

Characterization of the Function and Localization of the  $\alpha_{2A}$ -Adrenergic Receptor  
in the Bed Nucleus of the Stria Terminalis

By

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Dissertation

Submitted to the Faculty of  
the Graduate School of Vanderbilt University  
in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Molecular Physiology and Biophysics

August 2009

Nashville, TN

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To my mom and dad for their endless support

## ACKNOWLEDGEMENTS

I would like to acknowledge my family and friends, who have supported me throughout my PhD work. My mom, Vivan Shields, and dad, Justin Shields, have been extremely supportive of my education and career goals, and I would not be here without their love and direction. I would also like to thank my sister Cass Shields Noggle and brother-in-law Matthew Noggle, who moved to Nashville early in my graduate school years and have always welcomed me in their home for a good meal or night of relaxing with family. I am also thankful for having wonderful friends to keep life exciting during my free time outside of lab. I especially appreciate Lynn Martin Holiday, Renee Makowski, Donna Vleugels Hepper, Kimberly Ma, and Carolyn Drosey for their unwavering friendship and all the great laughs we have shared over the years. Also, I would like to thank my boyfriend Dan Grippo for the immense happiness and support he has brought me throughout the past year.

My graduate school experience would not be the same without the members of the Winder lab, who have been a great source of knowledge, inspiration, and friendship over the years. First, I would like to thank Dr. Danny Winder, who has been an amazing mentor. His expertise and guidance have truly helped make me a better scientist, and he has been pivotal in the success of my thesis project. I would also like to acknowledge Julie Healey and Elana Epstein for keeping the lab organized and running smoothly, in addition to completing their own research projects. I would like to thank Bob Matthews for his patience in teaching me stereotax surgeries, for his help in revising my

manuscripts, and his advice when I ran into issues with immunohistochemistry experiments. Additionally, Bob can design and build any gadget the lab needs, and I greatly admire his creativity in problem solving. I would like to show gratitude toward Thomas Kash and Zoe McElligott, who helped teach me how to operate field and whole cell electrophysiology rigs, and were always there to help with questions and problems. Also, I would like to thank Bill Nobis, Heather Gosnell, and Adeola Davis for their friendship, camaraderie and general help throughout my years in the lab.

My research depended very heavily on the HA  $\alpha_{2A}$ -adrenergic receptor knock in mice, and I owe a great deal of gratitude to Qin Wang, who developed these mice. Also, my thesis committee, consisting of Eric Delpire, Randy Blakely, Al Beth, Aurelio Galli, and Roger Colbran, has been extremely valuable in guiding my thesis work. Additionally, the expertise of Sam Wells and the staff of Cell Imaging Shared Resources have been instrumental in the success of my immunohistochemistry imaging work. Finally, I would like to thank Terry Dermody and the MSTP faculty for their continuous support throughout the MD/PhD program.

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## LIST OF ABBREVIATIONS

5-HT	serotonin
5-HT <sub>1A</sub> R	serotonin 1A receptor
$\alpha_1$ -AR	alpha1 adrenergic receptor
$\alpha_{1A}$ -AR	alpha <sub>1A</sub> adrenergic receptor
$\alpha_{1C}$ -AR	alpha <sub>1C</sub> adrenergic receptor
$\alpha_{1D}$ -AR	alpha <sub>1D</sub> adrenergic receptor
$\alpha_2$ -AR	alpha <sub>2</sub> adrenergic receptor
$\alpha_{2A}$ -AR	alpha <sub>2A</sub> adrenergic receptor
$\alpha_{2A}$ -AR KO	alpha <sub>2A</sub> -adrenergic receptor knockout mouse
$\alpha_{2B}$ -AR	alpha <sub>2B</sub> adrenergic receptor
$\alpha_{2C}$ -AR	alpha <sub>2C</sub> adrenergic receptor
ACSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
ADHD	attention-deficit hyperactivity disorder
AMPA	$\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole propionate
AMPA	AMPA receptor
$\beta$ -AR	beta adrenergic receptor
$\beta_1$ -AR	beta <sub>1</sub> adrenergic receptor
$\beta_2$ -AR	beta <sub>2</sub> adrenergic receptor
$\beta_3$ -AR	beta <sub>3</sub> adrenergic receptor
BDA	biotinylated dextran amine

BLA	basolateral nucleus of the amygdala
BNST	bed nucleus stria terminalis
Ca <sup>2+</sup>	calcium ion
CB1	cannabinoid 1 receptor
CB2	cannabinoid 2 receptor
CeA	central nucleus of the amygdala
Cl <sup>-</sup>	chloride ion
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
COMT	catechol-O-methyl-transferase
CPA	conditioned place avoidance
CPP	conditioned place preference
CRF	corticotropin-releasing factor
CRF1R	corticotropin releasing factor receptor 1
CRF2R	corticotropin releasing factor receptor 2
CRF BP	corticotropin releasing factor binding protein
CRF KO	CRF knock out
D1R	dopamine 1 receptor
D2R	dopamine 2 receptor
DA	dopamine
DAT	dopamine transporter
dBNST	dorsal BNST
DBH	dopamine beta-hydroxylase
DNAB	dorsal noradrenergic bundle

eEPSC	evoked excitatory postsynaptic current
eIPSC	evoked inhibitory postsynaptic current
EM	electron microscopy
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
ERK	extracellular signal-regulated kinase
FFA	free fatty acid
GABA <sub>A</sub> R	GABA <sub>A</sub> receptor
GAD	general anxiety disorder
GAD65	glutamate decarboxylase 65
GPCR	G-protein coupled receptor
HA	hemagglutinin
HA $\alpha_{2A}$ -AR KI	HA $\alpha_{2A}$ -adrenergic receptor knockin mouse
HPA axis	hypothalamic-pituitary-adrenal axis
ICV	intracerebroventricular
i.v.	intravenous
i.p.	intraperitoneal
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
K <sup>+</sup>	potassium ion
KA	kainite
KAR	kainite receptor
KI	knock in

KO	knock out
LC	locus coeruleus
LH	lateral hypothalamus
LTD	long term depression
LTP	long term potentiation
LTS	low threshold spike
MAO	monoamine oxidase
Mg <sup>2+</sup>	magnesium ion
mGluR	metabotropic glutamate receptor
Na <sup>+</sup>	sodium ion
NAc	nucleus accumbens
nAChR	nicotinic acetylcholine receptor
NE	norepinephrine
NET	norepinephrine transporter
NET KO	norepinephrine transporter knockout mouse
NMDA	N-methyl-D-aspartic acid
NMDAR	NMDA receptor
NPY	neuropeptide Y
NTS	nucleus tractus solitarius
PAG	periaqueductal gray
PBN	parabrachial nucleus
PBS	phosphate-buffered saline
PFC	prefrontal cortex

PLC	phospholipase C
PNMT	phenylethanolamine N-methyltransferase
PPR	paired-pulse ratio
PTSD	post-traumatic stress disorder
PVN	paraventricular hypothalamus
R <sub>m</sub>	membrane resistance
SERT	serotonin transporter
TH	tyrosine hydroxylase
TMT	2,5-dihydro-2,4,5-trimethylthiazoline
TTX	tetrodotoxin
vBNST	ventral BNST
VGLUT1	vesicular glutamate transporter 1
VGLUT2	vesicular glutamate transporter 2
VMAT	vesicular monoamine transporter
VNAB	ventral noradrenergic bundle
VTA	ventral tegmental area
WT	wild-type
Y2R	neuropeptide Y2 receptor



## CHAPTER 1

### INTRODUCTION

#### **Addiction**

Drug addiction can be defined as a chronically relapsing disorder characterized by compulsive drug use and loss of control over intake (Koob, 2008). In the United States alone, the economic cost of drug abuse in 2002 was estimated to be \$180.9 billion. This amount includes the loss in productivity from disability, death, and withdrawal from the workforce as well as the cost due to health and crime consequences. Loss in productivity accounted for about 71% of the economic cost of drug abuse, while health care costs totaled about \$16 billion (Harwood et al., 2004). The costs of addiction reach much further than one's pocketbook, however, and include emotional and physical devastation to the diseased individuals as well as their families, friends, and communities. There are currently few effective treatments for helping addicted individuals remain abstinent from drugs of abuse. Our lab is focused on understanding how the stress and reward systems of the brain interact, so that we may aid others in finding more effective treatments to prevent relapse in addicted individuals who are trying to become and remain drug free.

## Synaptic Transmission

Drugs of abuse are known to mediate many of their effects on individuals by altering normal physiologic mechanisms of signaling in the brain. The brain is made up of many networks of neurons which form synapses with one another in very organized, consistent patterns, resulting in various sets of nuclei that have similar connections and properties. These sets of nuclei are often grouped by scientists together as brain regions and subnuclei within each region. In a simple model, a neuron consists of an information-receiving end, the dendrite, a cell soma, and an information output end, the axon. One cell soma can have many dendrites and axons. A simple synapse is formed by one neuron's axon terminal (the presynaptic terminal) and another neuron's dendritic spine (the postsynaptic terminal). Other types of synapses also exist such as axoaxonal or axosomatic. When a neuron is excited, it depolarizes from a negative resting membrane potential and this signal propagates down the axon to the axon terminal (presynaptic terminal), leading to calcium ( $\text{Ca}^{2+}$ ) influx in the presynaptic terminal via opening of voltage-gated  $\text{Ca}^{2+}$  channels. Intracellular calcium levels thus rise, and  $\text{Ca}^{2+}$  signaling results in fusion of vesicles containing neurotransmitter with the cell membrane, resulting in release of neurotransmitter into the synaptic cleft (the space between the axon terminal and the dendritic spine). The neurotransmitter then binds to receptors on the postsynaptic side of the synapse, leading to either a hyperpolarization (inhibition) or depolarization (excitation) of the postsynaptic cell. If the cell is depolarized strongly enough, it too will fire an action potential and release neurotransmitter at its axons, thus propagating the

signal to yet other neurons (Kandel *et al*, 2000). Thus, “synaptic transmission” is a relay of information from one neuron to another via neurotransmitter-mediated signaling at a synapse.

There are three primary types of synaptic transmission, including excitatory, inhibitory, and modulatory. Excitatory transmission is primarily mediated by the neurotransmitter glutamate. Inhibitory transmission is primarily mediated by the neurotransmitter GABA, although the neurotransmitter glycine also mediates inhibitory transmission. Finally, many modulatory neurotransmitters such as norepinephrine (NE), corticotropin releasing factor (CRF), and dopamine (DA), can modulate glutamatergic and GABAergic transmission.

Glutamate is an excitatory neurotransmitter and leads to a depolarization at the postsynaptic terminal. Glutamate acts on both ionotropic and metabotropic glutamate receptors (Kandel *et al*, 2000). Kainate receptors (KAR),  $\alpha$ -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPA), and N-methyl-D-aspartic acid receptors (NMDAR) are the three ionotropic receptors and mediate fast excitatory transmission. These receptors are ion channels, and when activated by glutamate, result in the flux of  $\text{Na}^+$  and  $\text{K}^+$ , which depolarizes the postsynaptic membrane (Kandel *et al*, 2000). This depolarization can be measured as an excitatory postsynaptic potential (EPSP) or an excitatory postsynaptic current (EPSC). The NMDAR contains  $\text{Mg}^{2+}$  that blocks the pore at normal resting potentials. This  $\text{Mg}^{2+}$  block is only relieved when the membrane is sufficiently depolarized (usually, via activation of AMPARs and KARs). After the

Mg<sup>2+</sup> block is removed, glutamate can bind and open the NMDAR ion channel (Kandel *et al*, 2000). Importantly, NMDARs and some AMPARs can also flux Ca<sup>2+</sup> in addition to Na<sup>+</sup> and K<sup>+</sup>, resulting in activation of Ca<sup>2+</sup>-dependent intracellular signaling cascades. Glutamate also binds at metabotropic glutamate receptors (mGluRs), and these receptors are linked to G-coupled proteins, and are therefore considered to be modulatory in nature (Bellone *et al*, 2008).

In the adult brain, GABA and glycine are inhibitory neurotransmitters and their release leads to a hyperpolarization at the postsynaptic terminal. GABA is the primary inhibitory neurotransmitter in the brain, and binds at two receptors, GABA<sub>A</sub> and GABA<sub>B</sub> receptors. The GABA<sub>A</sub>R is an ionotropic receptor and mediates fast inhibitory transmission. Its activation leads to the influx of Cl<sup>-</sup>, which normally causes a hyperpolarizing response in adult neurons. This hyperpolarization can be measured as an inhibitory postsynaptic potential (IPSP) or an inhibitory postsynaptic current (IPSC). GABA<sub>B</sub>Rs are metabotropic, are therefore considered modulatory in nature (Kandel *et al*, 2000).

Finally, many neuromodulatory neurotransmitters exist that can alter glutamatergic and GABAergic mediated synaptic transmission. These neurotransmitters include dopamine (DA), norepinephrine (NE), serotonin (5-HT), corticotropin releasing factor (CRF), as well as many others, and all modulatory neurotransmitters act on receptors that are coupled to G-proteins (Kandel *et al*, 2000). NE, for example, acts on several receptors such as the α<sub>2</sub>-adrenergic receptor (α<sub>2</sub>-AR). NE acting through this receptor is known to depress excitatory transmission in regions such as the prefrontal cortex, bed nucleus of the stria

terminalis (BNST), and central nucleus of the amygdala (CeA) (Delaney *et al*, 2007; Egli *et al*, 2005; Ji *et al*, 2008). G-coupled protein receptors (GPCRs), via their coupling to G-proteins, exert their effects on neurons through second messenger signaling systems.  $\alpha_2$ -ARs, for example, are coupled to  $G_i$  proteins that lead to inhibition of adenylyl cyclase and decreased levels of the second messenger cAMP, resulting in signaling cascades that decrease glutamate release or the postsynaptic response to glutamate (Delaney *et al*, 2007; Egli *et al*, 2005; Ji *et al*, 2008). Additionally, glutamate and GABA can act on metabotropic GPCRs that alter transmission through these second messenger systems (Kandel *et al*, 2000).

Learning is thought to occur when changes in synaptic transmission become long term, rather than transient in nature. That is to say that a synaptic response remains altered, even hours, days, weeks, or months after the stimulus is gone (Kandel *et al*, 2000). These changes are due to altered levels or altered composition of receptors at the membrane surface, changes in enzyme activity, changes in neuronal dendritic spine structure, or altered release of neurotransmitter. Long term depression (LTD) and long term potentiation (LTP) are examples of these long term alterations at a glutamate synapse (Kandel *et al*, 2000).

### **Alterations in synaptic transmission by drugs of abuse**

As just described, the current theory maintains that sustained alterations in synaptic transmission in the brain are responsible for learning and memory.

The process of addiction involves many types of learning such as learning associations between drugs and the context in which the drugs are taken or received, as well as memory of both positive and negative experiences associated with drug intake. These associations lead to craving in situations such as when an addict is in the environment where he/she used to take or buy drugs, and craving is exacerbated by stress. Learned associations also include positive associations with experiencing drugs' effects and negative associations of withdrawal when the actions of drugs wear off or when drugs are not available.

Importantly, drugs of abuse hijack natural physiological signaling mechanisms by binding to endogenous receptors and transporters in the brain. For example, cocaine intake leads to alterations in dopamine (DA) release in the nucleus accumbens (NAc) and other regions of the brain by blocking the DA transporter (DAT) (Robbins *et al*, 2008). As a result, metabolic and molecular processes occur that attempt to counteract these changes and bring DA levels back to normal, and therefore maintain homeostasis. The term homeostasis encompasses the mechanisms that maintain stability within physiological systems and hold all parameters of the organism's internal milieu within limits that allow an organisms to survive (Koob and Le Moal, 2001). Chronic drug intake disrupts the ability of the brain to return to its normal homeostatic set point, leading to permanent changes in synaptic transmission over time (Koob *et al*, 2001). These changes have many physiologic consequences that lead to physiological and behavioral dependence on the drug of abuse (Koob *et al*, 2001). Thus, in many ways, drugs of abuse disrupt the brain's natural

homeostatic set points over time, a mechanism described as allostasis, or the process of achieving stability through change (Koob *et al*, 2001) The result is an allostatic state in which there is a chronic deviation of the regulatory systems in the body from their normal operating levels (Koob *et al*, 2001).

Drugs of abuse essentially hijack the brain's natural learning and memory mechanisms, as well as overwhelm the brain's ability to maintain homeostasis, leading to permanent changes in the brain that result in behavioral addiction. Thus, addiction is truly a disease of the brain.

### **Reward Pathway of the brain**

Addiction is a disease that alters the neural mechanisms involved in reward sensation and motivation. The prefrontal cortex (PFC), nucleus accumbens (NAc), and ventral tegmental area (VTA) are three brain regions that make up the basic reward pathway of the brain. Dopaminergic neurons from the VTA project to the NAc, and the VTA additionally sends a projection to the PFC. The PFC has glutamatergic projections that terminate in the NAc. The NAc additionally sends afferent fibers to the VTA (Kandel *et al*, 2000). Thus, these three regions are highly interconnected and make up the basic reward pathway (see Figure 1). Many other brain regions modulate this basic reward pathway, including areas involved in emotions, stress, and motivational behavior. These will be discussed in a later section. Importantly, drugs of abuse all have different mechanisms of action that lead to their common effect of activating the VTA, thereby allowing high levels of DA to be released into the NAc (Koob *et al*, 2001).

Figure 1

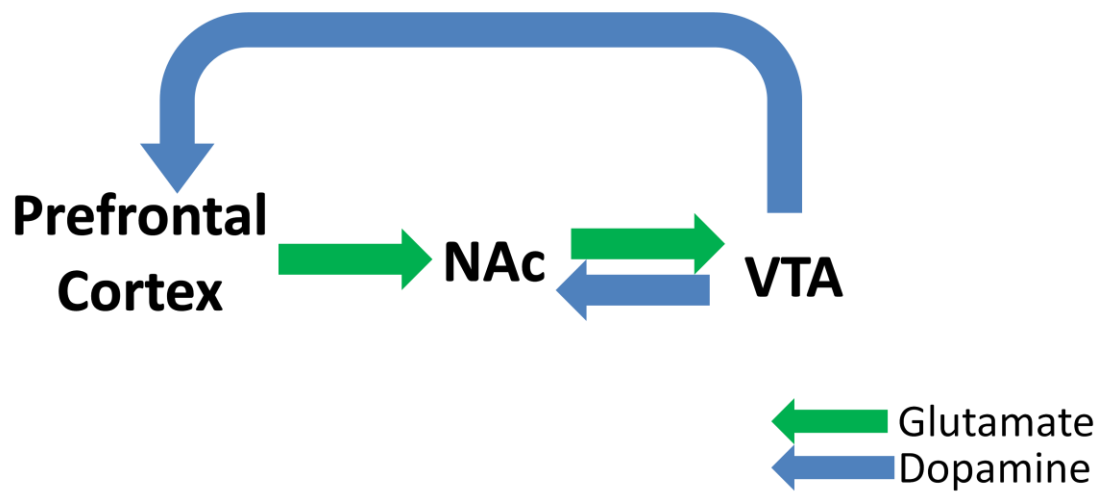


Figure 1. Basic reward pathway.



## **Mechanism of action for common drugs of abuse**

Common drugs of abuse include cocaine, amphetamines, alcohol, and opiates, and each have unique effects on the brain. Cocaine acts by blocking the DA, 5-HT, and NE transport proteins (DAT, SERT, and NET respectively), that are responsible for removing neurotransmitter from the synapse. Cocaine leads to an increase in DA in the NAc primarily by blocking the DAT, prolonging DA's presence in the synaptic cleft (Nestler, 2005). Amphetamines, on the other hand, reverse the DAT, causing an efflux of dopamine into the synaptic cleft (Koob *et al*, 2001). The effects of alcohol on the reward circuitry are more complex, but its effects are thought to be due to enhancement of the GABA<sub>A</sub> receptor function and decreased NMDAR function (Koob *et al*, 2001). Opiates act at endogenous opiate receptors including the  $\mu$ ,  $\delta$ , and  $\kappa$  opioid receptors, and the addictive properties of opiates are typically attributed to the effects of opiates on  $\mu$  opioid receptors (Koob *et al*, 2001). Similarly, nicotine acts at endogenous nicotine receptors (nAChRs), and cannabinoids act at the endogenous cannabinoid receptors, CB1 and CB2 (Koob *et al*, 2001).

## **Stages of Addiction**

### Initiation, continued use, and tolerance

Addiction is typically broken down into stages based on both biochemical changes in the brain and behavior. These stages include initiation, continued use, tolerance, withdrawal, dependence, abstinence, and relapse. The first time a person obtains and uses a drug is described as initiation. Typically, those who

continue to take drugs of abuse do so because the initial experience was pleasurable. For example, cocaine can induce intense feelings of euphoria and well-being, and can often increase the intensity of emotions and sexual feelings (Gawin, 1991; Johanson and Fischman, 1989). As an individual continues to use drugs, he/she often develops tolerance, in which greater and greater amounts of drug are needed to achieve the positive effects of the drug.

### Withdrawal and Dependence

Withdrawal is a negative state of being that is associated with cessation of drug intake and effectiveness. Dependence on a drug of abuse is described as adaptations that result in withdrawal symptoms when the drug of abuse is discontinued (Koob, 2009b). Physical symptoms of withdrawal depend on the drug being abused. For example, common signs of withdrawal for opiates can include sweating, vomiting, shaking, nausea, dilated pupils, agitation, diarrhea, restlessness, insomnia, and more (Perez 2008). Cocaine, on the other hand, may have less obvious physical symptoms of withdrawal, but symptoms include depressed mood, increased appetite, fatigue, restlessness, and agitation, nausea and vomiting, shaking, muscle pain, and disturbed sleep (Perez 2008).

Anxiety is a classic symptom of withdrawal, and severe anxiety during withdrawal is one reason many drug abusers continue using drugs. As users continue their use of a drug, they begin to do so to avoid the negative symptoms and signs associated with withdrawal, rather than for the pleasurable experience they had on their first few uses (Koob *et al*, 2001). When an individual has lost control over his/her drug intake, he/she is considered an addict. Addicted

individuals will continue to abuse drugs despite serious adverse social consequences such as loss of a job or dissolution of their marriage or other close relationships, and health consequences such as cancer or liver damage. Addiction is typically described as a compulsive uncontrollable use despite these types of social and health consequences (Koob, 2009a).

### Abstinence and Relapse

Individuals not using drugs for prolonged periods of time are said to be abstinent. Addicted individuals maintaining abstinence from drugs of abuse, however, are at great risk for relapse to drug seeking even many years after they quit taking drugs. Relapse is the return to drug seeking or drug intake by an addicted individual after a period of abstinence. Relapse tends to occur in part due to intense craving or desire for more drugs.

Relapse can occur due to re-exposure to the environment in which the addict abused drugs (context), exposure to drug cues such as drug paraphernalia, reexposure to the drug itself, and stress (Stewart, 2008). Stress has been implicated as a major driving force in drug addiction, playing a significant role in relapse to drug- and alcohol-seeking (Brown *et al*, 1995; Le *et al*, 2000; Sinha *et al*, 1999). In clinical studies, stress has been cited as the number one reason for relapse to drug addiction (Sinha *et al*, 1999).

### **Animal models of addiction**

Several rodent models have been developed to study and model addiction as seen in humans.

## Acquisition of drug preference and dependence, and effects of withdrawal

Conditioned place preference (CPP) is a model in which researchers attempt to determine an animal's preference or craving for a drug of abuse by pairing injection or availability of drug with one side (compartment) of a two-sided chamber. The chamber consists of two compartments with different contexts, such as differences in scent, color of walls, texture of flooring or lighting, such that the animal can tell one compartment from the other. Before the animal receives injections, it is allowed to roam freely between the two compartments and time spent in each side is monitored. Following drug pairing episodes of varying periods of time, the animal is again monitored for its time spent and activity in each side of the chamber. An increase in the drug-paired side is interpreted as the animal having obtained preference for the drug (Koob, 2008).

Self-administration is another model for examining drug preference. In this model, one of two levers available within an enclosed chamber is associated with an i.v. infusion of drug or some other mechanism of drug delivery. This lever is designated the active lever, and a second lever which does not result in any action if pressed is termed the inactive lever. In this paradigm, preference or desire for a drug can be measured by how much the animal presses the active lever to obtain the drug, and the ratio of active versus inactive lever pressing (Koob, 2008).

Once an animal has obtained preference for a drug of abuse, animals can then be tested for dependence by looking at somatic signs of withdrawal, and also by being placed into a chamber similar to that used for CPP and tested for

aversion to a side of the two-compartment chamber associated with withdrawal (Koob and Le Moal, 2008). For example, precipitated-withdrawal to opiate dependence is often accomplished by pairing a side of the chamber with naloxone injections. Naloxone is an opiate-receptor antagonist that quickly leads to the withdrawal state in an animal dependent on an opiate such as morphine (Delfs *et al*, 2000; Fuentealba *et al*, 2000). If the animal is forced to undergo withdrawal in only one side of the chamber, the animal will avoid that side of the chamber, thus indicating an aversion to withdrawal. Thus conditioned place aversion (CPA) measures the avoidance of the withdrawal-associated side of the chamber as compared to a pre-test in which the animal has no preference for either side of the chamber. The measure of avoidance in this model is correlated to the animal's aversion to the withdrawal state (Koob *et al*, 2008).

Many experimental models of anxiety have been developed, and these are often used to assess changes in an animal's anxiety levels following intake of drugs of abuse and during a withdrawal period. Two commonly used models are the elevated plus maze and open field test (Ramos, 2008). In the elevated plus maze model, animals are placed on an elevated maze that has open and closed arms resulting in the shape of a plus sign. Animals described as anxious when placed in the center of the maze typically stay in the closed arms of the maze; where as animals described as less anxious are more likely to explore the open arms (Rodgers and Dalvi, 1997). Similarly, animals can be monitored for their exploration in a novel, open field, environment. In this model, anxious animals stay toward the edges of the compartment, where as less anxious animals are

likely to spend more time in the center of the compartment (Prut and Belzung, 2003).

### Extinction

Abstinence is modeled in rodents by simply not providing the animal with drug for extended periods of time, and typically the animal is no longer placed in the environment where it previously obtained the drug. On the other hand, during extinction, the animal learns that a behavior previously required to obtain drug no longer provides the animal with the drug of abuse. Extinction can be modeled using either the CPP chamber or self-administration model (Shaham *et al*, 2003). In the CPP model, following acquisition of CPP, the animal is allowed back into the chamber over multiple sessions, but only saline is provided on the previously drug-paired side (as well as the previously non-drug-paired side (Shaham *et al*, 2003). Thus, the animal eventually loses preference for the previously drug-paired side. In the self-administration model, after acquiring preference for the drug-paired lever, the animal is placed back into the chamber over multiple sessions in which no drug is available. Eventually the animal learns that no drug will be delivered following pressing on the previously drug-paired lever, and the animal will lower responding on the lever system to that of pre-drug baseline responding (Shaham *et al*, 2003). In both of these models, when the animal learns that the previously learned behavior will no longer bring about drug acquisition and the animal stops attempts to obtain drug, it is said to have achieved extinction. Animals are often trained for extinction in order to further

understand learned behaviors; and importantly for treating addiction, animals are trained for extinction in order to learn more about relapse.

### Relapse and Craving

Relapse and craving can be modeled in animals in several ways. Animals may be primed with an injection of drug, and then placed back into a CPP chamber or self-administration chamber. An animal previously trained to lever-press for cocaine, for example, will reinstate lever pressing following a priming i.p. injection of cocaine (Koob, 2008). Animals will also reinstate drug-seeking when given cues associated with a drug (Koob, 2008). Stress-induced relapse is an important model for reinstatement of drug-seeking, but in response to a stressor rather than re-exposure to the drug (Shaham *et al*, 2003). People cite stress as the number one reason they relapse to drugs of abuse (Sinha *et al*, 1999). Thus, understanding the mechanisms that lead to stress-induced relapse are extremely important when thinking about treatment for addicts. Using the self-administration system, rats can be trained to lever press for access to a drug of abuse, such as an i.v. infusion of cocaine. Following extinction, if a rat is placed back into the chamber and given a footshock stress, it will immediately resume responding on the active lever (Erb *et al*, 1996; Shaham *et al*, 2000a; Shalev *et al*, 2001). Similarly, following a stressor, an animal will resume preference for the previously drug-paired side of a CPP chamber (Wang *et al*, 2001). Potential pharmacological therapies can be tested for their ability to prevent or induce reinstatement to drug-seeking in response to a stressor. These studies will be discussed in detail in a later section.

