubral group, and the serotonergic median and dorsal raphe nuclei (F H Lopes da Silva et al. 1990; Menno P Witter 2006).

The vSub serves as the output region of the hippocampal formation, providing major input to the entorhinal cortex (Swanson et al. 1978; Kloosterman et al. 2004). It also sends prominent projections to the medial and ventral orbitofrontal, as well as prelimbic and infralimbic cortices (Jay & M P Witter 1991; M P Witter & H J Groenewegen 1990). A retrosplenial cortex projection has also been described (M P Witter & H J Groenewegen 1990). In addition, a substantial subicular projection reaches the presubiculum and parasubiculum (Swanson & Cowan 1977; Swanson et al. 1978; van Groen & Wyss 1990).

The nucleus accumbens (NAc), particularly the caudo-medial portion, is heavily innervated by subicular fibers (H J Groenewegen et al. 1987; O'Mara et al. 2001; M P Witter & H J Groenewegen 1990; Aylward & Totterdell 1993). Another major subcortical target is the amygdaloid complex (Pitkänen et al. 2000) primarily the lateral, basal and accessory basal nuclei, as well as the bed nucleus of the stria terminalis (BNST), and the endopiriform nucleus (Canteras & Swanson 1992; F H Lopes da Silva et al. 1990). Projections to the thalamus mainly

target the nucleus reuniens, the nucleus interanteromedialis, the paraventricular nucleus and the nucleus gelatinosus (Canteras & Swanson 1992; Menno P Witter 2006; Ishizuka 2001)

The subiculum sends strong projections to the medial mammillary nucleus, as well as innervating the lateral hypothalamic region (Canteras & Swanson 1992). Importantly, the vSub also sends excitatory projections to multiple GABAergic areas that in turn inhibit the periventricular nucleus (PVN) of the hypothalamus; these include the bed nucleus of the stria terminalis (BNST), peri-PVN regions, subparaventricular zone, ventrolateral preoptic area and ventrolateral region of the dorsomedial hypothalamus (J P Herman & N K Mueller 2006). This disynaptic inhibition constitutes an important negative feedback to HPA activation in stress.

The strong subicular projection to the NAc is of particular interest in the context of this manuscript. Pyramidal neurons in the vSub send glutamatergic projections to the caudomedial portion of the NAc (O'Mara et al. 2001; M P Witter & H J Groenewegen 1990; Aylward & Totterdell 1993), which is connected to the VTA via the ventral pallidum (VP). In this way, activity of the ventral subiculum affects the population activity of VTA dopamine neurons (Floresco et al. 2001; Floresco et al. 2003). Importantly, NAc-projecting neurons in the vSub receive synaptic input from somatostatin-immunoreactive putative GABAergic interneurons (Aylward & Totterdell, 1993), which may express alpha-1 adrenoreceptors, as reviewed above. Thus, NE released in the vSub during stress may affect information processing in neurons controlling dopaminergic activity in the VTA.

### **1.4.3 Presentation of stressful stimuli or contexts activates the hippocampus and vSub**

The vSub is a forebrain structure central to orchestrating an organism's response to stressful stimuli, especially with psychogenic stressors (J P Herman & N K Mueller 2006; N K Mueller, C

M Dolgas, et al. 2004). Accordingly, the hippocampus is activated by many different stressful stimuli. Thus, c-fos expression in the hippocampus CA1-3 is increased by ether inhalation and open novelty stress (Emmert & J P Herman 1999), as well as swim and restraint stress (Figueiredo et al. 2003) (Cullinan et al. 1995) (Funk et al. 2006). While the vSub not been examined extensively in this regard, restraint stress-induced c-fos expression has also been reported in the vSub (Otake et al. 2002). In addition, stress increases the extracellular concentration of excitatory amino acids in the hippocampus, as measured by microdialysis (Moghaddam 1993; Lowy et al. 1995).

#### **1.4.4 vSub provides feedback inhibition to HPA axis stress response**

The hypothalamo-pituitary-adrenocortical (HPA) axis is a critical element of a vertebrate organism's adaptation to stressful stimuli. The end result of HPA activation is the release of glucocorticoid hormones (e.g., corticosterone in rats, cortisol in humans), which act to redistribute energy resources in the face of challenge (J P Herman & N K Mueller 2006). The vSub plays a central role in forebrain modulation of HPA activity, and as such it is critical in orchestrating an organism's response to stressful stimuli, especially with psychogenic stressors (J P Herman & N K Mueller 2006; N K Mueller, C M Dolgas, et al. 2004). The hippocampus is an important negative feedback modulator of HPA function. Thus, hippocampal lesions have been found to increase basal and stress-induced ACTH and corticosterone release in rats (J P Herman et al. 1992; J P Herman et al. 1989). On the other hand, stimulation of the hippocampus results in inhibition of HPA axis activity in animals as well as humans (Feldman & Weidenfeld 2001; Rubin et al. 1966). Furthermore, rats are reported to have a lower stress threshold following hippocampal lesions (Kant et al. 1984).

The ventral subiculum appears to be the critical element of the hippocampal formation with regards to HPA modulation. As reviewed above, it represents a major destination of CA1 projections and is the main output structure of the hippocampal formation. Furthermore, the overwhelming majority of hippocampal efferents to hypothalamic regions originate in the ventral subiculum; this suggests that ventral subiculum neurons are ideally situated to mediate HPA axis effects. In fact, this appears to be the case. Indeed, lesion studies in the rats have demonstrated that ablations that focused on the ventral subiculum/ventral CA1 region markedly elevated HPA axis output in response to restraint stress, without altering resting levels of corticosterone (J P Herman et al. 1995; J P Herman et al. 1998; Nancy K Mueller, C Mark Dolgas, et al. 2004).

Importantly, inhibition of HPA axis by the vSub appears to be stressor-selective, as vSub lesions in rats result in enhanced corticosterone response to restraint and novelty stress but not to ether exposure, which is known to activate the HPA axis (J P Herman et al. 1998). It has thus been suggested that vSub's control of HPA axis is relative to environmental context and recent experience (J P Herman & N K Mueller 2006).

#### **1.4.5 The vSub is important for learning that involves stressful stimuli**

The vSub is distinct in structure and function from the dorsal hippocampus, in that it has a major involvement in context-dependent processes, such as context-dependent fear conditioning (Fanselow 2000; Maren 1999) as well as processes that involve contextual relationships, such as extinction (Sinden et al. 1988), drug sensitization (Sun & Rebec 2003; Lodge & Anthony A Grace 2008), and stress (J P Herman & N K Mueller 2006; Valenti et al. 2011). Importantly, the vSub's connections to limbic regions – including the amygdala, prefrontal cortex, septum, and nucleus accumbens – point to its importance in emotional

processing; and indeed, the vSub is critical in the assignment of contextual relevance to emotional memories (Fanselow 2000; Maren 1999). Because stress can trigger symptoms in disorders affecting emotional processing, such as depression, anxiety disorders, post-traumatic stress disorder, schizophrenia and drug addiction, the effect of stress on the vSub is critical in understanding the pathophysiology of these diseases. It is therefore surprising how few studies have investigated this effect.

#### **1.4.6 The vSub regulates dopamine neuron population activity**

The vSub plays an important role in modulating midbrain dopamine neuron population activity in the VTA via its output to the nucleus accumbens (NAc) (Floresco et al., 2001, 2003). A single session of restraint stress is known to cause a sensitized behavioral and neurochemical response to amphetamine in rats (Pacchioni, Cador, Bregonzio, & Cancela, 2007; Pacchioni, Gioino, Assis, & Cancela, 2002), and the ventral subiculum mediates stress-induced increases in dopamine neuron population activity (Valenti et al. 2011), suggesting that stress alters control of the dopaminergic system. Thus the vSub provides a potential functional link between stress and dopaminergic pathophysiology of schizophrenia and drug abuse.

#### **1.4.7 Stress-induced alterations in hippocampal plasticity and morphology**

By producing increased fearful vigilance and anxiety in a threatening environment, stress can trigger short-term adaptive changes in the neural circuits that subservise cognition, decision making, anxiety and mood. However, acute and chronic stress can also create an imbalance in this circuitry (Bruce S McEwen et al. 2011).

The hippocampus is a brain structure particularly susceptible to damage by repeated stress (Bruce S McEwen 2010; Conrad et al. 1999; Krugers et al. 2010). In rats as well as primates, severe or prolonged stress can lead pyramidal neuron damage or cell loss (Uno et al. 1989; Mizoguchi et al. 1992). Less severe stress paradigms can lead to more subtle morphological changes. For example, repeated restraint stress in rats causes dendritic atrophy in CA3 pyramidal neurons in the hippocampus (Magariños & B S McEwen 1995; Sunanda et al. 1995; Y. Watanabe et al. 1992; Vestergaard-Poulsen et al. 2011; X.-D. Wang et al. 2011).

Stress also induces changes in hippocampal plasticity. Inhibitory avoidance, a learning paradigm involving stressful stimuli is known to induce long-term potentiation (LTP) in the hippocampus (Whitlock et al. 2006). On the other hand, stressful stimuli have been reported to suppress hippocampal LTP induced by high-frequency stimulation, a putative cellular mechanism underlying learning and memory. Acute stressors such as contextual fear conditioning and inescapable footshock stress block or decrease long-term potentiation (LTP) in the dorsal hippocampus and CA1 (Kim et al. 1996; Hirata et al. 2009; B. K. Ryan et al. 2010). On the other hand the same stressors have the opposite effect on hippocampal long-term depression (LTD) induced by low-frequency stimulation. Thus, acute stressors facilitate subsequent induction of LTD in CA1 (L. Xu et al. 1997; Yang et al. 2004). These changes in synaptic plasticity are NMDA-dependent, and transient, lasting for hours after acute stressor administration. In addition, the adrenal stress hormone corticosterone has been found to modulate synaptic plasticity in the hippocampus of rats in a biphasic manner. In the dentate gyrus as well as CA1, glucocorticoid (GR) receptors have been found to enhance LTP while mineralocorticoid (MR) receptors decrease LTP (Pavlidis et al. 1995; Pavlidis et al. 1996).

The hippocampus is an area where the neuronal population is continually replenished through neurogenesis (J. S. Snyder et al. 2009). Preclinical and clinical studies have shown that stress leads to reduced total hippocampal volume and atrophy and loss of neurons in this structure (Warner-Schmidt & Duman 2006). In particular, the rate of cell proliferation in the adult hippocampus is dramatically reduced by many different types of stressors, including predator odor (Galea et al. 2001), social stress (Czeh et al. 2001; Gould et al. 1997), acute and chronic restraint stress (Pham et al. 2003; Vollmayr et al. 2003; Rosenbrock et al. 2005), footshock stress (Malberg & Duman 2003; Vollmayr et al. 2003), and chronic mild stress (Alonso et al. 2004).

## **1.5 RATIONALE FOR PROPOSED EXPERIMENTS**

The hippocampal formation, and the vSub in particular, constitutes a central node in mediating the forebrain's stress response, particularly with psychogenic stressors (J P Herman & N K Mueller 2006; N K Mueller, C M Dolgas, et al. 2004). The vSub exerts an inhibitory influence over the hypothalamus, providing an important negative feedback to HPA activation in stress. In addition, the vSub plays an important role in controlling midbrain dopamine neuron population activity via its output to the NAc (Floresco et al. 2001; Floresco et al. 2003), providing a potential functional link between stress and dopaminergic pathophysiology of schizophrenia and drug abuse. However, it is not known how appropriate vSub activity is generated in response to acute and chronic stressful stimuli.

The vSub receives a major projection from two stress-related inputs, the LC and the BLA (Jones & R. Y. Moore 1977; Pikkarainen et al. 1999). As reviewed above, the noradrenergic



**Adapted from (Belujon & Anthony A Grace 2011).**

1. In Chapter 2, the effects of LC activation on the vSub are examined, and whether this activation is mediated via direct projections from the LC is evaluated. The response of vSub neurons to local application of NE and to electrical stimulation of the LC is tested using electrophysiological recordings in anesthetized rats. The mechanisms underlying changes in neuronal activity are further examined by pharmacological manipulations. Finally, to test whether the response to LC stimulation is mediated via the BLA, the response of vSub neurons to LC stimulation is tested after BLA inactivation.

2. In Chapter 3, the effects of BLA stimulation on vSub neurons is examined electrophysiologically. Moreover, the effect of BLA inactivation on spontaneous activity in the vSub is tested. Finally, the modulatory effect of LC input on BLA-evoked responses of the vSub is examined by paired stimulation of these two afferent regions.

3. Electrical footshock is known to phasically activate this circuit, and provides a way to elicit stress responses in anesthetized animals. Footshock also has a well-defined timecourse that allows for the examination of phasic responses of vSub neurons to stressful stimuli. In Chapter 4, the response of single vSub neurons to acute footshock and electrical LC stimulation is tested and the potential underlying noradrenergic mechanisms are examined. The relative contribution of the LC and the BLA is then assessed by testing the effect of inactivating these two structures on footshock responses.

4. A substantial body of literature has also established that the restraint stress model in rats causes long-lasting activation of these circuits. A single restraint session leads to increase in LC neuron activity (Abercrombie & Jacobs 1987b) and activates BLA and hippocampal neurons

(Chowdhury et al. 2000; Kovacs 1998; Reznikov et al. 2008). Importantly, this stressor also causes prolonged enhancement of responses to amphetamine in the NAc (Pacchioni et al. 2002; Pacchioni et al. 2007), which is an important downstream target of the vSub in regulating dopamine neuron activity. Indeed, inactivation of the vSub prevents restraint stress-induced activation of VTA dopamine DA neuron firing (Valenti et al. 2011). As reviewed above, the vSub controls the population activity of VTA DA neurons via outputs to the NAc. Thus, in contrast to footshock, restraint stress results in sustained activation of the system. In Chapter 5, we therefore examine the effect of restraint on NAc-projecting vSub neurons using a combination of anatomical tracing and immunolabeling for Fos, an immediate early gene (IEG) marker of neuronal activity.

## 1.6 HYPOTHESIS

We propose that the stressor-evoked NE signal generated by LC neurons is a critical element in initiating a stress response in the vSub. Specifically, we hypothesize that the vSub becomes hyperexcitable following stress-evoked NE release. As reviewed above, NE exerts complex effects on neurons in the dorsal hippocampus, where activation of synaptic alpha-1 receptors mediates a potent inhibition of spontaneous pyramidal neuron activity, while synaptic beta adrenoreceptors have been found to underlie a slow persistent activation (Curet & de Montigny 1988b; Dunwiddie et al. 1992). Furthermore, it has been hypothesized that postsynaptic alpha-2 receptors located at extrasynaptic sites mediate inhibitory effects on pyramidal neurons (Curet & de Montigny 1988a). We hypothesize that a similar distribution of receptors will be found in the vSub. Consequently, we expect to find both inhibitory and excitatory responses to LC

stimulation in this region. Stress causes a transient LC activation resulting in NE release in the vSub (Abercrombie & Jacobs 1987c), and we propose that the resulting activation of subicular adrenoceptors alters subsequent responses to NE in this region. This effect could be mediated by alterations in receptor number, changes in affinity of receptors for NE or changes in second messenger cascades mediated by adrenoceptors (Stone 1983). Indeed, acute presentation of various stressors has been reported to cause lasting changes in adrenoceptor expression. Thus, an increase in alpha-2 and decrease in beta receptor number has been reported following acute footshock in the cortex (Carter et al. 2004) and after acute restraint in the midbrain (U'Prichard DC & R. 1980).

Furthermore, we hypothesize that stress potentiates BLA afferent input to the vSub, thus increasing the excitability of this region by subsequent aversive stimuli. Norepinephrine is known to increase the evoked activity of neurons by potentiating afferent inputs in the somatosensory cortex (B D Waterhouse & Woodward 1980; B D Waterhouse, Moises & Woodward 1998a) as well as in the dorsal hippocampus (Segal et al. 1991). Additionally, norepinephrine is known to cause disinhibition of pyramidal cells by depressing excitatory input to interneurons in CA1 of the hippocampus. This has led to the idea that NE effectively acts as a “gating mechanism” (Devilbiss & B D Waterhouse 2000a) or “gain modulator” (G Aston-Jones & J. D. Cohen 2005), selecting functionally relevant inputs over spontaneous activity. We propose that prolonged stressful stimuli, such as restraint stress, effectively increase the gain of the aversive signal carried by BLA inputs to the vSub, via noradrenergic mechanisms.

## **2.0 ACTIVATION AND INHIBITION OF THE HIPPOCAMPAL VENTRAL SUBICULUM BY NOREPINEPHRINE AND LOCUS COERULEUS STIMULATION**

### **2.1 INTRODUCTION**

Stress is defined as any stimulus that disrupts an animal's ability to maintain homeostasis. The organism's stress response is a spectrum of neuroendocrine, cardiovascular, immune and autonomic changes precipitated by aversive stimuli or contexts (Kopin 1995). Recent advances have broadened our understanding of the stress response to include changes in brain function mediated by the central monoamine system, including norepinephrine (Sullivan et al. 1999; Joca et al. 2007; Y. Liu & S. Nakamura 2006). The ventral subiculum (vSub) of the hippocampal formation is a crucial element in the forebrain's stress response, particularly to psychogenic stressors (J P Herman & N K Mueller 2006; N K Mueller, C M Dolgas, et al. 2004), and is heavily innervated by noradrenergic neurons of the locus coeruleus (LC) (Oleskevich et al. 1989; Schroeter et al. 2000). The vSub is also implicated in processing of contextual information, which is significant in light of the fact that stress is a context-dependent phenomenon; i.e., the context in which the stressor is administered plays a major role in the adaptive response of the organism (Bouton & Bolles 1979). In addition, the vSub potently influences dopaminergic neuron activity (Floresco et al. 2001; Floresco et al. 2003), and prolonged stress is known to

trigger maladaptive responses to acute stress involving dopamine dysregulation, such as relapse in drug addiction, schizophrenia and depression.

Hippocampal neurons express 4 NE receptor subtypes: alpha-1 and -2 and beta-1 and -2 (Oleskevich et al. 1989; S. K. McCune et al. 1993; Pieribone et al. 1994; Nicholas et al. 1993). LC-mediated NE release has been reported to exert a complex set of effects on neurons in the dorsal hippocampus, causing inhibition via alpha-1 receptors, and activation via beta adrenoreceptors (Curet & de Montigny 1988b). Iontophoretic application of NE, on the other hand, has been found to cause primarily alpha-2 mediated inhibition in dorsal CA1 and CA3 (Curet & de Montigny 1988a). The vSub has one of the highest NE innervations in the brain (Schroeter et al. 2000; Oleskevich et al. 1989); however, the effect of NE on the vSub has not been examined.

The locus coeruleus-norepinephrine system is activated by a variety of stressors, including restraint, tailshock, footshock, hypotension, immune challenge, swim, water avoidance stress and social stress (Rita J Valentino & E. Van Bockstaele 2008). Furthermore, the behavioral effects of LC activation mimic the effects of acute stress exposure, and lesions of the LC block or attenuate neuroendocrine and behavioral stress responses (Ziegler et al. 1999). Given that noradrenergic neurons of the LC are potently activated by stressful stimuli, and that the vSub is one of the densest efferent targets of the LC, the way in which the vSub processes this input is critical to understanding its role in the brain's stress response. However, it is not known how vSub activity is generated in response to LC activation, and surprisingly few studies have addressed this question.

Here, the effects of LC activation on the vSub were examined, and whether this activation is mediated via direct projections from the LC was evaluated. The response of vSub

neurons to local application of NE and to electrical stimulation of the LC was also examined. Finally, to test whether the response to LC stimulation was mediated via the basolateral amygdala (BLA), the response of vSub neurons to LC stimulation was tested after BLA inactivation.