## **Stress**

Stress is defined in many ways, but all definitions convey the concept that stress is a demand placed on an animal. Some describe stress simply as any alteration in psychological homeostatic processes (Burchfield, 1979). Others define stress as any threat, either real or perceived, to the homeostasis and well-being of an organism (Morilak *et al*, 2005).

### **Acute, Chronic, Unconditioned, and Conditioned Stress**

Stressors can be acute or chronic, unconditioned or conditioned. Acute stressors are of short duration and typically have a defined onset and offset, and an animal's stress response recovers following cessation of the stressor (Morilak *et al*, 2005). Chronic stressors, on the other hand, can continue for indefinite periods of time, often leading an animal to experience prolonged anxiety (Hammack *et al*, 2009; Walker *et al*, 2003). Unconditioned stressors cause an animal distress without any previous learned behavior (Morilak *et al*, 2005). Examples of acute unconditioned stressors in rodent behavioral models include restraint stress, inescapable footshock stress, and forced-swim stress (Leao *et al*, 2009; Tanaka *et al*, 2000). Chronic unconditioned stress is often modeled in rodents using long term intermittent housing in a cold environment (Ma and Morilak, 2005), or using a series of unconditioned stressors such as cage tilt, wet bedding, food restriction, cold environment, footshock, forced swim, and restraint stress at random intervals over several days (Hammack *et al*, 2009; Mineur *et al*, 2006). Conditioned stressors come about when a previously unconditioned



stimulus, such as a tone, is paired with an unconditioned stress such as footshock (Garakani *et al*, 2006). The animal learns to associate the tone with the footshock, and will then have an anxiety-like response to the tone alone. Thus, stressors may be categorized based on their duration (acute or chronic) as well as whether or not they require learning of an association (conditioned or unconditioned).

### **Processive vs Systemic Stressors**

Stressors on an animal or person can also be described as processive or systemic (Morilak *et al*, 2005). Systemic stressors require no conscious thinking, and are real, imminent physical threats to health and well-being (Morilak *et al*, 2005). Thus, systemic stressors could be extreme heat or cold, massive blood loss, hypoglycemia, or any other stress that subconsciously activates the peripheral stress response (Morilak *et al*, 2005). Processive stressors, on the other hand, require higher order brain processing to evoke a stress response in an animal and depend upon perception, cognitive processing and interpretation of the stimulus (Morilak *et al*, 2005). For example, restraint stress is a prototypical processive stressor (Morilak *et al*, 2005). Importantly, both processive and systemic stressors activate the stress circuitry in the brain and periphery, albeit via different pathways and mechanisms. Both types of stressors importantly lead to activation of the paraventricular hypothalamus (PVN) which activates the peripheral stress system (Morilak *et al*, 2005).

## **Peripheral Stress Circuitry**

Although pathways and mechanism may differ, activation of the PVN is common to stress-evoking stimuli. When activated, the PVN releases corticotropin releasing factor (CRF) to the pituitary gland. In response the pituitary gland releases adrenocorticotrophic hormone (ACTH) to the adrenal glands. The adrenal glands then release cortisol into the bloodstream, which leads to systemic stress responses such as increased blood pressure, increased blood glucose, and decreased immune responses (Koob, 2008). Importantly, certain regions in the central stress circuitry contain receptors for cortisol and therefore provide a means of interaction and feedback between the central and peripheral stress circuits. These include a direct negative feedback inhibition of the PVN and pituitary gland by cortisol (Koob, 2008). Together, this circuitry is referred to as the hypothalamic-pituitary-adrenal (HPA) axis.

## **Central Stress Circuitry**

Many brain regions have been shown to be involved in stress and reward circuitry, and are implicated in addiction. These regions and their general role in stress and reward will be briefly reviewed here.

Activation of the VTA is critical for mediating natural reward, and the VTA is one of the major sources of DA in the reward pathway of the brain (Wise, 2009). DA in the brain is important for conditioned preferences, synaptic plasticity in learning and memory, as well as in reinforcement of response habits. Perturbations in VTA DA function are attributed to anhedonia and anxiety from

various stressors including withdrawal from drugs of abuse, and alterations in VTA function are critical in mediating relapse to drug-seeking in addicted individuals (Wise, 2009).

The NAc is also critically involved in reward processing, and mediates the addictive properties of drugs of abuse (Koob *et al*, 2001). Lesions of the NAc lead to decreased self-administration of cocaine, opiates, alcohol, and other drugs of abuse (Wang *et al*, 2008).

The limbic system, a set of regions involved in emotional regulation, is intricately involved in both stress and reward. Parts of the hypothalamus, septal area, nucleus accumbens, frontal cortex, and the amygdala make up the limbic system and these regions are highly interconnected (Feltenstein and See, 2008; Lopez *et al*, 1999). The limbic system is involved in fear and anxiety (Walker *et al*, 2003), and alterations in function of these regions by chronic stressors are thought to lead to anxiety disorders such as post-traumatic stress disorder (PTSD) (Shekhar *et al*, 2005). Drugs of abuse also alter synaptic transmission of various neurotransmission systems throughout the limbic circuitry, leading to anxiety and craving during withdrawal from drugs of abuse (Feltenstein *et al*, 2008).

The amygdala, a critical mediator of behavioral response to emotions, can be divided into several general regions. The central nucleus of the amygdala, CeA, is part of the extended amygdala critical for responses to short-term fear-evoking stimuli, and is one central source of the neuropeptide CRF (Walker and Davis, 2008b). The bed nucleus of the stria terminalis (BNST), which will be

discussed in detail in a later section, on the other hand, is critical in response to long-term fear- and anxiety-evoking stimuli (Walker *et al*, 2008b). The basolateral amygdala (BLA) is extremely important in the formation, consolidation, and extinction of fear and emotional memory, and is therefore critical in the initiation of response to stressful events (Akirav and Maroun, 2007). Thus, the BLA plays a pivotal role in classical conditioning of learned fear (Akirav *et al*, 2007).

The PFC is important for integration of affective states with autonomic and neuroendocrine regulation of stress, and it is critical in long-term fear extinction memory (Akirav *et al*, 2007; Hefner *et al*, 2008; Milad and Quirk, 2002). Importantly, the PFC regulates expression of amygdala-dependent memories (Quirk and Mueller, 2008). It can be subdivided into the prelimbic and infralimbic cortices, and the infralimbic cortex is important for consolidation of extinction learning in rodent models (Quirk *et al*, 2008).

Studies have shown that the insular cortex is important in processing emotional and homeostatic information, and is thought to be involved in drug addicts' negative affective states during abstinence (Contreras *et al*, 2007). For example, the insular cortex is critical for conscious aspects of needs and desires such as craving in drug-addicted individuals (Contreras *et al*, 2007, 2008; Naqvi *et al*, 2007).

The lateral hypothalamus (LH) is also involved in regulation of feeding behaviors and reward, and chemical activation of the LH can induce

reinstatement to drug-seeking via an orexin-containing projection to the VTA (Harris *et al*, 2005).

The periaqueductal gray (PAG) is a region of the midbrain involved in defensive behaviors, and the PAG regulates behaviors such as freezing, running, jumping, immobility and tachycardia, that are typical of a rodent under predator stress (Brandao *et al*, 2008). The PAG additionally is involved in regulating conscious recognition of pain via inhibition of ascending pain pathways (Wood, 2008).

Finally, the nucleus tractus solitarius (NTS) is highly involved in stress, anxiety, and stress-induced relapse to drug-seeking. This region contains a large NE projection to the limbic and reward circuitry, and is activated when an animal is under physiological or environmental stress (Walker *et al*, 2003). The locus coeruleus (LC) is also a large source of NE in the brain, and is activated under stress (Lopez *et al*, 1999), although its projections are not critical in mediating stress-induced relapse to drug-seeking (Olson *et al*, 2006).

## **Stress messengers**

### Corticotropin-releasing factor (CRF)

Corticotropin-releasing factor (CRF) is a 41 amino acid neuropeptide secreted in response to stress. There are two known CRF receptors, CRF1R and CRF2R, and each is made by a separate gene (Bale and Vale, 2004). CRF receptors are G<sub>s</sub> GPCRs that lead to activation of adenylate cyclase. Secretion of CRF by the PVN is important in activating the peripheral stress system. CRF

released into the hypophyseal portal system by the PVN acts on CRF1Rs in the anterior pituitary leading to the secretion of ACTH, which acts on the adrenal gland leading to secretion of the stress hormone cortisol into the blood stream (Koob, 2008). CRF is also expressed by extrahypothalamic brain regions such as the CeA and BNST as well as in hindbrain regions, and in peripheral tissues such as the gut, skin, and adrenal gland (Bale *et al*, 2004). CRF is inactivated via binding to the CRF binding protein (CRF BP) in the liver and circulation. Another hormone, urocortin, also binds to CRFRs and is involved in the stress response, although its distinct functions are not clear (Bale *et al*, 2004).

Central CRF has been implicated in many studies as a key stimulator of the stress response as well as a major player in mediating anxiety-like behaviors. Intracerebroventricular (ICV) infusions of CRF are known to decrease food intake, increase adrenal gland weight, reduce thymus and spleen weights, and increase cortisone and free fatty acid (FFA) levels. CRF KO mice, CRF1R KO mice, and CRF2R KO mice have confirmed the critical role of CRF in the stress response and the role of the CRF1R in mediating this response. The CRF2R, on the other hand, has been suggested to play a role in modulating the response mediated by CRF1Rs [for review, see (Bale *et al*, 2004)].

CRF is known to play a role in anxiety, and potentially the development of anxiety disorders. (Waddell *et al*, 2008). ICV infusion of the CRF antagonist  $\alpha$ -helical CRF9-41 increase latency and decrease time spent in the defense burying model of fear (Korte *et al*, 1994), and attenuate light-enhanced acoustic startle (de Jongh *et al*, 2003) and CRF-enhanced acoustic startle (Liang *et al*,

1992). Central infusions of CRF increase anxiety-like behaviors in the elevated plus maze (Moreau *et al*, 1997), potentiate the acoustic startle response (Swerdlow *et al*, 1986), decrease punished responding in the conflict test (Britton *et al*, 1985), decrease exploratory behavior in novel environments (Britton *et al*, 1982), and reduce exploratory behavior in a novel open field, increase circulating corticosterone levels, and disrupt social interaction behavior (Campbell *et al*, 2004). ICV infusions of CRF also block reinstatement to extinguished fear responses using a fear-potentiated startle model (Waddell *et al*, 2008). These and other anxiety measures have been shown to be mediated primarily via the CRF1R [for review see (Bale *et al*, 2004)]. CRF1R antagonists are currently being investigated for their anxiolytic role in alleviating anxiety disorders (Risbrough and Stein, 2006; Valdez, 2006). Importantly, extrahypothalamic CRF and in particular, amygdaloid CRF, is implicated in mediating effects of anxiety (Berridge and Dunn, 1989a; Gray, 1993).

ICV infusions of CRF antagonists have been shown to reverse ethanol and cocaine withdrawal-induced increases in anxiety. Alcohol-withdrawn rats injected with a CRF antagonist attenuate alcohol self-administration and show decreased anxiety in an elevated plus maze paradigm (Valdez *et al*, 2002). Additionally, animals pretreated daily with ICV injections of immunoserum raised against CRF display reduced anxiety-like behavior during cocaine withdrawal (Sarnyai *et al*, 1995).

Extrahypothalamic CRF has also been shown to mediate reinstatement of stress-induced drug-seeking behaviors. ICV CRF infusions can reinstate self-

administration of heroin and alcohol, and footshock stress- induced reinstatement to heroin- and alcohol-seeking can be attenuated by the CRF antagonists, alpha-helical CRF or d-phe-CRF (Le *et al*, 2000; Shaham *et al*, 1997). Similar findings were found in models of reinstatement for cocaine-seeking (Erb *et al*, 1998) and morphine-seeking (Lu *et al*, 2000), and CRF actions on reinstatement have been shown to be mediated by the CRF1R (Lu *et al*, 2000; Shaham *et al*, 1998). CRF antagonists do not, however, block drug-priming reinstatement to drug-seeking, implicating a selective role of CRF in stress interactions with drug-seeking (Erb *et al*, 1998; Shaham *et al*, 1997). CRF in the BNST specifically has been implicated in mediating stress-induced relapse to drug seeking behaviors and these studies will be reviewed in a later section.

### Norepinephrine (NE)

Norepinephrine (NE) has additionally been implicated in mediating the stress response. NE is synthesized by the enzyme dopamine beta-hydroxylase (DBH), which converts dopamine (DA) to NE. NE is packaged into synaptic vesicles at presynaptic terminals by the vesicular monoamine transporter (VMAT). Once released into the synapse, it is able to activate noradrenergic receptors (discussed later in this section). NE is cleared from the synapse via uptake by the NE transporter (NET) for recycling, or is rapidly degraded into metabolites by the enzymes catechol-O-methyl-transferase (COMT), monoamine oxidase (MAO) and phenylethanolamine N-methyltransferase (PNMT) (Kandel *et al*, 2000).



NE is converted to epinephrine by PNMT, and it is released into the bloodstream by the adrenal medulla where it can modulate peripheral stress responses. It is released in response to activation of the sympathetic nervous system and is an important part of the “flight or fight” response. Epinephrine increases heart rate, triggers release of glucose from energy stores, and increases blood flow to skeletal muscle and away from areas such as the digestive system. Thus, epinephrine released into the periphery prepares the animal to respond to a stressful situation (Kandel *et al*, 2000).

Centrally, NE is extremely important as a neuromodulator for mediating and modulating responses to processive and systemic stressors, focused attention, the sleep-wake cycle, motivation, and other functions. Many studies have shown that central NE is activated by a range of stressful stimuli such as immobilization, loud acoustic stimuli, electric foot-shock, cold exposure, and other stimuli (Morilak *et al*, 2005). NE is implicated in anxiety-related disorders and mediation of stress-induced relapse to drugs of abuse. Stress and withdrawal from drugs of abuse have been shown to increase NE levels throughout the brain (Koob, 2008). Additionally, mice lacking DBH have been shown to be resistant to the anxiogenic effects of cocaine (Schank *et al*, 2008).

There are two sources of NE in the brain, the locus coeruleus (LC) and the nucleus tractus solitarius (NTS) (Figure 3). The LC projects via the dorsal noradrenergic bundle to areas of the brain such as the thalamus, hippocampus, cerebellum, and prefrontal cortex, and areas of the hypothalamus. Early studies focused on the LC in response to stressful stimuli, and physiological stressors do

activate NE neurons in the LC (Page *et al*, 1992; Svensson, 1987; Valentino *et al*, 1993). Lesions of the LC, however, do not alter naloxone-induced CPA in opiate-dependent rats, or the expression of withdrawal symptoms following naloxone-precipitated withdrawal (Caille *et al*, 1999).

The NTS is a much smaller NE nucleus and projects via the ventral noradrenergic bundle (VNAB) to areas implicated in anxiety and stress-behaviors such as the amygdala, BNST, and PVN. Lesions of the VNAB have been shown to inhibit footshock-induced reinstatement of morphine-seeking (Wang *et al*, 2001), and block opiate withdrawal-induced conditioned place aversion (Aston-Jones *et al*, 1999) implicating the NE from the NTS in stress and anxiety behaviors. Additionally, mice lacking DBH were used to demonstrate that viral restoration of DBH in the NTS but not LC restored CPP for morphine, thus implicating NE from the NTS, but not LC, as critical for drug-seeking behaviors (Olson *et al*, 2006).

Figure 2. Illustration of norepinephrine (NE) projections in the brain

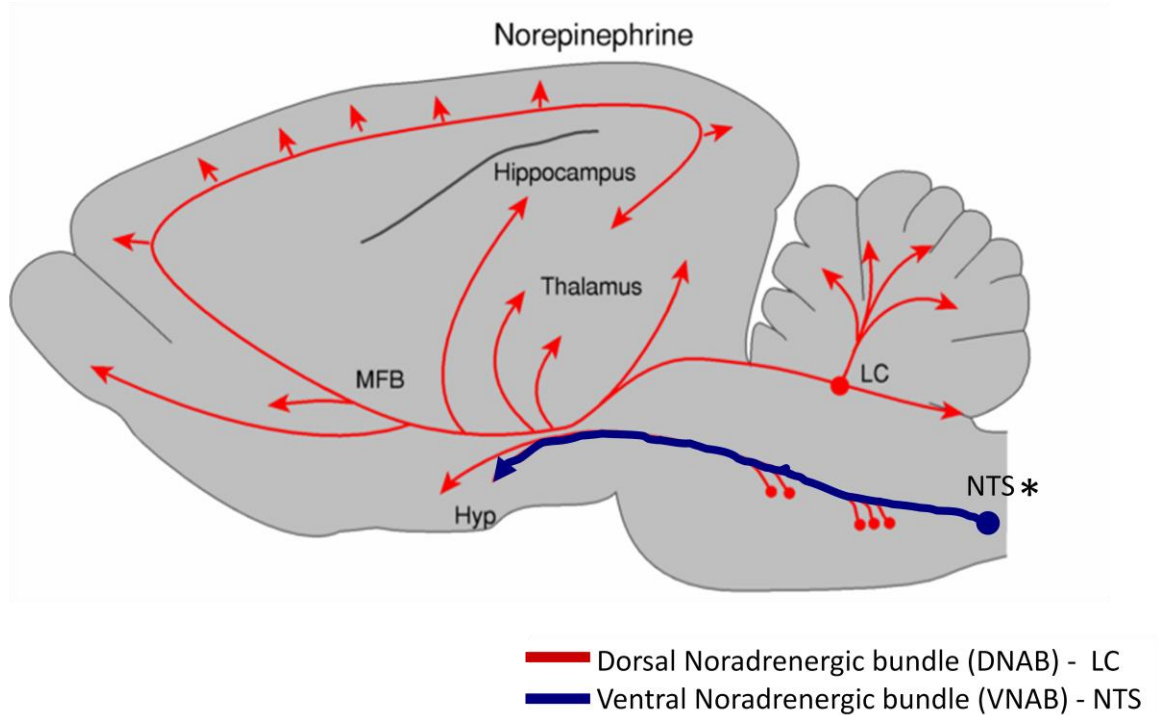


Figure 2. Illustration demonstrating projections of NE from the locus coeruleus through the dorsal noradrenergic bundle and from the nucleus tractus solitarius through the ventral noradrenergic bundle. Adapted from Fundamental Neuroscience (Zigmond, 1999).

### *β-adrenergic receptors*

NE acts on several G-protein coupled adrenergic receptors (ARs), found throughout the brain and periphery. These include the  $\alpha_1$ -ARs,  $\alpha_2$ -ARs, and  $\beta$ -ARs. The  $\beta$ -ARs are composed of three isoforms,  $\beta_1$ -ARs,  $\beta_2$ -ARs, and  $\beta_3$ -ARs, and all are  $G_s$ -coupled GPCRs (Koob, 2008). Thus, activation of these receptors leads to activation of adenylate cyclase. Alcohol withdrawal signs and symptoms are alleviated in both human and rodent models by administration of  $\beta$ -AR antagonists (Romach and Sellers, 1991; Trzaskowska and Kostowski, 1983). Increased anxiety following cocaine and morphine is also blocked by  $\beta$ -AR antagonists (Harris and Aston-Jones, 1993). Also, systemic injection of  $\beta$ -AR antagonists attenuates stress-induced reinstatement to cocaine seeking (Leri *et al*, 2002) as well as anxiogenic responses to cocaine administration (Rudoy and Van Bockstaele, 2007; Schank *et al*, 2008). ICV administration of  $\beta$ -AR antagonists can also attenuate stress-induced behaviors such as restraint stress-induced decreases in exploration on the elevated plus maze (Gorman and Dunn, 1993).

### *α<sub>1</sub>-adrenergic receptors*

$\alpha_1$ -ARs are  $G_q$  coupled GPCRs and mediate their signaling through the phospholipase C (PLC) 2<sup>nd</sup> messenger cascade. There are three subtypes of the  $\alpha_1$ -AR,  $\alpha_{1A}$ -ARs,  $\alpha_{1C}$ -ARs, and  $\alpha_{1D}$ -ARs (Koob, 2008).  $\alpha_1$ -AR antagonists can reduce signs of withdrawal from alcohol in rodents (Trzaskowska *et al*, 1983). Additionally, the  $\alpha_1$ -AR antagonist prazosin can reduce heroin self-administration in rats given extended access (Greenwell *et al*, 2009), and can attenuate self-

administration of cocaine in those given long term access (Wee *et al*, 2008; Zhang and Kosten, 2007). The  $\alpha_1$ -AR antagonist prazosin can also attenuate ethanol self-administration in ethanol-dependent rats (Walker *et al*, 2008a). Thus,  $\alpha_1$ -ARs appear to play a significant role in mediating aspects of addiction.

#### *$\alpha_2$ -adrenergic receptors*

$\alpha_2$ -ARs are  $G_i$  coupled GPCRs and their activation leads to inhibition of adenylate cyclase. The  $\alpha_2$ -AR family is comprised of three isoforms,  $\alpha_{2A}$ -ARs,  $\alpha_{2B}$ -ARs, and  $\alpha_{2C}$ -ARs, in mammals (Bylund *et al*, 1994). The  $\alpha_{2A}$ -AR has a widespread mRNA distribution in the brain including, but not limited to, regions involved in mediation of stress and anxiety, such as the NTS, amygdala, hippocampus, hypothalamus, and cortex (Scheinin *et al*, 1994; Tavares *et al*, 1996; Wang *et al*, 1996). The  $\alpha_{2C}$ -AR has a similar though not as widespread mRNA distribution as compared to the  $\alpha_{2A}$ -AR, whereas  $\alpha_{2B}$ -AR mRNA expression is mainly limited to the thalamus (Scheinin *et al*, 1994; Tavares *et al*, 1996; Wang *et al*, 1996). Both  $\alpha_{2C}$ -ARs and  $\alpha_{2A}$ -ARs are suggested to act as autoreceptors regulating the release of NE (Altman *et al*, 1999; Bucheler *et al*, 2002; Devoto *et al*, 2004; Hein *et al*, 1999; Trendelenburg *et al*, 1999; Trendelenburg *et al*, 2001), although data also implicates  $\alpha_2$ -ARs as heteroreceptors regulating the release of other neurotransmitters such as 5-HT (Ansah *et al*, 2003; Gobert *et al*, 1998; Maura *et al*, 1992; Numazawa *et al*, 1995; Scheibner *et al*, 2001), DA (Bucheler *et al*, 2002; Gobert *et al*, 1998; Ihalainen and Tanila, 2002), and glutamate (Carey and Regehr, 2009; Delaney *et al*, 2007; Glass *et al*, 2001; Milner *et al*, 1998; Milner *et al*, 1999; Yamanaka *et al*, 2006).

Interestingly, peripheral administration of blood-brain-barrier permeant, but not impermeant,  $\alpha_2$ -AR agonists such as clonidine and lofexidine blocks stress-induced reinstatement of heroin-seeking (Shaham *et al*, 2000b), cocaine-seeking (Erb *et al*, 2000), speedball- (mixture of heroin and cocaine) seeking (Highfield *et al*, 2001), nicotine-seeking (Zislis *et al*, 2007), and alcohol-seeking in rodent models (Le *et al*, 2005). Lofexidine also decreases alcohol self-administration (Le *et al*, 2005). ICV injections of the  $\alpha_2$ -AR agonist clonidine also blocks stress-induced reinstatement to heroin-seeking (Shaham *et al*, 2000b). These studies implicate central  $\alpha_2$ -ARs and NE rather than peripheral  $\alpha_2$ -ARs and NE in mediating stress-induced relapse to drug-seeking (Shaham *et al*, 2000b).

It is clear that activation of central  $\alpha_2$ -ARs decreases anxiety during withdrawal and can prevent stress-induced relapse to drug seeking behaviors (Morilak *et al*, 2005). In agreement with these findings, many studies have reported that the  $\alpha_2$ -AR antagonist yohimbine evokes anxiety in both human (Holmberg and Gershon, 1961; Murburg *et al*, 1991; Redmond and Huang, 1979) and animal studies (Davis *et al*, 1979; Holmes *et al*, 2002; Lang and Gershon, 1963). Cain and others found that yohimbine can facilitate extinction of conditioned fear in mice (Cain *et al*, 2004), while other groups concluded that yohimbine has no effect on extinction of conditioned fear (Mueller *et al*, 2009). Other studies have shown that yohimbine can enhance development of CPP for morphine (Zarrindast *et al*, 2002). Additionally, intraperitoneal (IP) injections of yohimbine can reinstate methamphetamine-seeking following extinction in the self-administration model (Shepard *et al*, 2004), as well as alcohol-seeking (Le *et*

*al*, 2005; Marinelli *et al*, 2007; Richards *et al*, 2009) and cocaine-seeking (Lee *et al*, 2004). Thus, yohimbine, through its putative action at  $\alpha_2$ -ARs appears to enhance anxiety-like behaviors.

Other studies, however, have suggested that the anxiogenic effects of yohimbine are partially mediated through 5-HT<sub>1A</sub> receptors. Powell and others have shown that yohimbine disrupts prepulse inhibition of acoustic startle in rats through the 5-HT<sub>1A</sub> receptor, and not  $\alpha_2$ -ARs, as the effects of yohimbine were blocked by a 5-HT<sub>1A</sub>-R antagonist but not an  $\alpha_2$ -AR agonist, and the effect of yohimbine was not mimicked by the more selective  $\alpha_2$ -AR antagonist atipamezole (Powell *et al*, 2005). Studies looking at selectivity of yohimbine indicates that yohimbine has about a 10-fold selectivity for the  $\alpha_{2A}$ -AR over the 5-HT<sub>1A</sub>-R, whereas atipamezole displays negligible effects on the 5-HT<sub>1A</sub>-R (Newman-Tancredi *et al*, 1998). Thus it is likely that blockade of  $\alpha_2$ -ARs cannot account for all of the anxiogenic effects of yohimbine, though further work is needed to differentiate the roles of yohimbine and  $\alpha_2$ -ARs in stress and anxiety.

## **The Bed Nucleus of the Stria Terminalis (BNST)**

### **General anatomy of BNST**

The BNST, a part of the extended amygdala, is a very diverse region, involved in many physiological and behavioral functions such as modulation of stress, anxiety, and fear responses, relapse to drug seeking, maternal behavior,

reproductive behavior, aggression and defensive behaviors, analgesia and regulation of pain, and regulation of fluid homeostasis.

The BNST has been divided into multiple subnuclei as described in Tables 1 and 2. Our laboratory focuses on regulation of the anterior division of the BNST, as this region has been shown to be involved in anxiety and relapse to drug-seeking. The laboratory uses a simplified classification of subregions within the anterolateral BNST, with the subregions being the dorsal anterolateral, dorsal anteromedial, and ventral subregions. The dorsal anterolateral region encompasses the oval and juxtacapsular nuclei, and the dorsal anteromedial region encompasses the central core of the BNST. Our ventral BNST recordings and localization encompass the subcommissural, fusiform, dorsomedial, and parastriatal nuclei. Ju and Swanson have extensively characterized these subnuclei anatomically (Ju and Swanson, 1989b; Ju *et al*, 1989c), and much work has been done in recent years to further identify the efferents and afferents of various subnuclei (Alheid *et al*, 1998; Dong *et al*, 2000; Dong *et al*, 2001a; Dong *et al*, 2001b; Dong and Swanson, 2004a, b, 2006a, b, c).