## 2.2 MATERIALS AND METHODS

*Surgery.* All procedures were performed in accordance with the United States Public Health Service *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Male Sprague Dawley rats (300-400 g) were housed 2 per cage with food and water available ad libidum. Rats were anesthetized with urethane (1.5 g/kg i.p.) and placed in a stereotaxic device (David Kopf Instruments, Tujunga, CA). The skull was exposed, and holes were drilled in the skull overlaying the vSub, the LC and/or the BLA. Coordinates (rostral from bregma; lateral from midline) were determined using a stereotaxic atlas (Paxinos and Watson, 1997): vSub: -6.0, 4.6; BLA: -3.5, 5.0; LC (see *LC stimulation* below).

*Extracellular single unit recording.* Recordings were performed using microelectrodes constructed from omegadot (WPI) borosilicate glass tubing using a microelectrode puller (Narishige, Tokyo, Japan) as previously described (Goto and Grace, 2006). Briefly, microelectrodes were filled with 2% pontamine sky blue dye dissolved in 2 M NaCl. The recording electrode impedance measured in situ ranged between 6 and 14 MOhms. Electrical potentials were amplified using an extracellular amplifier (Fintronics Inc., Orange, CT), and monitored on an oscilloscope. Data were fed to a PC and recorded using custom-designed

software (Neuroscope) with a data acquisition board interface (Microstar Laboratories, Bellevue, WA). Following recording, dye was ejected from the electrode by applying a constant negative current in order to mark the recording placement.

*Iontophoresis.* NE was applied by microiontophoresis onto vSub neurons using 5-barrel microelectrodes. One barrel was filled with a NE solution (0.5M NE, 100 mM NaCl, pH 4.0) , two barrels were filled with 3 M NaCl solution for current balancing, and one barrel was filled with a glutamate solution (10 mM Glu, 150 mM NaCl, , pH 8.0) to activate neurons with low spontaneous firing rates. NE was applied in a dose-response fashion (5, 10, 20, 40 nA) using current balancing and controlling for warm-up effects (Bloom 1974) as previously described (D M Buffalari & A A Grace 2007). The response was defined in terms of firing rate, and firing pattern.

*LC stimulation.* The LC (A: -12.6, M/L: +1.1, D/V: -6.0 from bregma, at 10°) was stimulated using current pulses (0.25 ms duration) delivered via a bipolar concentric electrode at current amplitudes between 500-800 mA.. Train stimulation (4 pulses at 20 Hz) was delivered to mimic bursts of spikes during phasically activated LC responses. Responses were characterized in terms of firing rate and firing pattern within 4 sec of LC stimulation, relative to a 4 sec pre-stimulation baseline. Stimulation pulses were delivered every 10 sec.

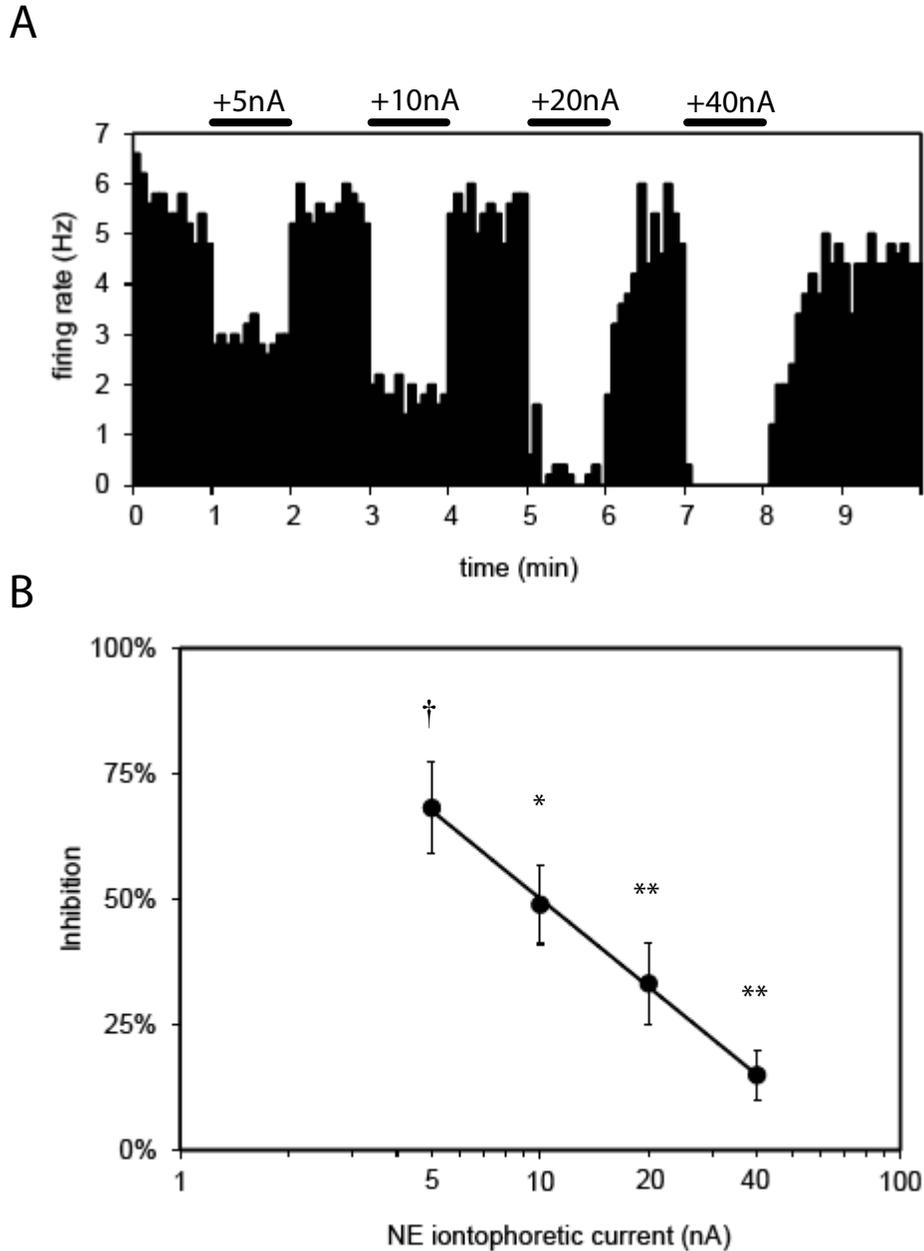
*Drug application.* Once the neuronal response was characterized, the alpha-2 antagonist idazoxan (1.0 mg/kg i.v.), alpha-1: antagonist prazosin (1 mg/kg i.v) or the beta antagonist propranolol (1.0 mg/kg i.v.) was administered (D M Buffalari & A A Grace 2007) via a lateral tail vein. Drugs were applied systemically in order to adequately block afferent-evoked input to distal dendrites, which cannot be reliably blocked by local iontophoretic application. Moreover,

it is not reliable to block iontophoretic agonist administration with iontophoretic antagonist, given the greater distribution of the more hydrophilic transmitter.

*BLA inactivation.* In a subset of experiments, a cannula guide (Plastics One, Roanoke, VA) was lowered into the BLA (from bregma: -3.5 caudal, 5.0 lateral) of urethane anesthetized rats. Once baseline responses to LC stimulation are established, the effect of BLA inactivation by TTX infusion was tested. A cannula was inserted into the guide, so that the tip of the cannula extended 1 mm past the tip of the cannula guide. 0.5  $\mu$ L of TTX (1.0  $\mu$ M) was infused over 30 seconds using a Hamilton syringe. Two minutes following the infusion, the response to LC stimulation was tested again.

## 2.3 RESULTS

*NE iontophoresis inhibits vSub neurons primarily via  $\alpha$ -2 receptors.* Spontaneously active neurons were isolated in the vSub, and baseline activity was recorded for at least one minute. For neurons with a spontaneous firing rate of less than 1 Hz, glutamate was applied iontophoretically by turning off the glutamate retention current. Subsequently, four doses (5 nA, 10 nA, 20 nA, and 40 nA) of NE were applied iontophoretically. In all neurons tested, NE dose-dependently inhibited spontaneous firing (N = 10; Figure 2.1). A two-way repeated ANOVA revealed a significant effect of NE on firing rate compared to baseline ( $p < 0.001$ ), but no main effect of NE dose ( $p = 0.163$ ). However, there was a significant interaction between treatment (NE vs. baseline) and NE dose ( $p < 0.001$ ).



**Figure 2.1** Iontophoretically applied NE dose-dependently inhibits the firing of vSub neurons.

**A.** A firing rate histogram showing a representative neuron showing dose-dependent inhibition by NE. NE ejection currents are indicated above the figure. **B.** Group data showing inhibition as a percentage of baseline firing. †  $p=0.065$ ; \*  $p<0.01$ , \*\*  $p<0.001$ .

In 5 additional neurons, after the response to 3 doses of NE (5 nA, 10 nA, and 20 nA) was recorded, the  $\alpha$ -2 receptor antagonist idazoxan was injected (1.0 mg/kg i.v.), and the response to NE was re-tested. Idazoxan eliminated the inhibitory effect of iontophoretic NE. Before drug application, NE significantly inhibited firing rate compared to baseline (two-way RM ANOVA,  $p < 0.05$ ); systemic idazoxan blocked this inhibition (two-way RM ANOVA / Holm-Sidak test,  $p < 0.05$ ; Figure 2.2A). In another 5 neurons, the effect of the  $\alpha$ -1 receptor antagonist prazosin (1.0 mg/kg i.v.) was tested on 3 doses of NE (5 nA, 10 nA, and 20 nA). Prazosin partially blocked the inhibitory effect of NE applied at 5 nA, at the lowest iontophoretic current of NE (two-way RM ANOVA / Holm-Sidak test,  $p < 0.05$ ; Figure 2.2B)

*LC stimulation produced excitatory and inhibitory effects on vSub neurons.* Spontaneously active vSub neurons were isolated using single barrel recording electrodes and responses to electrical stimulation of the LC were tested. Average z-score histograms were calculated for each neuron, based on at least 25 trials, and using 100 ms bins. Activation following train stimulation was defined as z-scores greater than 2.0 in at least 5 bins during 1.0 seconds following stimulation. Inhibition was defined as z scores below -2.0 in at least 5 bins during 1.0 seconds following stimulation. Of 32 vSub neurons tested, 16% showed inhibition within 1.0 second following LC burst stimulation (baseline FR =  $1.2 \pm 0.7$  Hz, LC simulation FR =  $4.1 \pm 2.3$  Hz;  $p < 0.05$ , paired t-test; Figure 2.3A); whereas 38% showed activation within this period (baseline FR =  $3.55 \pm 1.4$  Hz, LC simulation FR =  $9.98 \pm 3.3$  Hz;  $p < 0.05$ , paired t-test; Figure 2.3B). Both types of responses were long lasting, persisting for an average of  $0.8 \pm 0.3$  seconds after LC stimulation.

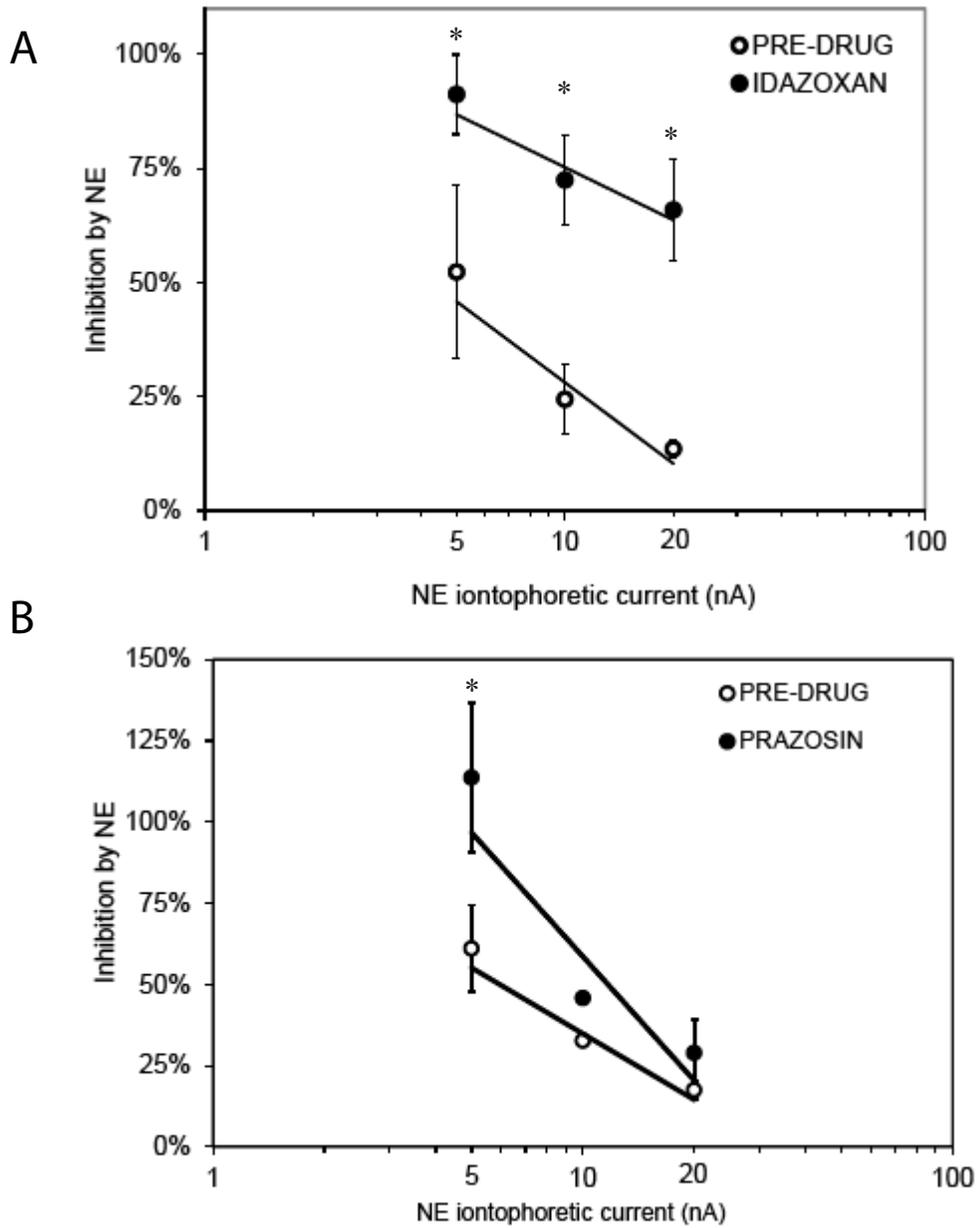


Figure 2.2 Pharmacology of NE-induced inhibition in the vSub.

NE-induced inhibition of vSub neuron firing is A. blocked by systemic alpha-2 antagonist idazoxan (1 mg/kg) at all currents, and B. partially attenuated by systemic alpha-1 antagonist prazosin (1 mg/kg) at the lowest iontophoretic current. (\* indicates difference between pre- and post-drug condition,  $p < 0.05$ )

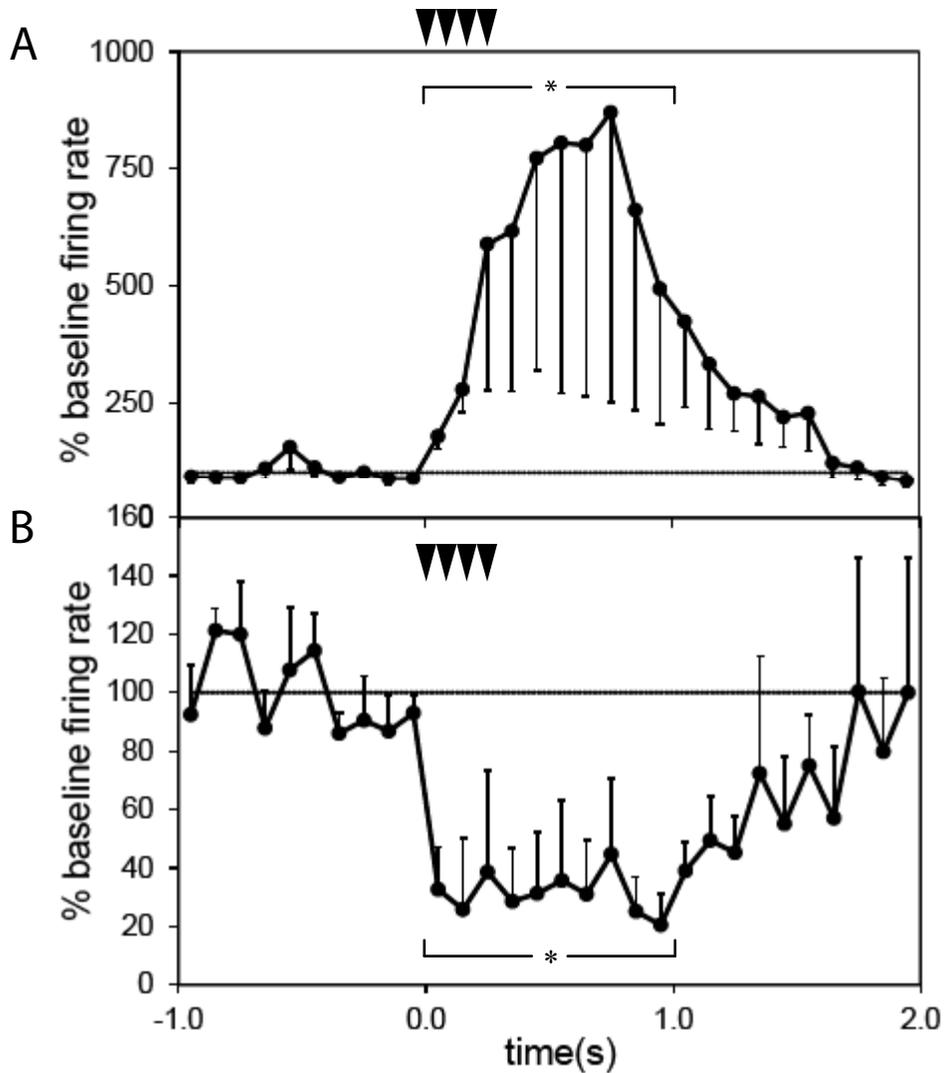
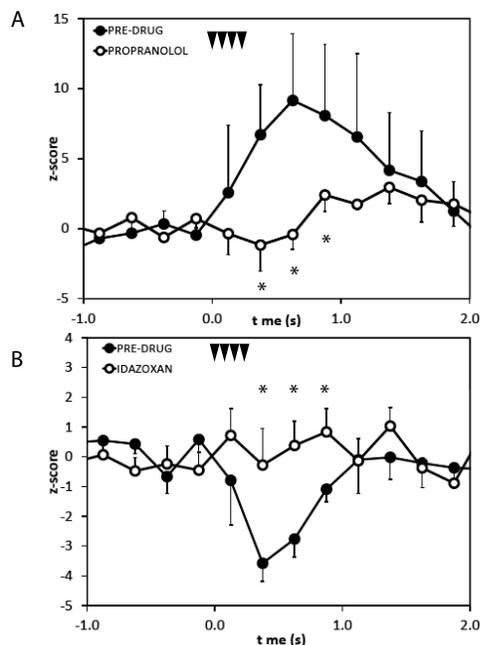


Figure 2.3 LC stimulation resulted in activation or inhibition of neurons in the vSub.

LC stimulation resulted in activation in 38% (A), and inhibition in 16% (B) of vSub neurons tested. Each plot shows the average normalized firing rate of the neurons in response to burst stimulation of the LC (delivered at  $t = 0$ ), represented as a percentage of the 10 second baseline. ( \* indicates difference between baseline and post-LC stimulation FR,  $p < 0.05$ )



**Figure 2.4 Pharmacology of LC stimulation-evoked responses in vSub neurons.**

**A. LC stimulation induced excitation is blocked by propranolol. B. LC stimulation induced inhibition is blocked by idazoxan. (\* indicates difference in FR between pre- and post-drug condition,  $p < 0.05$ )**

In 5 additional neurons activated by LC stimulation, the  $\beta$  receptor antagonist propranolol (1 mg/kg i.v.) was injected after the baseline response was established. Propranolol blocked the LC evoked activation (two-way RM ANOVA / Holm-Sidak test,  $p < 0.05$ ; Figure 2.4A). In another 4 neurons, the effect of  $\alpha$ -2 receptors antagonist idazoxan was tested on LC-evoked inhibition. Idazoxan blocked the inhibitory effect of LC stimulation. (two-way RM ANOVA / Holm-Sidak test,  $p < 0.05$ ; Figure 2.4B).

In addition to its direct projections to the vSub, LC stimulation also will affect neuron activity in BLA (Buffalari & Grace); a region that also innervates the vSub. In order to determine whether part of the response to LC stimulation was mediated via BLA-vSub afferents,

the response of vSub neurons to LC stimulation was tested before and after TTX infusion in the BLA. BLA inactivation did not affect activation of vSub neurons by LC stimulation (Figure 2.5).

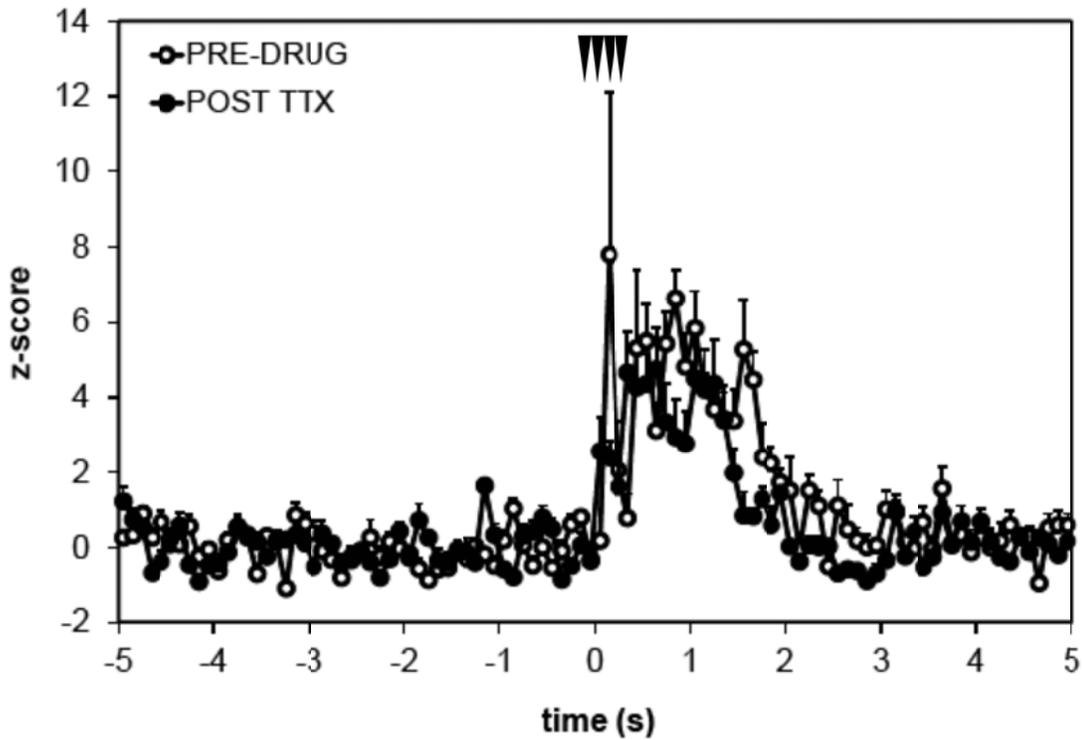


Figure 2.5 BLA inactivation does not block LC-induced excitation of vSub neurons.

## 2.4 DISCUSSION

We found that LC-stimulated NE release and direct application of NE into vSub resulted in different patterns of vSub neuronal activity. Direct NE application inhibited firing of all neurons examined, whereas LC stimulation inhibited some and activated other cells. We further characterized mechanisms underlying inhibition and activation by using pharmacological manipulations, and showed that they involve alpha-2 and beta adrenergic receptors, respectively.

The vSub is characterized as a central region involved in stress responses. Given the known involvement of the NE system in stress, the response of the vSub to direct and indirect NE stimulation was evaluated. Thus, the response of the vSub to LC stimulation can be mediated either by direct action of NE on vSub neurons, indirectly through another LC target such as the BLA, or possibly a combination of these mechanisms. In this manuscript, we compare the effects of direct LC stimulation with direct iontophoretic application of NE to vSub neurons. If the response of vSub neurons to LC stimulation is mediated by NE acting locally in the vSub, we would expect to observe correlated responses to iontophoretically applied NE and to LC stimulation.

Iontophoretic NE strictly inhibited the firing of vSub neurons, and this was mediated via the activation of alpha-2 receptors. Thus, the alpha-2 receptor antagonist idazoxan significantly reversed NE-mediated inhibition at all iontophoretic currents of NE tested, effectively shifting the dose-response curve to the right, as expected for a competitive antagonist. In addition, the alpha-1 receptor antagonist prazosin partially blocked the inhibition produced by iontophoretic NE, suggesting that alpha-1 receptors contribute relatively less compared to other adrenergic receptors to the inhibitory effect of NE. This result is consistent with the action of iontophoretic NE as reported in the dorsal hippocampus. In experiments examining the action of NE on dorsal CA1 and CA3 neurons, Curet and de Montigny found that NE potently suppresses neuron firing; and that idazoxan, and to a lesser extent prazosin, block this effect, at the same doses of the antagonists (1 mg/kg) (Curet & de Montigny 1988a).

Whereas most studies into the effects of alpha-2 receptors have addressed the presynaptic alpha-2 autoreceptor located on NE terminals that inhibits NE release (Arima et al. 1998), alpha-2 receptors have also been identified at postsynaptic sites in the central nervous

system (J. E. Freedman & Aghajanian 1985; U'Prichard et al. 1980; D M Buffalari & A A Grace 2007).

In contrast to iontophoretically applied NE, electrical stimulation of the LC resulted in inhibition of firing in 16 % of neurons and activation in 38 % . Both types of responses were apparently mediated by noradrenergic receptors, since administration of the alpha-2 antagonist idazoxan blocked the inhibitory responses and the beta antagonist propranolol blocked the excitatory responses. This confirms that the effect of LC stimulation on the vSub resulted from activation of NE neurons.

Studies of the effects of NE on CA3 pyramidal neurons of the dorsal hippocampus, (Curet & de Montigny 1988b) reported that electrical stimulation of the LC also inhibited neuronal firing. However, in contrast to the current results, administration of idazoxan increased the effectiveness of this inhibition, rather than blocked it. Although the reason for this difference is not readily apparent, there are several potential factors that could account for this difference. Thus, in our studies the LC was activated phasically with a burst of pulses, whereas Curet and de Montigny applied continuous 1 Hz stimulation to the LC, which would be expected to facilitate NE accumulation and presynaptic inhibition of NE release. Second, we recorded spontaneously active vSub neurons, whereas the CA3 neurons in the Curet and de Montigny study were activated by iontophoretic application of acetylcholine. Finally, it may be that the difference may be related to differences between the vSub and the dorsal hippocampus. The increase in inhibition caused by idazoxan in the dorsal hippocampus suggests that this effect is mediated by the blockade of pre-synaptic alpha-2 autoreceptors, which control the release of NE into the synaptic cleft (Arima et al. 1998). On the other hand, the reversal of inhibition by idazoxan in the vSub suggests that blockade of presynaptic inhibition of NE release may not play as

significant of a role. The fact that idazoxan blocked the inhibition caused by both iontophoretic NE as well as by LC stimulation in the vSub supports the conclusion that LC stimulation-induced inhibition is mediated by the action of NE released from LC terminals onto post-synaptic alpha-2 receptors.

The current study showed that beta adrenoreceptors are the primary mediators of excitatory responses to LC-evoked NE release in the vSub. Indeed, beta receptors are typically excitatory in nature and are positively linked to adenylyl cyclase and cyclic-AMP via activation of Gs proteins (Pfeuffer 1977; Ross et al. 1978). Moreover, NE stimulation of beta receptors has also been shown to mediate excitatory responses of hippocampal neurons to NE in the slice preparation (Dunwiddie et al. 1992) and of BLA neurons in vivo (D M Buffalari & A A Grace 2007). In the majority of neurons described in the Curet and de Montigny study, LC-evoked inhibition was followed by a period of activation (Curet & de Montigny 1988b). Similar to the LC-evoked activation of vSub neurons in our experiments, this activation of CA3 neurons was blocked by systemic application of propranolol. However, in the vSub, LC stimulation evoked inhibition or activation in separate populations of neurons. This suggests that, in the vSub, post-synaptic alpha-2 and beta receptors may be differentially distributed among separate neural populations.

In contrast to LC stimulation-induced inhibition, LC stimulation-induced activation was not mimicked by iontophoretic NE in the vSub. This indicates that the activation results from NE acting on beta adrenoreceptors located outside the vSub, or along the distal portion of vSub neurons where beta receptors would be inaccessible to iontophoretically applied NE. While the subcellular localization of adrenergic receptors has not been studied in the vSub, beta receptors

have been observed primarily on the dendrites of granule cells in the dentate gyrus (Milner et al. 2000).

The BLA receives a strong LC projection, and is known to be activated during stress (Cullinan et al. 1995; Rosen et al. 1998; Dayas et al. 2001; Chowdhury et al. 2000; Akirav et al. 2001). In addition, the vSub is activated by BLA inputs. In order to evaluate whether part of the excitatory response of the vSub to LC stimulation occurred via the BLA, the BLA was inactivated by TTX microinfusion. BLA inactivation did not block the increase in firing caused by LC stimulation, suggesting that this response is mediated by  $\beta$  receptors outside of the BLA.

The modulation of vSub activity by NE has important implications for understanding central responses to stressors and how stress can influence regulation of the dopamine system. As reviewed above, the context-dependent vSub activity drives the activation of dopamine neuron populations in the VTA via a projection involving the NAc – VP – VTA circuit. Since stress-induced LC activation causes NE release in the vSub as well as other forebrain regions, knowledge of the downstream impact of this release is critical to our understanding of this system. Our findings suggest that stress-induced LC activation can cause inhibition of some vSub neurons, and activation of others. This shift may represent an adaptive response between contextual representations, with alpha-2 receptor-mediated inhibition suppressing ongoing hippocampal activity, and beta receptor stimulation providing a context-selective activation of a subset of the network. Such a broad inhibition overlaid with a selective activation may contribute to the adaptive nature of the response to stressors.

### **3.0 LC STIMULATION MODULATES BASOLATERAL AMYGDALA-EVOKED ACTIVATION OF THE HIPPOCAMPAL VENTRAL SUBICULUM**

#### **3.1 INTRODUCTION**

The ventral subiculum (vSub) of the hippocampal formation is a crucial element in the forebrain's response to stress, in particular to psychogenic stressors (J P Herman & N K Mueller 2006; N K Mueller, C M Dolgas, et al. 2004), and it is heavily innervated by noradrenergic neurons of the locus coeruleus (LC) (Oleskevich et al. 1989; Schroeter et al. 2000). LC neurons are activated by a variety of stressors (Ceccatelli et al. 1989; M. A. Pezzone et al. 1993; Passerin et al. 2000; Rasmussen & Jacobs 1986; Abercrombie & Jacobs 1987a; Grant et al. 1988; G Aston-Jones et al. 1991). This activation plays a major role in the neuroendocrine and behavioral responses to stress (Ziegler et al. 1999).

The LC also projects to the basolateral nucleus of the amygdala (BLA), another region critically implicated in the stress response. Activation of LC inputs has been shown to cause primarily inhibitory responses in BLA neurons (D M Buffalari & A A Grace 2007; F. J. Chen & Sara 2007); however, chronic cold stress increases excitatory actions of the LC on the BLA (Deanne M Buffalari & Anthony A Grace 2009). The BLA, in turn, sends projections to the vSub (Pikkarainen et al. 1999), which suggests that some of the responses of the vSub to stress may be mediated through this input.

In this study, the effects of BLA stimulation on vSub neurons is examined. Moreover, the effect of BLA inactivation on spontaneous activity in the vSub is tested. Finally, the modulatory effect of LC input on BLA-evoked responses of the vSub is examined by paired stimulation of these two afferent regions.

### 3.2 MATERIALS AND METHODS

*Surgery.* All procedures were performed in accordance with the United States Public Health Service *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Male Sprague Dawley rats (300-400 g) were housed 2 per cage with food and water available ad libidum. Rats were anesthetized with urethane (1.5 g/kg i.p.) and placed in a stereotaxic device (David Kopf Instruments, Tujunga, CA). The skull was exposed, and holes were drilled in the skull overlaying the vSub, the LC and the BLA. Coordinates (rostral from bregma; lateral from midline) were determined using a stereotaxic atlas (Paxinos and Watson, 1997): vSub: -6.0, 4.6; BLA: -3.5, 5.0; LC (see *LC stimulation* below).

*Extracellular single unit recording.* Recordings were performed using microelectrodes constructed from Omegadot (WPI) borosilicate glass tubing using a microelectrode puller (Narishige, Tokyo, Japan) as previously described (D M Buffalari & A A Grace 2007). Briefly, microelectrodes were filled with 2% pontamine sky blue dye dissolved in 2 M NaCl. The recording electrode impedance measured in situ ranged between 6 and 14 MOhms. Electrical potentials were amplified using an extracellular amplifier (Fintronics Inc., Orange, CT), and monitored on an oscilloscope. Data were fed to a PC and recorded using

custom-designed software (Neuroscope) with a data acquisition board interface (Microstar Laboratories, Bellevue, WA). Following recording, dye was ejected from the electrode by applying a constant negative current in order to mark the recording placement.

*BLA stimulation.* Bipolar, concentric stimulating electrodes were lowered into the BLA. Recordings did not begin until a minimum of 30 minutes after lowering of the stimulating electrodes. vSub neurons responsive to BLA stimulation were isolated using a search-stimulate protocol: Single-pulse stimuli (50-800uA, 0.25ms, 0.1 Hz) were delivered to the BLA while the recording electrode was lowered through the vSub to identify responsive neurons. Neurons were characterized as having presumed orthodromic monosynaptic, orthodromic polysynaptic, or antidromic responses to EC stimulation. Responses were defined as orthodromic if they had an onset latency of < 20ms, showed a failure to substantially change response latency to increases in current intensity, their onset latency remained fairly consistent with approximately 1-5ms of inter-trial variability in evoked spike latency, and they follow paired pulses at 50Hz but not 400Hz. After a neuron was identified as having a presumed orthodromic monosynaptic response, the stimulation intensity was adjusted so that the evoked probability was close to 50%, and a baseline BLA-evoked firing probability was established using at least 50 trials.

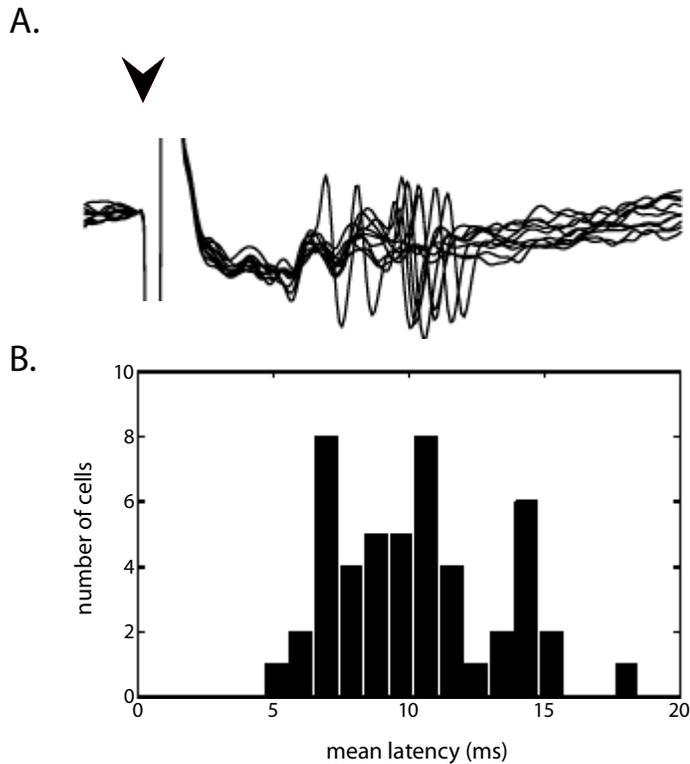
*LC stimulation.* The LC (A: -12.6, M/L: +1.1, D/V: -6.0 from bregma, at 10°) was stimulated using current pulses (0.25 ms duration) delivered via a bipolar concentric electrode at current amplitudes between 500-800 uA. Train stimulation (4 pulses at 20 Hz) was delivered to mimic bursts of spikes during phasically activated LC responses. After baseline BLA-evoked firing probability was established in BLA neurons, stimulation bursts were delivered preceding BLA stimulation at varying intervals (0.25, 0.5, 1.0, 2.0 sec), every 10 sec. Each LC pre-stimulation interval was tested sequentially, with at least 50 trials for each.

*BLA inactivation.* In another set of experiments, a guide cannula (Plastics One, Roanoke, VA) was lowered into the BLA (from bregma: -3.5 caudal, 5.0 lateral) of urethane anesthetized rats (N=8). The effect of BLA inactivation by TTX infusion was tested. A cannula was inserted into the guide, so that the tip of the cannula extended 1 mm past the tip of the cannula guide. Spontaneously active vSub neurons were isolated using extracellular recording (described above). After at least 1 minute baseline recording, 0.5  $\mu$ L of TTX (1.0  $\mu$ M) was infused over 30 seconds using a Hamilton syringe into the BLA, and the recording was continued for at least 2 minutes.

*Statistical analysis.* Paired t-test was used to calculate differences between two conditions in the BLA inactivation experiment. One-way ANOVA / Holm-Sidak test was used to determine differences in evoked probability between baseline and LC pre-stimulation conditions.

### 3.3 RESULTS

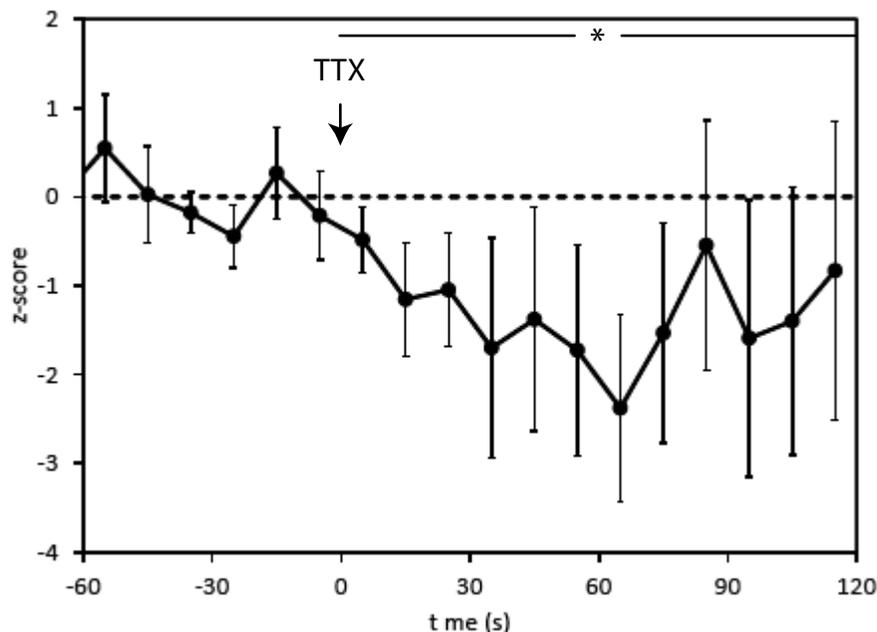
*Stimulation of the BLA resulted in activation of vSub neurons.* The activity of vSub neurons was recorded extracellularly while electrically stimulating the BLA. BLA stimulation resulted in evoked action potential spikes at short latencies (N = 48;  $10.2 \pm 2.9$  ms) in vSub neurons (Figure 3.1B). The latency of evoked spikes was characterized by inter-trial variability characteristic of an orthodromic monosynaptic response (Figure 3.1A).