Recently, Choi and others have shown that different subregions of the BNST differentially modulate the HPA axis. Lesions of the posterior BNST elevated plasma ACTH and corticosterone levels in response to acute stress, and increased stress-induced PVN c-fos mRNA and CRF mRNA relative to controls (Choi *et al*, 2007). Lesions of the dorsomedial and fusiform nuclei in the anteroventral BNST, on the other hand, attenuated the plasma corticosterone response and decreased c-fos mRNA in the PVN in response to acute stress



(Choi *et al*, 2007). More recent studies by this group, however, did not support critical roles for the posterior and anteroventral BNST in response to chronic variable stress (Choi *et al*, 2008a; Choi *et al*, 2008b). These data begin to suggest that the posterior BNST nuclei are normally involved in inhibition of the HPA axis, whereas anteroventral nuclei activate the HPA axis (Choi *et al*, 2007), although more work needs to be done to elucidate the roles of various subregions in the BNST on HPA axis activation.

Our lab has primarily focused on afferents and efferents to the dorsal anterolateral (dBNST) and ventral (vBNST) regions of the BNST.

Table 1. Anterior Division of the Bed Nucleus of the Stria Terminalis  
(adapted from Ju and Swanson, 1989)

<u>Anterior Division</u>	Subnuclei	Description of Location	Cytoarchitecture
Anterodorsal		Dorsal to the AC, contiguous with the anteroventral area rostral and caudal to the AC	Dense population of small and round/oval shaped cells; although central core contains high density of large triangular and multipolar cells
Anterolateral		Borders the anterodorsal region rostrally and the posterior division caudally; merges with anteroventral area without clear boundary	
	Oval	Lies dorsal to the AC, mid-level rostro-caudally, just medial to the internal capsule	Uniform medium-sized round-oval neurons
	Juxtacapsular	Narrow vertical strip of cells just medial to the internal capsule	Smaller but similar to neurons in oval nucleus
	Rhomboid	Caudal and ventral to the oval and juxtacapsular nuclei; lies between internal capsule and ventromedial running stria terminalis	Uniform sized neurons that are round-to-multipolar; contains substance P and somatostatin-immuoreactive neurons
Anteroventral		Bordered dorsally by AC, ventrally and medially by the substantia innominata and preoptic areas, caudally by the lateral and medial hypothalamic areas	
	Subcommissural zone	Just ventral to the AC, similar to region just dorsal to the AC	Generally small neurons
	Fusiform	Lies along ventrolateral border of the anteroventral area	Small to medium-sized, oval-to-spindle-shaped neurons
	Dorsomedial	Bordered caudally and laterally by the dorsolateral nucleus; adjacent to the anteroventral area anteriorly	Ill-defined nucleus; contained scattered medium-sized multipolar cells that are oriented dorsomedial to ventrolateral
	Dorsolateral	Ventral to the AC, lateral to the dorsomedial nucleus, and dorsomedial to the magnocellular nucleus	Medium-sized cells; many triangular or multipolar
	Magnocellular	Rostrally, it is ventrolateral to crossing of the AC, lateral to the dorsolateral nucleus; extends dorsolaterally to ventral border of the anterolateral area	Darkly stained, large and plump cells
	Ventral	Triangular are ventral to the magnocellular nucleus; caudally becomes narrow	Medium-sized multipolar neurons
	Related nuclei	Parastrial and posteriodorsal	Lie at interface between preoptic area and BNST

Table 2. Posterior Division of the Bed Nucleus of the Stria Terminalis  
(adapted from Ju and Swanson, 1989)

<b><u>Posterior Division</u></b>	<b>Subnuclei</b>	<b>Description of Location</b>	<b>Cytoarchitecture</b>
	Principal	Sausage-shaped nucleus; rostrally, is ventral to tip of LV; caudally, spreads ventrally and medially	Round-oval small densely packed neurons
	Interfascicular	Scattered cells that lie among the fibers of the stria terminalis and lateral to the principal nucleus	Round-oval small densely packed neurons
	Transverse	Mid-dorsoventral levels in lateral portion of posterior diffusion; thin sheet of cells within the interfascicular and lateral principal nuclei	More heterogeneous in shape and size than interfascicular and principal nuclei
	Premedullary	Narrow sparse region of cells ventral to the obliquely running fibers of the stria at the caudal end of the BNST	Transversely oriented fusiform nuclei
	Dorsal	Ventral to the lateral ventricle, extends anteriorly over the anterodorsal area, caudally runs continuously with the stria extension of the BNST	Dense small round neurons
	Cell-sparse zone	Ventral to the floor of the LV, medial to the dorsal nucleus	Few cells
	Related cell groups		
		Strial extension of the BNST – extends caudally along the dorsal border of the stria terminalis	
		Magnocellular neurosecretory cells – scattered within BNST along lateral border of fornix and stria medullaris and medial edge of internal capsule and stria terminalis	
		Bed nucleus of the AC – lateral to the region where the fornix passes caudal to the AC	Small densely packed neurons with a few larger cells scattered

AC = anterior commissure

LV = lateral ventricle

### **Afferents to the BNST** (see Figure 3 for a simplified illustration)

Previous anatomical studies characterizing the inputs to the BNST have been completed almost exclusively in rat brain, with sparse studies also completed in cat, monkey, and opossum.

Anterograde and retrograde tracer studies, as well as *in vivo* electrophysiology studies, have been used to identify glutamatergic afferents to the BNST. Glutamatergic inputs to the lateral BNST include the infralimbic cortex (Massi *et al*, 2008; McDonald, 1998; McDonald *et al*, 1996; McDonald *et al*, 1999; Room *et al*, 1985; Shin *et al*, 2008; Vertes, 2004; Weller and Smith, 1982), insular cortex (Shin *et al*, 2008; Weller *et al*, 1982), the basolateral amygdala (Dong *et al*, 2001a; McDonald, 1991; McDonald and Culbertson, 1986; Shin *et al*, 2008; Walker *et al*, 2003), as well as the ventral subiculum of the hippocampus (Canteras and Swanson, 1992; Cullinan *et al*, 1993; Dong *et al*, 2001a; Weller *et al*, 1982), lateral hypothalamus (Dalsass and Siegel, 1987; Goto *et al*, 2005), and parabrachial nucleus of the hypothalamus (PBN) (Alden *et al*, 1994; Pritchard *et al*, 2000; Saper and Loewy, 1980).

While most studies examining infralimbic inputs to the BNST have been completed using anterograde tracer studies (McDonald, 1998; McDonald *et al*, 1996; McDonald *et al*, 1999; Room *et al*, 1985; Vertes, 2004), Massi and others have shown that the infralimbic cortex directly enhances activation of dopaminergic neurons in the VTA through a relay in the BNST, and that this activation is modulated by cannabinoid CB1-receptors in the BNST (Massi *et al*, 2008).

Other groups have used retrograde tracers to examine inputs to the BNST, and found that not only the infralimbic cortex, but also the insular cortex projects to the BNST (Shin *et al*, 2008; Weller *et al*, 1982). Two other groups completed anterograde tracer studies in rat confirming this projection (McDonald *et al*, 1999; Yasui *et al*, 1991).

In the adult brain, glutamatergic projections from one region to the other typically contain only one of two types of vesicular glutamate transporters (VGLUT1 or VGLUT2), as VGLUT1 and VGLUT2 have complementary patterns of expression in the brain (Fremeau *et al*, 2001; Herzog *et al*, 2001). VGLUTs are responsible for packaging glutamate into presynaptic vesicles (Hayashi *et al*, 2001). VGLUT1 is typically found in cortical regions such as the infralimbic cortex and insular cortex, as well as the BLA and hippocampus; whereas thalamic and striatal regions and hypothalamic areas such as the parabrachial nucleus are shown to express primarily VGLUT2 (Boulland *et al*, 2004; Fremeau *et al*, 2001; Herzog *et al*, 2001; Islam and Atoji, 2008; Varoqui *et al*, 2002). Therefore, VGLUTs are potentially good markers to identify subsets of glutamatergic inputs to the BNST.

The BNST also receives a strong GABAergic input from the CeA (Dong *et al*, 2001a; McDonald, 1991; McDonald *et al*, 1999; Price and Amaral, 1981; Shin *et al*, 2008; Sun *et al*, 1991; Weller *et al*, 1982; Zahm *et al*, 1999), and this projection contains the neuropeptide CRF (Asan *et al*, 2005; Erb *et al*, 2001; Sakanaka *et al*, 1986).

The BNST receives noradrenergic projections from both the NTS and LC. In fact, the BNST, and in particular, the vBNST, receives the densest innervation of NE in the brain (Forsay and Gysling, 2004). Most NE in the BNST arises from the NTS (Delfs *et al*, 2000; Geerling and Loewy, 2006; Ricardo and Koh, 1978; Roder and Ciriello, 1994), and this projection is critical in stress-induced reinstatement to drug-seeking behaviors (Aston-Jones *et al*, 1999; Olson *et al*, 2006; Shaham *et al*, 2000b; Wang *et al*, 2001). The BNST also receives a much more modest projection from the LC (Dahlstrom and Fuxe, 1964; Forsay *et al*, 2004; Forsay *et al*, 2000; Weller *et al*, 1982), although this projection is not critical for stress-induced reinstatement to drug-seeking (Olson *et al*, 2006; Shaham *et al*, 2000b; Wang *et al*, 2001). Additionally, the A1 noradrenergic cell group from the ventrolateral medulla projects to the BNST (Woulfe *et al*, 1990).

Finally, the BNST receives dopaminergic innervations (Eliava *et al*, 2003; Phelix *et al*, 1992a) from the VTA (Hasue and Shammah-Lagnado, 2002; Zahm *et al*, 2001) and PAG (Hasue *et al*, 2002; Meloni *et al*, 2006; Shin *et al*, 2008), and dopamine has been shown to modulate neurons in the BNST (Kash *et al*, 2008). The BNST also receives a serotonergic input from the raphe nucleus (Phelix *et al*, 1992a, b).

Figure 3

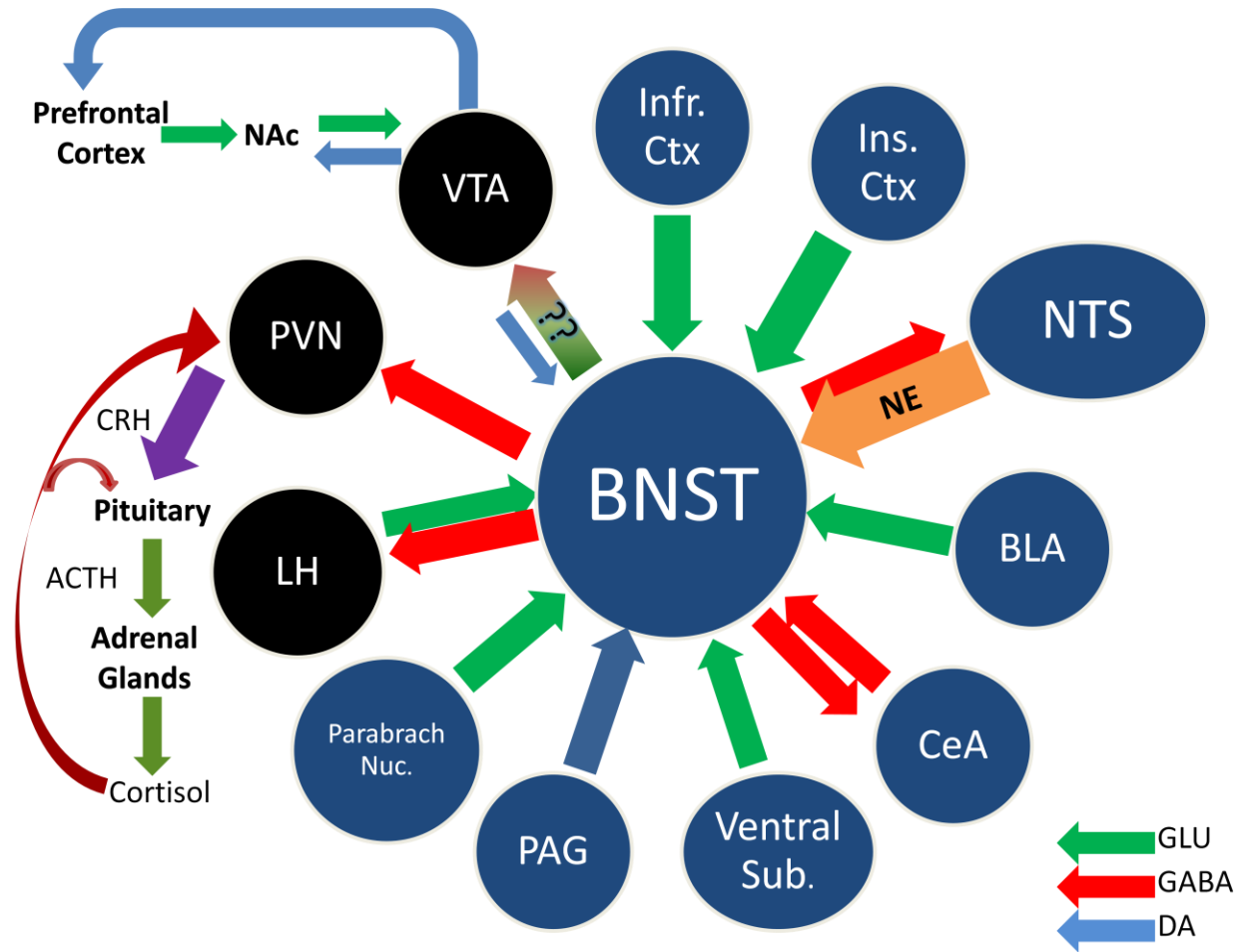


Figure 3. Illustration of afferents and efferents in the BNST that are related to stress and reward processing.

**Efferents of the BNST** (see Figure 3, page 39 for a simplified illustration )

Anterograde tracer studies examining projections from the anterolateral and juxtacapsular nuclei of the BNST show that the BNST sends projections to the NTS, PBN, VTA, CeA, LH, BLA, PAG, and NTS, and infralimbic cortex (Dong *et al*, 2000; Dong *et al*, 2004a). All of these regions innervate the BNST (see previous section), indicating reciprocal connections between these nuclei and the BNST.

Both retrograde and anterograde tracer studies demonstrate a projection from the BNST to the NTS that is predominantly ipsilateral (Kang and Lundy, 2009; van der Kooy *et al*, 1984). Many studies demonstrate a projection from the BNST to the PBN, which is an important projection concerning gustatory sensation and feeding (Dong *et al*, 2000; Kang *et al*, 2009; Moga *et al*, 1989; Saggiu and Lundy, 2008; Tokita *et al*, 2009). The BNST additionally sends a GABAergic projection to the PVN, and this projection is critical in regulation of the HPA axis by the BNST (Cullinan *et al*, 1993; Dunn, 1987; Herman *et al*, 1994).

The BNST also contains a reciprocal connection with the VTA (Dong *et al*, 2004a; Georges and Aston-Jones, 2001). Output from the BNST is typically assumed to be GABAergic due to the large density of GABAergic interneurons present (McDonald, 1983; Sun and Cassell, 1993), and limited functional studies support a GABAergic projection from the BNST to the VTA (Eiler and June, 2007). Other studies, however, indicate that an excitatory projection from the BNST to the VTA exists (Dumont and Williams, 2004; Georges and Aston-Jones, 2002). Additionally, a CRF(+) projection from the BNST to the VTA has been



identified (Rodaros *et al*, 2007). Despite the controversy over the makeup of the projection from the BNST to the VTA, it is generally accepted that activation of the BNST results in overall excitation of the VTA.

The BNST also contains a dense reciprocal connection with the CeA. Together, the CeA and BNST are considered to make up the extended amygdala. Like the projection from the CeA to the BNST, the projection from the BNST to the CeA (Dong *et al*, 2000; Dong *et al*, 2004a) is considered to be a GABAergic CRF(+) projection (Smith and Aston-Jones, 2008).

Finally, studies show that the anterolateral BNST also projects to multiple other regions in the brain, where it may modulate function. These regions include the NAc, prelimbic cortex, substantia innominata, thalamocortical feedback loops, ventral CA1 area of the hippocampus, medial amygdaloid nuclei, midbrain reticular nucleus, as well as well as many other areas of the BNST itself (Dong *et al*, 2004a). The BNST is a very diffuse region with many subnuclei. Additional studies completed in the magnocellular division of the BNST, posterior BNST, and medial BNST indicate that regions of the BNST are also important for control of reproductive behavior, social behavior, feeding behavior, and maternal behavior, in addition its involvement in the stress and reward pathway (Dong *et al*, 2004b, 2006a, b, c).

## **Role of the BNST in mediating anxiety and relapse to drug seeking behaviors**

The BNST has long been implicated in mediating responses to stress, and in particular anxiety, where anxiety is a response to an unconditioned stressor or long-term conditioned stressor (the closely related nucleus, the CeA, on the other hand is thought to be responsible for fear behavior, where fear is the response to a short-term conditioned stressor) (Alheid, 2003; Heimer, 2003; Henke, 1984; Walker *et al*, 2003). Both restraint stress and stimulation of the BNST produce similar changes in locomotor, exploratory, and stereotypic behavior, and stimulation of the BNST also leads to vigorous escape behavior and biting (Casada and Dafny, 1991). Similarly, lesion of the BNST in rats prior to a forced swim stress causes increased immobility and decreased escape behaviors, without changes in general motor behavior in an open field test, thus implicating the BNST in modulating responses to uncontrollable stress (Pezuk *et al*, 2008; Schulz and Canbeyli, 2000). Studies also demonstrate a role for the BNST, but not the CeA, in light-enhanced startle, where light is an unconditioned stressor (Davis *et al*, 1997). Additionally, inactivation of the BNST but not the more lateral amygdaloid nuclei, results in inhibition of TMT-induced freezing behavior (TMT is a component of fox odor (Fendt *et al*, 2003). Lesions of the BNST also reduce freezing and escape latency to an inescapable shock (Hammack *et al*, 2004; Schulz and Canbeyli, 1999). Thus, the BNST appears to play a role in behavioral responses to unconditioned stressors [but see (Gray *et al*, 1993; Pezuk *et al*, 2008; Treit *et al*, 1998)].

The BNST may also play a role in contextual fear responses to acute conditioned stressors, as lesions of the BNST produce anxiolytic-like effects in the Vogel conflict test and in contextual fear conditioning (Resstel *et al*, 2008; Sullivan *et al*, 2004). Lesions of the BNST also prevent enhanced classical eyeblink conditioning following footshock stress (Bangasser *et al*, 2005), and inactivation of the BNST impairs retention in a one-trial step-through inhibitory avoidance task (Liang *et al*, 2001). These studies demonstrate a role for the BNST in stress during learning. Finally, the BNST is known to also be involved in acute responses to visceral and somatic pain (Deyama *et al*, 2007).

Anxiety related to chronic stress, conditioned or unconditioned, also appears to be mediated by the BNST. Interestingly, Pego and others showed that chronic unpredictable stress increases measures of anxiety in the elevated plus maze, and also leads to increased volume and dendritic hypertrophy in the BNST (Pego *et al*, 2008). Lesions of the BNST block long term sensitization to the startle reflex and conditioned freezing to context (rather than cue) (Davis *et al*, 1997; Sullivan *et al*, 2004). Lesions of the BNST also block sensitization of the acoustic startle reflex produced by repeated stress (Gewirtz *et al*, 1998). Additionally, lesions of the BNST attenuate reinstatement of extinguished fear, but not fear conditioning to a short-term conditioned stimulus (Waddell *et al*, 2006). Data further supporting this role for the BNST show that lesions of the BNST do not block fear-potentiated startle or conditioned freezing using an explicit cue, suggesting that the BNST is not involved in modulating stress

responses to acute conditioned fear stimuli (Hitchcock and Davis, 1991; LeDoux *et al*, 1988).

The BNST is clearly involved in mediating anxiety-like responses to stressful unconditioned stimuli and long term responses to conditioned stimuli, and studies have also demonstrated a role for the BNST in stress-induced relapse to and withdrawal from drugs of abuse. Relapse to cocaine-seeking is blocked when the BNST is inhibited by infusion of GABA agonists (McFarland *et al*, 2004). Additionally, lesions of the BNST will block conditioned place aversion (CPA) to precipitated morphine withdrawal (Nakagawa *et al*, 2005). Finally, morphine-dependent abstinent rats, but not non-dependent rats, show elevated Fos expression in the BNST, and this expression negatively correlates with food preference in abstinent animals (morphine abstinent rats show reduced CPP for food-associated environments as compared to non-dependent rats) (Harris and Aston-Jones, 2003, 2007). Thus, it is clear that the BNST plays an important role in stress-induced relapse to drug-seeking, as well as anxiety stress and altered hedonic values during withdrawal (Aston-Jones and Harris, 2004).

### **Cell types, neurotransmitters in the BNST and their roles in stress and relapse**

Glutamate and GABA are classic neurotransmitters, providing excitatory and inhibitory neurotransmission in the BNST. Using *in vitro* whole cell recordings of BNST brain slices, Rainnie first demonstrated that electrical stimulation of cells in the BNST could evoke both excitatory and inhibitory post-synaptic potentials (EPSP and IPSC respectively), and showed that EPSPs could

be abolished by application of an AMPAR antagonist, while IPSPs could be nearly abolished by a GABA<sub>A</sub>-R antagonist (Rainnie, 1999). These and other findings were confirmed by Egli and Winder (2003). These investigators went on to show that a substantial number of cells exhibited a low-threshold spike (LTS) driven by T-type Ca<sup>2+</sup> channels, an I<sub>h</sub> channel-dependent depolarization, and inward rectification in response to increasing current injection steps. Egli and others also found regional differences in cells exhibiting these properties: cells in the vBNST typically exhibited a faster  $\tau$ , exhibited a LTS (74.5% vs only 22.9% in the dBNST), and were less likely to exhibit an I<sub>h</sub>-current (15.7% vs 48.6% in the dBNST) (Egli and Winder, 2003). Hammock and Rainnie have gone further to describe three types of neurons in the dorsal anterolateral BNST, designated Types I, II, and III, based on characterization of electrical properties (Hammock *et al*, 2007). Dumont and Williams recorded from projection neurons in the vBNST that were retrogradely labeled from the VTA. They found that these neurons exhibited different properties from non-labeled neurons in the vBNST and had a higher membrane resistance (R<sub>m</sub>), lower membrane capacitance, and were inwardly-rectifying as compared to nonlabeled neurons (Dumont *et al*, 2004).

Glutamatergic transmission can be regulated by a variety of glutamate receptors, including the ionotropic AMPARs and NMDARs which act as cation channels when activated, leading to depolarization and excitability changes; as well as metabotropic glutamate receptors (mGluRs) which are GPCRs. Group I mGluRs (mGluR1 and mGluR5) act via the G<sub>q</sub> coupled pathway, where as group

II (mGluR 2 and mGluR3) and group III mGluRs (mGluR4, mGluR6, mGluR7, and mGluR8) act via  $G_{i/o}$  coupled pathways. Fast excitatory transmission in the BNST can be abolished with AMPAR and NMDAR antagonists (Egli *et al*, 2003; Rainnie, 1999). High frequency stimulation can invoke an NMDAR-dependent LTP in the BNST which does not require the NR2A subunit of the NMDAR, and the early portion of this LTP is reduced by acute exposure to ethanol in a  $GABA_A$ R-dependent manner (Weitlauf *et al*, 2004; Weitlauf *et al*, 2005).

Dumont additionally showed that chronic morphine treatment selectively increases AMPA-dependent EPSCs in the dBNST of neurons projecting to the VTA (Dumont *et al*, 2008). Finally, rats trained to self-administer cocaine or food pellets show an increase in excitability of vBNST neurons via alterations in AMPAR/NMDAR ratios (Dumont *et al*, 2005). In animals receiving cocaine or food reward passively, however, these alterations were not observed, suggesting that the change in the AMPAR/NMDAR ratio could not have resulted from direct actions of these stimuli on the synapse but were likely mediated by alterations in synapses of the reward circuitry (Dumont *et al*, 2005).

Activation of group II and group III mGluRs has been shown to presynaptically decrease glutamate transmission in the BNST (Grueter and Winder, 2005; Muly *et al*, 2007), and activation of group II mGluRs leads to LTD, indicating a role for them in synaptic plasticity of the BNST (Grueter *et al*, 2005). Moreover, activation of mGluR5, a type I mGluR, leads to an extracellular signal-regulated kinase (ERK) 1-dependent LTD (Grueter *et al*, 2006a; Grueter *et al*, 2008). Additionally, this LTD is postsynaptically maintained as interference with

postsynaptic machinery via a dynamin-inhibitory peptide disrupts the late phase of the LTD (Grueter *et al*, 2008). The early phase of this LTD is CB1 receptor dependent (Grueter *et al*, 2006a). mGluR5 LTD in this region is disrupted 24 hours but not 10 days following multiple cocaine exposures through animals self-administering cocaine or via multiple noncontingent i.p. injections (Grueter *et al*, 2006a; Grueter *et al*, 2008). Grueter and colleagues were additionally able to show that cocaine promotes activation of the mGluR signaling pathway *in vivo* in the BNST resulting in occlusion or desensitization of mGluR5 (Grueter *et al*, 2008).

GABAergic transmission in the BNST has not been as extensively characterized as glutamatergic transmission in this region, though 70-90% of neurons in the BNST are thought to be GABAergic (Cullinan *et al*, 1993; McDonald, 1983; Sun *et al*, 1993) and the BNST receives a dense GABAergic input from the CeA (Dong *et al*, 2001a; Price *et al*, 1981; Weller *et al*, 1982). Studies to date are limited in their exploration of alterations in GABAergic transmission by stress or drugs of abuse. Neurotransmitters such as NE have been shown to modulate GABAergic transmission, however, and these studies will be discussed later in this section.

The BNST contains several neurotransmitters including DA, NE, and 5HT, that can potentially modulate glutamatergic and GABAergic synaptic transmission in this region. NE will be discussed later in this section. DA is distributed throughout the BNST, with its highest concentration found in the dorsolateral and ventral BNST, as inferred by the density of TH labeling (Phelix

*et al*, 1992a). Acute exposure to various drugs of abuse increases DA in the BNST (Carboni *et al*, 2000), and causes a DA-dependent activation of neurons in the BNST (Valjent *et al*, 2004). D1R antagonists but not D2R antagonists can reduce alcohol-motivated responding in rats trained to self-administer alcohol (Eiler *et al*, 2003). On the other hand, D1R antagonists injected in the dBNST of rats increase the rate of cocaine self-administration, indicating a role for D1Rs in self-administration (Epping-Jordan *et al*, 1998). DA application enhances excitatory transmission in the dBNST through activation of D1 and D2 receptors, and this enhancement is activity dependent and requires downstream activation of CRF1Rs (Kash *et al*, 2008). Both *in vivo* and *ex vivo* cocaine have been shown to induce a DA receptor- and CRF1R-dependent enhancement of NMDAR dependent short-term potentiation (Kash *et al*, 2008). The effect of DA on inhibitory transmission has not been examined.

The BNST receives a serotonergic input from the raphe nucleus, which is concentrated more heavily in the medial BNST and innervates CRF(+) nuclei of the BNST (Phelix *et al*, 1992a, b). Infusion of a 5-HT<sub>1R</sub> agonist into the BNST reduces the acoustic startle response, indicating an anxiolytic role for 5-HT in the BNST (Levita *et al*, 2004). Alterations in 5-HT fiber density in the BNST have also been suggested following cocaine exposure in hamsters (DeLeon *et al*, 2002). Rainnie has shown that 5-HT can postsynaptically modulate excitability of cells in the medial and lateral dBNST, and that 5-HT can also modulate neurons presynaptically, although the response of cells was variable (Rainnie, 1999). The



hyperpolarization response that many cells in the lateral dBNST exhibit in response to 5-HT is likely mediated through the 5-HT<sub>1A</sub>R (Levita *et al*, 2004).