**Figure 3.1 A. BLA simulation activates vSub neurons.**

**Overlay of 10 traces showing short-latency spikes evoked by BLA stimulation in a representative vSub neuron. B. Histogram showing the distribution of average spike latencies evoked by BLA stimulation in 48 vSub neurons.**

*BLA inactivation decreased basal firing in vSub neurons.* In order to assess the contribution of BLA activity to the basal activation state of vSub neurons, the effect of BLA inactivation with sodium channel blocker TTX on spontaneous firing in the vSub was examined. After at least 1 minute baseline recording, TTX was infused into the BLA and the recording was continued for at least 2 minutes. BLA inactivation suppressed spontaneous firing of vSub neurons (Figure 3.2; baseline FR =  $5.1 \pm 1.2$ ; post-TTX FR =  $1.4 \pm 0.3$ ;  $p = 0.03$ , paired t-test).



**Figure 3.2** Effect of BLA inactivation on spontaneous firing of vSub neurons.

Infusion of TTX into the BLA (arrow) resulted in a suppression of firing in the vSub. (\* indicates significant change from baseline,  $p=0.03$ , t-test)

*LC pre-stimulation modulated BLA-evoked firing of vSub neurons.* Burst stimulation was delivered to the LC at varying intervals (0.25, 0.5, 1.0, 2.0 sec) before BLA stimulation, in order to examine the time course of LC effects. BLA-evoked responses were potentiated in 5 neurons (Figure 3.3A;  $p < 0.05$ , one way RM ANOVA, Holm-Sidak test) and suppressed in 6 neurons (Figure 3.3B;  $p = 0.01$ , one way RM ANOVA, Holm-Sidak test). The potentiation of BLA-evoked firing was significant at the 0.25 and 0.5 sec time points after LC stimulation, while suppression occurred at 0.25, 0.5 and 1.0 sec after LC stimulation. BLA-evoked firing measured 120 seconds following the LC pre-stimulation protocol (recovery) were not significantly different from pre-stimulation baseline.

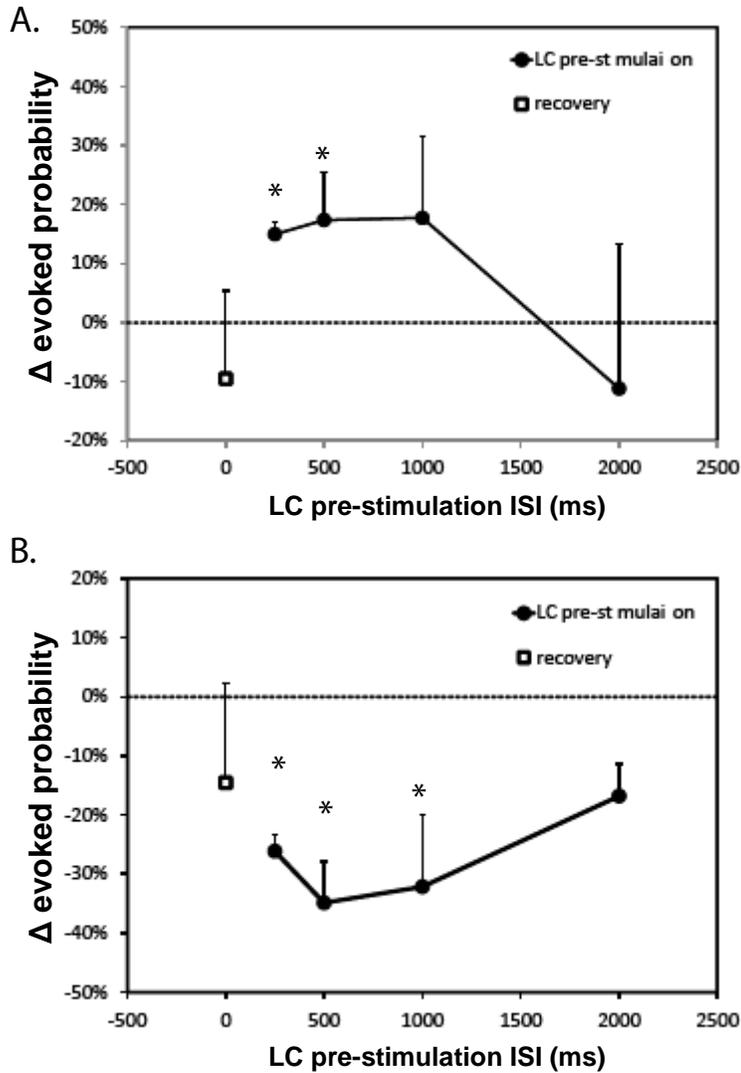


Figure 3.3 BLA-evoked firing of vSub neurons is modulated by LC pre-stimulation.

LC modulation of BLA-evoked firing was measured by the change in evoked firing probability from pre-stimulation baseline. LC burst stimulation was delivered at varying intervals (0.25, 0.5, 1.0, 2.0 secs) before BLA stimulation. A. In 5 neurons, BLA-evoked firing was potentiated by NE pre-stimulation. B. In 6 neurons, NE pre-stimulation resulted in depression of BLA-evoked firing. BLA-evoked firing tested after the LC pre-stimulation protocol (recovery) did not show change from baseline. (\* indicates significant change from baseline ( $p < 0.05$ , one-way RM ANOVA, Holm-Sidak test))

### 3.4 DISCUSSION

The BLA is known to play a major role in mediating the response to stressors (J. LeDoux 2003) and is known to project directly to the vSub, suggesting that this input may be important in conveying stress signals to the subiculum. We found that BLA stimulation effectively drives neurons in the vSub, in a manner consistent with monosynaptic orthodromic responses. Furthermore, the BLA appears to exert a tonic excitatory influence over the vSub, since BLA inactivation suppressed vSub spontaneous firing.

Given the importance of the LC and the BLA in stress responses, the interaction of LC inputs to BLA-evoked drive of the vSub was tested. Pre-stimulation of the LC potentiated BLA-evoked responses in approximately 45% percent of vSub neurons and suppressed responses in 55% percent of neurons. This is consistent with previous studies showing the role of NE in the modulation of neuronal evoked activity in other regions. Thus, a number of studies document the function of NE in either increasing or decreasing evoked activity in the same neurons (Foote et al. 1975; Segal et al. 1991; B D Waterhouse & Woodward 1980; B D Waterhouse et al. 1984; B D Waterhouse, Moises & Woodward 1998b; B D Waterhouse et al. 2000; Doze et al. 1991; Devilbiss & B D Waterhouse 2000b). In the BLA, NE primarily suppresses responses evoked by stimulation of cortical inputs; however chronic cold stress results in NE-mediated potentiation of these responses (Deanne M Buffalari & Anthony A Grace 2009).

Modulation of BLA inputs to the vSub by the LC may be mediated through the action of NE on adrenergic receptors. Beta adrenergic receptors are known to be expressed in the hippocampus (Nicholas et al. 1993; Hillman, Doze, et al. 2005; Hillman, Knudson, et al. 2005), and their post-synaptic effect is typically excitatory (Pfeuffer 1977; Ross et al. 1978). In the dentate gyrus they are thought to be primarily expressed on the dendrites of granule cells

(Milner et al. 2000), which is the principal glutamatergic neuron in this hippocampal subfield. While adrenergic receptor subtypes have not been characterized in the vSub, a similar dendritic expression pattern would also allow beta receptors to potentiate synaptic inputs to vSub neurons, in a manner similar to that which was observed with BLA afferents. On the other hand, LC stimulation-evoked suppression of BLA responses may be mediated by pre- or postsynaptic alpha-2 receptors, which have been reported to mediate inhibitory responses to LC stimulation in the dorsal hippocampus (Curet & de Montigny 1988b). Another possibility is that the BLA input is suppressed by GABAergic interneurons in the vSub. Indeed, a subset of hippocampal inhibitory interneurons express alpha-1 receptors, the activation of which depolarizes these cells, thereby increasing inhibition of their targets (Pieribone et al. 1994; Papay et al. 2006; Bergles et al. 1996). Further research is needed to examine the pharmacological mechanisms of LC actions on BLA-evoked inputs in the vSub, in order to understand how this input functions during stress.

Taken together, these findings suggest that the BLA provides a tonic excitatory influence over the vSub, and furthermore may be involved in activating the vSub during stress. Moreover, this input is modulated by the LC. Because the LC is also known to exert actions on the BLA, the net effect of these inputs during stress appears to be complex, and may depend on the type of stressor, history of chronic stress, as well as the context under which it is administered. Thus, under basal conditions, stress-induced activation of the LC would suppress BLA-vSub evoked responses, potentially mediating a homeostatically suppressed response to novel situations. In contrast, if the stressor is maintained, the system is set to provide a co-ordinated potentiation of vSub drive that may relate to the learned context of the event.

## **4.0 NORADRENERGIC INPUT FROM THE LOCUS COERULEUS MEDIATES ACTIVATION AND INHIBITION OF THE HIPPOCAMPAL VENTRAL SUBICULUM BY FOOTSHOCK**

### **4.1 INTRODUCTION**

Stress is defined as any stimulus that disrupts an animal's ability to maintain homeostasis. The organism's stress response is a spectrum of neuroendocrine, cardiovascular, immune and autonomic changes precipitated by aversive stimuli or contexts (Kopin 1995). Recent advances have broadened our understanding of the stress response to include changes in brain function mediated by the central monoamine system, including norepinephrine (Sullivan et al. 1999; Joca et al. 2007; Y. Liu & S. Nakamura 2006).

The locus coeruleus-norepinephrine system is activated by a variety of stressors, including restraint, tailshock, footshock, hypotension, immune challenge, swim, water avoidance stress and social stress (Rita J Valentino & E. Van Bockstaele 2008). Furthermore, the behavioral effects of LC activation mimic the effects of acute stress exposure, and lesions of the LC block or attenuate neuroendocrine and behavioral stress responses (Ziegler et al. 1999). Given that noradrenergic neurons of the LC are potently activated by stressful stimuli, that the vSub is one of the densest efferent regions of the LC, and that the vSub is considered to be a central component in the stress response (J P Herman & N K Mueller 2006), the way in which

the vSub processes NE input is critical to understanding its role in the brain's stress response. However, it is not known how vSub activity is altered in response to LC activation, and surprisingly few studies have addressed this question.

The ventral subiculum (vSub) of the hippocampal formation is a crucial element in the forebrain's stress response, particularly with psychogenic stressors (J P Herman & N K Mueller 2006; N K Mueller, C M Dolgas, et al. 2004), and is heavily innervated by noradrenergic neurons of the locus coeruleus (LC) (Oleskevich et al. 1989; Schroeter et al. 2000). The vSub is also implicated in processing of contextual information, which is significant since stress is a context-dependent phenomenon; i.e., the context in which the stressor is administered plays a major role in the adaptive response of the organism (Bouton & Bolles 1979). In addition, the vSub potently influences dopaminergic neuron activity (Floresco et al. 2001; Floresco et al. 2003), and prolonged stress is known to trigger maladaptive responses to acute stress involving dopamine dysregulation, such as relapse in drug addiction, schizophrenia and depression.

In this experiment, neural pathways involved in this response were investigated. First, the response of single vSub neurons to acute footshock and electrical LC stimulation was tested and the potential underlying noradrenergic mechanisms were examined. Given that the LC projects to the basolateral amygdala (BLA) a stress-reactive structure that also innervates the vSub (Pitkänen et al. 2000), the relative contribution of the LC and the BLA was then assessed by testing the effect of inactivating these two structures on footshock responses.

## 4.2 MATERIALS AND METHODS

*Surgery.* All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Male Sprague Dawley rats (300-400 g) were housed 2 per cage with food and water available ad libidum. Rats were anesthetized with urethane i.p. (1.5 g/kg) and placed in a stereotaxic device (David Kopf Instruments, Tujunga, CA). The skull was exposed, and holes were drilled in the skull overlaying the vSub, the LC and/or the BLA. Coordinates (rostral from bregma; lateral from midline) were determined using a stereotaxic atlas (Paxinos and Watson, 1997): vSub: -6.0, 4.6; BLA: -3.5, 5.0; LC (see *LC stimulation* below).

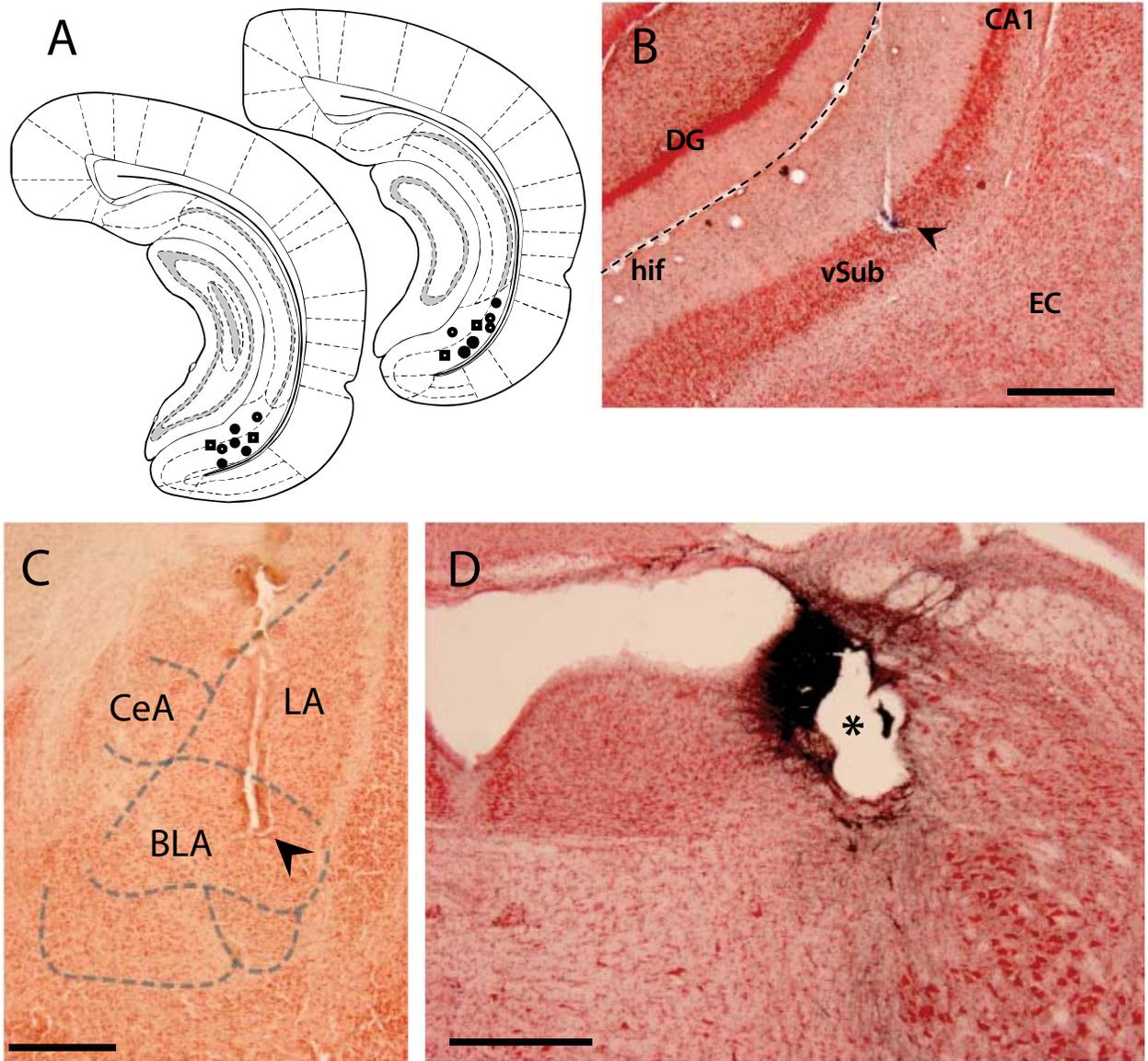
*Extracellular single unit recording.* Recording was performed using microelectrodes constructed from borosilicate glass tubing using a vertical microelectrode puller (Narishige PE-2, Tokyo, Japan) as previously described (Goto and Grace 2006). Briefly, microelectrodes were filled with 2% pontamine sky blue dye dissolved in 2 M NaCl. The recording electrode impedance measured in situ ranged between 6 and 14 MOhms. Electric potentials were amplified using an extracellular amplifier (Fintronics Inc., Orange, CT), and monitored on an oscilloscope. Data were fed to a PC and recorded using custom-designed software (Neuroscope) with a data acquisition board interface (Microstar Laboratories, Bellevue, WA). Following recording, dye was ejected from the electrode by applying a constant negative current in order to mark the recording placement.

*Effects of footshock on vSub neuron activity.* The sciatic nerve was activated indirectly using constant current, 250  $\mu$ sec duration pulses delivered at 0.2 Hz by two 26-gauge needles inserted into the medial aspect of the contralateral hind paw (Mana & A A Grace 1997).

*LC stimulation.* The LC (A: -12.6, M/L: +1.1, D/V: -6.0 from bregma, at 10°) was stimulated using current pulses (0.25 ms duration) delivered via a bipolar concentric electrode at current amplitudes between 500-800 uA. Train stimulation (4 pulses at 20 Hz) was delivered to mimic bursts of spikes during phasically activated LC responses. Responses were characterized in terms of firing rate and firing pattern within 4 sec of LC stimulation, relative to a 4 sec pre-stimulation baseline. Stimulation pulses were delivered every 10 sec.

*LC inactivation.* In order to determine if LC activity is required for footshock-evoked responses in the vSub, LC neuron firing was suppressed by local infusion of the alpha-2 adrenergic agonist clonidine (4.8 µg/0.5µl) into the LC, at doses effective at reducing hippocampal NE levels as measured by microdialysis (R. D. Mair et al. 2005). A guide cannula (Plastics One, Roanoke, VA) was lowered into the LC (A: -12.6, M/L: +1.1, D/V: -6.0 from bregma, at 10°), and spontaneously active neurons were isolated in the vSub. A cannula was inserted into the guide, so that the tip of the cannula extended 1 mm past the tip of the guide cannula. After baseline responses to footshock were established, clonidine was infused over 30 seconds using a Hamilton syringe. . Two minutes following the infusion, the response to footshock stimulation was again tested.

*BLA inactivation.* In a subset of experiments, a guide cannula (Plastics One, Roanoke, VA) was lowered into the BLA (from bregma: -3.5 caudal, 5.0 lateral) of urethane anesthetized rats. Once baseline responses to footshock stimulation were established, the effect of BLA inactivation by TTX infusion was tested. A cannula was inserted into the guide, so that the tip of the cannula extended 1 mm past the tip of the guide cannula. 0.5 µL of TTX (1.0 µM) was infused over 30 seconds using a Hamilton syringe. Two minutes following the infusion, the response to footshock stimulation was again tested.