The BNST also contains several neuropeptides including galanin (Morilak *et al*, 2003), neurotensin (Day *et al*, 2002; Ju *et al*, 1989b; Rainnie, 1999), orexin (Baldo *et al*, 2003; Nambu *et al*, 1999), enkephalin (Ju *et al*, 1989b; Kozicz, 2002; Rainnie, 1999), neuropeptide Y (NPY) (Walter *et al*, 1991), substance P (Walter *et al*, 1991), and oxytocin (Uhl-Bronner *et al*, 2005; Wilson *et al*, 2005). Many of these are known to modulate synaptic transmission in the BNST. NPY, a neuropeptide suggested to have anxiolytic effects (Bannon *et al*, 2000; Heilig *et al*, 1989), is known to inhibit GABAergic transmission in the BNST, likely by regulating GABA release via activation of the Y2R (Kash and Winder, 2006). Galanin in the BNST is anxiogenic, as a galanin antagonist can attenuate anxiety behaviors and ACTH plasma levels in response to immobilization stress (Morilak *et al*, 2003). Orexins have also been implicated in stress and anxiety and relapse to drug-seeking (Boutrel *et al*, 2005; Harris *et al*, 2005; Lawrence *et al*, 2006), although the role of orexin in the BNST has not been elucidated. Enkephalin is another neuropeptide in the BNST that is known to be involved in reward, positive mood, and euphoria, and the BNST contains high levels of the endogenous  $\mu$ -opioid receptor, which mediates the addictive properties of opiate drugs (Koob *et al*, 2001).

Perhaps most interesting is a large set of data pointing to the role of the stress messengers CRF and NE in the BNST on anxiety, stress of withdrawal from drugs of abuse, and stress-induced relapse to drug-seeking.

## CRF

The stress neuropeptide CRF in the BNST is critical for anxiety-like responses to stressful stimuli as well as for relapse to drug-seeking behaviors. Numerous studies have identified cell bodies expressing CRF throughout the BNST (Cummings *et al*, 1983; Ju and Han, 1989a; Moga *et al*, 1989; Olschowka *et al*, 1982; Phelix *et al*, 1992b; Sakanaka *et al*, 1987). Additionally, the BNST receives a CRF(+) projection from the CeA (Sakanaka *et al*, 1986). CRF mRNA is increased in the BNST following exposure to chronic mild uncontrollable stress (Kim *et al*, 2006). Infusion of the CRF antagonist,  $\alpha$ -helical CRF9-41, blocks reinstatement of extinguished fear (Waddell *et al*, 2008). Lesions of the BNST block CRF-enhanced acoustic startle, and infusions of a CRF antagonist into the BNST block CRF-enhanced acoustic startle and CRF-enhanced startle to bright lights, but not fear-potentiated startle (Davis *et al*, 1997; Lee and Davis, 1997). Additionally, infusions of CRF into the BNST mimic anxiety effects produced by ICV infusions of CRF (Lee *et al*, 1997). Infusions of CRF enhance retention of a one-trial step-through inhibitory avoidance task (Liang *et al*, 2001), and additionally lead to anorexia in food-deprived rats (Ciccocioppo *et al*, 2003). CRF antagonists injected into the BNST, on the other hand, attenuate stress-induced behaviors such as submissive-defensive behaviors following social defeat (Jasnow *et al*, 2004). Intra-BNST infusions of CRF increase anxiety as measured by the elevated plus maze, and these effects are blocked by co-infusion of a CRF1R, but not a CRF2R, antagonist (Sahuque *et al*, 2006). Rats also demonstrate conditioned place aversion (CPA) to a CRF-paired

environment, and this aversion is blocked by both CRF1R and CRF2R antagonists infused into the BNST (Sahuque *et al*, 2006).

CRF in the BNST also plays a role in relapse to cocaine-seeking, as infusion of the CRF antagonist D-Phe CRF12-41 block stress-induced reinstatement of cocaine-seeking (Erb and Stewart, 1999). Injections of CRF1R antagonists in the BNST also block stress-induced reinstatement of morphine CPP (Wang *et al*, 2006). Interestingly, ethanol-withdrawn animals have increased levels of CRF in the BNST, and this is reversed when animals are given access to ethanol but not control diet (Olive *et al*, 2002).

CRF projections from the CeA to the BNST are thought to be critical in CPA and relapse to drug-seeking behaviors (Smith *et al*, 2008). Lesions of these projections can block stress-induced reinstatement to cocaine-seeking behavior (Erb *et al*, 2001). Also, lesions of the CeA reduce opiate-withdrawal induced Fos activation in the BNST, but BNST lesions have no effects on Fos in the CeA, thus indicating a role for the projection of the CeA to the BNST in effects of opiate withdrawal (Nakagawa *et al*, 2005).

Electrophysiology studies looking at the effects of CRF on synaptic transmission in the BNST have also shed some light on how CRF modulates this region. In whole-cell recordings in the vBNST, bath application of CRF enhances the amplitude of evoked and miniature IPSCs (eIPSCs and mIPSCs, respectively), but does not alter the paired-pulse ratio (PPR) of eIPSCs or the frequency of mIPSCs (Kash *et al*, 2006). These and other findings suggest that the effects of CRF are mediated by postsynaptic CRF1Rs (Kash *et al*, 2006).

Interestingly, it appears that CRF and DA interact in the BNST. Whereas activation of CRF1R does not appear to alter eEPSCs in the vBNST, as demonstrated by lack an effect of the CRF1R agonist urocortin I (Kash *et al*, 2006); data from Kash and others does demonstrate an activity-dependent enhancement by DA on excitatory transmission in the dBNST that requires downstream activation of the CRF1R (Kash *et al*, 2008).

NE (see Table 3)

The BNST has one of the densest innervations of NE in the brain (Forray *et al*, 2004; Phelix *et al*, 1992a), and this projection comes primarily from the NTS, the NE nucleus responsible for stress-induced relapse to drug-seeking behaviors (see section on Afferents to the BNST). Immobilization stress, as well as chronic morphine treatment and withdrawal, significantly increase NE release into the BNST as measured by *in vivo* microdialysis (Cecchi *et al*, 2002a; Fuentealba *et al*, 2000; Pacak *et al*, 1995). Additionally, exposure to trimethylthiazoline (TMT), a component of fox odor, leads to increased NE in the BNST, and lesion of the BNST blocks freezing in response to the odor (Fendt *et al*, 2005). Infusion of NE into the BNST enhances retention in a one-trial step-through avoidance task (Liang *et al*, 2001). Also, increased NE in the BNST elicited by visceral and somatic pain stimuli is important for the negative affective component of these types of pain (Deyama *et al*, 2007).

*In vivo* recordings in the BNST of anesthetized rats have shown that infusion of NE in the BNST suppresses neuronal firing (Casada and Dafny, 1993). Additionally, microdialysis studies in *ex vivo* minislices of the BNST show

that NE inhibits glutamate release (Forsay *et al*, 1999), and that NMDA, an agonist at NMDARs, increase NE release (Aliaga *et al*, 1995; Forsay *et al*, 1995). In field recordings in the dorsal BNST, bath application of NE causes both excitation and inhibition of glutamatergic transmission, whereas bath application of NE in the vBNST always results in a depression of glutamatergic transmission (Egli *et al*, 2005). Further work using whole recordings demonstrate that NE causes a depression of glutamate transmission in the dorsal BNST, and with prolonged application leads to an  $\alpha_1$ -AR dependent LTD (McElligott and Winder, 2008). Work by Dumont and Williams looking at inhibitory transmission revealed that NE causes an increase in spontaneous GABA<sub>A</sub>-mediated IPSCs, and this is not altered in rats sacrificed following naloxone-precipitated withdrawal from morphine (Dumont *et al*, 2004).

Thus, NE appears to play a significant role in regulation of the BNST, and in particular anxiety and addiction behaviors. The adrenergic receptor subtypes mediating these effects have been parsed out to some extent. Current findings will be reviewed next.

Table 3. Known effects of norepinephrine (NE) in the BNST.

NE	Behavioral Findings	Electrophysiological Findings
	<p>Increases in NE in the BNST are elicited by</p> <ul style="list-style-type: none"> <li>• Immobilization stress (Cecchi <i>et al</i>, 2002a; Pacak <i>et al</i>, 1995)</li> <li>• Chronic morphine treatment and withdrawal (Fuentelba <i>et al</i>, 2000)</li> <li>• Exposure to TMT (Fendt <i>et al</i>, 2005)</li> <li>• Visceral and somatic pain stimuli (Deyama <i>et al</i>, 2007)</li> </ul> <p>Infusion of NE into the BNST</p> <ul style="list-style-type: none"> <li>• Enhances retention in a one-trial step-through avoidance task (Liang <i>et al</i>, 2001)</li> <li>• Suppresses neuronal firing in <i>in vivo</i> recordings of anesthetized rats (Casada <i>et al</i>, 1993)</li> </ul>	<p>NE causes</p> <ul style="list-style-type: none"> <li>• Inhibition of glutamate release in <i>ex vivo</i> minislices of the BNST in studies employing microdialysis (Forsay <i>et al</i>, 1999)</li> <li>• Both excitation and inhibition of glutamatergic transmission in field recordings in the dorsal BNST (Egli <i>et al</i>, 2005)</li> <li>• A depression of excitatory transmission that results in an <math>\alpha_1</math>-AR dependent LTD with prolonged NE application in whole cell recordings (McElligott <i>et al</i>, 2008)</li> <li>• A depression of glutamatergic transmission in the ventral BNST (Egli <i>et al</i>, 2005)</li> <li>• An increase in sIPSCs, that is unaltered in rats following naloxone-precipitated withdrawal from morphine (Dumont <i>et al</i>, 2004)</li> </ul> <p>NMDA infused into the BNST increases release of NE in microdialysis studies (Aliaga <i>et al</i>, 1995; Forsay <i>et al</i>, 1995)</p>

*β-adrenergic receptors (see Table 4)*

Infusion of a mixture of  $\beta_1$ -AR and  $\beta_2$ -AR antagonists into the BNST attenuates immobilization stress-induced reduction in open arm exploration in the elevated plus maze (Cecchi *et al*, 2002a). In animals showing high-reactivity to opioid withdrawal as measured by teeth chattering, eye twitching, and somatic signs, infusion of the  $\beta_1$ -AR antagonist betaxolol into the BNST dose-dependently reduces teeth chattering to levels similar to animals with low-reactivity to opioid withdrawal (Cecchi *et al*, 2007). Betaxolol injected into the BNST also blocks CPA to morphine withdrawal in high-reactivity animals (Cecchi *et al*, 2007). Additionally, infusion of  $\beta$ -AR antagonists into the BNST attenuates stress-induced reinstatement to cocaine-seeking (Leri *et al*, 2002), as well as opiate-withdrawal-induced CPA (Delfs *et al*, 2000). Intra-BNST infusion of the  $\beta_{1/2}$ -AR antagonist timolol prevents the development of behavioral sensitization to amphetamine (Colussi-Mas *et al*, 2005).  $\beta$ -AR antagonists in the BNST also block the affective component of pain elicited by visceral and somatic pain stimuli (Deyama *et al*, 2009; Deyama *et al*, 2008).  $\beta$ -ARs do not appear to be involved, however, in stress and learning as measured by retention of a one-trial step-through inhibitory avoidance task (Liang *et al*, 2001).

Electrophysiological recordings have been used to examine the effects of  $\beta$ -ARs on transmission in the BNST. Egli and others (2005) have shown that the  $\beta_{1/2}$ -AR agonist isoproterenol causes an increase in the N2 amplitude in field recordings, and an increase in sharp microelectrode EPSP slope, with no change in eEPSC amplitude in whole cell recordings in the dBNST. Interestingly, there

was no effect of isoproterenol in field recordings in the vBNST. They also saw a dual effect of NE on excitatory transmission, and found that the increase in excitatory transmission caused by application of NE is blocked by the  $\beta_{1/2}$ -AR antagonist timolol, but not the  $\beta_1$ -AR antagonist betaxolol. Application of the  $\beta_2$ -AR antagonist ICI-118,551 blunted the NE-induced increase of excitatory transmission. Interestingly, when application of NE caused an increase in excitatory transmission, subsequent application of the  $\beta_{1/2}$ -AR agonist isoproterenol led to a further increase in transmission. On the other hand, when NE led to a decrease in excitatory transmission, application of isoproterenol had no effect. These studies demonstrate a role for postsynaptic modulation of BNST neurons by activation of  $\beta_2$ -AR receptors in the dorsal, but not ventral, BNST (Egli *et al*, 2005). Dumont and Williams found that the  $\beta_{1/2}$ -AR antagonist propranolol reduced a NE-mediated increase in GABA<sub>A</sub>-IPSCs during acute morphine withdrawal, but that propranolol had no effect on this measure in control slices, demonstrating recruitment of  $\beta$ -ARs during opioid withdrawal (Dumont *et al*, 2004).



Table 4. Known functions of  $\beta$ -adrenergic receptors in the BNST

Adrenergic Receptor	Behavioral Findings	Electrophysiological Findings
$\beta$ -ARs	<p>Infusion of <math>\beta_{1/2}</math>-AR antagonists in the BNST</p> <ul style="list-style-type: none"> <li>• attenuates immobilization stress-induced reduction in open arm exploration in the elevated plus maze (Cecchi <i>et al</i>, 2002a)</li> <li>• attenuates stress-induced reinstatement to cocaine-seeking (Leri <i>et al</i>, 2002) and opiate withdrawal-induced CPA (Delfs <i>et al</i>, 2000)</li> <li>• prevents the development of behavioral sensitization to amphetamine (Colussi-Mas <i>et al</i>, 2005)</li> <li>• blocks the affective component of pain elicited by visceral and somatic pain stimuli (Deyama <i>et al</i>, 2009; Deyama <i>et al</i>, 2008)</li> </ul> <p>Infusion of a <math>\beta_1</math>-AR antagonist in the BNST</p> <ul style="list-style-type: none"> <li>• reduces signs of opioid withdrawal (Cecchi <i>et al</i>, 2007)</li> <li>• blocks CPA to morphine withdrawal in high-reactivity animals (Cecchi <i>et al</i>, 2007)</li> </ul>	<p>NE can both increase and decrease excitatory transmission in the BNST (Egli <i>et al</i>, 2005)</p> <ul style="list-style-type: none"> <li>• The <math>\beta_{1/2}</math>-AR antagonist timolol blocks the increase in excitatory transmission elicited by NE in field recordings, and the <math>\beta_2</math>-AR antagonist ICI-118,551 blunts this NE induced increase in excitatory transmission, but the <math>\beta_1</math>-AR antagonist betaxolol has no effect (Egli <i>et al</i>, 2005)</li> <li>• When NE increases excitatory transmission, isoproterenol further increases transmission, but when NE decreases excitatory transmission, isoproterenol has no effect (Egli <i>et al</i>, 2005)</li> </ul> <p>The <math>\beta_{1/2}</math>-AR agonist isoproterenol in the dorsal BNST</p> <ul style="list-style-type: none"> <li>• increases the N2 amplitude in field recordings</li> <li>• increases the EPSP slope in sharp microelectrode recordings</li> <li>• has no effect on eEPSC amplitude in whole cell recordings (Egli <i>et al</i>, 2005)</li> </ul> <p>The <math>\beta_{1/2}</math>-AR agonist isoproterenol in the ventral BNST</p> <ul style="list-style-type: none"> <li>• has no effect on N2 amplitude in field recordings (Egli <i>et al</i>, 2005)</li> </ul> <p>The <math>\beta_{1/2}</math>-AR antagonist reduces a NE-mediated increase in GABA<sub>A</sub>-IPSCs during acute morphine withdrawal, but not in control conditions (Dumont <i>et al</i>, 2004)</p>

*$\alpha_1$ -adrenergic receptors (see Table 5)*

Involvement of  $\alpha_1$ -ARs in the BNST has also been demonstrated in some studies. Blockade of  $\alpha_1$ -ARs in the BNST blocks the reduction in open-arm exploration in the elevated plus maze induced by acute immobilization stress, as well as reduces plasma levels of ACTH following immobilization stress (Cecchi *et al*, 2002a). Additionally, infusion of the  $\alpha_1$ -AR antagonist prazosin into the BNST impairs retention in a one-trial step-through avoidance task (Liang *et al*, 2001). Prazosin can also decrease self-administration in rats given long term, but not short term, access to cocaine, and rats given long term extended access to cocaine show decreased  $\alpha_1$ -AR-like immunoreactivity in neurons of the BNST, (Wee *et al*, 2008).

*In vivo* microdialysis studies show that infusion of prazosin in the BNST reduces NE but not glutamate or GABA levels in the BNST; and that prazosin increases  $K^+$ -induced glutamate release and decreases  $K^+$ -induced NE release (Forray *et al*, 1999) in the BNST. Activation of  $\alpha_1$ -ARs in whole cell recordings increases spontaneous IPSC frequency in the vBNST of animals exposed to morphine (Dumont *et al*, 2004). Using whole cell recordings, McElligott and Winder (2008) have shown that activation of  $\alpha_1$ -ARs in the BNST leads to LTD of excitatory transmission, and that blockade of  $\alpha_1$ -ARs can inhibit NE-induced LTD. Interestingly, whereas  $\alpha_1$ -AR mediated LTD is intact following acute cocaine exposure in mice, both NET KO and  $\alpha_{2A}$ -AR KO mice show disrupted  $\alpha_1$ -AR mediated LTD (McElligott *et al*, 2008), indicating a potential disruption of  $\alpha_1$ -AR function following chronic but not acute stress.

*$\alpha_2$ -adrenergic receptors (see Table 5)*

Infusion of  $\alpha_2$ -AR agonists in the BNST leads to decreased anxiety and drug-seeking. Injection of the  $\alpha_2$ -AR agonist clonidine into the BNST disrupts both acquisition and expression of fear-potentiated startle, and well as light-enhanced startle (Schweimer *et al*, 2005). Clonidine in the BNST also blocks increases in NE release elicited by TMT exposure (Fendt *et al*, 2005).  $\alpha_2$ -AR agonists in the BNST also block opiate withdrawal-induced CPA (Delfs *et al*, 2000), as well as stress-induced reinstatement of morphine-seeking (Wang *et al*, 2001). Activation of  $\alpha_2$ -ARs in the BNST also decreases extracellular NE levels in chronic morphine treated and 48hr withdrawn rats (Fumentalba *et al*, 2000). Interestingly, activation of  $\alpha_2$ -ARs decreases extracellular glutamate only in control, but not morphine-treated or withdrawn rats, suggesting morphine and withdrawal from morphine may lead to an increase in glutamatergic activity in the BNST (Fumentalba *et al*, 2000).

Forray and others have done multiple studies in the BNST examining the effect of  $\alpha_2$ -AR agonists on extracellular NE and glutamate levels using *in vivo* microdialysis, and have shown that infusion of the  $\alpha_2$ -AR agonist UK-14,304 in the BNST decreases extracellular NE and glutamate levels, while an  $\alpha_2$ -AR antagonist increases NE and glutamate levels (Forray *et al*, 1999).  $\alpha_2$ -AR agonists also decrease  $K^+$ -induced release of NE and glutamate, whereas  $\alpha_2$ -AR antagonists have the opposite effect (Forray *et al*, 1997, 1999). Work using minislices containing vBNST also demonstrated that  $\alpha_2$ -AR agonists decrease  $K^+$ -induced release of NE and glutamate in the BNST (Forray *et al*, 1995).

Using field and whole cell recordings, Egli and others have shown that the  $\alpha_2$ -AR agonist UK-14,304 causes a depression of excitatory transmission in both the dorsal and ventral BNST, and that this effect is absent in the dBNST and greatly reduced in the vBNST of  $\alpha_{2A}$ -AR KO mice (Egli *et al*, 2005). Application of UK-14,304 causes an increase in PPR, suggesting that  $\alpha_2$ -ARs modulate glutamatergic transmission presynaptically (Egli *et al*, 2005).

Limited studies have examined the role of  $\alpha_2$ -ARs on inhibitory transmission in the BNST. *In vivo* microdialysis studies in the BNST have shown that the  $\alpha_2$ -AR agonist UK-14,304 does not alter extracellular GABA levels in this region (Forray *et al*, 1999). Additionally, clonidine, a partial  $\alpha_2$ -AR agonist, does not alter GABA<sub>A</sub> receptor-mediated spontaneous transmission in the vBNST (Dumont *et al*, 2004). Further work is needed, however, to fully examine the potential role of  $\alpha_2$ -AR activation on modulation of GABAergic transmission.

Table 5. Known functions of  $\alpha$ -adrenergic receptors in the BNST

Adrenergic Receptor	Behavioral Findings	Electrophysiological Findings
$\alpha_1$ -ARs	<p>Infusion of <math>\alpha_1</math>-AR antagonists in the BNST</p> <ul style="list-style-type: none"> <li>• blocks the reduction in open-arm exploration in the elevated plus maze induced by acute immobilization stress (Cecchi <i>et al</i>, 2002a)</li> <li>• impairs retention in a one-trial step-through avoidance task (Liang <i>et al</i>, 2001)</li> <li>• decreases self-administration in rats given long-term, but not short-term, access to cocaine (Wee <i>et al</i>, 2008)</li> </ul> <p>Rats given long-term extended access to cocaine show decreased <math>\alpha_1</math>-AR-like immunoreactivity in neurons of the BNST (Wee <i>et al</i>, 2008)</p>	<p>The <math>\alpha_1</math>-AR antagonist prazosin</p> <ul style="list-style-type: none"> <li>• reduces NE but not glutamate or GABA levels in the BNST with <i>in vivo</i> microdialysis experiments (Forray <i>et al</i>, 1999)</li> <li>• increases K<sup>+</sup>-induced glutamate release and decreases K<sup>+</sup>-induced NE release in the BNST in <i>in vivo</i> microdialysis experiments (Forray <i>et al</i>, 1999)</li> <li>• inhibits NE-induced LTD in whole cell recordings (McElligott <i>et al</i>, 2008)</li> </ul> <p><math>\alpha_1</math>-AR agonists in the BNST</p> <ul style="list-style-type: none"> <li>• increases sIPSC frequency in the ventral BNST of animals exposed to morphine (Dumont <i>et al</i>, 2004)</li> <li>• causes an LTD of excitatory transmission in the dorsal BNST in whole cell recordings (McElligott <i>et al</i>, 2008)</li> </ul> <p><math>\alpha_1</math>-AR-mediated LTD is absent in NET KO and <math>\alpha_{2A}</math>-AR KO mice, but is intact following acute exposure to cocaine in wild type mice (McElligott <i>et al</i>, 2008)</p>
$\alpha_2$ -ARs	<p>Infusion of <math>\alpha_2</math>-AR agonists in the BNST</p> <ul style="list-style-type: none"> <li>• disrupts acquisition and expression of fear-potentiated startle and light-enhanced startle (Schweimer <i>et al</i>, 2005)</li> <li>• blocks opiate-withdrawal induced CPA (Delfs <i>et al</i>, 2000)</li> <li>• attenuates stress-induced reinstatement of morphine-seeking (Wang <i>et al</i>, 2001)</li> <li>• decreases extracellular NE levels in chronic morphine treated and 48 hr withdrawn rats (Fuentealba <i>et al</i>, 2000)</li> <li>• decreases extracellular glutamate levels in control, but not morphine-treated or withdrawn rats (Fuentealba <i>et al</i>, 2000)</li> <li>• blocks increases in NE release elicited by TMT exposure (Fendt <i>et al</i>, 2005)</li> </ul>	<p><math>\alpha_2</math>-AR agonists in the BNST</p> <ul style="list-style-type: none"> <li>• decrease extracellular NE and glutamate levels but do not alter GABA levels in <i>in vivo</i> microdialysis studies (Forray <i>et al</i>, 1999)</li> <li>• decrease K<sup>+</sup>-induced release of NE and glutamate (<math>\alpha_2</math>-AR antagonists have the opposite effect) in <i>in vivo</i> microdialysis studies (Forray <i>et al</i>, 1999) and in minislices containing the ventral BNST (Forray <i>et al</i>, 1995)</li> <li>• depress excitatory transmission in the dorsal BNST and this effect is absent in the <math>\alpha_{2A}</math>-AR KO mice (Egli <i>et al</i>, 2005)</li> <li>• depress excitatory transmission in the ventral BNST and this effect is diminished in the <math>\alpha_{2A}</math>-AR KO mice (Egli <i>et al</i>, 2005)</li> <li>• cause an increase in the PPR of excitatory transmission in whole cell recordings (Egli <i>et al</i>, 2005)</li> <li>• have no effect on GABA<sub>A</sub>-receptor mediated sIPSCs in the ventral BNST</li> </ul>

Finally, despite data implicating a tonic NE tone in the BNST (Forray *et al*, 1999), effects of  $\alpha_2$ -AR antagonists alone have not been examined using field or whole cell electrophysiology methods to determine the effects of potential tonic  $\alpha_2$ -AR activity in the BNST.

#### *Potential role of the $\alpha_{2A}$ -AR subtype*

To date, no studies have examined which subtype of the  $\alpha_2$ -AR mediates behavior and physiological findings of  $\alpha_2$ -AR agonists and antagonists in the BNST. A lack of effect of UK-14,304 in the  $\alpha_{2A}$ -AR KO mice suggests this subtype may be responsible, although  $\alpha_1$ -AR mediated LTD is also absent in this mouse (McElligott *et al*, 2008), indicating potential desensitization of remaining ARs, and therefore  $\alpha_{2C}$ -AR contributions to the effect of UK-14,304 cannot be ruled out. Similar findings in hippocampal and cortical tissue from  $\alpha_{2A}$ -AR KO mice have shown that  $\alpha_2$ -AR agonist-mediated inhibition of NE is disrupted in these mice, thus indicating an autoreceptor role of the  $\alpha_{2A}$ -AR (Trendelenburg *et al*, 1999; Trendelenburg *et al*, 2001). Therefore, the  $\alpha_{2A}$ -AR has been hypothesized to regulate stress and relapse behaviors due to its ability to regulate NE release (Stewart, 2000; Trendelenburg *et al*, 2001).

Recently, however, data has indicated that  $\alpha_{2A}$ -ARs may also regulate neurotransmission in the brain heterosynaptically, by modulating neurotransmitters other than NE. Gilsbach and others found that mice expressing the  $\alpha_{2A}$ -AR only under the DBH-promotor (and therefore presumably only in noradrenergic cells) could not rescue behavioral deficits in  $\alpha_{2A}$ -AR KO mice that are typically attributed to  $\alpha_{2A}$ -AR autoreceptors, such as analgesia,

hypothermia, sedation and anesthetic-sparing (Gilsbach *et al*, 2009). In the CeA, a structure closely related to the BNST,  $\alpha_2$ -ARs heterosynaptically regulate glutamate transmission via a direct interaction of the  $\beta\gamma$  subunit of the G-protein coupled receptor (GPCR) complex with release machinery (Delaney *et al*, 2007). Carey and Regehr have also demonstrated that activation of the  $\alpha_2$ -AR on climbing fiber-Purkinje cell synapses in the cerebellum can alter associative learning by disrupting LTD elicited via near-simultaneous activation of parallel fiber-Purkinje cell and climbing fiber-Purkinje cell synapses (Carey *et al*, 2009). They demonstrate that  $\alpha_2$ -ARs heterosynaptically modulate glutamatergic transmission selectivity at climbing fiber-Purkinje cell synapses via a presynaptic mechanism (Carey *et al*, 2009). Additionally,  $\alpha_{2A}$ -ARs in the prefrontal cortex mediate depression of excitatory transmission, although likely through a postsynaptic mechanism (Ji *et al*, 2008). The  $\alpha_{2A}$ -AR selective agonist guanfacine improves performance on working memory tasks via inhibition of postsynaptic cAMP-HCN channel signaling in the prefrontal cortex (Wang *et al*, 2007), and other studies support these findings (Barth *et al*, 2008; Carr *et al*, 2007).

Studies using electron microscopy to localize the  $\alpha_{2A}$ -AR also support a potential heterosynaptic role for the receptor. In the NTS, ventrolateral medulla, and hippocampus,  $\alpha_{2A}$ -ARs have been noted on presynaptic autoreceptor and heterosynaptic terminals, as well as postsynaptically, and on glial cells (Glass *et al*, 2001; Milner *et al*, 1998; Milner *et al*, 1999). Thus, it is possible that  $\alpha_2$ -ARs may regulate excitatory synaptic transmission in the BNST indirectly by regulating NE release (as a presynaptic autoreceptor), directly by regulating

glutamate release (as a presynaptic heteroreceptor), and/or by altering the response to glutamate postsynaptically (as a postsynaptic receptor) (see Figure 4).