**Figure 4.1** Histological confirmation of electrode placements.

**A.** Recording electrode placements within the vSub. ● marks locations of neurons activated by LC stimulation and footshock; ○ marks locations of neurons inhibited by LC stimulation and footshock; □ marks the location of neurons net showing a response to these stimuli. **B.** Representative example of a dye deposit (arrowhead) marking recording electrode placement within the vSub in a Nissl-stained section. **C.** Example of cannula placement (arrowhead) within the BLA in a Nissl-stained section. **D.** Electrode placement (\*) within the LC in an anti-dopamine beta-hydroxylase (DBH) immunolabeled and Nissl-stained section; blue-black BDH staining of noradrenergic neurons indicates location of the LC. CeA – central nucleus of the

amygdala; LA – lateral nucleus of the amygdala; DG – dentate gyrus of the hippocampus; hif – hippocampal fissure; EC – entorhinal cortex. Scale bar = 0.5 mm.

### 4.3 RESULTS

*Effects of footshock on vSub neuron activity in anesthetized rats.* Spontaneously active neurons were isolated in the vSub of urethane anesthetized rats. After a stable baseline was recorded, the response to at least 5 trials of footshock was tested. Neurons were categorized based on their firing rate (FR) during the 5 second footshock relative to baseline FR. Of 38 neurons tested, 18 (48%) were activated (footshock-activated (FA); baseline FR =  $3.1 \pm 0.7$  Hz, footshock FR  $7.0 \pm 0.9$  Hz,  $p < 0.001$ , t-test), 5 (13%) were inhibited (footshock-inhibited (FI); baseline FR =  $6.9 \pm 2.3$  Hz, footshock FR =  $2.8 \pm 1.7$  Hz,  $p = 0.008$ , t-test), while 15 (39%) did not show a significant change in firing (baseline FR =  $1.8 \pm 0.4$  Hz, footshock FR =  $1.9 \pm 0.4$  Hz,  $p = 0.326$ ) to footshock (Figure 4.2).

*Footshock response was correlated with response to LC stimulation.* In a subset of the neurons tested for response to footshock, response to electrical stimulation of the LC was also examined. The response of every neuron to each stimulus was represented as a single measure, calculated as a z-score based on the neurons' average firing rate during the 5 second footshock, and during one second beginning at the start of LC stimulation, relative to each pre-stimulus baseline. When the responses of the vSub neurons to footshock were regressed against responses to LC stimulation, a significant positive correlation emerged (Figure 4.3;  $N = 33$ , constant coefficient =  $4.0 \pm 1.9$ ; slope coefficient =  $1.9 \pm 0.4$ ;  $R^2 = 0.39$ ,  $p < 0.001$ ). Thus, FI

neurons tended also to be inhibited by LC stimulation; and, conversely, FA neurons tended to be activated by LC stimulation.

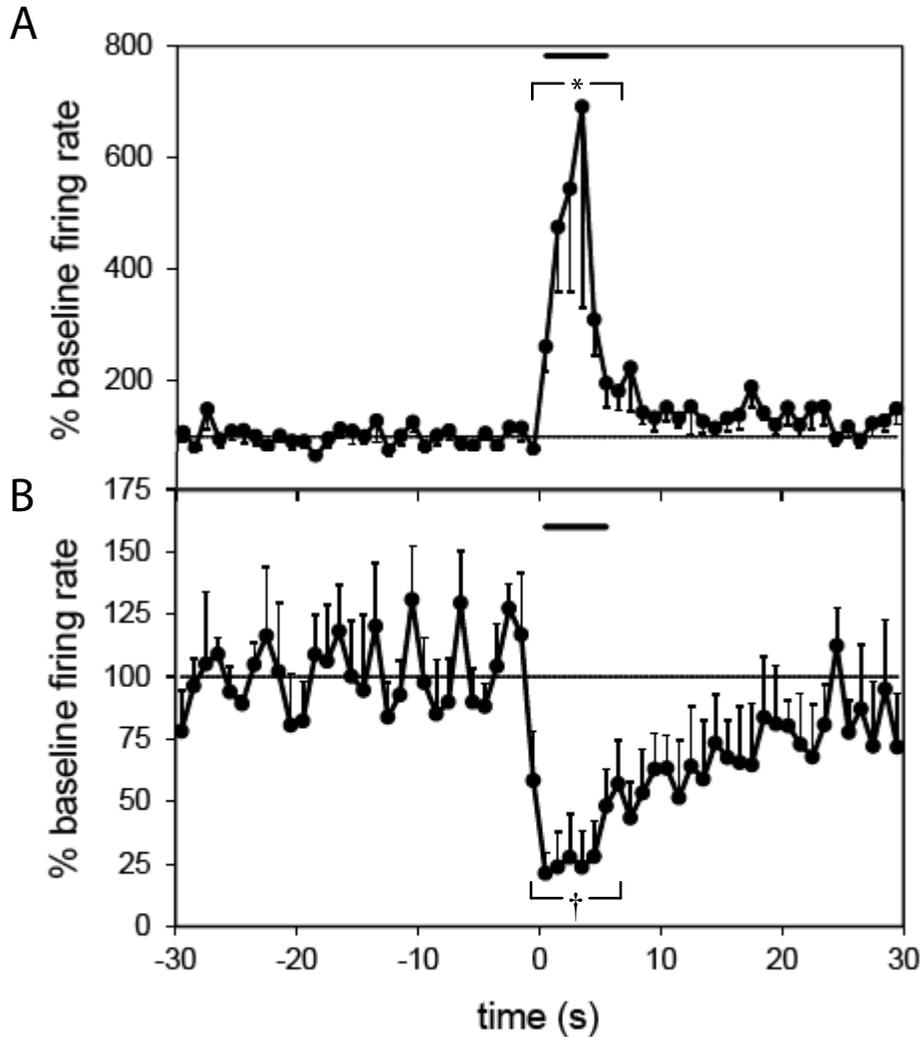
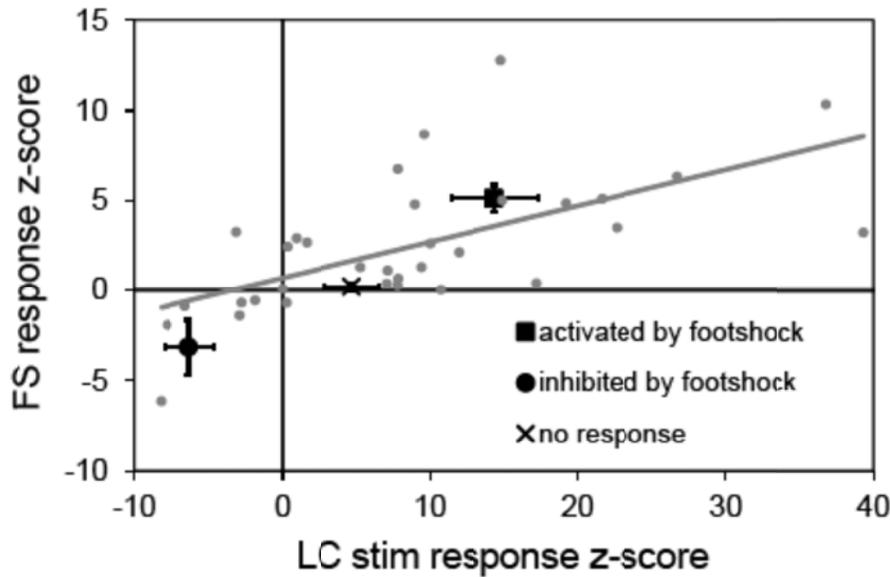


Figure 4.2 Footshock resulted in activation or inhibition of neurons in the vSub.

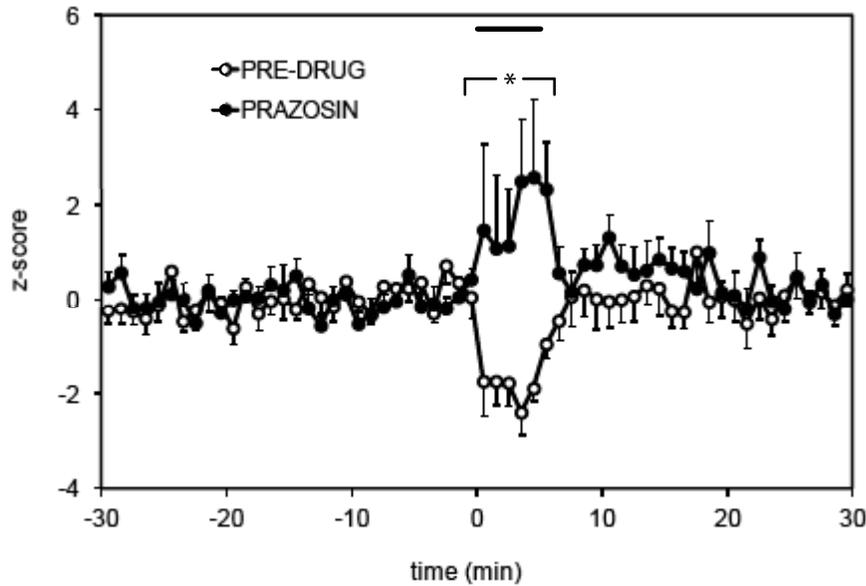
Footshock stimulation resulted in activation in 48% (A), and inhibition in 13% (B) of vSub neurons tested. Each plot shows the average firing rate of the neurons in response to a 5 second footshock (delivered at  $t = 0$ ), represented as a percentage of the 30 second baseline. (\* indicates significant difference ( $p < 0.001$ ) between baseline FR and footshock FR in FA neurons; † indicates significant difference ( $p = 0.008$ ) between baseline FR and footshock FR in FI neurons.)



**Figure 4.3 Responses to LC stimulation and to footshock are correlated.**

The response to footshock was correlated with the response to LC stimulation in vSub neurons tested with both stimuli. Response to each stimulus is represented as z-score based on the firing rate of the neuron during the 5 second footshock, and 1 second beginning at the start of LC stimulation, relative to baseline.

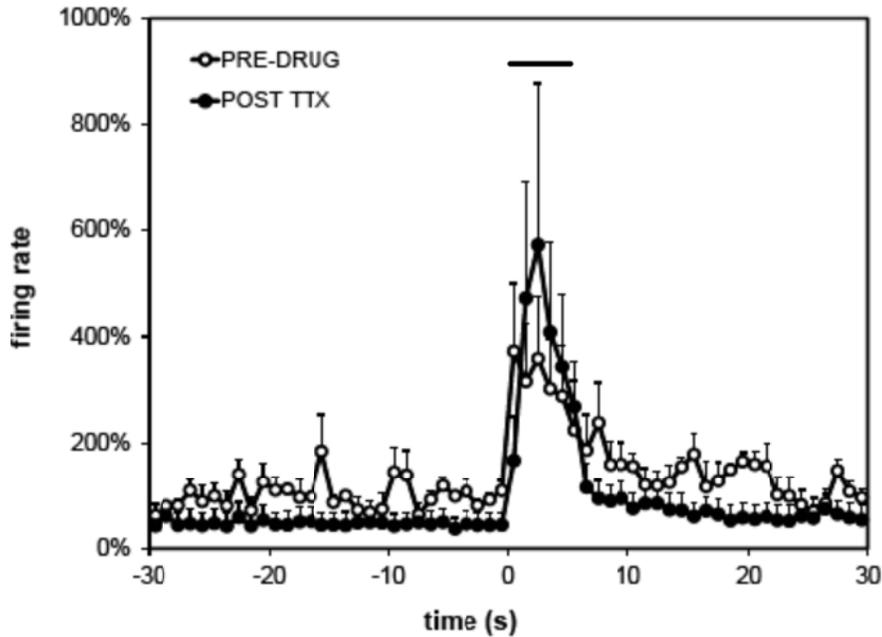
*Alpha-1 receptor blockade reversed the inhibitory responses to footshock.* In a separate population of 10 neurons inhibited by footshock, the effect of the alpha-1 noradrenergic antagonist prazosin on footshock-induced inhibition was examined. After establishing a pre-drug response, 1 mg/kg prazosin was injected i.v. and the response to footshock was re-tested. Prazosin reversed the footshock-induced inhibition, revealing instead an activating response (Figure 4.4). A two way repeated measure ANOVA revealed a significant effect of prazosin versus the pre-drug condition ( $p=0.0497$ ), and the Holm-Sidak post-hoc analysis showed a statistically significant difference in the footshock response between prazosin and the pre-drug baseline ( $p=0.006$ ).



**Figure 4.4 Footshock-evoked inhibition is alpha-1 receptor-dependent.**

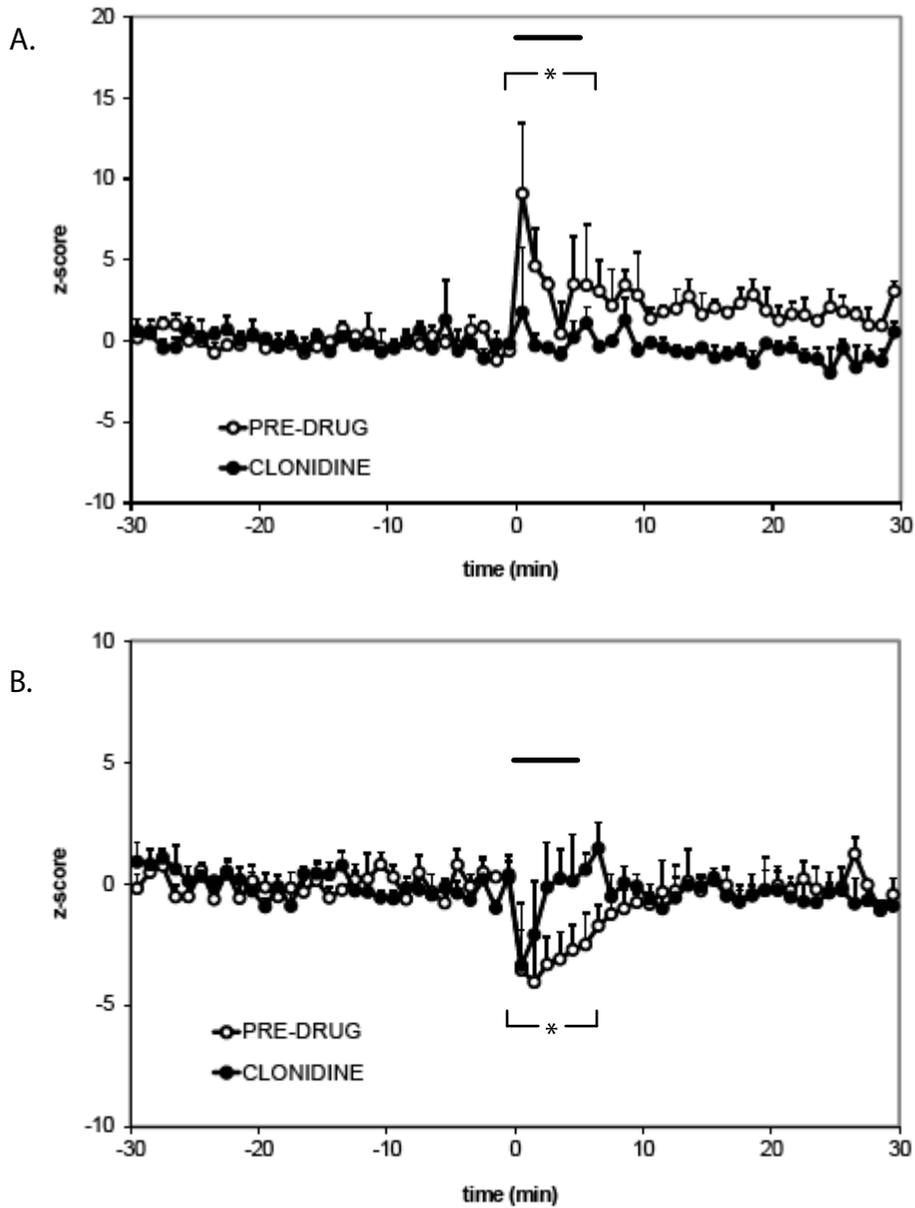
Systemic application of alpha-1 noradrenergic receptor antagonist prazosin (1 mg/kg) reversed the inhibitory effect of footshock, revealing an underlying footshock-evoked excitation. Response is expressed as z-score relative to the 30 second baseline. 5 second footshock was delivered at  $t = 0$ . (\* indicates significant difference in firing rate following prazosin injection (two-way RM ANOVA, Holm-Sidak test,  $p=0.006$ ), compared with the pre-drug condition.)

*BLA activity is not required for footshock-evoked activation of vSub neurons.* The BLA is activated by footshock and is capable of driving vSub neurons (as shown in Chapter 3 of this document). In order to examine the possibility that the footshock-evoked activation observed in the vSub was mediated via the BLA, we tested the response to footshock before and after TTX infusion into the BLA. BLA infusion did not significantly affect the activation of vSub neurons by footshock (Figure 4.5). A two way repeated measure ANOVA showed no effect of TTX infusion ( $p = 0.130$ ).



**Figure 4.5** Inactivation of the BLA with TTX did not block footshock-induced activation of vSub neurons.

*Footshock-evoked responses are mediated via the LC.* In order to determine if LC activity is required for footshock-evoked responses in the vSub, LC neuron firing was suppressed by cannula infusion of the alpha-2 adrenergic agonist clonidine into the LC. Footshock responses were tested before and after clonidine infusion. In neurons activated by footshock before clonidine infusion, LC inactivation blocked footshock-evoked responses (Figure 4.6A; N=5; Two-way repeated ANOVA, Holm-Sidak test, significant difference in footshock response between TTX and pre-drug baseline,  $p < 0.05$ ). On the other hand, in neurons inhibited by footshock before clonidine infusion, LC inactivation resulted in partially suppressed footshock-evoked responses (Figure 4.6B; N=5; Two-way repeated ANOVA, Holm-Sidak test, significant difference in footshock response between TTX and pre-drug baseline,  $p < 0.05$ ).



**Figure 4.6 Footshock-evoked responses in vSub neurons require LC activity.**

Inactivation of the LC by clonidine infusion suppressed footshock-induced activation (A) and partially suppressed inhibition (B) in two separate populations of vSub neurons. (\* indicates difference between pre-drug baseline and clonidine conditions,  $p < 0.05$ )

#### 4.4 DISCUSSION

The ventral subiculum is an important forebrain structure in regulating the behavioral response of an animal to environmental challenges (J P Herman & N K Mueller 2006; O'Mara et al. 2001), and contains one of the densest NE innervations of the brain (Oleskevich et al. 1989). It is also known to be a key modulator of the dopamine signal in the VTA via its output via the NAc – VP –VTA circuit (Floresco et al. 2001; Floresco et al. 2003). As such, the vSub presents an intriguing neural substrate for the link between stress and the dopaminergic pathophysiology of disorders such as drug abuse and schizophrenia. However, while neuronal responses to stressful stimuli in other subfields of the hippocampal formation have been examined experimentally, the effect of stressors on the vSub has remained poorly understood. In this manuscript, we examined the responses of single vSub neurons to footshock, and the relative contribution of the LC and the BLA in mediating these responses.

*Footshock responses in the vSub are regulated by alpha-1 adrenergic receptors.*

Footshock resulted in the activation of 48% (footshock-activated; FA), and inhibited the firing of 13% (footshock-inhibited; FI) of spontaneously active neurons in the vSub. Blockade of alpha-1 noradrenergic receptors with systemic prazosin injection reversed the inhibitory responses in FI neurons, revealing an underlying footshock-induced activation. This result suggests that FA and FI neurons may not represent static populations, but rather that the state of alpha-1 activation may determine the type of response a neuron shows to footshock.

Alpha-1 receptors are generally known to increase neuronal excitability via the Gq/11 class of G proteins and activation phospholipase C beta, which leads to IP3-mediated Ca<sup>++</sup> release from intracellular stores, as well as activation of PKC (Wozniak M et al. 2000). mRNAs of alpha-1A/D subtype are expressed throughout the hippocampal formation including

the dentate gyrus, Ammon's horn, and the subiculum (S. K. McCune et al. 1993; Pieribone et al. 1994), particularly on interneurons (Hillman et al. 2007). It is noteworthy that somatostatin-expressing interneurons in area CA1 that also express alpha-1 receptors (Hillman, Knudson, et al. 2005; Hillman, Doze, et al. 2005) correspond to a population of GABAergic interneurons targeting both the basal and apical dendrites of pyramidal cells (Somogyi & Klausberger 2005). While it is not known whether the same interneuron type exists in the subiculum (O'Mara et al. 2001), footshock-induced inhibition of pyramidal neurons in the vSub may be mediated by alpha-1 receptors located on GABAergic interneurons. By increasing firing of the interneurons, alpha-1 activation would, in turn, suppress pyramidal neuron activity.

*Footshock responses in the vSub are mediated by NE release from the LC.* It is well-established that neurons in the LC are activated by footshock, and that footshock results in NE release in the hippocampus, measured by microdialysis (Hajós-Korcsok et al. 2003). In this study, we demonstrate that LC activity is required for the full expression of footshock-evoked responses in both FA and FI neurons in the vSub. Thus, LC inactivation by clonidine infusion blocked footshock-evoked activation in FA neurons, and partially blocked footshock-evoked inhibition in FI neurons.

## **5.0 RESTRAINT STRESS ACTIVATES C-FOS IN NUCLEUS ACCUMBENS-PROJECTING NEURONS OF THE HIPPOCAMPAL VENTRAL SUBICULUM**

### **5.1 INTRODUCTION**

Stressful stimuli elicit many adaptive brain responses; however, stress can also trigger pathological responses, particularly in the dopamine system. Stressful events can lead to relapse in drug addiction, and can precipitate symptoms in schizophrenia patients, conditions that are thought to involve dopaminergic pathophysiology. It is thus of interest to study how stress impacts the physiology of the dopamine system.

The ventral subiculum (vSub) is a region that is believed to mediate the central effects of stress. Thus, it is a potent feedback modulator of the HPA axis (J P Herman et al. 1995), and lesions of this region lead to elevated neuroendocrine responses to stress (J P Herman et al. 1995; J P Herman et al. 1998; Nancy K Mueller, C Mark Dolgas, et al. 2004). Accordingly, stress has also been shown to activate hippocampal circuits. In rats, stress increases extracellular concentration of excitatory amino acids in the hippocampus (Moghaddam 1993; Lowy et al. 1995). In addition, multiple stressors, including restraint, swim stress as well as novelty stress have been found to increase the hippocampal expression of Fos, an immediate early gene marker of neuronal activity (Emmert & J P Herman 1999; Figueiredo et al. 2003)

Previous studies from our laboratory and others (Otake et al. 2002), have shown that, in rats, restraint stress activates neurons in the vSub.

While the dopamine system plays a critical role in reward-related behaviors, it is also activated by stressful stimuli (Imperato et al. 1993; Piazza & Le Moal 1998). In rats, restraint stress has been shown to increase the population activity (i.e. the number of dopamine neurons firing spontaneously) of dopamine neurons in the ventral tegmental area (VTA) (Valenti et al. 2011). Importantly, inactivation of the vSub abolished this effect. The vSub is known to regulate the population activity of dopamine neurons in VTA through a vSub-NAc-ventral pallidal-VTA circuit (Floresco et al. 2001; Floresco et al. 2003). This suggests that NAc-projecting neurons in the vSub are activated by stress.

Immediate early genes, such as c-fos, are important elements in intracellular signal transduction. They encode transcription factors that help mediate neural responses to extracellular stimuli. Fos, a protein encoded by c-fos, is present in most neurons at relatively low levels, but its expression can be induced dramatically by a variety of physiological and pharmacological stimuli. In particular stressful experiences are known to markedly activate c-fos expression. C-fos mapping has thus been proposed as a useful tool for characterizing trans-synaptically activated neurons in different regions of the brain (Morgan and Curran 1991, Sagar et al 1988, Lillrank et al. 1996).

In this study, we investigated whether NAc-projecting vSub neurons are activated by restraint stress using a combination of retrograde tracing and Fos immunocytochemistry. First, retrograde tracer Fluorogold (FG) was used to label vSub neurons projecting to the NAc. Then, rats were subjected to acute restraint stress, and the brain tissue was immunolabeled for FG and Fos. In order to determine if NAc-projecting vSub neurons were activated by restraint

stress, the number of FG/Fos double-labeled neurons in the vSub was quantified. Our results indicate that restraint stress leads to an increase in the number of double-labeled neurons in the vSub. Thus, stress-induced activation of the vSub can impact dopamine cell activity in the VTA via outputs to the NAc.

## 5.2 MATERIALS AND METHODS

*Animals.* Experimental protocols were approved by the University of Pittsburgh Institutional Animal Care and Use Committee, and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Adult male Sprague–Dawley rats (300–350 g BW; Harlan Laboratories, Indianapolis, IN, USA) were individually housed in stainless steel cages in a controlled environment (20–22 °C, 12 hour light-dark cycle; lights off at 1900 h) with *ad libitum* access to water and chow (Purina 5001 Bethlehem, PA, USA).

*Iontophoretic tracer delivery.* Rats were anesthetized by isoflurane inhalation (Halocarbon Laboratories, River Edge, NJ; 1–3% in oxygen) and oriented into a Kopf stereotax in the flat skull position. An incision was made in the scalp, and a small hole was drilled bilaterally in the skull to expose the cortical surface overlying the NAc. Using negative pressure, a pulled glass micropipette (approximately 20  $\mu\text{m}$  outer tip diameter) was backfilled with a 1–2% solution of FluoroGold (FG; Fluorochrome; Denver, CO) diluted in 0.1 M cacodylic acid. The micropipette was attached to the arm of the stereotax and lowered into the caudomedial NAc using stereotaxic coordinates (from bregma: +1.5 anterior,  $\pm$ 1.0 lateral, -6.0 ventral). A microwire connected to a current source (Stoelting) and lowered into the tracer solution was used to deliver a 0.5  $\mu\text{A}$  retaining current to minimize molecular diffusion of tracer from the pipette

tip as it was lowered into the brain. FG was iontophoresed into the NAc using a 7 s pulsed positive current of 5  $\mu$ A for a duration of 5 min (FG) (Bienkowski & Linda Rinaman, 2011). The iontophoresis was performed bilaterally in all animals except for one Restraint rat and one Control rat, in order to assess the uptake of tracer into the contralateral hemisphere. The micropipette tip was left in place for 5 min after iontophoresis, and then was withdrawn. The skin over the skull was closed with stainless steel clips and rats were injected with 1 mg of analgesic (Ketofen; 0.5 ml, s.c.). Rats were returned to their home cage after regaining consciousness and full mobility.

*Restraint stress exposure.* After a 7–14 day post-surgery survival time, 6 FG-injected rats were exposed to 30 min restraint stress (Restraint rats) in a clear plastic cylindrical tube (Kent Scientific Corporation, Torrington, CT, USA), and 5 FG-injected rats served as unrestrained controls (Control rats). Restraint rats were placed individually into a polyethylene cage with fresh bedding, transported to an adjacent room, put into the restrainer, and then left undisturbed in the restrainer within the transport cage for 30 min. At the end of restraint, rats were removed from the restrainer and returned to their home cage for an additional 60 min. Rats were then deeply anesthetized and perfused with fixative (see *Perfusion and histology*) This timing of perfusion results in maximal stress-induced neural Fos expression, which generally peaks 60 min after treatment-induced neural stimulation (Kovacs 1998). CONTROL rats were not handled for at least 24 hrs before before perfusion and were perfused either before or after restraint procedures and perfusions were complete. Restraints and perfusions were always performed during the light phase of the rats' diurnal cycle.

*Perfusion and histology.* Rats were anesthetized with sodium pentobarbital (Nembutal, Hospira, Inc., Lake Forest, IL, USA; 100 mg/kg, i.p.). Rats were then perfused transcardially with 50 mL of saline followed by 250 mL of fixative (4% paraformaldehyde in 0.1 M phosphate buffer with L-lysine and sodium metaperiodate) (McLean & Nakane 1974). Rats were decapitated and brains were post fixed in situ overnight at 4 °C, and then removed from the skull and cryoprotected in 20% sucrose solution for 24–72 h. Coronal 35 µm-thick tissue sections were cut using a freezing microtome, and sections were collected sequentially into 6 adjacent sets and stored in cryoprotectant.

*Fos Immunocytochemistry.* One set of sections from each rat was removed from storage and rinsed in buffer (0.1 M sodium phosphate, pH 7.4). Tissue sections were initially processed for immunoperoxidase localization of Fos protein using a rabbit polyclonal antiserum (1:50,000; provided by Dr. Philip Larsen, Denmark), diluted in buffer containing 0.3% Triton-X100 and 1% normal donkey serum. The specificity of this antibody for Fos has been reported (Rinaman et al. 1997). After rinsing, sections were processed with Vectastain Elite ABC reagents (Vector Laboratories, Burlingame, CA, USA) and reacted with nickel sulfate-intensified diaminobenzidine (DAB) to generate a blue-black reaction product in the nuclei of Fos-positive cells.

*FG Immunocytochemistry.* Following Fos immunocytochemistry, sections were rinsed in buffer and incubated overnight in primary antiserum to localize the FG tracer, rabbit anti-FG (1:30,000; Chemicon International), diluted in buffer containing 0.3% Triton X100 and 1% normal donkey serum. After rinsing, sections were incubated in biotinylated secondary donkey anti-goat or anti-rabbit IgG (1:500) and Vectastatin ABC Elite reagents followed by a

non-intensified DAB-hydrogen peroxide reaction to produce brown immunoprecipitate localizing the neural tracer delivery site and retrogradely-labeled neurons. Immunostained tissue sections were then rinsed in buffer, mounted onto Superfrost Plus microscope slides (Fisher Scientific), allowed to dry overnight, dehydrated and defatted in graded ethanols and xylene, and coverslipped using Cytoseal 60 (VWR).

*Quantification of FG and Fos labeling within the vSub.* A neuron was counted as FG-positive if it contained brown cytoplasmic immunoreactivity and had a visible nucleus. A neuron was considered Fos-positive if it contained blue-black nuclear immunolabeling, regardless of intensity. The criteria for double-labeled neurons included both the presence of brown cytoplasmic immunoreactivity, and blue-black nuclear labeling. The total number of double labeled neurons was quantified in sections immunolabeled for Fos and FG, in Restraint and Control cases. In each experimental case, all sections containing FG-positive neurons within the ventral cornu Ammonis and ventral subiculum (rostro-caudal level -4.2 to -7.4, relative to bregma) were analyzed bilaterally (region of interest, ROI). There were between 12 and 15 sections in total, per case. In one representative Restraint, and one representative Control case, all Fos-positive and all FG-positive neurons were counted.

*Statistical analysis.* A two way ANOVA was used to assess differences in the number of double labeled neurons between groups, with rat status (Restraint vs. Control) and the hemisphere (left vs. right) as independent variables. Post-hoc testing was done using the Holm-Sidak method.

### 5.3 RESULTS

*Iontophoretic FG injection sites.* FG was successfully iontophoresed in all 10 rats. Iontophoresis sites produced spherical tracer deposit sites that were localized in the caudo-medial portion of the NAc, medial to the anterior commissure, and ventral to the lateral ventricle (Figure 5.1A). Most tracer delivery sites encompassed parts of the NAc shell, and there was none or minimal FG labeling in the adjacent medial and lateral septum. The tracer deposits were distributed rosto-caudally from +2.0 mm to +1.0 mm, from bregma.

*Distribution of FG labeled neurons in the hippocampal formation.* Retrograde labeling was observed almost exclusively within the vSub the adjacent ventral portion of the CA1 within the hippocampal formation; the entorhinal and piriform cortices were also moderately labelled. The brown FG immunoreaction product labelled the somata, as well as large apical dendrites of neurons in the pyramidal cell layer in the most densely labeled portions of the vSub (Figure 5.2). In cases where iontophoretic tracer deposits were made in the more caudal portion of the NAc, the densest retrograde labeling occurred in the distal portion of the vSub, with little or no labeling in the CA1 (Figure 5.1B). On the other hand, more rostral FG deposits led to retrograde transport that was more sparsely distributed between the proximal vSub and CA1 (Figure 5.1C). Cases in which the tracer was delivered unilaterally (N=2), little to no FG labeling was observed in the contralateral hippocampal formation. Additionally, no differences in FG labeling were observed between cases where FG iontophoretic deposits were concentrated in the shell and in the core of the NAc.

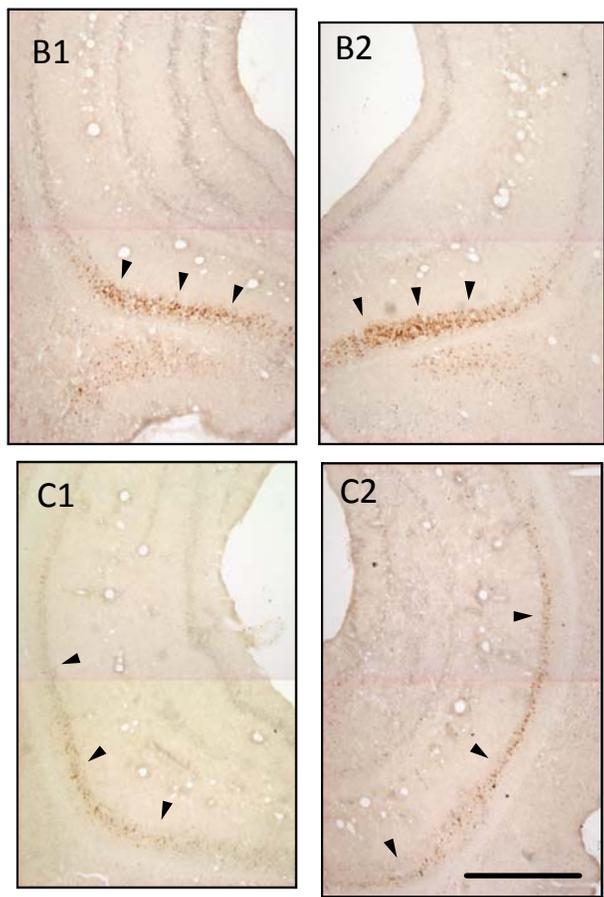
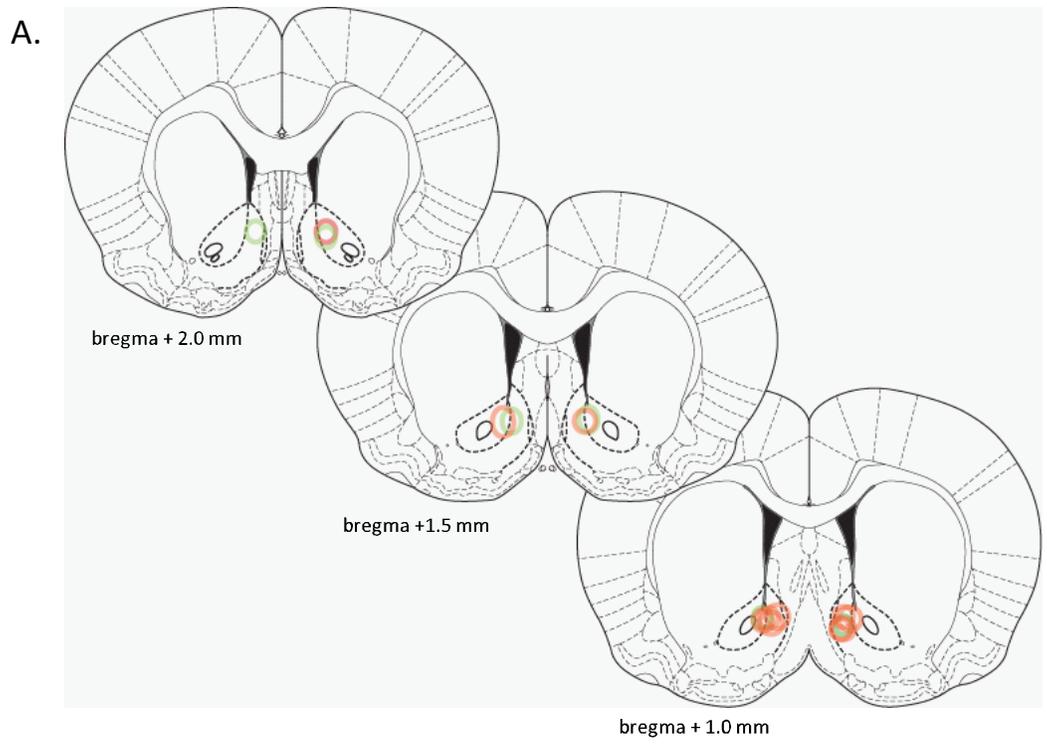


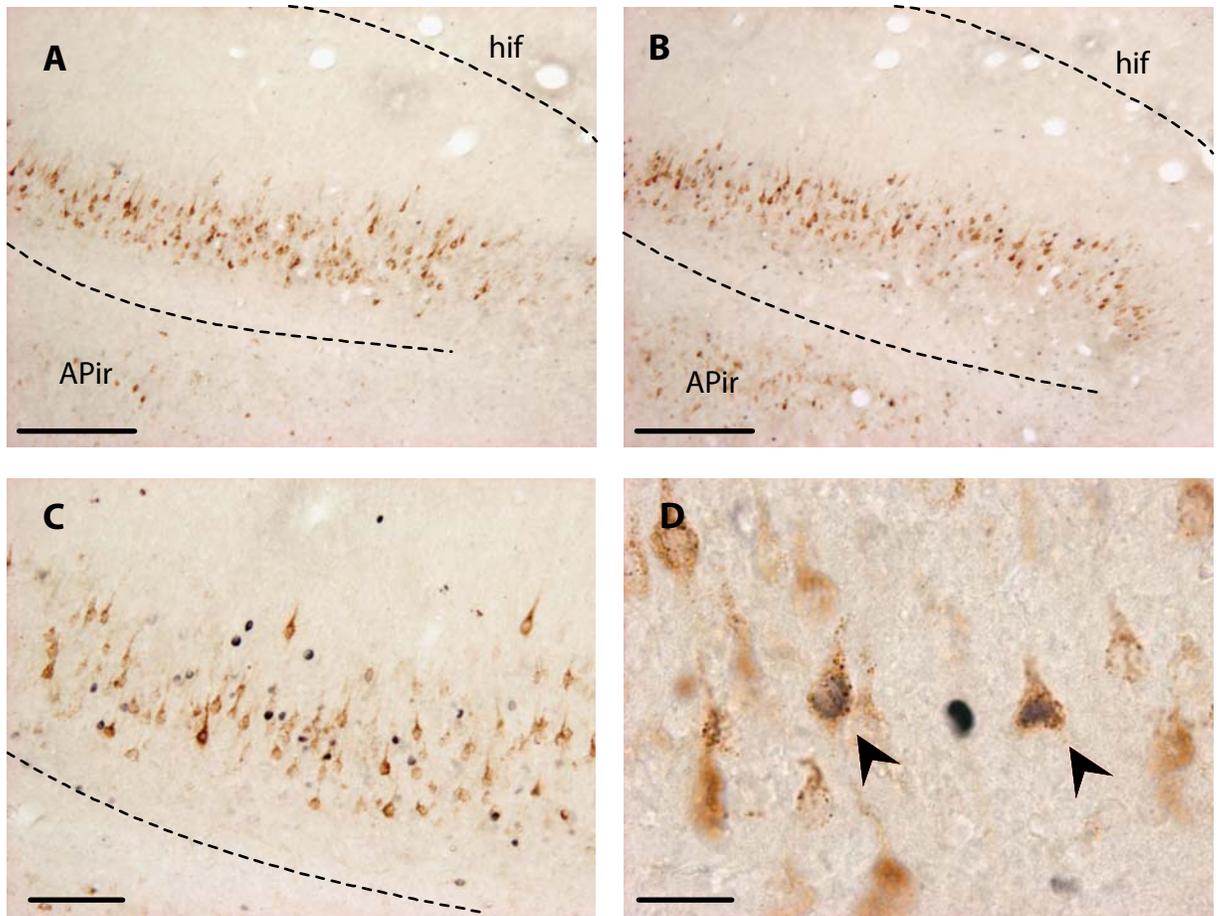
Figure 5.1 Placement of iontophoretically-delivered FG deposits in the NAc.