#### Potential CRF-NE interactions in the BNST

Finally, several authors have proposed that CRF and NE interact in the brain, and data indicate that NE may regulate CRF release through activation of the  $\alpha_1$ -AR (Berridge and Dunn, 1989b; Forray *et al*, 2004; Smith *et al*, 2008; Stewart, 2000), although an interaction between  $\alpha_{2A}$ -ARs or  $\beta$ -ARs and CRF cannot be ruled out. Authors have pointed to the BNST as a potential region for this interaction (Forray *et al*, 2004; Stewart, 2000), yet no studies to date have looked at co-distribution of ARs and CRF.



Figure 4

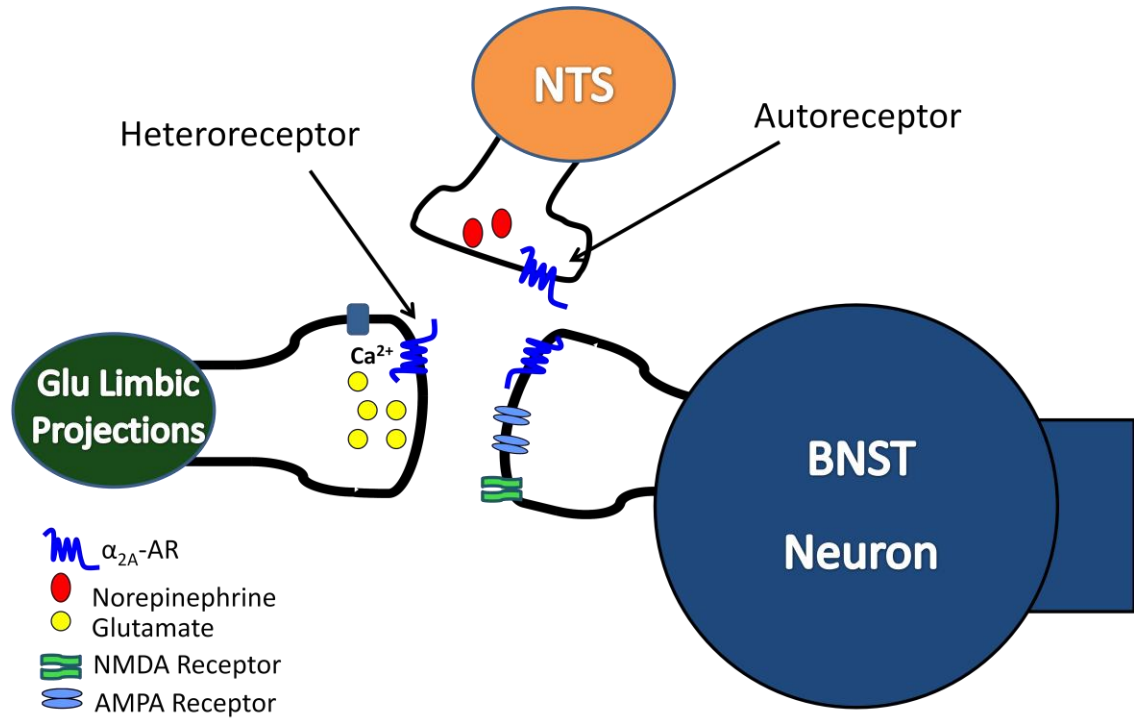


Figure 4.  $\alpha_{2A}$ -ARs may potentially regulate glutamate transmission in the BNST indirectly by altering NE release at NE terminals (as an autoreceptor), or more directly by regulating release of glutamate at glutamatergic terminals (as a heteroreceptor), or by altering the postsynaptic response to glutamate (as a postsynaptic receptor).

## **Summary**

The BNST is a critical relay in mediating behavioral and physiological responses to stress and anxiety. Stressors such as withdrawal from drugs of abuse lead to increases in NE in the BNST, and  $\alpha_2$ -AR agonists infused in the BNST block stress-induced reinstatement to many drugs of abuse.

Electrophysiology studies have examined the role of  $\alpha_2$ -ARs on excitatory transmission in the BNST, but the role of the  $\alpha_{2A}$ -AR subtype is currently unclear. In addition, although studies using the  $\alpha_2$ -AR antagonist yohimbine indicate that systemic injections of yohimbine indeed elicit anxiety-like behaviors, the exact mechanism of the action of yohimbine is debated. Finally, most studies elucidating the anatomical inputs to the BNST have been conducted in rat, and despite many behavioral and physiology studies conducted in mouse, no anatomical studies have confirmed these inputs to the BNST in the mouse model.

## **Hypothesis**

NE modulates glutamatergic and GABAergic transmission of cells in the dBNST through heterosynaptic  $\alpha_{2A}$ -ARs.

## **Specific Aims**

Specific Aim 1 To test the hypothesis that activation of  $\alpha_{2A}$ -ARs in the dBNST modulates glutamatergic and GABAergic transmission of cells in the dBNST.

Specific Aim 2 To test the hypothesis that  $\alpha_{2A}$ -ARs localize to heterosynaptic terminals in the dBNST.

Specific Aim 3 To test the hypothesis that yohimbine affects glutamate transmission in the dBNST through a non- $\alpha_2$ -AR dependent mechanism.

## CHAPTER II

### THE $\alpha_2$ AR HETEROSYNAPTICALLY MODULATES GLUTAMATERGIC TRANSMISSION IN THE BNST

This chapter describes work completed toward the fulfillment of Aims I and II of my thesis project.

#### **Introduction**

Stress has been implicated as a major driving force in drug addiction, playing a significant role in relapse to drug- and alcohol seeking (Brown *et al*, 1995; Le *et al*, 2000; Sinha *et al*, 1999). Relapse to drug-seeking can be modeled in rodents using behavioral paradigms such as stress-induced reinstatement of drug-seeking (Shaham *et al*, 2000a) and conditioned place preference (Wang *et al*, 2001). The bed nucleus of the stria terminalis (BNST), part of a region known as the extended amygdala, has been suggested to play an important role in general anxiety and relapse to drug abuse (Walker *et al*, 2003). The BNST is well-situated to process inputs from other brain regions involved in stress and reward pathways, such as the insular and infralimbic cortices, hippocampus, and the central and basolateral nuclei of the amygdala (Chiba *et al*, 2001; Shin *et al*, 2008; Weller *et al*, 1982). The BNST sends projections to many brain regions, including the hypothalamus (Conrad and Pfaff, 1976; Swanson and Cowan, 1979), a region involved in the stress response; and the ventral tegmental area (VTA) (Georges *et al*, 2002; Swanson *et al*, 1979), a region involved in reward and motivation. Thus, the BNST is an important center

for processing inputs from stressful stimuli and integrating them into stress and reward circuitry.

The noradrenergic system is a key mediator of the stress response, and has been implicated in relapse to drug addiction. The BNST receives one of the densest noradrenergic (NE) inputs in the brain (Brownstein *et al*, 1974). This input arises primarily from the nucleus tractus solitarius (NTS) which makes up part of the ventral noradrenergic bundle (VNAB) (Aston-Jones *et al*, 1999). Lesions of the VNAB result in the inhibition of footshock-induced reinstatement of morphine-seeking (Wang *et al*, 2001) and heroin-seeking (Shalev *et al*, 2001). Withdrawal from chronic morphine treatment increases extracellular norepinephrine (NE) levels in the rat BNST (Fuentelba *et al*, 2000). Lesions of the VNAB have also been shown to block opiate withdrawal-induced conditioned place aversion (Aston-Jones *et al*, 1999). Also, mice lacking the enzyme dopamine beta-hydroxylase (DBH) do not exhibit morphine-induced conditioned place preference (CPP). Viral restoration of DBH in the NTS but not the locus coeruleus (the source of NE in the dorsal noradrenergic bundle) of these mice can restore CPP for morphine, indicating the importance of NE in this paradigm (Olson *et al*, 2006). Importantly, NE has been shown to suppress neuronal firing in the BNST and studies indicate that the BNST output is under noradrenergic tone (Casada *et al*, 1993; Dumont *et al*, 2004; Egli *et al*, 2005; Forray *et al*, 1997). In total, these data strongly implicate NE in the BNST in stress-reward related behaviors.

NE is known to act through  $\beta$ -adrenergic receptors ( $\beta$ -ARs),  $\alpha_1$ -adrenergic receptors ( $\alpha_1$ -ARs), and  $\alpha_2$ -adrenergic receptors ( $\alpha_2$ -ARs) in the BNST (Dumont *et al*, 2004; Egli *et al*, 2005; Matsui and Yamamoto, 1984; McElligott *et al*, 2008). Evidence suggests that  $\alpha_2$ -ARs within the BNST play important roles in stress-addiction interactions. Peripheral administration of blood-brain-barrier permeant  $\alpha_2$ -AR agonists blocks foot-shock induced reinstatement of heroin seeking (Shaham *et al*, 2000b), and alcohol-seeking in rats (Le *et al*, 2005). Intra-BNST injections of  $\alpha_2$ -AR agonists block morphine withdrawal-induced conditioned place aversion (Delfs *et al*, 2000), and stress-induced reinstatement of morphine-conditioned place preference (Wang *et al*, 2001). Intra-BNST injection of the  $\alpha_2$ -AR agonist clonidine also blocks stress-induced freezing behavior induced by the innate stressor trimethylthiazoline, a component of fox feces (Fendt *et al*, 2005).

Previous electrophysiology studies have implicated  $\alpha_2$ -ARs in the modulation of glutamatergic and potentially GABAergic transmission in the BNST. The non-subtype specific  $\alpha_2$ -AR agonists UK-14,304 and clonidine cause a decrease in evoked excitatory transmission in the BNST and this effect is absent in brain slices prepared from  $\alpha_{2A}$ -AR knockout (KO) mice (Egli *et al*, 2005). Paired-pulse ratios of evoked synaptic responses are increased by UK-14,304, suggesting a presynaptic modulation of glutamate release by  $\alpha_2$ -ARs (Egli *et al*, 2005). Although NE is known to modulate both glutamatergic and GABAergic transmission in the BNST through  $\alpha_1$ -AR and  $\beta$ -ARs (Dumont *et al*, 2004; Egli *et al*, 2005; McElligott *et al*, 2008), the modulation of inhibitory transmission by  $\alpha_2$ -ARs in naïve mice has not been extensively studied. The  $\alpha_2$ -

AR partial agonist clonidine does not alter GABA<sub>A</sub> receptor-mediated spontaneous transmission in the ventral BNST, suggesting that  $\alpha_2$ -ARs may not modulate GABA transmission (Dumont *et al*, 2004).

There are three known  $\alpha_2$ -AR subtypes,  $\alpha_{2A}$ -AR,  $\alpha_{2B}$ -AR, and  $\alpha_{2C}$ -ARs, in mammalian brain tissues (Bylund *et al*, 1994). The  $\alpha_{2A}$ -AR has a widespread mRNA distribution in the brain including, but not limited to, regions involved in mediation of stress and anxiety, such as the NTS, amygdaloid complex, hypothalamus, and cortex (Scheinin *et al*, 1994; Wang *et al*, 1996). The  $\alpha_{2C}$ -AR has a similar though not as widespread mRNA distribution as compared to the  $\alpha_{2A}$ -AR, whereas  $\alpha_{2B}$ -AR mRNA expression is mainly limited to the thalamus (Scheinin *et al*, 1994; Wang *et al*, 1996). Studies have implicated the  $\alpha_{2A}$ -AR as a major subtype utilized by NE to modulate neuronal function in the brain, although the  $\alpha_{2C}$ -AR also plays a role in this modulation (Trendelenburg *et al*, 2001). Egli and others have shown that the effects of the  $\alpha_2$ -AR agonist UK-14,304 on excitatory transmission are absent in the dBNST and dramatically reduced in the vBNST of the  $\alpha_{2A}$ -AR knockout mouse (Egli *et al*, 2005). However, additional data indicate that actions of other ARs in BNST are also absent in these knockout mice, raising the possibility that these phenotypes may be more related to autoreceptor rather than heteroreceptor roles of the  $\alpha_{2A}$ -AR (Egli *et al*, 2005; McElligott *et al*, 2008). Similarly, hippocampal and cortical tissue from  $\alpha_{2A/D}$ -AR KO mice show substantial loss of  $\alpha_2$ -AR agonist-mediated inhibition of NE release, suggesting that these mice have disruptions in autoreceptor-mediated inhibition of NE (Trendelenburg *et al*, 1999;

Trendelenburg *et al*, 2001). Recently, however, research in mice expressing the  $\alpha_{2A}$ -AR in only adrenergic neurons (autoreceptors), has suggested that many functions previously attributed to the role of autoreceptors such as analgesia, hypothermia, sedation, and anesthetic-sparing, may actually be mediated by  $\alpha_{2A}$ -ARs on non-noradrenergic (heterosynaptic) neurons (Gilsbach *et al*, 2009). Studies in the central nucleus of the amygdala also point to a heterosynaptic function of  $\alpha_2$ -ARs in the brain (Delaney *et al*, 2007). We have therefore focused our current studies on the  $\alpha_{2A}$ -AR subtype, with the hypothesis that heterosynaptic  $\alpha_{2A}$ -ARs modulate acute synaptic transmission in the BNST.

We have taken advantage of a hemagglutinin  $\alpha_{2A}$ -AR knockin (HA  $\alpha_{2A}$ -AR KI) mouse to localize the  $\alpha_{2A}$ -AR within the BNST. Using the  $\alpha_{2A}$ -AR selective agonist guanfacine, we have characterized the ability of  $\alpha_{2A}$ -ARs to modulate both excitatory and inhibitory transmission in the dorsal BNST. We provide evidence that the  $\alpha_{2A}$ -AR functions in the BNST as a heteroreceptor regulating glutamate release presynaptically, with a more modest action on GABAergic transmission.

## **Experimental Procedures**

All procedures were performed according to Institutional Animal Care and Use Committee approved procedures.



## **Light Microscopy Level Immunohistochemistry Studies**

### Fluorescent Immunohistochemistry

A minimum of 10 adult male 6-14 week old C57BL/6J mice (Jackson Laboratory), a minimum of 10 male and female 6-14 week old HA-tagged  $\alpha_{2A}$ -AR knockin (KI) mice (backcrossed to C57BL/6 background for 10 generations, provided by Qin Wang at University of Alabama), and two male 6-14 week old NET KO mice (provided by Randy Blakely at Vanderbilt University) were transcardially perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde for 2-3 days at 4°C, and were then transferred to 30% sucrose until fully impregnated.

Following post-fixation, 30  $\mu$ m coronal or 30  $\mu$ m sagittal sections of brain were sliced on a cryostat (Leica CM3050S). Sections containing the BNST were then free-floated for immunolabeling. Sections were blocked with 4% normal donkey serum containing 0.2% Triton-X-100 in PBS. Sections were then incubated with primary antibody for 48 hours at 4°C, followed by incubation with cyanine dye- or Alexa fluorophore-conjugated secondary antibody for 24 hours at 4°C. Sections were mounted on slides, sealed with PolyAquamount, and left overnight to dry.

#### *Reagents used*

Mouse anti-HA from Chemicon was used at 1:500-1:1000, guinea-pig anti-VGLUT1 from Chemicon was used at 1:8000, mouse anti-NET contributed from Randy Blakely was used at 1:1000, rabbit anti-TH from Chemicon was used at 1:1000. Secondary antibodies used from Jackson ImmunoResearch included: cy2- and cy3-conjugated donkey anti-mouse (1:1000), cy5-conjugated donkey

anti-guinea pig (1:2000), and cy3-conjugated donkey anti-rabbit (1:1000).

Secondary antibodies used from Molecular Probes included: Alexa 568-conjugated goat anti-mouse IgG2b and Alexa 488-conjugated goat anti-mouse IgG1.

In the first immunohistochemistry experiment (Figure 2), mouse anti-HA was used at 1:500 and cy2-conjugated donkey anti-mouse was used at 1:1000. In the second immunohistochemistry experiment (Figure 4A-C), mouse anti-HA (IgG1) was used at 1:1000 and mouse anti-NET (IgG2b) was used at 1:1000. Alexa-488-conjugated goat anti-mouse IgG1 was used at 1:1000, and Alexa-568-conjugated goat anti-mouse IgG2b was used at 1:1000. In the third immunohistochemistry experiment (Figure 4D-F, 5D-F), mouse anti-HA was used at 1:1000 and rabbit anti-TH was used at 1:1000. Cy2-conjugated donkey anti-mouse was used at 1:2000, and cy3-conjugated donkey anti-rabbit was used at 1:1000. In the final immunohistochemistry experiment (Figure 4G-I, 5A-C), mouse anti-HA was used at 1:1000 and guinea pig anti-VGLUT1 was used at 1:8000. Cy2-conjugated donkey anti-mouse was used at 1:1000, and cy5-conjugated donkey anti-guinea pig was used at 1:2000.

### Imaging

Images were taken with an LSM510 confocal microscope using a 10x objective and a 63x/1.4 NA planapochromat objective (Carl Zeiss, Inc.). Laser excitation was kept constant between genotypes and primary/no primary controls. High-power (63x) images (optical sections) were taken at 0.5  $\mu\text{m}$  focus intervals. Under conditions identical for those used to obtain sample images,

we imaged microscopic fluorescent latex beads (Multispeck, Molecular Probes, Inc.) with spectral properties similar to the three sample probes (Cy2, Cy3, and Cy5) in order to calibrate the three-channel registration and subsequently compensate for instrument-induced positional shifts in the data. Measured shifts were minor and used to apply precise offsets to the image data using the LSM software, thus providing accurate probe positions to within the diffraction limit of the microscope (less than 0.25  $\mu\text{m}$  in x and y). Using CorelDraw12, images in Figure 2 were converted to monochrome, and intensity, brightness, and contrast were altered to allow clear presentation of signal. Using ImageJ software, Figures 4 and 5 were subtracted for background and were then altered in brightness and contrast to allow clear presentation of signal.

### **Electrophysiological recordings in BNST slices**

#### Animals

6-12 week old male C57BL/6J mice (The Jackson Laboratory) or HA  $\alpha_{2A}$ -AR KI mice were housed in cages of two to five animals on a 12 h light/dark cycle with food and water *ad libitum*. The number of animals used in each experiment is indicated in the text and figure legends.

#### Brain slice preparation

Methods were as described previously (Grueter *et al*, 2005; Grueter *et al*, 2006b). Briefly, mice were decapitated under isofluorane anesthesia. Brains were quickly removed and placed in an ice-cold, low-sodium/high-sucrose dissecting solution. Hemisected (300  $\mu\text{m}$ ) coronal brain slices containing anterior portions of BNST (bregma 0.26–0.02 mm) were prepared on a Leica vibratome and

transferred to either an interface chamber (field potential recordings) or a holding chamber (whole cell recordings) containing oxygenated and heated artificial cerebrospinal fluid (ACSF) composed of the following (in mM): 124 NaCl, 4.4 KCl, 2.5 CaCl<sub>2</sub>, 1.3 MgSO<sub>4</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, 10 glucose, and 26 NaHCO<sub>3</sub>.

### Field potential recordings

Field potential recordings were performed as previously reported (McElligott *et al*, 2008). Briefly, slices were transferred immediately after slicing to interface chambers where they rested for at least 30 min in a humidified and oxygenated environment while continuously being perfused with oxygenated and heated (approximately 28–30°C) ACSF at a rate of 2 ml/min. Next, 25 µM picrotoxin was added to the bath to block GABA<sub>A</sub> receptors and slices were rested another 30 min prior to recording. Picrotoxin was included during the entirety of all experiments to isolate excitatory transmission. Recording electrodes of approximately 1 MΩ resistance were pulled on a Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA) and filled with ACSF. A bipolar Nichrome (A-M Systems, Carlsborg, WA) stimulating electrode was placed dorsal to the recording electrode within the dorsal lateral BNST such that stimulation of the field resulted in two distinguishable negative shifts in potential: N1 (the TTX sensitive fiber volley estimate) and N2 (CNQX sensitive synaptic response) as previously reported (Egli *et al*, 2005; Grueter *et al*, 2005; Weitlauf *et al*, 2004). The amplitude (voltage) of the N2 was measured at a stimulation intensity that resulted in a voltage approximately 50% of the maximum N2 response. Slices were stimulated at a frequency of 0.05 Hz. Field potentials were

recorded using Clampex 8.2 (Molecular Devices, Sunnyvale, CA). All drugs were bath applied at their final concentrations.

#### *Reagents used*

UK-14,304 (Sigma) stock solution was made in DMSO and added directly to the ACSF.

#### *Analysis of Field Recordings*

All recorded data were analyzed via Clampfit 9.0 (Molecular Devices). All field recordings contain a 20 min baseline recording prior to agonist application (with the exception of one 10  $\mu$ M UK-14,304 experiment completed in wildtype C57BL/6J, which had a 10 min baseline) and all data points were normalized to the baseline 5 min prior to the agonist application. Plotted time courses for field experiments are represented as 1 min averages. Minutes 21-25 post-drug application were also averaged for statistical analysis and comparison to baseline.

#### Whole-cell voltage-clamp recordings

Whole-cell recordings were performed as previously reported (Grueter *et al*, 2006a; Grueter *et al*, 2005; Kash *et al*, 2006). Briefly, following slicing on the vibratome, slices were allowed to recover for a minimum recovery period of 60 minutes in a submerged holding chamber (25°C) containing oxygenated ACSF. Slices were then removed from the holding chamber and placed in the recording chamber, where they were continuously perfused with oxygenated (95% O<sub>2</sub>/5% CO<sub>2</sub>) and heated (approximately 24-25°C) ACSF at a rate of 2 ml/min.

### *Experiments examining excitatory transmission in the BNST*

Electrodes of 2.5–7.0 M $\Omega$  were filled with the following for experiments examining excitatory transmission (in mM): 117 Cs gluconate, 20 HEPES, 0.4 EGTA, 5 TEA, 2 MgCl, 4 Na<sub>2</sub>ATP, 0.3, Na<sub>2</sub>GTP (pH 7.2-7.4, Osm 290-295). Two experiments looking at the effect of 1  $\mu$ M UK-14,304 were conducted using electrodes filled with (in mM): 135 K<sup>+</sup>-gluconate, 5 NaCl, 10 HEPES, 0.6 EGTA, 4 Na<sub>2</sub>ATP, 0.4 Na<sub>2</sub>GTP (pH 7.2-7.4, Osm 290-295), but no difference was noted, so results were combined. EPSCs of 100–400 pA were recorded at a frequency of 0.17 Hz while voltage-clamped at –70 mV in the presence of the GABA<sub>A</sub> receptor antagonist, picrotoxin (25  $\mu$ M). After whole-cell configuration was achieved, cells were allowed to equilibrate a minimum of 10 minutes before baseline recordings were started. Postsynaptic parameters were monitored continuously throughout the duration of the experiments. Data are represented as an average of the peak amplitudes of ten sweeps (1 min). A paired-pulse ratio (PPR) was acquired by applying a second stimulus of equal intensity 50 ms after the first stimulus, where  $PPR = EPSC_2/EPSC_1$ .

### *Experiments examining inhibitory transmission in the BNST*

For IPSC experiments, electrodes of 2.5–7.0 M $\Omega$  were filled with 70 K<sup>+</sup>-gluconate, 70 Cs gluconate, 1 EGTA, 5 HEPES, 4 Na<sub>2</sub>ATP, 0.3 Na<sub>2</sub>GTP (pH 7.2-7.4, Osm 290-295). IPSCs of 100-400 pA were recorded at frequency of 0.10Hz while voltage-clamped at -70 mV in the presence of 4 mM kynurenic acid to block excitatory transmission. After whole-cell configuration was achieved, cells were allowed to equilibrate a minimum of 10 min before baseline recordings were

started. Postsynaptic parameters were monitored continuously throughout the duration of the experiments. Data are represented as an average of the peak amplitudes of six sweeps (1 min). A paired-pulse ratio (PPR) was acquired by applying a second stimulus of equal intensity 50 ms after the first stimulus, where  $PPR = EPSC_2/EPSC_1$ .

#### *Reagents used*

UK-14,304 (Sigma) stock solution was made in DMSO, guanfacine hydrochloride (Tocris) stock solution was made in water, picrotoxin (Tocris) stock solution was made in DMSO, and atipamezole hydrochloride (Pfizer Animal Health) and kynurenic acid (Sigma) were added directly to ACSF.

#### *Analysis of whole-cell recordings*

Recorded data were analyzed via Clampfit 9.2 (Molecular Devices). Recordings contain a 5-10 min baseline recording prior to agonist application, and all data points were normalized to the baseline 5 min prior to agonist application. Plotted time courses for whole-cell experiments are represented as 1 min averages. Minutes 16-20 post-drug application were also averaged for statistical analysis and comparison to baseline.

#### Statistics

All data points were reported as the mean +/- SEM and significance (determined by paired and unpaired Student's *t*-test). Experiments examining a difference as compared to baseline were analyzed using a paired Student's *t*-test.

Experiments comparing relative effects of a drug across separate conditions were analyzed using an unpaired student's *t*-test (Figure 1B comparing effects of

UK-14,304 between HA KI and WT mice, Figure 7 comparing effects of guanfacine between excitatory and inhibitory transmission). Results are reported in the text and figure legends. Significant differences were defined as having a  $p < 0.05$ .

## **Results**

### **The HA $\alpha_{2A}$ -AR KI mouse contains functional $\alpha_{2A}$ -ARs**

The HA  $\alpha_{2A}$ -AR KI mouse is a recently described line (Lu *et al*, 2009) in which an  $\alpha_{2A}$ -AR with an N-terminal HA tag is inserted into the endogenous allele. Lu *et al*. have demonstrated that the N-terminal HA tag on the  $\alpha_{2A}$ -AR does not appreciably alter receptor density or coupling (Lu *et al*, 2009). Thus this mouse affords a model for genotypically-controlled analysis of  $\alpha_{2A}$ -AR expression and localization.

We have previously reported that the  $\alpha_2$ -AR agonist UK-14,304 causes a depression in excitatory transmission in the BNST and that this response is absent in slices prepared from the  $\alpha_{2A}$ -AR KO mouse (Egli *et al*, 2005). To grossly assess whether  $\alpha_2$ -AR function in the BNST is normal in the knock-in line, we assessed the ability of the  $\alpha_2$ -AR agonist UK-14,304 (10  $\mu$ M) to depress synaptic transmission. Using extracellularly recorded field potentials (Egli *et al*, 2005; Grueter *et al*, 2005; McElligott *et al*, 2008; Weitlauf *et al*, 2005) we show that 10  $\mu$ M UK-14,304 depresses excitatory transmission in both wildtype mice (68.4  $\pm$  9.9% from baseline,  $n = 4$ ,  $p < .01$ ,  $t = 7.50$ ,  $df = 3$ ) and HA KI mice



(90.7 +/- 5.1% from baseline, n = 4, p < .001, t = 17.47, df = 3), and that the HA KI mouse has functional  $\alpha_2$ -ARs, as the response to 10  $\mu$ M UK-14,304 was not different from that observed in slices prepared from wildtype animals (p > .07, t = -2.14, df = 5) (Figure 5B).

Figure 5

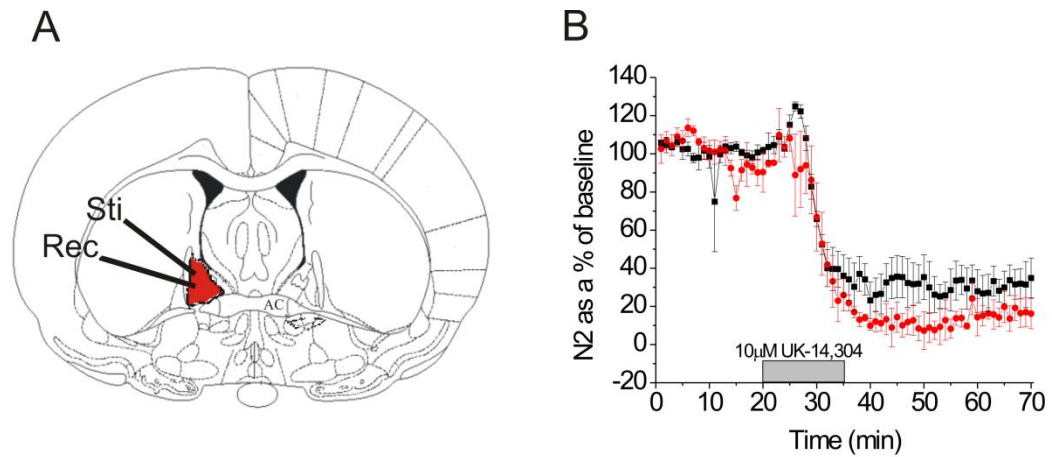


Figure 5. The HA  $\alpha_{2A}$ -adrenergic receptor knockin mouse (HA  $\alpha_{2A}$ -AR KI) contains functional  $\alpha_{2}$ -ARs. A) Coronal brain section demonstrating placement of stimulating and recording electrodes for field electrophysiology experiments. B) A 10 min 10  $\mu$ M UK-14,304 application causes a depression in excitatory transmission in the HA  $\alpha_{2A}$ -AR KI (red, n = 4) that is not different from wild type C57BL/6J mice (black n = 4).