(Figure 5.1 caption continued from the page before) Circles represent the extent of FG deposits in Control animals (green), and Restraint animals (red). B, C. Example of the distribution of retrogradely labeled neurons in the left (1) and right (2) ventral hippocampal formation corresponding to cases where FG was iontophoresed in the caudal (B, +1.0 from bregma) and rostral (C, +2.0 from bregma) portions of the NAc. Arrowheads indicate the dense concentration of FG immunolabeling in the distal portion of the vSub in B, and a more sparse distribution of FG immunolabeling in the proximal vSub and the CA1 in C. Scale bar = 1 mm.

*Restraint stress induces Fos expression in NAc-projecting neurons in the vSub.*

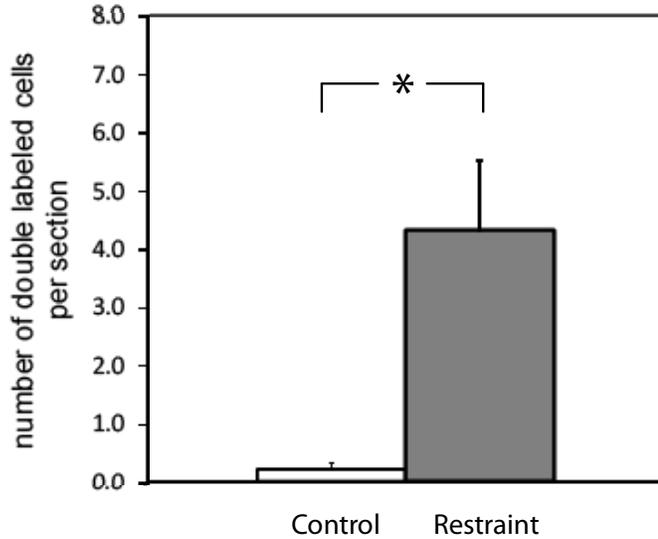
The number of double-labeled neurons within the ROI was significantly increased in Restraint cases, compared to non-restrained Controls (Figure 5.3). A two-way ANOVA revealed a difference between experimental groups (Restraint  $4.3 \pm 1.2$ ; Control  $0.2 \pm 0.1$  double labeled cells per section,  $p < 0.01$ ) but no significant difference between the left and right hemispheres. FG / Fos double-labeled neurons were most densely concentrated in the middle portion of the vSub, approximately -6.0 mm from bregma (Figure 5.4).

In order to estimate the proportion of NAc-projecting neurons expressing Fos, and the proportion of Fos-expressing neurons projecting to the NAc, the total number of Fos-positive and FG-positive neurons was counted within the ROI in one representative Control case and one representative Restraint case. In the Restraint case, 1100 were Fos-positive, 2776 were FG-positive, and 102 were double labeled, accounting for 9.3% of Fos-positive and 3.7% of FG positive neurons. In the Control case, 119 were Fos-positive, 1500 were FG-positive, and 9 were double labeled, accounting for 7.6% of Fos-positive and 0.6% of FG-positive neurons.



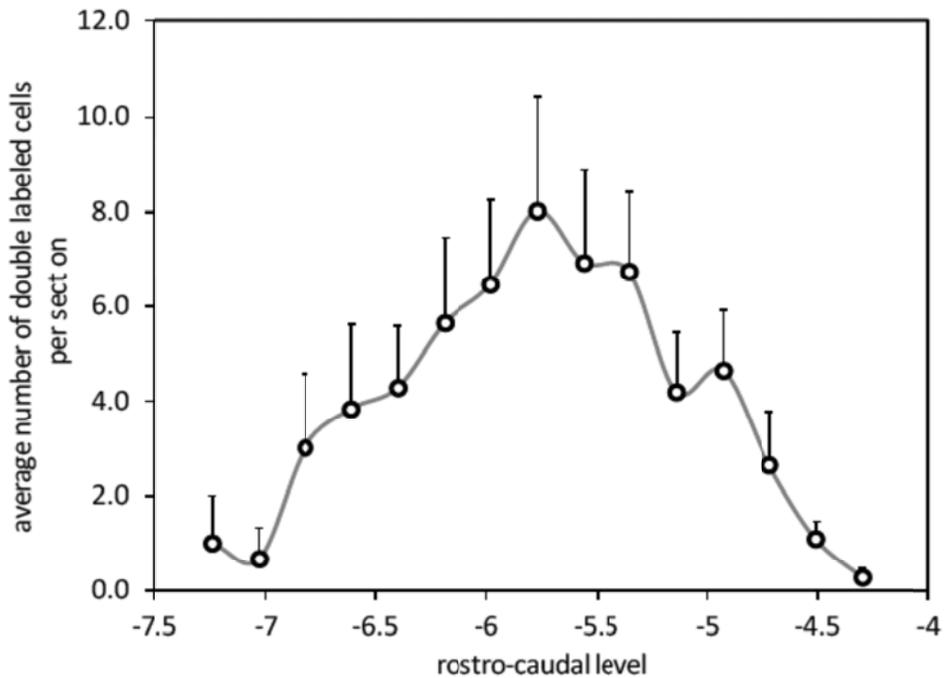
**Figure 5.2** Examples of FG / Fos double immunolabeled sections.

Example of double immunolabeled sections from Control (A) and Restraint (B) cases. Blue-black nuclear labeling is visible in the Restraint case, but virtually absent in the Control case. C. Higher magnification of a double immunolabeled section from a Restraint case. D. Example of two double-labeled neurons (arrowheads) in a Restraint section viewed under high magnification. APir – amygdalopiriform transition area; hif – hippocampal fissure. Scale bar = 250  $\mu\text{m}$  (A & B), 100  $\mu\text{m}$  (C), 25  $\mu\text{m}$  (D).



**Figure 5.3** Restraint stress induced Fos expression in NAc-projecting vSub neurons.

Acute restraint increased the number of FG / Fos double-labeled neurons in the vSub, compared with unhandled rats. (\* indicates significant difference between the mean number of double labeled neurons per section in each group,  $p < 0.01$ , t-test)



**Figure 5.4** Distribution of double-labeled neurons in the rostro-caudal axis of the ROI.

FG / Fos double-labeled neurons were most densely concentrated in the middle portion of the vSub, approximately -6.0 mm from bregma.

## 5.4 DISCUSSION

The hippocampus is known to be strongly activated following a variety of stressors. Using combined Fos immunohistochemistry and retrograde tracing, it is possible to identify specific subsets of activated cells and trace their projections. In this study, we showed that restraint stress activated a distinct population of neurons projecting from the hippocampal ventral subiculum to the nucleus accumbens. This suggests that stress may impact on dopamine cell activity in the VTA through vSub-NAc-VP-VTA (Floresco et al. 2001; Floresco et al. 2003).

The distribution of retrogradely labeled neurons in the hippocampal formation was consistent with previous studies showing that the caudo-medial portion of the NAc receives the densest innervations from the vSub (H J Groenewegen et al. 1987; O'Mara et al. 2001; M P Witter & H J Groenewegen 1990; Aylward & Totterdell 1993). The morphology of FG immunoreactive neurons was consistent with large pyramidal cells that constitute the population of projection neurons in the vSub. Fos-positive neurons were distributed throughout the ventral hippocampal formation in Restraint cases, but were very sparsely distributed in Control animals. This is consistent with previous studies, showing that restraint stress activates Fos immunoreactivity in the hippocampal neurons (Funk et al. 2006; Cullinan et al. 1995; Otake et al. 2002) and particularly, the vSub (Otake et al. 2002).

The vSub is critical in mediating the effects of stress, as well as in modulating dopamine cell activity in the VTA via outputs to the NAc. This finding led us to hypothesize that restraint stress would activate neurons in the vSub that project to the NAc. Indeed, there was an increase in the number of Fos-positive, NAc-projecting neurons in the vSub of Restraint rats compared with Controls. While this increase was statistically significant ( $p < 0.01$ ), it was estimated that only a small proportion (less than 5%) of NAc-projecting neurons expressed Fos in this study.

On the other hand, almost 10% of activated neurons in the vSub projected to NAc. Thus, stress-induced activation of the vSub can impact dopamine cell activity in the VTA by driving the NAc – VP – VTA circuit.

## **6.0 GENERAL DISCUSSION**

### **6.1 SUMMARY OF RESULTS AND CONCLUSIONS**

These studies support the role of the vSub in mediating aspects of the response to stress, and elucidate the mechanisms underlying the effect of stressful stimuli on this structure through noradrenergic input from the LC, and from the BLA. An examination of the responses of vSub neurons to increased NE within the vSub as well as to the activation of LC neurons, both of which are known to occur during stress, revealed that vSub neurons are sensitive to these manipulations, and that these responses are correlated with responses to footshock in single neurons.

Iontophoretic application of NE was found to potently inhibit the firing of vSub neurons, and this was mediated by the activation of alpha-2 receptors. NE is released in the vSub during stress from terminals of LC neurons which densely innervate this region. Therefore, the effect of electrically stimulating this input was examined. In contrast to iontophoretically applied NE, LC stimulation resulted in inhibition of firing in 16 % of neurons and activation in 38 %. These effects were apparently mediated through NE release from the LC, as both types of responses were blocked by specific noradrenergic receptor antagonists. Thus, administration of the alpha-2 antagonist idazoxan blocked the inhibitory responses, and the beta antagonist propranolol blocked the excitatory responses to LC stimulation. The fact that both increasing NE

concentration in the vSub through iontophoresis and LC stimulation caused alpha-2 mediated inhibition suggests that the inhibitory effects of LC stimulation are mediated by alpha-2 receptors located in the vSub. However, in contrast to LC stimulation-induced inhibition, LC stimulation-induced activation was not mimicked by iontophoretic NE in the vSub. This indicates that the activation results from NE acting on beta adrenoreceptors located outside the vSub, or along the distal portion of vSub neurons where beta receptors would be inaccessible to iontophoretically applied NE. Regardless of their site of action, the current study confirmed previous studies showing that beta adrenoreceptors are the primary mediators of excitatory responses to LC-evoked NE release in the vSub.

The vSub was also found to respond to footshock, an acute stressor known to activate the LC noradrenergic system. Footshock resulted in the activation of 48% (footshock-activated; FA), and inhibited the firing of 13% (footshock-inhibited; FI) of spontaneously active neurons in the vSub. Blockade of alpha-1 noradrenergic receptors reversed the inhibitory responses in FI neurons, revealing an underlying footshock-induced activation. This result suggests that the response to footshock in vSub neurons may be controlled by two opposing, and competing influences: an alpha-1 mediated inhibitory influence and an activating input. Importantly, when footshock responses were regressed against responses to LC stimulation, a positive correlation was revealed, suggesting that footshock responses are mediated in part through this input to the vSub.

The BLA is another brain region involved in the stress response, and it sends projections to the vSub. Electrical stimulation of the BLA was found to elicit firing in vSub neurons in a manner consistent with orthodromic monosynaptic activation. Inactivation of the BLA, on the other hand, resulted in a suppression of basal firing in the vSub. This suggests that the BLA may

contribute to the driving of stress responses in vSub neurons. Therefore, the role of the BLA in mediating the activating effects of LC stimulation and footshock was examined. BLA inactivation did not block the increase in firing caused by LC stimulation or footshock, indicating that these responses are mediated independent of the BLA. However, while the BLA does not appear to play a role in footshock responses in the vSub, this input may be important in mediating responses to other stressors, and in providing emotional valence to contextual representations encoded by the vSub. Interestingly, the LC input was found have a modulatory effect on BLA-evoked responses in the vSub, facilitating responses in 5 neurons, while suppressing responses in 6 others. In this way, stress-evoked LC activation may gate BLA input to the vSub.

Another function of the ventral subiculum is to control dopamine neuron population activity in the VTA through output to the NAc – VP – VTA network. This role of the vSub has implications for understanding the pathophysiology of schizophrenia and drug abuse; conditions that are characterized by a hyper-responsive dopamine system, and are aggravated by stress. In order to assess the effect of stressors on NAc-projecting neurons in the vSub, a combination of retrograde tracing and Fos immunocytochemistry was used to investigate whether this particular population of vSub efferents is activated by restraint stress. Indeed, restraint stress activated a distinct population of neurons projecting from the vSub to the NAc. Although less than 5% of NAc-projecting neurons were activated by restraint, almost 10% of activated neurons in the vSub projected to NAc. This suggests that stress may impact on dopamine cell activity in the VTA through vSub-NAc-VP-VTA circuit.

## **6.2 POTENTIAL MECHANISMS OF EFFECTS INDUCED BY IONTOPHORETIC NE IN THE VSUB**

We hypothesize that the inhibitory actions of iontophoretic NE are mediated primarily by post-synaptic alpha-2 receptors. This is supported by our data showing that the alpha-2 receptor antagonist idazoxan significantly reversed NE-mediated inhibition at all iontophoretic currents of NE tested. Hippocampal neurons are known to express alpha-2 receptors at both pre- and post-synaptic sites, suppressing post-synaptic activity and afferent input, respectively. Iontophoretic NE, however, exerts its actions primarily at the somatic region of the neuron, and is unlikely to significantly affect presynaptic receptors, which are located along the large dendritic tree of vSub pyramidal neurons.

In addition, the alpha-1 receptor antagonist prazosin partially blocked the inhibition produced by iontophoretic NE, suggesting that alpha-1 receptors contribute relatively less compared to other adrenergic receptors to the inhibitory effect of NE. This result is consistent with the action of iontophoretic NE as reported in the dorsal hippocampus. Alpha-1 receptors are thought to be expressed primarily by inhibitory GABAergic interneurons in the hippocampus, and alpha-1 receptor activation depolarizes these neurons, thereby presumably increasing inhibition of hippocampal pyramidal cells. It should be noted that the tip of the iontophoretic micropipette is only approx. 5 um in diameter, and the somata of a pyramidal neuron is on the order of 50 um; therefore, intophoretic application of drug is not likely to affect elements much beyond the soma. Therefore, as mentioned above, iontophoretically applied NE is unlikely to diffuse far enough to exert its action on a significant population of other neurons that are located distal to the recording site.

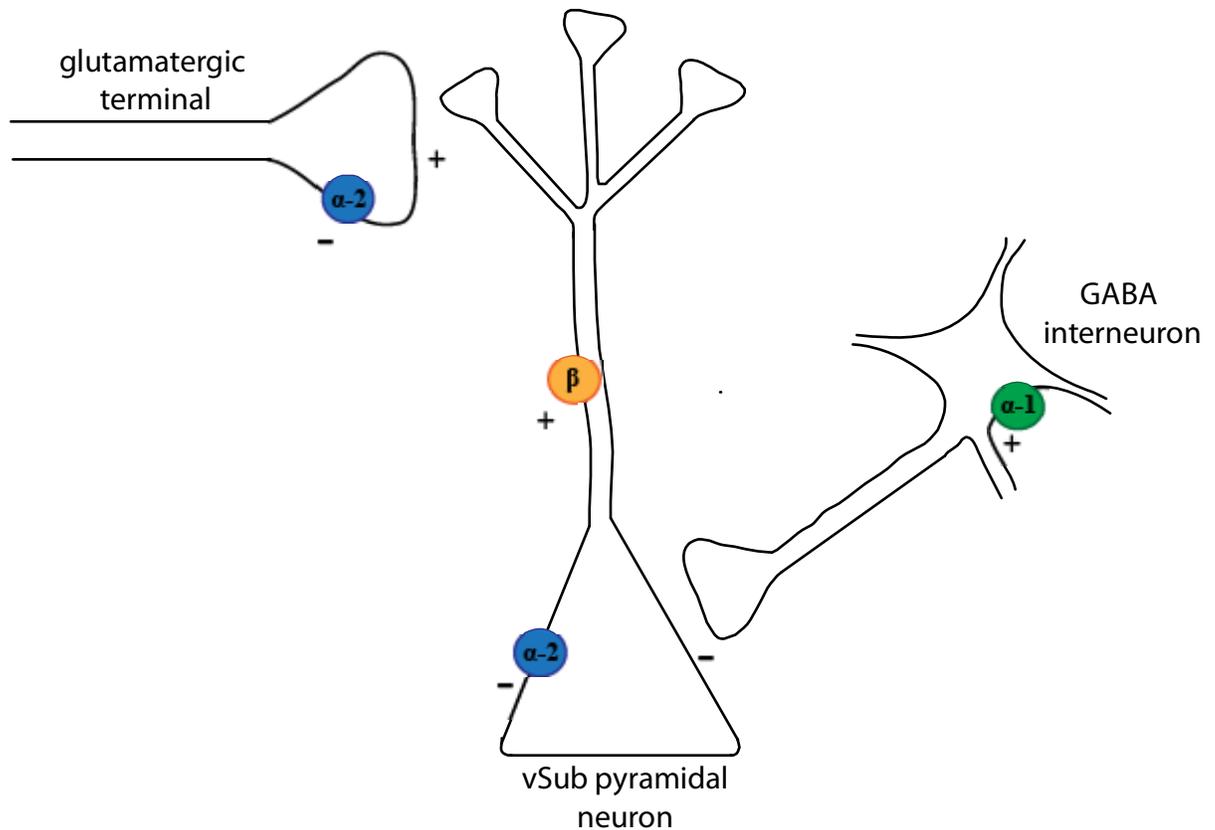
### **6.3 POTENTIAL MECHANISMS OF LC MODULATION OF VSUB NEURONAL FIRING IN THE VSUB**

LC stimulation activated 38% of spontaneously active neurons in the vSub, while inhibiting 16%. The inhibitory action of LC stimulation in these studies is thought to be mediated by alpha-2 receptors, either pre- or postsynaptically. Accordingly, LC-evoked inhibition was blocked by alpha-2 receptor antagonist idazoxan. Activation of LC input by phasic bursts is known to release high concentrations of NE in target regions; this, combined with the fact that alpha-2 receptors mediate the inhibitory effects of ionophoretically released NE in the vSub, suggests that these two effects have a common mechanism. However, LC stimulation-evoked NE release can also affect alpha-2 receptors at pre-synaptic sites. Therefore, it is possible that the inhibitory effects of LC stimulation are mediated by suppression of afferent inputs to vSub neurons by pre-synaptic alpha-2 receptors. Another potential mechanism is the activation of alpha-1 receptors on inhibitory GABA interneurons, which would increase inhibition of hippocampal pyramidal cells. However, this mechanism would not be sensitive to alpha-2 receptor antagonism which blocked LC stimulation-evoked suppression in the vSub; therefore it is not thought to be involved in mediating this effect.

On the other hand, the activating effects of LC stimulation are thought to be mediated by beta adrenergic receptors; accordingly, the beta receptor antagonist propranolol blocked these responses. Indeed, beta receptors are generally known to exert excitatory effects postsynaptically. Since these activating effects were not mimicked by iontophoretic application of NE in the vSub, it appears that it is mediated by beta receptors located in regions other than the immediate site of iontophoresis. This may include afferent regions to the vSub, or sites along the distal portion of vSub neurons where beta receptors are inaccessible to iontophoretically applied

NE. To address the first possibility, the effect of BLA inactivation was tested on LC stimulation-evoked activation. Our experiments showed that BLA inactivation did not block these responses, suggesting that they are not mediated through this input. This result is not surprising, since in unstressed rats, NE release from the LC in the BLA has been reported to primarily inhibit this region, with only a small proportion of neurons being activated through a beta adrenergic mechanism. However, chronic cold stress was reported to increase the excitatory effects of NE in the BLA, suggesting this input to the vSub may play a role in responses to LC stimulation under chronic stress conditions.