### **The $\alpha_{2A}$ -AR is highly expressed in the BNST**

We examined the distribution of the  $\alpha_{2A}$ -AR in the knockin mouse at the light microscopy level utilizing an anti-HA antisera. High levels of HA labeling (presumptive  $\alpha_{2A}$ -AR immunoreactivity) were found in the locus coeruleus, NTS, hippocampus, and lateral septum, as expected based on previous findings (Glass *et al*, 2001; Lee *et al*, 1998; Milner *et al*, 1998; Scheinin *et al*, 1994; Wang *et al*, 1996) (see Figure 6A). In addition, intense labeling was observed in the BNST. The  $\alpha_{2A}$ -AR immunoreactivity was present throughout both the dorsal and ventral portions of the anterior BNST, with brightest labeling in the stria terminalis (Figure 6B). Wildtype tissue incubated with the mouse anti-HA antibody did not show appreciable immunolabeling (Figure 6C).

Figure 6

A

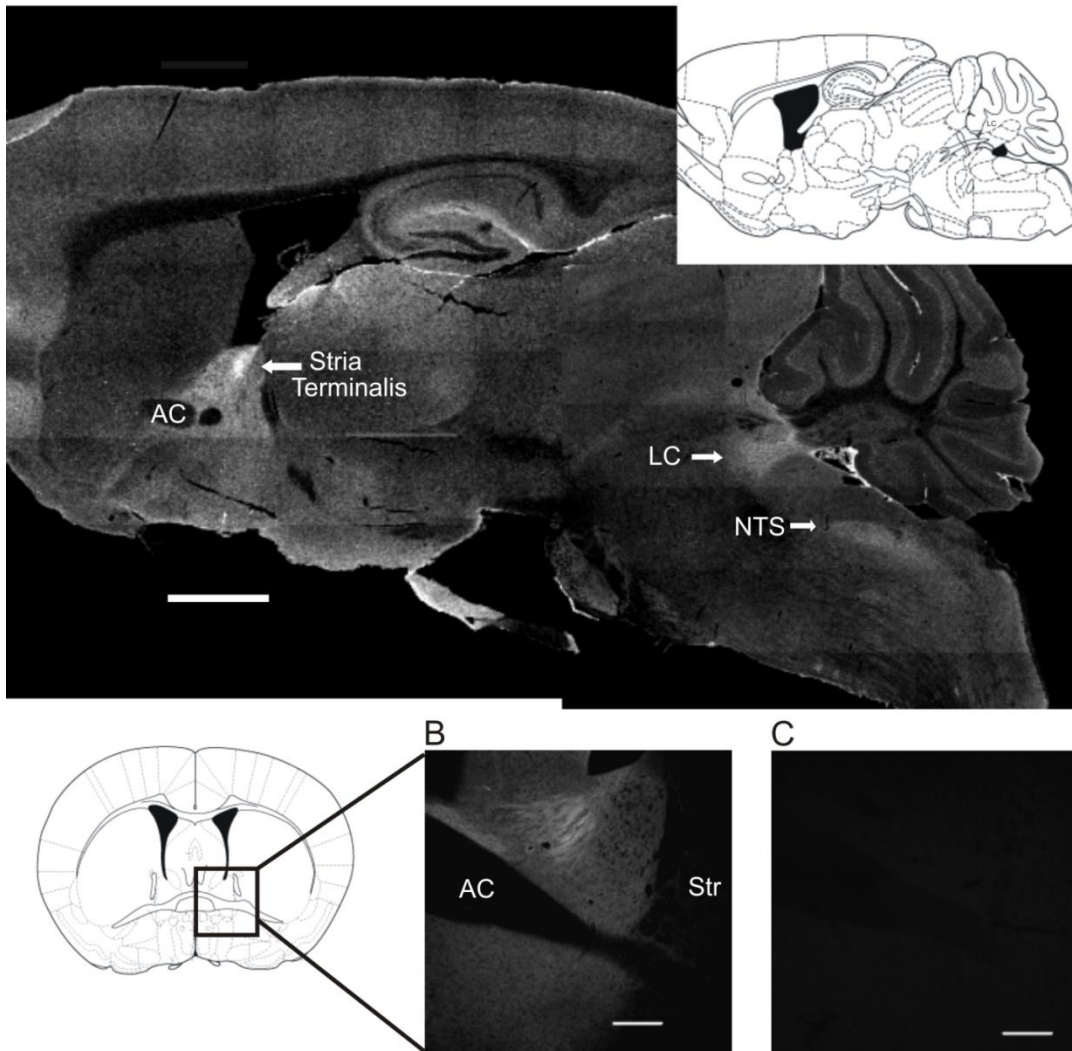


Figure 6

$\alpha_{2A}$ -ARs are enriched in the BNST. A) Parasagittal section of the HA  $\alpha_{2A}$ -AR knockin (KI) mouse showing HA labeling, and corresponding Franklin-Paxinos image at 0.96 mm lateral to midline (scale bar = 1 mm). B) Coronal section of the HA  $\alpha_{2A}$ -AR KI mouse bed nucleus of the stria terminalis (BNST) showing labeled HA (scale bar = 200  $\mu$ m), and corresponding Franklin-Paxinos image at (+) 0.14 mm from bregma. C) Corresponding wildtype C57BL/6J mouse image showing lack of labeling with HA antibody (scale bar = 200  $\mu$ m).

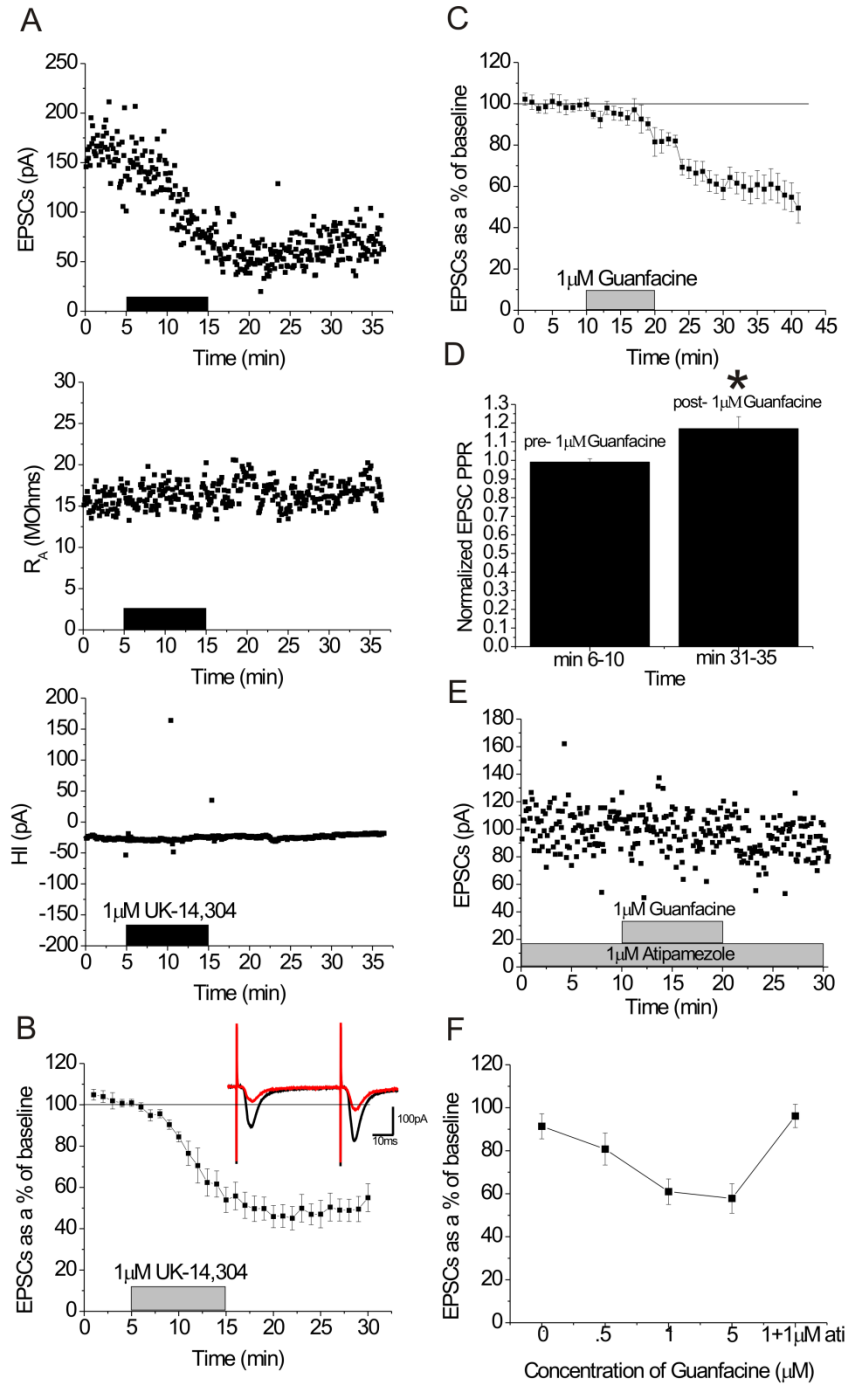
## The $\alpha_{2A}$ -AR selective agonist guanfacine depresses glutamatergic transmission in the dorsal BNST

Next, we used whole cell patch clamp electrophysiology in slices prepared from wildtype mice to assess the likelihood that previously observed effects of  $\alpha_2$ -AR agonists in the BNST are mediated by the  $\alpha_{2A}$ -AR subtype. First, we replicated previous findings and showed that the  $\alpha_2$ -AR agonist UK-14,304 (1  $\mu$ M) causes an acute depression (51.0  $\pm$  5.9% from baseline, n = 9, p < .0001, t = 9.55, df = 8) of excitatory transmission (Figure 7A-B). We then examined the effects of application of the  $\alpha_{2A}$ -AR selective agonist guanfacine. Guanfacine is an  $\alpha_2$ -AR agonist with 60-fold selectivity for the  $\alpha_{2A}$ -AR subtype over  $\alpha_{2B}$ -ARs (Uhlen and Wikberg, 1991) and 22-fold selectivity over  $\alpha_{2C}$ -ARs (Uhlen *et al*, 1992). Guanfacine elicited a concentration-dependent acute decrease in excitatory synaptic transmission in BNST (500 nM, 19.1  $\pm$  7.4% from baseline, n = 6, p < .05, t = 2.71, df = 5; 1  $\mu$ M, 39.9  $\pm$  5.9% from baseline, n = 9, p < .001, t = 6.04, df = 8; 5  $\mu$ M, 42.2  $\pm$  6.8% from baseline, n = 10, p < .001, t = 6.13, df = 9) (Figure 7C, 7F). The effect of 1  $\mu$ M guanfacine was blocked by the  $\alpha_2$ -AR selective antagonist atipamezole (n = 4, p > .30, t = 1.19, df = 3) (Figure 7E-F). Thus these data further suggest a role for the  $\alpha_{2A}$ -AR subtype in modulation of glutamatergic transmission in the BNST.

To begin to assess synaptic mechanisms involved in the effects elicited by guanfacine, we assessed paired-pulse responses before and after guanfacine application. Changes in the paired-pulse ratio (PPR) are indicative of alterations in the probability of transmitter release. We observed that PPR of evoked

glutamate responses increased after guanfacine application (1  $\mu$ M,  $p < .05$ ,  $t = -2.55$ ,  $df = 8$ ; 500 nM,  $p < .01$ ,  $t = -4.30$ ,  $df = 5$ ) (Figure 7D).

Figure 7



### Figure 7

$\alpha_2$ -AR agonists depress excitatory transmission in the dorsal BNST (dBNST). A) Representative experiment showing the effect of 1  $\mu$ M UK-14,304 on excitatory transmission in the dBNST. pA = pico-amps, HI = holding current,  $R_A$  = access resistance. B) 1  $\mu$ M UK-14,304 depresses excitatory transmission in the dBNST (n = 9). Inset: representative 5 min average traces pre (black)- and post (red)-UK-14,304. C) 1  $\mu$ M guanfacine depresses excitatory transmission in the dBNST (n = 9). D) 5 min average paired-pulse ratios pre- and post- 1  $\mu$ M guanfacine. E) Representative experiment showing the effect of 1  $\mu$ M guanfacine in the presence of 1  $\mu$ M atipamezole in the dBNST. F) Dose-response curve for effect of guanfacine on excitatory transmission in the dBNST (n = 3 vehicle, n = 9 for 500 nM, n = 9 for 1  $\mu$ M, n = 10 for 5  $\mu$ M), also effect of 1  $\mu$ M guanfacine in the presence of 1  $\mu$ M atipamezole (n = 4).



## **The $\alpha_{2A}$ -AR is not distributed similarly with noradrenergic terminal markers in the BNST**

We used immunohistochemistry in attempt to morphogenically localize the  $\alpha_{2A}$ -AR in parallel with our functional assessments. This receptor subtype has been proposed to play a role as an autoreceptor at noradrenergic synapses to modulate NE release (Altman *et al*, 1999; Hein *et al*, 1999; Stewart, 2000; Trendelenburg *et al*, 2001). However, the acute depression of glutamate transmission observed here would most easily be explained by a heterosynaptic role of the receptor. Alternatively, the receptor could be regulating glutamate transmission indirectly by regulating endogenous NE release. The distribution of the  $\alpha_{2A}$ -AR (as assessed by HA immunoreactivity in the knock-in mouse), however, was very distinct from the distribution of immunoreactivity for the NE transporter (NET) (Figure 8A-C). Additionally, the  $\alpha_{2A}$ -AR is much more broadly distributed throughout the BNST than tyrosine hydroxylase (TH), a marker for both dopaminergic and noradrenergic terminals (Figure 8D-F and Figure 9D-F). The broad light microscopy level distribution of  $\alpha_{2A}$ -AR immunoreactivity outside that of TH and NET immunoreactivity suggests that the receptor may indeed exist as a heteroreceptor on non-noradrenergic terminals.

## **The $\alpha_{2A}$ -AR is distributed similarly to glutamatergic terminal markers in the BNST**

Next, we looked at the co-distribution at the light microscopy level of the  $\alpha_{2A}$ -AR and immunoreactivity for the glutamatergic terminal marker vesicular glutamate transporter 1 (VGLUT1). VGLUT1 is diffusely distributed throughout the BNST in a pattern grossly similar to the  $\alpha_{2A}$ -AR (Figure 8G-I, Figure 9A-C). Qualitatively, it appears that the  $\alpha_{2A}$ -AR colocalizes considerably with VGLUT1 (Figure 9A-C).

Figure 8

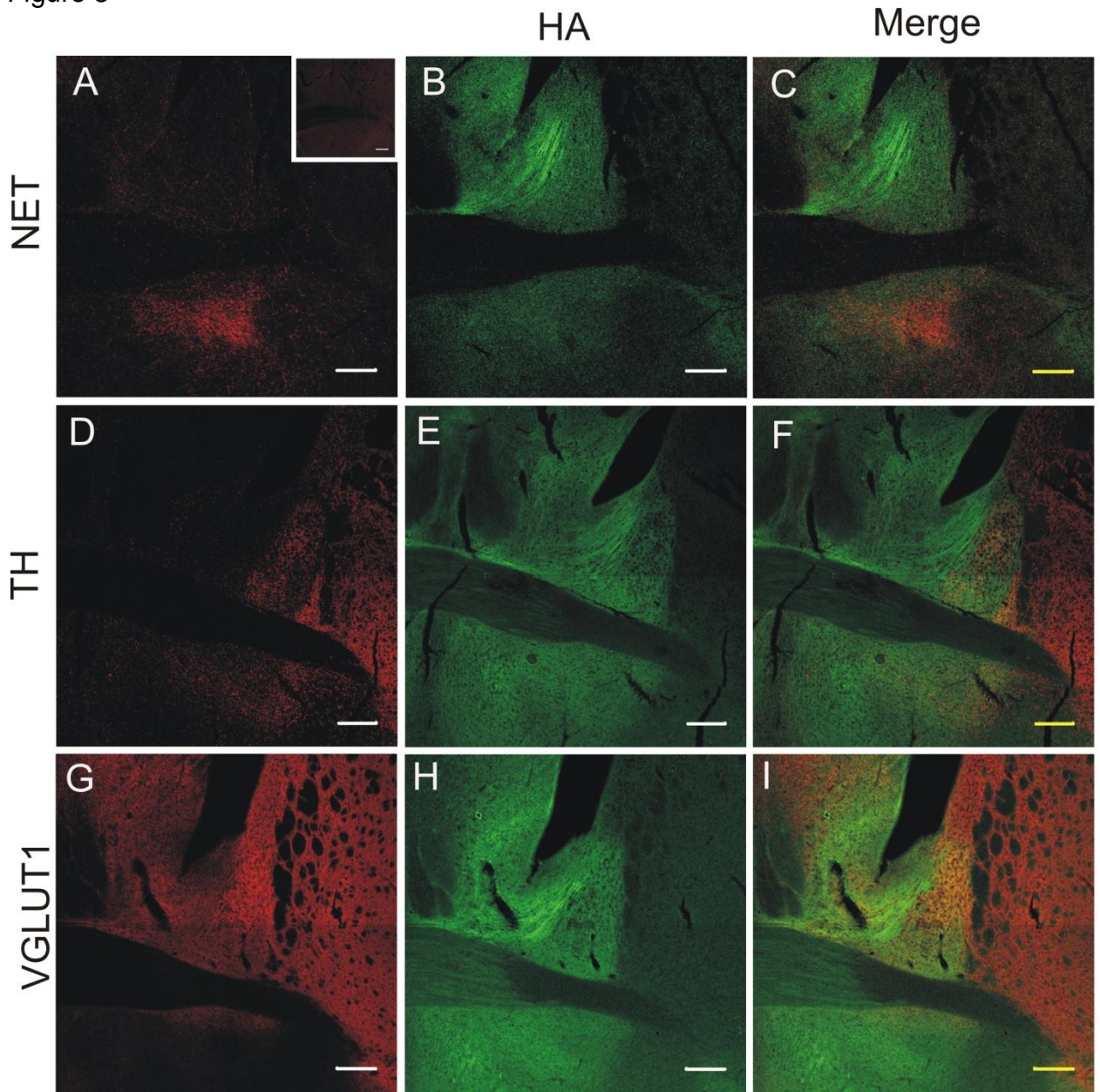


Figure 8

$\alpha_{2A}$ -AR distribution in the BNST is dissimilar to norepinephrine transporter (NET) and tyrosine hydroxylase (TH) distribution but is similar to vesicular glutamate transporter 1 (VGLUT1) distribution. (A-I) 2x2 tiles of coronal sections containing the BNST, scale bars represent 200  $\mu$ m. A) NET labeling in the BNST with inset of the NET KO mouse. B) HA labeling in the BNST. C) Merge of NET and HA. D) TH labeling in the BNST. E) HA labeling in the BNST. F) Merge of TH and HA. G) VGLUT1 labeling in the BNST. H) HA labeling in the BNST. I) Merge of VGLUT1 and HA.

Figure 9

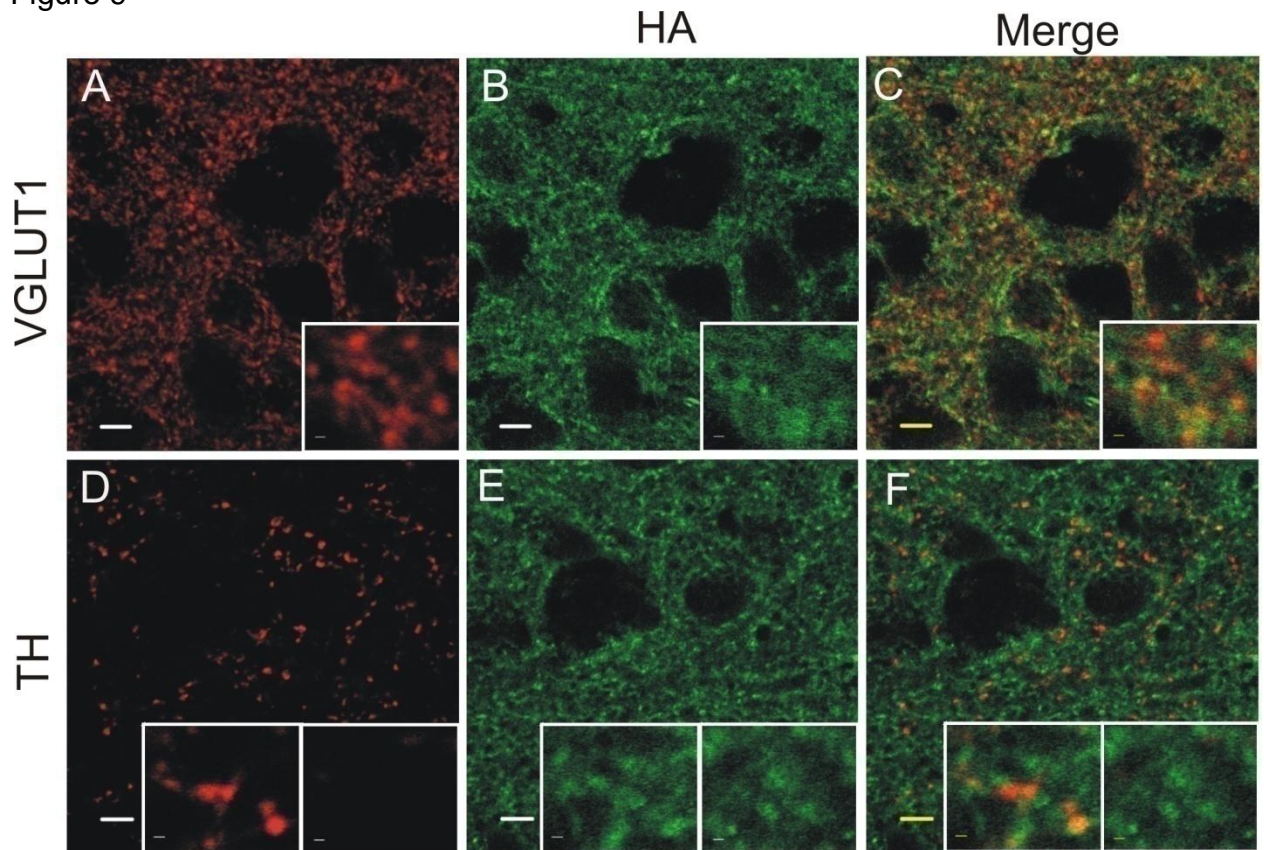


Figure 9

$\alpha_{2A}$ -AR distribution in the dorsal lateral BNST is broader than the distribution of tyrosine hydroxylase (TH), but is similar to distribution of vesicular glutamate transporter 1 (VGLUT1). (A-F) High power image of coronal sections containing the BNST, scale bars represent 5  $\mu$ m. Inset scale bars are 0.5  $\mu$ m. Inset boxes measure 6.9  $\mu$ m (width) x 5.6  $\mu$ m (height). A) VGLUT1 labeling in the dBNST. B) HA labeling in the dBNST. C) Merge of VGLUT1 and HA. D) TH labeling in the dBNST. E) HA labeling in the dBNST. F) Merge of TH and HA.

## **The $\alpha_{2A}$ -AR selective agonist guanfacine depresses GABAergic transmission in the dBNST**

Due to the widespread expression of the  $\alpha_{2A}$ -AR in the BNST, we examined the ability of guanfacine to regulate fast inhibitory transmission. Here, we examined the ability of the  $\alpha_{2A}$ -AR specific agonist guanfacine to regulate evoked inhibitory postsynaptic currents (eIPSCs) in the dBNST. We found that guanfacine elicited a concentration-dependent acute albeit modest decrease in inhibitory synaptic transmission in BNST (500 nM, 0.9 +/- 6.5% from baseline, n = 3, p > .8, t = 0.27, df = 2; 1  $\mu$ M, 23.7 +/- 4.7% from baseline, n = 5, p < .01, t = 8.35, df = 4; 5  $\mu$ M, 39.4 +/- 5.5% from baseline, n = 4, p < .01, t = 6.69, df = 3) (Figure 10A, 10C). Interestingly, there was no change in PPR as seen with EPSCs, with a trend for a decrease in PPR (Figure 10B).

We show that guanfacine decreases both excitatory and inhibitory transmission in the dBNST. Whereas 500 nM guanfacine had a significant effect on EPSCs, it did not produce a change in IPSCs. At 1  $\mu$ M guanfacine, the effect on EPSCs was significantly greater than the effect on IPSCs (p < .05, t = -2.19, df = 12) (Figure 11). Therefore, under our recording conditions, it is possible that activation of the  $\alpha_{2A}$ -AR produces greater changes at excitatory synapses than inhibitory synapses, thereby causing an overall reduction in BNST excitability.

Figure 10

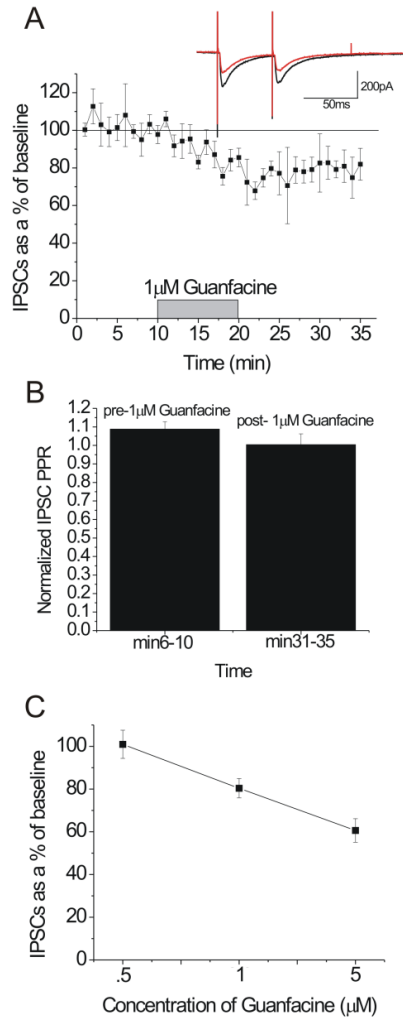


Figure 10

The  $\alpha_{2A}$ -AR agonist guanfacine depresses inhibitory transmission in the dBNST. (A) 1  $\mu$ M guanfacine depresses excitatory transmission in the dBNST (n = 5) Inset: representative 5 min average traces pre (black)- and post (red)-guanfacine. B) 5 min average paired-pulse ratios pre- and post- 1  $\mu$ M guanfacine. C) Dose-response curve for the effect of guanfacine on inhibitory transmission in the dBNST (n = 3 for 500 nM, n = 5 for 1  $\mu$ M, n = 4 for 5  $\mu$ M).



Figure 11

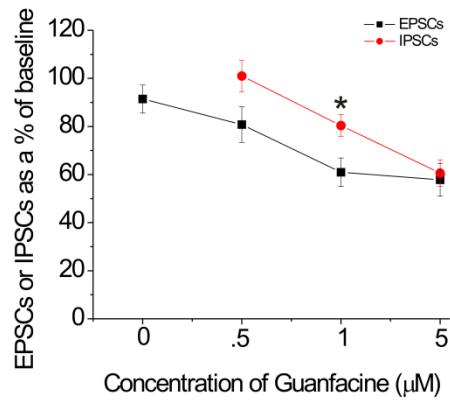


Figure 11

Overlay of guanfacine dose-response curves showing increased efficacy of guanfacine on excitatory transmission over inhibitory transmission.

## **Discussion**

### **The HA $\alpha_{2A}$ -AR KI mouse contains normally distributed and functional $\alpha_{2A}$ -ARs in the mouse brain**

The HA  $\alpha_{2A}$ -AR KI mouse contains a 9 amino acid N-terminal hemagglutinin tag on the  $\alpha_{2A}$ -AR (Lu *et al*, 2009). This tag does not grossly alter function of the  $\alpha_{2A}$ -AR *in vitro*, and the receptor is grossly normally distributed in the mouse brain (Lu *et al*, 2009). A parasagittal section of HA  $\alpha_{2A}$ -AR KI mouse brain demonstrates high levels of  $\alpha_{2A}$ -AR in the LC, NTS, hippocampus, and stria terminalis, as expected based on previous studies (Glass *et al*, 2001; Lee *et al*, 1998; Milner *et al*, 1998; Scheinin *et al*, 1994; Wang *et al*, 1996). Moreover, our data suggest that these receptors remain functional in the BNST, consistent with a previous report on this mouse (Lu *et al*, 2009).

### **The HA $\alpha_{2A}$ -AR KI mouse provides a unique method to specifically label $\alpha_{2A}$ -ARs in brain tissue**

HA is an exogenous sequence not found in normal brain tissue, and thus should not cross-react with other receptors or proteins in the brain. As shown in Figure 6B-C, mouse anti-HA specifically labels the HA presented on the HA-tagged  $\alpha_{2A}$ -AR and does not show appreciable immunolabeling in the WT mouse. Previous studies examining localization of the  $\alpha_{2A}$ -AR in rodent brain tissue have used antibodies targeting various portions of the  $\alpha_{2A}$ -AR. Studies conducted in the NTS by Glass and others took advantage of a goat anti-  $\alpha_{2A}$ -AR from Santa Cruz (Glass *et al*, 2001), which is an antibody against the C-terminus of the receptor. Additionally, Milner and others have published studies in the



hippocampus and ventrolateral medulla using an antibody developed against a 47 amino acid chain of the 3<sup>rd</sup> intracellular loop of the rat  $\alpha_{2A}$ -AR fused to GST (Milner *et al*, 1998; Milner *et al*, 1999). Our data confirm aspects of the previous studies, demonstrating the presence of the  $\alpha_{2A}$ -AR in VGLUT1-(+) and TH-(+) compartments within the BNST.

### **The $\alpha_{2A}$ -AR selective agonist guanfacine depresses excitatory transmission in the BNST**

Using *in vivo* microdialysis techniques, Forray and others have shown that in the BNST, the  $\alpha_2$ -AR agonist UK-14,304 depresses extracellular glutamate and K<sup>+</sup>-induced glutamate release, and that the  $\alpha_2$ -AR antagonist RX821002 has opposite effects (Forray *et al*, 1999). We previously reported, using field and whole-cell electrophysiology approaches, that the  $\alpha_2$ -AR agonist UK-14,304 depresses glutamatergic transmission in the BNST (Egli *et al*, 2005). This effect is absent in the  $\alpha_{2A}$ -AR knockout mouse (Egli *et al*, 2005), implicating the  $\alpha_{2A}$  AR subtype as potentially responsible for this depression of glutamatergic transmission. It is also possible though, that multiple ARs in the  $\alpha_{2A}$ -AR knockout mouse are desensitized due to high levels of extracellular NE in this knockout mouse, as  $\alpha_1$ -AR-mediated LTD is also absent in these mice (McElligott *et al*, 2008). Therefore, we cannot rule out that the  $\alpha_{2C}$ -AR contributes to some effects seen with UK-14,304 (Trendelenburg *et al*, 1999).

Using whole-cell electrophysiology to explore the specific role of the  $\alpha_{2A}$ -AR in modulation of glutamate transmission in the BNST, we applied the  $\alpha_{2A}$ -AR selective agonist guanfacine to cells in the dBNST. We found that guanfacine

depresses glutamatergic transmission in a dose-dependent manner, and that this effect can be blocked with the  $\alpha_2$ -AR selective antagonist atipamizole. Thus, in total these data strongly suggest that activation of the  $\alpha_{2A}$ -AR acutely depresses glutamate release in the BNST.

### **$\alpha_{2A}$ -ARs depress excitatory transmission by presynaptically modulating glutamate release**

The  $\alpha_{2A}$ -AR is discussed as an autoreceptor modulating NE release (Altman *et al*, 1999; Hein *et al*, 1999; Stewart, 2000; Trendelenburg *et al*, 2001), and it is conceivable that effects on glutamate transmission are the result of an  $\alpha_{2A}$ -AR-mediated altered release of NE. To examine this possibility, we used immunohistochemistry to co-label the HA-tagged  $\alpha_{2A}$ -AR and markers of noradrenergic terminals, NET and TH. Interestingly, the  $\alpha_{2A}$ -AR is much more broadly distributed than either NET or TH in the BNST, making modulation of NE release by the  $\alpha_{2A}$ -AR unlikely to provide the sole, or perhaps even major, explanation for the acute effects seen with application of guanfacine. In fact, the  $\alpha_{2A}$ -AR has a very similar distribution to the glutamatergic terminal marker, VGLUT1. In electrophysiological experiments we observed an increase in the paired pulse ratio (PPR) upon application of guanfacine, thus suggesting that the  $\alpha_{2A}$ -AR regulates glutamate transmission by decreasing glutamate release probability.

Other areas of the extended amygdala, such as the central nucleus of the amygdala (CeA), also show heterosynaptic regulation of glutamate transmission by  $\alpha_2$ -ARs, but via a distinct mechanism involving direct interaction of the  $\beta\gamma$

subunit of the G-protein coupled receptor complex with release machinery (Delaney *et al*, 2007). In contrast,  $\alpha_{2A}$ -AR mediated depression of excitatory transmission in the prefrontal cortex has been theorized to be postsynaptic due to an absence of effect on PPR, although regulation similar to that seen in the CeA cannot be ruled out based on current findings (Ji *et al*, 2008). The  $\alpha_{2A}$ -ARs are also known to presynaptically modulate L-type calcium channels in the retina (Dong *et al*, 2007). In other areas including the NTS (Glass *et al*, 2001), ventrolateral medulla (Milner *et al*, 1999), and hippocampus (Milner *et al*, 1998), evidence consistent with expression of heterosynaptic presynaptic  $\alpha_{2A}$ -ARs has been reported. Using a combination of various techniques, we demonstrate that the  $\alpha_{2A}$ -AR is also located heterosynaptically on glutamate terminals and modulates excitatory transmission in the BNST by presynaptically altering glutamate release, thus adding to findings demonstrating the complex role of the  $\alpha_{2A}$ -AR in modulation of transmission in the brain. Our findings are consistent with the proposal by Gilsbach and others that many  $\alpha_{2A}$ -AR-mediated modulations of function in the brain are via  $\alpha_{2A}$ -AR heteroreceptors, not autoreceptors (Gilsbach *et al*, 2009).

### **$\alpha_{2A}$ -ARs modulate GABAergic transmission in the BNST**

NE is known to modulate both inhibitory and excitatory transmission in the BNST. For example, activation of the  $\alpha_1$ -AR causes LTD at excitatory synapses (McElligott *et al*, 2008), and also increases IPSC frequency at inhibitory synapses that are shown to project to the VTA via a retrograde label (Dumont *et*

*al*, 2004). Therefore, we must consider the possibility that  $\alpha_{2A}$ -ARs regulate inhibitory transmission in the BNST in addition to their effects on excitatory transmission. Dumont and Williams saw no effect of the  $\alpha_2$ -AR agonist clonidine or the  $\alpha_2$ -AR antagonist yohimbine on spontaneous IPSCs in the ventral BNST (Dumont *et al*, 2004). Also, Forray and others saw no effect of the  $\alpha_2$ -AR agonist UK-14,304 on GABA basal extracellular levels using *in vivo* microdialysis approaches in the BNST (Forray *et al*, 1999). Here, however, we show that the  $\alpha_{2A}$ -AR selective agonist depresses inhibitory transmission in the BNST in a dose-dependent manner, but does not alter PPR. These data suggest that the  $\alpha_{2A}$ -AR may modulate inhibitory transmission in the BNST through a postsynaptic mechanism, although we cannot rule out that there is some direct interaction between the  $\alpha_{2A}$ -AR and release machinery as seen with excitatory transmission in the CeA (Dumont *et al*, 2004). Also, at present we cannot rule out the possibility that the greater effect of guanfacine on eIPSCs at higher concentrations is partially due to recruitment of  $\alpha_{2C}$ -ARs in addition to  $\alpha_{2A}$ -ARs.

### **$\alpha_{2A}$ -ARs gate excitatory drive to the BNST, and regulate inhibitory transmission in the BNST via distinct mechanisms**

Taken together, our data indicate that the  $\alpha_{2A}$ -AR plays a particularly important role in modulation of BNST output. This is not the first instance in which  $\alpha_{2A}$ -ARs have been suggested to be differentially localized within a region. In the NTS, ventrolateral medulla, and hippocampus,  $\alpha_{2A}$ -AR-like immunoreactivity has been noted on presynaptic autoreceptor and heterosynaptic terminals, as well as postsynaptically, and on glial cells (Glass *et*

*al*, 2001; Milner *et al*, 1998; Milner *et al*, 1999). Here, we find that the  $\alpha_{2A}$ -AR is broadly associated with at least two compartments, TH(+) and VGLUT(+) terminals.

Based on current findings, guanfacine appears to have a higher potency at excitatory than inhibitory synapses. Excitatory input into the BNST comes from insular cortex, infralimbic cortex, the ventral subiculum and the basolateral amygdala (Dong *et al*, 2001a; Massi *et al*, 2008; Weller *et al*, 1982), other regions known to be involved in stress-reward circuitry, and regions known to express mRNA for the  $\alpha_{2A}$ -AR (Scheinin *et al*, 1994; Wang *et al*, 1996). Thus, the  $\alpha_{2A}$ -AR potentially gates these inputs, effectively decreasing excitatory drive to the BNST during events known to release NE, such as chronic stressors, withdrawal from drugs of abuse, and other social stressors (Aston-Jones *et al*, 1999; Cecchi *et al*, 2002a; Cecchi *et al*, 2002b; Delfs *et al*, 2000; Fendt *et al*, 2005).

The main inhibitory projection to the BNST is from a closely related structure, the central nucleus of the amygdala (CeA), a region also involved in fear and anxiety states. Whereas the BNST and CeA are very homologous structures with similar afferents and efferents, it is hypothesized that the BNST is involved in unconditioned prolonged anxiety states (Walker *et al*, 2003) whereas the CeA is involved in conditioned fear to distinct sensory cues (Campeau and Davis, 1995; Kim and Davis, 1993; Wilensky *et al*, 2006). Others, however, have considered that these two structures have more anatomical similarity than differences and suggested they be grouped together as the extended amygdala

(Dong *et al*, 2000; Dong *et al*, 2004a, b; Holstege *et al*, 1985; Hopkins and Holstege, 1978; Veening *et al*, 1984). Therefore, the activation of  $\alpha_{2A}$ -ARs may modulate functional signaling between or within these two parts of the extended amygdala.

Additionally, the BNST is made up of a large network of GABAergic interneurons. Thus, modulation of inhibitory transmission by activation of  $\alpha_2$ -ARs may also influence output of the BNST. For example, the BNST is thought to contain an excitatory projection to the VTA (Georges *et al*, 2002; Massi *et al*, 2008), and Dumont and others' work has suggested that NE triggers GABA<sub>A</sub>-mediated inhibition of these fibers (Dumont *et al*, 2004). Here, we have demonstrated that  $\alpha_2$ -ARs do modulate inhibitory transmission in this region.

$\alpha_{2A}$ -AR knockout mice have been shown to have enhanced anxiety and depression-related phenotypes (Schramm *et al*, 2001), leading to the hypothesis that the  $\alpha_{2A}$ -AR may play a protective role for an animal under stressful conditions. Studies demonstrate that  $\alpha_2$ -AR activation in the BNST mediates inhibition of stress-induced reinstatement to various drugs of abuse (Delfs *et al*, 2000; Wang *et al*, 2001) and inhibition of stress-induced behavior by predator odor (Fendt *et al*, 2005). These findings support a stress-protective function of  $\alpha_{2A}$ -ARs in the BNST. Thus, activation of the  $\alpha_{2A}$ -AR may in fact serve to lessen the effects of stress by dampening signaling from inputs to the BNST such as the infralimbic cortex, insular cortex, and BLA, regions known to be activated under stressful events such as withdrawal from drugs of abuse.

## **The BNST may play a role in the therapeutic effects of guanfacine on anxiety-related disorders**

Clinical data indicate that stress is a common reason patients relapse to using drugs of abuse (Sinha *et al*, 1999). Finding therapeutic agents to help addicts with anxiety and craving are extremely limited at this time. Guanfacine, an  $\alpha_{2A}$ -AR selective agonist, is FDA-approved for treatment of some anxiety disorders including post-traumatic stress disorder (PTSD) and chronic Tic disorder. Guanfacine is also prescribed as a centrally-acting hypertensive agent to reduce blood pressure in patients struggling with hypertension (Kisicki *et al*, 2007), and is used for treatment of patients suffering from schizophrenia (McClure *et al*, 2007). Guanfacine is currently undergoing clinical trial for its role in preventing stress-induced relapse to cocaine, smoking, and alcohol use (Clinicaltrials.gov). Additional clinical trials looking at its efficacy in treating PTSD, ADHD, and schizophrenia are also underway (Posey and McDougle, 2007).

Guanfacine is a partial agonist at the  $\alpha_{2A}$ -AR, and data suggest that partial agonism at this receptor subtype results in fewer unwanted side effects such as sedation in the potential treatment of anxiety disorders (Tan *et al*, 2002). Additionally, in disorders such as addiction, PTSD, or ADHD, where chronic therapeutics are needed, an agonist that does not readily induce desensitization of its target may be beneficial. Guanfacine desensitizes  $\alpha_{2A}$ -ARs much less readily than clonidine, likely explaining the longer duration of action by guanfacine clinically despite the two partial agonists having a similar half-life (Lu *et al*, 2009). Thus, guanfacine is a promising potential therapy for anxiety

disorders and relapse in addiction. We theorize that the effects of guanfacine on  $\alpha_{2A}$ -ARs in the BNST are partially responsible for its effectiveness as a therapeutic agent in anxiety- and addiction-related behaviors.



## CHAPTER III

### CHARACTERIZATION OF CORTICAL AND AMYGDALAR AFFERENTS TO THE BNST IN C57BL/6J MICE

This chapter describes work completed toward the fulfillment of Aim II of my thesis project.

#### **Introduction**

The bed nucleus of the stria terminalis (BNST) is a region thought to play a key role in general anxiety and stress-induced relapse to drugs of abuse (Walker *et al*, 2003). The BNST is an important center for processing inputs from stressful stimuli and integrating them into the reward and stress circuitry, thereby affecting behavior. Previous anatomical studies in rat brain have shown that the BNST receives projections from brain regions involved in processing stress and anxiety-provoking stimuli. These regions include the insular and infralimbic cortices, the basolateral nucleus of the amygdala, as well as the hippocampus, central nucleus of the amygdala, and nucleus tractus solitarius (Dong *et al*, 2001a; Hurley *et al*, 1991; McDonald *et al*, 1999; Shin *et al*, 2008; Takagishi and Chiba, 1991; Vertes, 2004; Walker *et al*, 2003; Weller *et al*, 1982; Yasui *et al*, 1991). The BNST is reciprocally connected with many of these regions (Dong *et al*, 2001b; Erb *et al*, 2001). The BNST sends projections to various regions of the hypothalamus (Conrad *et al*, 1976; Swanson *et al*, 1979), a region involved in

the peripheral stress response; and the ventral tegmental area (VTA) (Georges *et al*, 2002; Swanson *et al*, 1979), a region involved in reward and motivation. Thus, the BNST is highly integrated with stress and reward pathways in rat brain.

Whereas anatomical studies have been conducted almost exclusively in rat, much behavioral work and electrophysiological studies examining BNST function have been carried out in mouse (Egli *et al*, 2005; Grueter *et al*, 2006a; Kash *et al*, 2008; McElligott *et al*, 2008; Weitlauf *et al*, 2004). The degree to which connections within brain regions of the rat are conserved in mouse is currently unknown. Examining afferents to the BNST in the mouse model will help confirm conclusions reached using functional data. In this study, we have begun to characterize the inputs to the BNST in mice. We have chosen to focus on select cortical and amygdalar inputs known to innervate the BNST in rat, as these inputs are important in the processing and expression of stress and anxiety states.

The BNST receives the densest noradrenergic innervation in the brain (Brownstein *et al*, 1974), and this input arises primarily from the nucleus tractus solitarius (NTS) (Aston-Jones *et al*, 1999). Numerous studies have shown that the NTS is critical for stress-induced relapse to drug-seeking behaviors (Aston-Jones *et al*, 1999; Olson *et al*, 2006; Shalev *et al*, 2001; Wang *et al*, 2001). Additionally, studies have demonstrated a role for NE in the BNST in the mediation of stress-related behaviors. Injection of  $\beta$ -AR antagonists or  $\alpha_2$ -AR agonists into the BNST can block morphine withdrawal-induced conditioned place aversion (CPA) (Delfs *et al*, 2000), and stress-induced reinstatement of

morphine conditioned place preference (CPP) (Wang *et al*, 2001).

Electrophysiology studies have implicated  $\alpha_2$ -ARs in the presynaptic modulation of glutamatergic transmission in the BNST (Egli *et al*, 2005). There are three subtypes of the  $\alpha_2$ -AR, the  $\alpha_{2A}$ -AR,  $\alpha_{2B}$ -AR, and  $\alpha_{2C}$ -AR, and the  $\alpha_{2A}$ -AR and  $\alpha_{2C}$ -AR subtypes are widely expressed in the BNST (Scheinin *et al*, 1994; Wang *et al*, 1996). Recent studies have implicated heterosynaptic  $\alpha_{2A}$ -ARs as mediating alterations in glutamate transmission (see Chapter II).

In these studies, we use a transgenic knock in mouse model, in which a hemagglutinin tagged  $\alpha_{2A}$ -AR is knocked into the endogenous  $\alpha_{2A}$ -AR, to begin to address the potential regulation of specific inputs to the BNST by  $\alpha_{2A}$ -ARs.

## **Experimental Procedures**

### **Animals**

Eight to twenty week-old hemagglutinin-tagged  $\alpha_{2A}$ -AR knockin (HA  $\alpha_{2A}$ -AR KI) male and female mice (bred in-house on a C57BL/6 background, provided by Qin Wang at the University of Alabama) were used for the present studies. The HA KI mice have been shown to have normal physiology and anatomy (Lu *et al*, 2009)(also see Chapter II, Figures 5 and 6). No differences between female and male animals were noted in the results of the current studies, so results were combined. All animal procedures were conducted according to an approved protocol from the Institutional Animal Care and Use Committee of Vanderbilt

University, which certifies compliance with the National Institutes of Health and the U.S. Department of Agriculture standards for humane animal utilization.

### **Anterograde Labeling of BNST Neurons**

Mice were anesthetized with tribromoethanol (2.5% solution in saline, 0.19 ml/10 g, i.p.). Ophthalmic ointment (Puralube Vet) was applied to the eyes to prevent corneal damage. The fur on the top of the head was shaved off and the exposed skin was scrubbed sequentially with 70% ethanol, 10% povidone iodine, and then again with 70% ethanol. The animal was mounted in a stereotaxic apparatus (Kopf Instruments) and placed on a warming pad kept at 39°C. The skin over the skull was cut and deflected enough to expose the bregma and lambda sutures. Local anesthetic cream containing benzocaine was applied to the wound. The head was leveled with respect to the intersections of bregma and lambda with the midline suture. Holes were drilled in the skull directly above the right insular cortex, BLA, or infralimbic cortex according to the atlas of Franklin and Paxinos. A sterilized glass pipette with a 30-40  $\mu\text{m}$  diameter tip was filled with 10% biotinylated dextran amine (BDA) (10,000 MW, lysine fixable, Molecular Probes) in .1 M phosphate buffer. The dura was cut and the syringe needle lowered into the insular cortex (AP +0.02, L 3.66, D 4.16), BLA (AP -1.34, L 3.45, D 4.67), or infralimbic cortex (AP +1.78, L 0.22, D 2.91). The BDA was iontophoresed over 10 min at 7 sec on/7 sec off at +3.0  $\mu\text{A}$ , and the pipette then removed under negative current. After withdrawing the pipette, the wound was closed with sutures and the animal was hydrated with a saline

injection (1 ml, s.c.) followed by freshly dissolved ampicillin (0.45 mg/0.1 ml per animal, s.c.). The animal was kept warm during recovery from anesthesia and was given an analgesic when ambulatory (buprenorphin, 0.1 mg/kg, s.c.). Postsurgery, animals were housed singly or with other injected littermates, and weighed and injected with analgesic twice per day for up to 6 days or until the animal gained weight. Animals that lost more than 20% of their body weight or showed signs of uncontrolled pain, stress, or dehydration were euthanized. Ten days post-surgery, healthy animals were then processed for immunohistochemistry.

### **Fluorescent Immunohistochemistry**

Ten days following injection of BDA, animals were injected with 0.25 ml pentobarbital, and once they were unresponsive to foot and tail pinch, were transcardially perfused with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Brains were post-fixed in 4% paraformaldehyde for 2-3 days at 4°C, and were then transferred to 30% sucrose until fully equilibrated. Following post-fixation, 30-100 µm coronal sections of brain were sliced on a cryostat (Leica CM3050S). Sections containing the region to be studied were then free-floated for immunolabeling.

Brain sections from BDA injected mice were washed with PBS, then incubated with cyanine three conjugated streptavidin for two hours at room temperature. Following three 10 minute washes in PBS, slices were mounted on slides, sealed with PolyAquamount, and left overnight to dry.

Slices colabeled with VGLUT1 or HA were incubated in the donkey anti-streptavidin and then washed as described above. The slices were then blocked with 4% normal donkey serum containing 0.2% Triton-X-100 in PBS. Slices were then incubated with primary guinea pig anti-the vesicular glutamate transporter 1 (VGLUT1) (Chemicon, 1:1000) or primary mouse anti-HA (Covance, 1:1000) for 48 hours at 4°C, followed by three 10 minute washes in PBS, and then incubated with cyanine dye conjugated donkey anti-guinea pig or donkey anti-mouse (Jackson ImmunoResearch) for 24 hours at 4°C. Following three 10 minute washes in PBS, sections were mounted on slides, sealed with PolyAquamount, and left overnight to dry.

## **Imaging**

Images were taken with an LSM510 confocal microscope using a 10x objective and a 63x/1.4 NA planapochromat objective (Carl Zeiss, Inc.). Laser excitation was kept constant between genotypes and primary/no primary controls. High-power (63x) images (optical sections) were taken at 0.5µm focus intervals. Under conditions identical for those used to obtain sample images, we imaged microscopic fluorescent latex beads (Multispeck, Molecular Probes, Inc.) with spectral properties similar to the three sample probes (Cy2, Cy3, and Cy5) in order to calibrate the three-channel registration and subsequently compensate for instrument-induced positional shifts in the data. Measured shifts were minor and used to apply precise offsets to the image data using the LSM software, thus providing accurate probe positions to within the diffraction

limit of the microscope (less than 0.25  $\mu\text{m}$  in x and y). Figure 12 was adjusted using the Autoequalize feature in CorelDRAW Graphics Suite 12. Figures 12B, 12E, and 12H were then increased in brightness by +15 using CorelDRAW Graphics Suite 12 in order to illuminate landmarks in the slices more clearly. Using ImageJ software, Figures 13 and 14 were subtracted for background and were then adjusted in brightness and contrast to allow clear presentation of signal.

## **Results and Discussion:**

### **BDA injections in the insular cortex**

Four mice were successfully injected with BDA into the insular cortex. ANG-15 is shown as a representative mouse for the insular cortex injection (Figure 12B-C). All successful injections had a dense BDA deposit in the insular cortex. Previous studies in rat indicate that the dysgranular, but not agranular insular cortex projects to the BNST (McDonald *et al*, 1999). We cannot make this distinction in these mice, as the injection site appears to spread throughout both dysgranular and agranular divisions in all mice. We did, however, see an input to the BNST from the insular cortex (Figure 12C), consistent with previous studies in rat (McDonald, 1998; McDonald *et al*, 1999; Shin *et al*, 2008; Weller *et al*, 1982; Yasui *et al*, 1991). Figure 12B shows the injection site in the insular cortex, in the same coronal plane as the BNST. The corresponding Franklin and Paxinos atlas image is shown in Figure 12A. On close inspection, one can see

the tracking of fibers from the insular cortex over to the BNST (Figure 12B) This closely resembles previously published data demonstrating the projection track in rat (McDonald, 1998). Figure 12C shows fibers projecting to the BNST. In all insular cortex injected mice, these fibers appeared to be thin and beaded in the BNST, consistent with previous studies (Takagishi *et al*, 1991).

### **BDA injections in the infralimbic cortex**

Seven mice were injected with BDA into the infralimbic cortex, and injection sites were confirmed using the Franklin and Paxinos atlas. Of these, 5 mice showed labeling of fibers in the BNST, with heavier projections to the ventral BNST and more sparse projections to the dorsal lateral BNST. Like projections from the insular cortex, projections from the infralimbic cortex were thin and beaded in appearance. ANG61 is illustrated as a representative mouse with injection of BDA into the infralimbic cortex. Figure 12E shows the injection site in the infralimbic cortex, with the corresponding Franklin and Paxinos coronal section in Figure 12D. Figure 12F shows fibers in the BNST of this same mouse. These findings are consistent with previous studies conducted in rat (Dong *et al*, 2001a; Hurley *et al*, 1991; Massi *et al*, 2008; McDonald *et al*, 1999; Shin *et al*, 2008; Takagishi *et al*, 1991), as well as cat (Room *et al*, 1985) and monkey (Chiba *et al*, 2001).