Another possibility is that LC stimulation-evoked activation in the vSub is mediated by beta receptors located along the distal portion of vSub neurons. This would account for the inability of iontophoretic NE, which is applied near the soma, to activate these receptors. While the subcellular localization of these receptors has not been studied in the vSub, beta receptors have been observed primarily on the dendrites in the dorsal hippocampus. This suggests the hypothesis that dendritic beta receptors in vSub pyramidal neurons potentiate synaptic inputs to these cells during stress-induced LC activation. The hypothesized distribution of adrenergic receptors in the vSub is summarized in Figure 6.1.



**Figure 6.1 Presumed distribution of adrenergic receptors in the vSub.**  
 (+) indicates activation of post-synaptic neuron, (-) indicates inhibition.

#### **6.4 POTENTIAL MECHANISMS OF FOOTSHOCK- AND RESTRAINT-EVOKED ACTIVITY IN THE VSUB**

Similar in its effects to LC stimulation, footshock activated 48% (footshock activated; FA), and suppressed 13% (footshock inhibited; FI) of neurons in the vSub. Furthermore, responses to footshock were correlated with responses to LC stimulation in single neurons. This suggests that footshock responses may be mediated by activation of the LC.

In FI neurons, blockade of alpha-1 receptors reversed the footshock-induced inhibition, revealing instead an activating response. This result suggests that FA and FI neurons may not represent static populations, but rather that footshock-responsive neurons may be dynamically modulated by alpha-1 activation. As mentioned above, activation of alpha-1 receptors depolarizes GABA interneurons, thereby increasing inhibitory input onto pyramidal neurons in the vSub. One possibility would be that FI neurons are selectively innervated by a population of alpha-1 expressing GABA interneurons. Footshock-evoked NE release from the LC would then activate these interneurons, thereby inhibiting FI cells.

Importantly, footshock responses may also be mediated by glutamatergic input to the vSub. Indeed, one function of the hippocampal formation is to continually encode episodic memories based on multimodal sensory input. Accordingly, various sensory cortical areas send projections to this structure, most notably to the entorhinal cortex (EC). The EC projects directly to the vSub, as well as indirectly through the multisynaptic hippocampal circuit. This sensory input, modulated by the LC noradrenergic signal, could be involved in mediating footshock responses. One possible scenario is that this activating sensory signal is facilitated by beta adrenergic receptors on FA neurons and suppressed by alpha-1 receptors on interneurons projecting to FI neurons, as discussed above. Further experiments investigating the noradrenergic pharmacology – including alpha-2 and beta receptors – is needed to fully understand the underlying mechanisms of footshock responses in the vSub.

In this study, we found that 30 min acute restraint stress also activated a distinct population of neurons projecting from the vSub to the NAc. Although less than 5% of NAc-projecting neurons were activated by restraint, almost 10% of activated neurons in the vSub projected to the NAc. Like footshock, restraint is also known to activate LC inputs to the vSub.

However, unlike the phasic responses to footshock we observed in the vSub, restraint-induced Fos expression reflects activation of the system over a longer time window. There are several potential mechanisms of restraint-induced activation. One possibility is that activation of beta adrenergic receptors by NE released from LC terminals activates vSub neurons during restraint stress. This hypothesis is supported by our finding that LC stimulation activates vSub neurons via beta adrenergic receptors. We would then expect that antagonism of beta receptors with systemic propranolol prior to restraint would block Fos expression in the vSub.

## **6.5 POTENTIAL MECHANISMS OF LC MODULATION OF BLA-EVOKED ACTIVITY IN THE VSUB**

We hypothesize that the BLA drives activity in the vSub via a monosynaptic glutamatergic projection. This hypothesis is supported by anatomical studies, which describe substantial projections from the BLA to the vSub. Furthermore, the main projection cells of the BLA are thought to be glutamatergic pyramidal neurons. Finally, our experiments show that vSub neurons are activated at short latencies by electrical stimulation of the BLA, in a manner consistent with a monosynaptic orthodromic response. Conversely, inactivation of the BLA depressed spontaneous firing in the vSub, suggesting that BLA drive to this structure contributes to the basal activity level in the vSub, even in anesthetized rats. In addition to a direct projection, we cannot discount multisynaptic connections between the BLA and the vSub. Indeed, the BLA projects to hippocampal subfields upstream of the vSub, which is the main output structure of the hippocampal formation. This also supports our hypothesis that the BLA functions to drive the vSub when activated by relevant stressful stimuli.

We further hypothesize that the LC activity modulates BLA inputs to the vSub through actions of NE released in the vSub and acting on adrenergic receptors. In our experiments, LC pre-stimulation facilitated BLA-evoked responses in 5 neurons, while suppressing these responses in 6 other neurons. Our experiments with LC stimulation show that LC stimulation-evoked increases in firing are mediated by beta receptors in the vSub; however, iontophoretically delivered NE does not activate these neurons. As discussed above, one explanation for this discrepancy is that beta receptors are localized on the distal portion of dendrites in vSub pyramidal neurons, where NE cannot diffuse from the site of iontophoresis before it is cleared by the norepinephrine transporter. This would also allow beta receptors to potentiate synaptic inputs to vSub neurons, in a manner similar to that which we observed with BLA afferents. On the other hand, LC stimulation-evoked suppression of BLA responses may be mediated by pre- or postsynaptic alpha-2 receptors. This is supported by our data, which show that alpha-2 receptors are responsible for mediating the inhibitory effects of LC stimulation and iontophoretic NE in the vSub. Our data cannot distinguish between the action of pre- and postsynaptic receptors. Thus, suppression of BLA inputs may be a result of NE acting on postsynaptic alpha-2 receptors, which would decrease the excitability of vSub neurons. Alternately, NE may act to suppress input from the BLA via activation of pre-synaptic alpha-2 receptors. Another possibility is that the BLA input is suppressed by GABAergic interneurons in the vSub. As discussed above, a subset of hippocampal inhibitory interneurons express alpha-1 receptors, the activation of which depolarizes these cells, thereby increasing inhibition of their targets. Indeed, we found that inhibitory responses to footshock stimulation are mediated through alpha-1 receptors. While responses to footshock are not mediated through the BLA, this result suggests that the circuitry described above may exist in the vSub. Further experiments

examining the pharmacology of LC modulation of BLA-evoked responses in the vSub are necessary to fully understand these mechanisms.

## **6.6 POSSIBLE MEANS OF STRESS-INDUCED ALTERATIONS IN NE MODULATION**

There are multiple ways in which chronic stress exposure could alter the neurophysiological actions of NE on vSub neurons. Many studies have shown that chronic stress exposure enhances NE efflux in terminal regions caused by acute stress (Nisenbaum et al. 1991, Pacak et al. 1992, Gresch et al. 1994, Jedema et al. 1999). Furthermore, alterations in the molecular actions of NE at postsynaptic targets may play a role in the behavioral effects seen after chronic stress. One way this may happen is through changes in NE receptors at terminal sites; indeed, changes in receptor number, in affinity of receptors for NE, and in the way in which receptors affect second messenger cascades have been reported following stress exposure (Stone et al. 1983). Thus, increased alpha-2 receptor expression has been reported in the cortex following acute footshock (R. M. Cohen et al. 1986). On the other hand, restraint stress decreases beta receptors in hypothalamus (Glavin et al. 1983). Furthermore various stressors, including inescapable tail shock, loud noise or restraint (H. E. W. Day, Kryskow, Stanley J Watson, Huda Akil & Campeau 2008a), as well as corticosterone (H. E. W. Day, Kryskow, Stanley J Watson, Huda Akil & Campeau 2008b) increase the expression of alpha-1 receptor mRNA in the hippocampus.

Changes in the adrenergic receptor number have also been reported with chronic stressors. Thus, cortical beta-receptors expression is decreased following repeated immobilization, handling, variable stress, and tailshock (Stone & Platt 1982; Nomura et al. 1981;

C. Stanford et al. 1984; Basso et al. 1993) and food deprivation (Stone 1983). Footshock, restraint, and repeated handling have also been shown to increase alpha-2 receptors in cortex (Stone 1981; Lynch et al. 1983; C. Stanford et al. 1984).

In addition, changes in receptor sensitivity and signaling of intracellular cascades have been reported after stress exposure. Decreased cyclic-AMP responses to beta receptor stimulation have been observed after repeated handling stress (Baamonde et al. 2002), while chronic tail pinch stress leads to alpha-2 receptor desensitization (Gómez et al. 1998). Chronic restraint stress also results in desensitized beta and alpha-2 receptors (Bellavía & Gallará 1998), as well as alpha-1 receptor desensitization and suppressed cyclic-AMP response to receptor stimulation (Stone 1987). In contrast, chronic variable stress (García-Vallejo et al. 1998) and chronic cold exposure (Nisenbaum & Abercrombie 1993) lead to alpha-2 receptor hypersensitivity, suggesting that these molecular changes are stressor-dependent.

Taken together, these studies illustrate the complex nature of stress-induced changes in NE receptors' composition. Any combination of the effects outlined above may influence the way whereby target neurons respond to NE. Stress-induced regulation of NE receptor expression and sensitivity in the vSub has not yet been studied.

## **6.7 THE ACTION OF GLUCOCORTICOIDS IN THE HIPPOCAMPUS, AND STRESS**

The hippocampal formation, and particularly the vSub, is an important forebrain negative feedback modulator of the HPA axis and expresses high levels of mineralocorticoid and glucocorticoid receptors. Stressful stimuli result in the release of adrenal hormones

(corticosterone in rats), which are responsible for many aspects of the stress response. It is therefore possible that some of the stress responses observed in this study are mediated by glucocorticoid receptors. Our data argue against this possibility however. First, footshock responses were positively correlated with responses to LC stimulation in our experiments, and footshock-evoked inhibition was found to be mediated by alpha-1 noradrenergic receptors. These observations point to the LC noradrenergic system rather than the neuroendocrine system in mediating these responses. Second, adrenalectomy does not alter the pattern of c-fos expression induced by acute and repeated restraint stress in the cortex, hippocampus, hypothalamus, septum, and brainstem (Melia et al. 1994). We therefore hypothesize that the restraint-induced increase in Fos expression observed in NAc-projecting vSub neurons was also mediated by the LC noradrenergic system. Nonetheless, hippocampal corticosteroid receptors are known to be influenced by the noradrenergic system (Bruce S McEwen 2007), and corticosterone regulates the expression of adrenergic receptors (H. E. W. Day, Kryskow, Stanley J Watson, Huda Akil & Campeau 2008b). Specifically, hippocampal expression of alpha-1 receptor mRNA is increased by corticosterone. Since alpha-1 receptors were found to mediate footshock-evoked inhibition in our experiments, this provides a mechanism whereby the neuroendocrine system may modulate the responsivity of the vSub to acute stress.

## **6.8 ACTIONS OF NE IN THE VSUB: CONSIDERATION OF CONDITIONING AND HIPPOCAMPAL PLASTICITY**

The hippocampal formation, and especially the ventral subiculum, are known to be critically involved in memory and learning processes (Sinden et al. 1988), and particularly in conditioning

paradigms involving contextual cues (Fanselow 2000; Maren 1999). Importantly, the vSub also plays a role in learning that involves drug-related cues. Thus, activation of the ventral subiculum is necessary and sufficient to elicit reinstatement of extinguished psychostimulant-seeking behaviors (Vorel et al. 2001; Taepavarapruk & Phillips 2003; Sun & Rebec 2003; Rogers & See 2007). Furthermore, context-induced reinstatement of cocaine-seeking behavior in rats requires specifically the ventral hippocampal formation that includes the subiculum (Lasseter et al. 2010). As reviewed above, stress also constitutes an important predisposing condition in drug addiction.

We suggest that NE release in the vSub functions not only in modulation of acute stress responses, but also in synaptic plasticity underlying stress-related learning. As reviewed above, LTP and LTD are known to occur in the hippocampus, and are thought to constitute cellular mechanisms underlying learning and memory formation. Additionally, stress has been shown to suppress hippocampal LTP (Kim et al. 1996; Hirata et al. 2009; B. K. Ryan et al. 2010) and facilitate LTD (L. Xu et al. 1997; Yang et al. 2004) in this region. This effect is partially explained by the action of corticosterone, which modulates synaptic plasticity in the hippocampus of rats in a receptor-dependent manner, enhancing LTP via GRs and decrease LTP via MRs (Pavlidis et al. 1995; Pavlidis et al. 1996).

Interestingly, adrenoreceptors have also been reported to affect synaptic plasticity in the hippocampus, primarily in a facilitatory manner. Thus, beta and alpha-1 receptors have been found to mediate the role of NE in facilitating LTP and suppressing LTD in CA1 slices (Katsuki et al. 1997). Beta receptors, in particular, have been implicated in enhancement of LTP in the hippocampus (Edagawa et al. 2005) and neocortex (Inaba et al. 2009). Furthermore, NE, acting via beta receptors was found to be responsible for both facilitating LTP in hippocampus and

conditioned responding to predator stress in mice, by regulating AMPA receptors (Hu et al. 2007).

Thus an intriguing picture emerges, where stress generally suppresses hippocampal excitability through the HPA axis, while facilitating stress-related learning via adrenoreceptors. Importantly, our studies indicate that beta and alpha-1 receptors are activated by stressful stimuli, suggesting that chronic stress may induce alterations in synaptic plasticity in specific inputs to the vSub via these receptors.

## **6.9 POTENTIAL BEHAVIORAL IMPLICATIONS OF STRESS-INDUCED MODULATION OF VSUB NEURONAL ACTIVITY BY NE AND THE BLA**

We hypothesize that inputs from the LC and the BLA regulate context representations in vSub neural networks. Under basal, low-stress conditions, contextual representations in the vSub drive the NAc – VP – VTA circuit to maintain appropriate behaviors with DA feedback. However, in the presence of a stressor, activation of the LC inputs results in inhibition of vSub activity through activation of alpha-1 and alpha-2 adrenoreceptors, which effectively disengages the current behavioral pattern. At the same time, activation of beta adrenergic receptors can activate a different set of inputs that constitute a stress context, which in turn, may drive a new adaptive behavioral pattern. Regulation of adrenergic receptor expression may be critical in defining dynamic populations of vSub neurons encoding specific contexts. The LC NE system may also function to generate new contextual representations by regulating synaptic plasticity.

While the BLA does not appear to play a role in footshock-induced activation of the vSub, this input may be important in mediating responses to other stressors, and in providing

emotional valence to contextual representations encoded by the vSub. Our results support the role of the LC NE system in modulating these functions. Furthermore, the LC NE system is known to primarily inhibit BLA neurons under basal conditions (D M Buffalari & A A Grace 2007), while chronic stress increases NE-induced activation in this region (Deanne M Buffalari & Anthony A Grace 2009). Thus, chronic stress may engage the BLA drive of vSub neurons, increasing the activation of the system.

## 6.10 CLINICAL RELEVANCE

The severely detrimental effects of stress exposure on neurological processes have been revealed through numerous clinical and pre-clinical studies. Whereas stress often triggers adaptive responses and enables a rapid reaction to threatening or harmful stimuli, it can also precipitate maladaptive changes. This manuscript focuses in particular on the mechanisms whereby stress influences the dopaminergic pathophysiology of schizophrenia and drug abuse.

*Clinical implications for drug abuse.* Stress increases the likelihood of relapse to drug-taking and drug-seeking behaviors (Shaham et al. 2000). Understanding the interaction between stress and drug use, and associated neuroadaptations, is key for developing therapies to combat substance use disorders (Logrip et al. 2011). As reviewed above, the vSub is a critical element in mediating the brain's stress response, and plays an important role in contextual modulation of emotional processing, suggesting a role in context-dependent aspects of drug addiction. Many clinical studies have documented that individuals undergoing stressful challenges show an increased vulnerability to drug addiction. Brain stress systems are thought to play a significant role in generating the negative emotional state characteristic of drug dependence and relapse

(George F Koob 2008; Belujon & Anthony A Grace 2011). The recruitment of brain stress systems during the progression to drug dependence suggests that anxiety disorders, characterized by heightened stress responses, may predispose individuals to develop or worsen addictive disorders. Our findings suggest that an imbalance between alpha-mediated inhibition and beta-mediated activation of the vSub during stress results in over-activation of the DA system. Mesolimbic dopamine release is critically involved in addictive processes including sensitization, suggesting a mechanism for cross-sensitization between stress and drug abuse.

*Implications for schizophrenia.* The vSub regulates population activity of midbrain dopamine neurons providing a possible link between stress and DA dysregulation in schizophrenia. The hippocampal formation has been a centerpiece of neuropathologic investigations of schizophrenia. Numerous imaging, histopathological and molecular studies suggest that neuronal pathology exists in the hippocampal formation of patients with schizophrenia (Anthony A Grace 2010; Lodge & Anthony A Grace 2011; Weinberger 1999; Lipska et al. 1995; Lipska & Weinberger 2000). We hypothesize that an imbalance in vSub responses to LC NE, which causes exaggerated responses to stress, may impair context-dependent decision making in schizophrenia. Indeed, stress can precipitate psychotic episodes (Pani et al. 2000, Moghaddam, 2002) in schizophrenia patients. Patients with schizophrenia also show impairments in choosing appropriate responses based on context, and deciding between relevant and irrelevant stimuli (Luck & Gold 2008). Understanding a role of vSub in mediating these phenomena may explain these deficits occurring in schizophrenia.

## 6.11 EXPERIMENTAL CAVEATS AND POSSIBLE CONFOUNDS

One significant confounding variable for all electrophysiological studies described here is the use of anesthetic. Urethane may alter neuronal activity levels, stimulation-evoked responses, as well as disrupt hippocampal rhythms and synchrony. Reduced basal firing in the vSub under anesthesia also makes 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.

Electrophysiological recordings in these experiments revealed activation of some neurons and inhibition of others in response to both LC stimulation and footshock. Many factors could account for the heterogeneity of these responses. One possibility is that experimental factors such as level of anesthesia may explain these differential responses. Indeed, urethane is has been reported to increase circulating levels of corticosterone in rats (Maggi & Meli, 1986; Zaretsky, Molosh, Zaretskaia, Rusyniak, & DiMicco, 2010), which in turn could lead to changes in stress responsivity. However, several lines of evidence argue against this interpretation. First, the level of anesthesia was carefully controlled by adjusting the anesthetic dose based on body mass of each animal, and by monitoring the pedal withdrawal reflex and breathing of the animal throughout the experiment. Second, heterogeneous responses were observed in multiple neurons recorded in close proximity within the same animal, suggesting that properties of single neurons, rather than the state of the animal, determined the direction of responses. Finally, application of noradrenergic receptor antagonists blocked or altered responses to LC stimulation and footshock, suggesting that differential noradrenergic receptor expression and localization determined neuronal responses to these stimuli.

As discussed in the introduction, in addition to NE, LC neurons contain a variety of different neuropeptides. Therefore, we cannot ensure that the release of neurotransmitter from LC terminals evoked by LC stimulation is limited to NE. Consequently the effects seen after LC stimulation may be mediated in part by peptide release. However, supporting the theory that NE is mediating these effects, are the data demonstrating that the effects of LC stimulation can, in part, be replicated by applying NE via iontophoresis. More importantly, application of noradrenergic receptor antagonists blocked LC stimulation evoked responses.

In addition to activating projections to the vSub, electrical stimulation of the BLA may activate other efferent pathways, including those leading to the LC through the central amygdala and the BNST (Luppi et al. 1995). These projections, while sparse, have been shown to activate the LC during stress via corticotropin-releasing factor (CRH) (Curtis et al. 2002; E. J. Van Bockstaele et al. 1998). Because of the short latency of the vSub response to BLA stimulation, it is unlikely that indirect LC activation contributed to this response.

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