Additionally, four mice were injected with BDA in the prelimbic cortex, which is dorsal to the infralimbic cortex, and none of these mice showed fibers projecting into the BNST (data not shown). This is consistent with studies in rat,



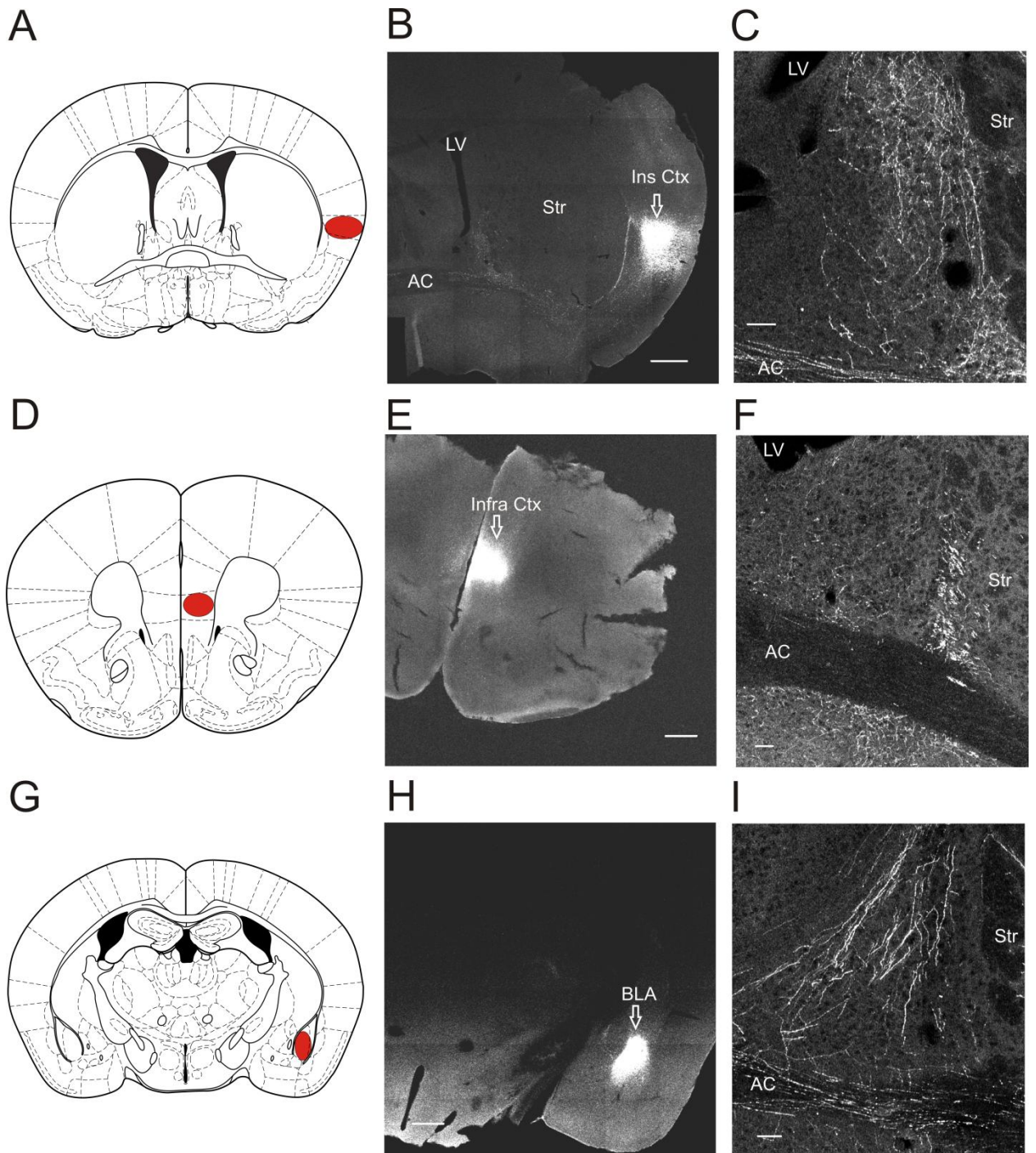
which do not show projections from the prelimbic cortex to the BNST, but do demonstrate fibers passing through the striatum just lateral to the dBNST as we saw in our studies (McDonald *et al*, 1996; McDonald *et al*, 1999; Sesack *et al*, 1989; Vertes, 2004). Four mice were injected with BDA ventral to the infralimbic cortex, in the dorsal penduncular cortex. This region has been shown to project very lightly to the BNST in rat (McDonald *et al*, 1999). Consistent with this, only sparse fibers were seen in the BNST in these mice (data not shown).

### **BDA injections in the BLA**

Four mice contained successful basolateral amygdala (BLA) injections without spread of tracer outside the medial/lateral boundaries of the BLA, and with minimal spread to the striatum dorsal to the BLA in the injection tract. ANG25 is shown as a representative mouse with an injection of BDA into the BLA (Figure 12G-I). The injection site is shown in Figure 12H, with the corresponding Franklin Paxinos atlas image in Figure 12G. The BNST of this mouse is shown in Figure 12I. Projections from the BLA to the BNST go through the stria terminalis in rat (McDonald, 1991), and our findings in mouse are consistent with this and other previous studies in rat and opossum (Dong *et al*, 2001a; McDonald, 1991; McDonald *et al*, 1986; Shin *et al*, 2008; Walker *et al*, 2003). Interestingly, these fibers were much thicker and smoother than fibers coming from the insular cortex, and did not have the thin beaded appearance like those of the insular cortex (see Figure 12C). Five mice were injected with BDA into the dorsal endopiriform cortex, just lateral to the BLA, and two mice were

injected with BDA in the striatum just dorsal to the BLA. These mice did not show appreciable fibers projecting to the BNST (data not shown).

Figure 12



## Figure 12

The insular cortex, infralimbic cortex, and the basolateral amygdala project to the BNST. A) Franklin Paxinos image of coronal plane 30, corresponding to Figure 12B. Red sphere indicates insular cortex. B) Tiled coronal image demonstrating injection of BDA into the insular cortex. Scale bar = 500  $\mu\text{m}$ . C) Tiled image illustrating BDA(+) fibers in the BNST projecting from the insular cortex. Scale bar = 50  $\mu\text{m}$ . D) Franklin Paxinos image of coronal plane 15, corresponding to Figure 12E. Red sphere indicates infralimbic cortex. E) Tiled coronal image demonstrating injection of BDA into the infralimbic cortex. Scale bar = 500  $\mu\text{m}$ . F) Tiled image illustrating BDA(+) fibers in the BNST projecting from the infralimbic cortex. Scale bar = 50  $\mu\text{m}$ . G) Franklin Paxinos image of coronal plane 40, corresponding to Figure 12H. Red sphere indicates basolateral amygdala (BLA). H) Tiled coronal image demonstrating injection of BDA into the BLA. Scale bar = 500  $\mu\text{m}$ . I) Tiled image illustrating BDA(+) fibers in the BNST projecting from the BLA. Scale bar = 50  $\mu\text{m}$ .

## **Projections from the insular cortex, infralimbic cortex, and BLA contain the excitatory terminal marker VGLUT1**

The insular cortex, infralimbic cortex, and basolateral amygdala are known to carry excitatory projections to the BNST in the rat. Also, Adamec has shown that there is a monosynaptic excitatory projection from the BLA to the BNST in the cat (Adamec, 1989). The vesicular glutamate transporters VGLUT1 and VGLUT2 are known to store glutamate in presynaptic terminals, making them good markers of glutamatergic fibers and terminals (Boulland *et al*, 2004; Hayashi *et al*, 2001; Herzog *et al*, 2001; Takamori *et al*, 2000). Cortical regions primarily express vesicular glutamate transporter 1 (VGLUT1), with low levels of VGLUT2. Noncortical regions such as the pons, thalamus, and striatum express primarily VGLUT2, and have low levels of VGLUT1 (Boulland *et al*, 2004; Fremeau *et al*, 2001; Islam *et al*, 2008; Kaneko and Fujiyama, 2002; Varoqui *et al*, 2002). To confirm that the insular cortex, infralimbic cortex, and BLA are glutamatergic, we used VGLUT1 as a glutamatergic marker and colabeled BNST sections from BDA injection mice with VGLUT1. Representative images are illustrated from mouse ANG15 (insular cortex), ANG61 (infralimbic cortex), and ANG64 (BLA). As shown in Figure 13A-I, each of the three regions studied have projections to the BNST that colocalize with VGLUT1, thus confirming that these projections are glutamatergic in mouse.

Figure 13

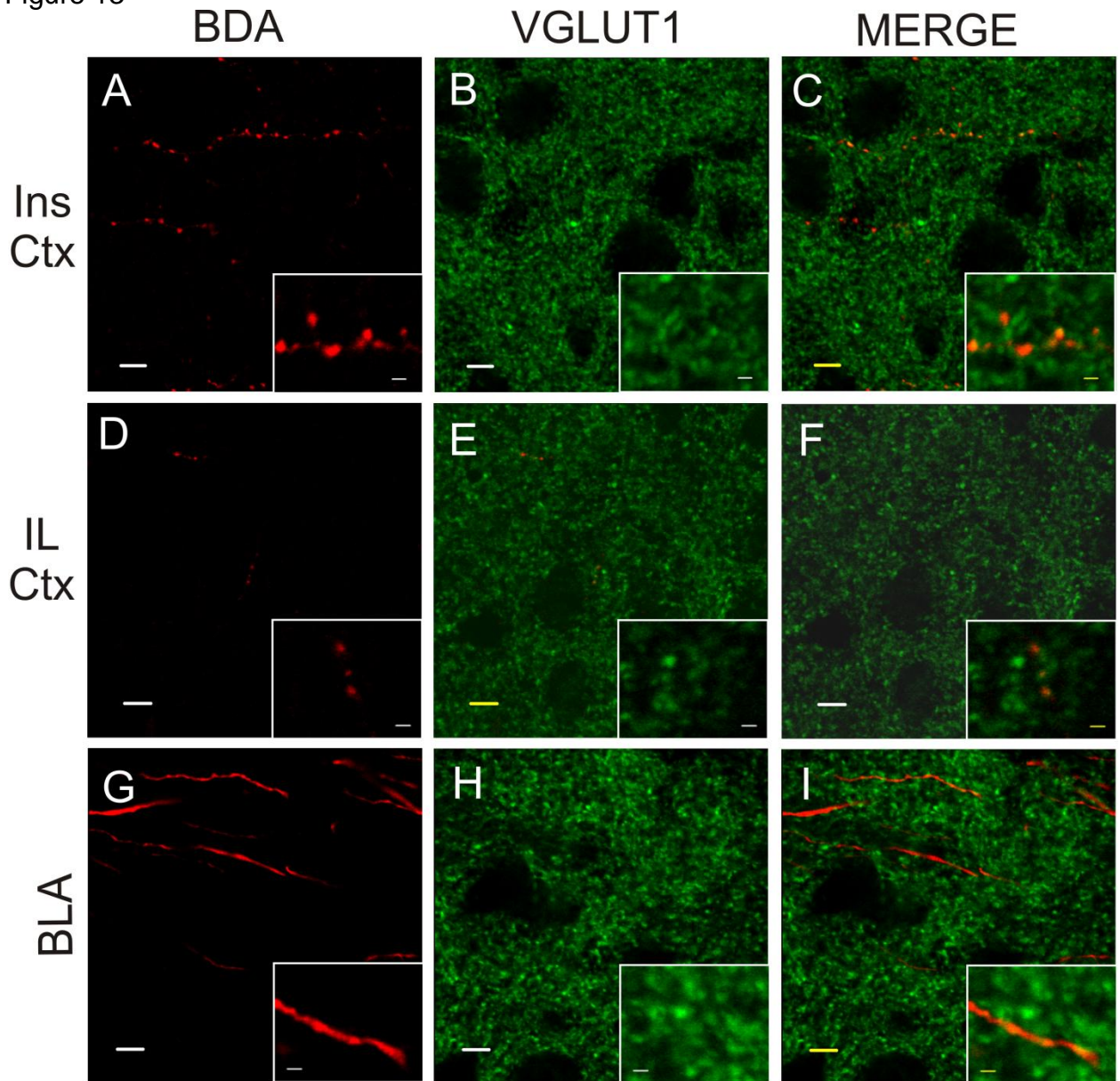


Figure 13. The insular cortex, infralimbic cortex, and BLA projections to the dorsal BNST are VGLUT1-containing. A-C) BDA projection from insular cortex A) BDA fibers B) VGLUT1 C) Merge D-F) BDA projection from infralimbic cortex D) BDA fibers E) VGLUT1 C) Merge G-I) BDA projection from the BLA. G) BDA fibers H) VGLUT1 I) Merge. Scale bars = 5  $\mu$ m. Insets, scale bars = 1  $\mu$ m.

## **Projections from the Insular Cortex, Infralimbic Cortex, and BLA potentially contain the $\alpha_{2A}$ -AR**

Recent data suggests that the  $\alpha_{2A}$ -AR plays an important role in modulation of glutamatergic transmission in the BNST (see Chapter II). Functional and immunohistochemical studies indicate that the  $\alpha_{2A}$ -AR regulates glutamatergic transmission in part via presynaptic heteroreceptors on glutamatergic terminals. Using the HA tagged  $\alpha_{2A}$ -AR KI mouse, we have begun to examine the potential distribution of  $\alpha_{2A}$ -ARs on fibers from the insular cortex, infralimbic cortex, and BLA. Qualitatively, the  $\alpha_{2A}$ -AR appears to colocalize on insular cortical, infralimbic cortical, and BLA fibers of the BNST (Figure 14A-I). Future studies examining localization of the  $\alpha_{2A}$ -AR on these projections should be conducted to confirm current findings, as the density of the  $\alpha_{2A}$ -AR precludes us from conducting reliable quantification of our data set.



Figure 14

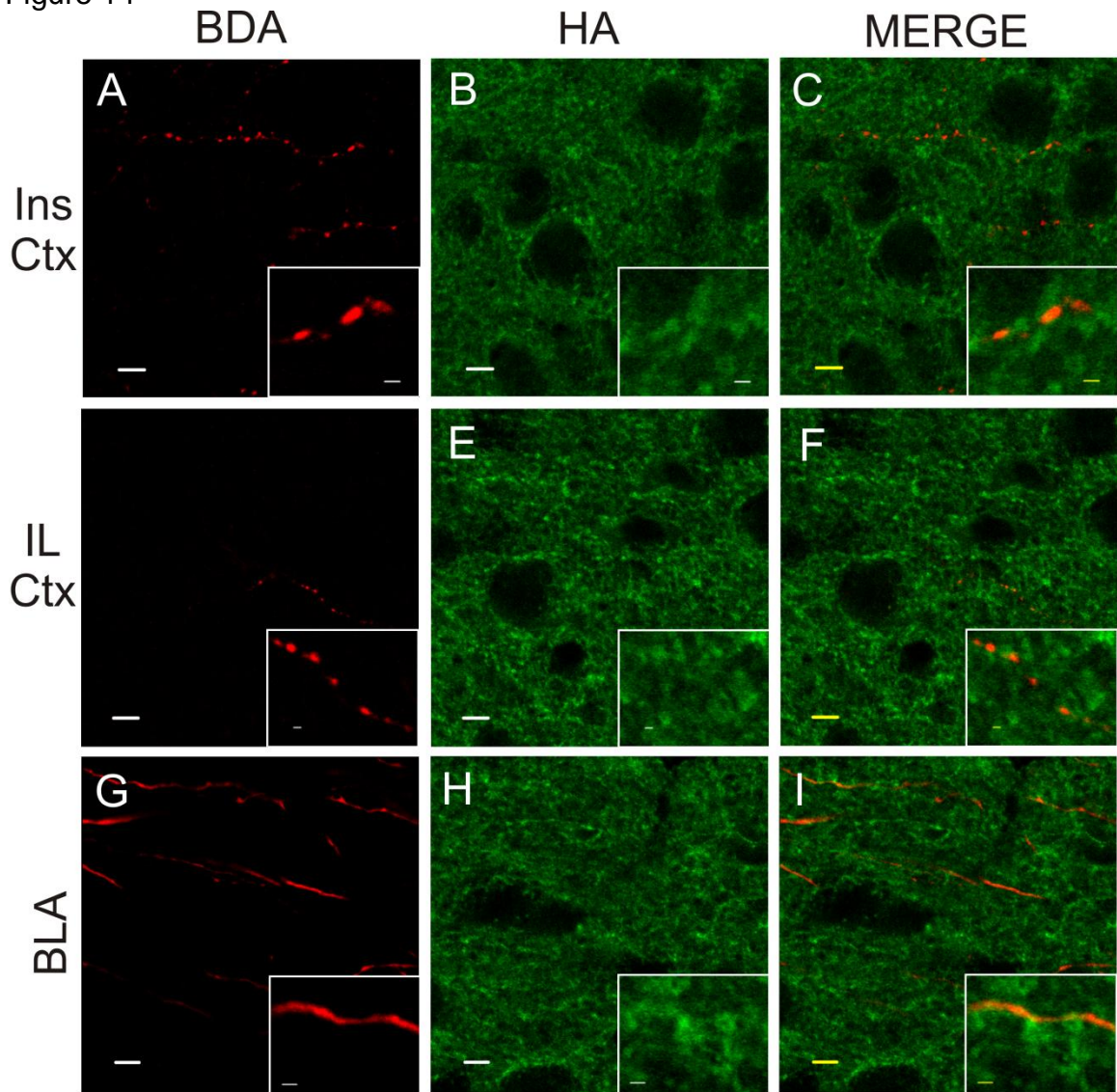


Figure 14. The insular cortex, infralimbic cortex, and BLA projections to the dorsal BNST are potentially  $\alpha_{2A}$ AR-containing. A-C) BDA projection from insular cortex. A) BDA fibers. B) HA. C) Merge. D-F) BDA projection from infralimbic cortex. D) BDA fibers. E) HA. C) Merge. G-I) BDA projection from the BLA. G) BDA fibers H) HA. I) Merge. Scale bars = 5  $\mu$ m. Insets, scale bars = 1  $\mu$ m.



## **General Discussion**

### **Insular cortex, infralimbic cortex, and BLA projections to the BNST in mouse and rat are phylogenetically conserved**

Our initial studies examining inputs to the BNST in the mouse model demonstrate anatomical projections from insular cortex, infralimbic cortex, and basolateral amygdala to the BNST in the C57BL/6J background mouse model. Additionally, injections made in regions surrounding these three sites, including the prelimbic cortex, striatum, and dorsal endopiriform cortex, did not project to the BNST. These findings suggest that projections to the BNST in rat and mouse are phylogenetically conserved. This is important, as much behavioral, biochemical, and biophysical work has been completed across various rat and mouse strains, and many conclusions have been drawn from findings across the two rodent models.

The BNST is a region known to be highly involved in stress and anxiety, as well as in relapse behaviors of addiction (Koob, 2008; Walker *et al*, 2003). Others have extensively reviewed studies implicating the BNST in stress and anxiety, and addictive behaviors (Choi *et al*, 2007; Forray *et al*, 2004; Koob, 2008; Smith *et al*, 2008; Stewart, 2000; Walker *et al*, 2008b; Walker *et al*, 2003).

Our current study begins to confirm that projections in rat brain to the BNST involved in anxiety and addiction are also present in mice. These projections include the BLA, a region involved in fear learning and extinction of learned behavior (Davis, 2006; Quirk *et al*, 2008; Walker *et al*, 2008b). The infralimbic and insular cortex are two additional regions known to modulate BNST

output in rodent models. The infralimbic cortex is important in consolidation of extinction learning in rodent models and regulates expression of amygdala-dependent memories [for review, see (Quirk *et al*, 2008)]. Others have shown the infralimbic projection to the BNST is directly involved in regulation of dopaminergic signaling in the ventral tegmental area, a critical region in the reward pathway that is disrupted by drugs of abuse (Massi *et al*, 2008). Here, we have begun to characterize the infralimbic projection to the BNST in mouse brain. Finally, we have demonstrated that in the mouse, as in rat, the insular cortex projects to the BNST. This region is known to be involved in processing of emotional and homeostatic information, and is thought to be involved in drug addicts' negative affective state during abstinence (Contreras *et al*, 2007). Additionally, lesion of the insula in humans has been reported to reduce feelings of craving (Naqvi *et al*, 2007). Thus, in the mouse model, the BNST receives input from regions highly involved in determining emotional significance of external stimuli.

### **The BLA, infralimbic cortex, and insular cortex projections to the BNST potentially contain $\alpha_{2A}$ -ARs**

Current studies indicate potential regulation of the insular cortex and BLA glutamatergic projections by activation of  $\alpha_{2A}$ -ARs. Further studies, however, are needed to confirm these findings, likely at the electron microscopy level as the density of the  $\alpha_{2A}$ -AR at the light microscopy level makes quantification of results unreliable in mouse brain tissue. Qualitative findings, however, indicate that the  $\alpha_{2A}$ -AR may be localized to fibers projecting from the insular cortex and BLA.

Recent data from our lab demonstrate that  $\alpha_{2A}$ -ARs regulate glutamate transmission in part by heterosynaptically modulating glutamate release at presynaptic glutamatergic terminals (see chapter II). Importantly, we show that the  $\alpha_{2A}$ -AR colocalizes with VGLUT1 at the light microscopy level (see chapter II). Here we show that projections from the insular cortex, infralimbic cortex, and BLA are VGLUT1(+), and that the  $\alpha_{2A}$ -AR potentially localizes to fibers on projections from these regions. If confirmed, these findings indicate that the  $\alpha_{2A}$ -AR regulates a subset of glutamatergic inputs to the BNST, and therefore is likely an important regulator of BNST output under stressful conditions such as during withdrawal from drugs of abuse.

### **Conclusions**

Injections of the anterograde tracer BDA into brain regions involved in processing stress and anxiety states, including the infralimbic and insular cortices and basolateral amygdala, demonstrate that each of these regions innervates the BNST in mouse brain. Additionally, the infralimbic and insular cortices and BLA are VGLUT1-containing. These anatomical findings support a broad array of functional and behavioral data implicating the BNST in stress and anxiety and relapse to drug-seeking behaviors in the mouse model.

## CHAPTER IV

### YOHIMBINE DEPRESSES TRANSMISSION IN THE DORSAL BNST VIA AN $\alpha_2$ -AR INDEPENDENT PROCESS

This chapter describes work completed toward the fulfillment of Aim III of my thesis project.

#### **Introduction**

As discussed previously in the thesis introductory chapter, yohimbine is a putative  $\alpha_2$ -AR antagonist that has been widely used to evoke anxiety in rodent models. It is clear that activation of central  $\alpha_2$ -ARs decreases anxiety during withdrawal and can prevent stress-induced relapse to drug-seeking behaviors (Morilak *et al*, 2005). In agreement with these findings, many studies have reported that the  $\alpha_2$ -AR antagonist yohimbine evokes anxiety in both humans (Holmberg *et al*, 1961; Murburg *et al*, 1991; Redmond *et al*, 1979) and animal studies (Davis *et al*, 1979; Holmes *et al*, 2002; Lang *et al*, 1963). Cain and others found that yohimbine can facilitate extinction of conditioned fear in mice (Cain *et al*, 2004), while other groups concluded that yohimbine has no effect on extinction of conditioned fear (Mueller *et al*, 2009). Other studies have shown that yohimbine can enhance development of CPP for morphine (Zarrindast *et al*, 2002). Additionally, intraperitoneal (i.p.) injections of yohimbine can reinstate methamphetamine-seeking following extinction in the self-administration model (Shepard *et al*, 2004), as well as alcohol-seeking (Le *et al*, 2005; Marinelli *et al*,

2007; Richards *et al*, 2009) and cocaine-seeking (Lee *et al*, 2004). IP yohimbine can also reinstate food-seeking in animals trained to lever press for food (Ghitza *et al*, 2006). Thus, yohimbine appears to enhance anxiety-like behaviors through its putative action on  $\alpha_2$ -ARs.

Other studies, however, have suggested that the anxiogenic effects of yohimbine are partially mediated through 5-HT<sub>1A</sub> receptors. Powell and others have shown that yohimbine disrupts prepulse inhibition of acoustic startle in rats through the 5-HT<sub>1A</sub> receptor, and not  $\alpha_2$ -ARs, as the effects of yohimbine were blocked by a 5-HT<sub>1A</sub>-R antagonist but not an  $\alpha_2$ -AR agonist, and the effect of yohimbine was not mimicked by the more selective  $\alpha_2$ -AR antagonist atipamezole (Powell *et al*, 2005). Studies looking at selectivity of yohimbine indicates that yohimbine has about a 10-fold selectivity for the  $\alpha_{2A}$ -AR over the 5-HT<sub>1A</sub>-R, where as atipamezole has negligible effects on the 5-HT<sub>1A</sub>-R (Newman-Tancredi *et al*, 1998). Thus, it is likely that blockade of  $\alpha_2$ -ARs cannot account for all of the anxiogenic effects of yohimbine, but further work is needed to differentiate the roles of yohimbine and  $\alpha_2$ -ARs in stress and anxiety.

Studies to date have looked at the effects of yohimbine on fear extinction, but not on reward-related memory. To further assess the role of yohimbine on reward-related memories and behavior, Davis and others looked at the role of yohimbine in extinction of reward-related memories formed by exposure to cocaine (Davis *et al*, 2008). Using a cocaine-induced CPP model, they showed that yohimbine impairs extinction of cocaine CPP, and that this effect is exacerbated in the  $\alpha_{2A}$ -AR knock out mice (Davis *et al*, 2008). Additionally, the

more selective  $\alpha_2$ -AR antagonist, atipamezole, does not alter cocaine CPP extinction and did not replicate effects of yohimbine on extinction of fear conditioning (Davis *et al*, 2008). These results contribute to studies suggesting that some effects of yohimbine are not mediated through the  $\alpha_2$ -AR.

To further investigate whether the effects of yohimbine are mediated solely through the  $\alpha_2$ -AR, we looked at the actions of yohimbine and atipamezole on excitatory transmission in the dorsal BNST. We found that yohimbine greatly depresses excitatory transmission in the BNST, that the effect of yohimbine is unaltered in the  $\alpha_{2A}$ -AR KO mouse, and that the effect of yohimbine is not replicated by atipamezole. This work was published with the behavioral work completed by Adeola Davis and others (Davis *et al*, 2008).

## **Experimental Procedures**

### **Animals**

Experiments on C57BL/6J mice were conducted using males obtained from The Jackson Laboratory (Bar Harbor, ME) aged 8–12 weeks. Male  $\alpha_{2A}$ -AR KO mice were generated as previously described (Altman *et al*, 1999) and backcrossed onto a C57BL/6J genetic background for a minimum of eight generations. KO and WT littermate controls were bred from heterozygous parents to minimize any potential genotype-related maternal abnormalities (Millstein and Holmes, 2007). Mice were housed on a 12 hour light/ dark cycle in groups of two to five with *ad libitum* access to food and water. All procedures were approved by the Vanderbilt University and NIAAA Animal Care and Use

Committees and were in accordance with the Animal Welfare Act and the guidelines outlined in “Using Animals in Intramural Research.”

### **Whole cell recordings**

Brain slices from the dorsal BNST were prepared as previously described (McElligott *et al*, 2008). Briefly, mice were retrieved from the colony and allowed to rest in sound attenuating boxes for a minimum of 1 hour, after which they were anesthetized (isoflurane) and decapitated in a separate room. Three-hundred-micrometer coronal slices were cut on a VT1000S vibratome (Leica Microsystems) in a 1°C–4°C, oxygenated (95% O<sub>2</sub>, 5% CO<sub>2</sub>), high-sucrose/low Na<sup>+</sup> artificial cerebral spinal fluid (ACSF; in mM: 194 sucrose, 20 NaCl, 4.4 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 10 glucose, 26 NaHCO<sub>3</sub>).

Following slicing, hemisected slices were allowed to rest submerged in a holding chamber filled with oxygenated and heated (28°C) ACSF for at least 30 min. After this incubation time, an individual slice was moved to the recording chamber where it was submerged in oxygenated and heated (28°C) ACSF with added picrotoxin (25 μM included for the entirety of all experiments) to isolate currents evoked by glutamate receptor activation at a rate of 2 mL/min. A bipolar Nichrome (A-M Systems) stimulating electrode was placed dorsally to the recording electrode within the dBNST. Patch electrodes (3–6 MΩ) were pulled on a Flaming/Brown microelectrode puller (Sutter Instruments) and filled with either cesium (Cs)-gluconate intracellular solution (in mM: Cs-gluconate 135, NaCl 5, MgCl<sub>2</sub> 2, HEPES 10, EGTA 0.6, Na<sub>2</sub>ATP 4, Na<sub>2</sub>GTP 0.4). In all whole-

cell experiments, cells were clamped at  $-70$  mV throughout and excitatory post-synaptic currents (EPSCs) were recorded using Clampex 9.2 (Molecular Devices). Series resistance was monitored throughout each experiment, and a change greater than 20% resulted in the exclusion of the experiment from the data set. EPSCs were evoked at a frequency of 0.167 Hz, and 100–400 pA EPSCs were recorded. Consistent with the field experiments, drugs were bath applied at their final concentrations.

A 5-10 min baseline was acquired prior to drug application, and all points were normalized to minutes 8–10 within each. Baseline is an average of 2 min before drug application, and the experimental value is 24–25 min following drug application. Points are 1-min averages on plotted time course.

## **Results**

Central NE derives from two primary sources, the VNAB and the DNAB. Lesion of the VNAB impairs extinction learning (Cole and Robbins, 1987). The VNAB projections terminate in the lateral hypothalamus and the extended amygdala, including the BNST (Aston-Jones *et al*, 1999). Thus, we examined the effects of yohimbine and atipamezole on glutamatergic transmission in the dorsal BNST. We previously reported that the  $\alpha_2$ -AR agonists clonidine and UK-14,304 elicit an acute depression of glutamatergic transmission in this region (UK-14,304,  $41.7 \pm 7.7\%$  of baseline,  $P < 0.05$ ) (Egli *et al*, 2005). We have replicated these data and show a representative experiment for comparison (Figure 15A). Paradoxically, we have shown here that a 30 min 10  $\mu$ M



application of yohimbine in the dBNST unexpectedly caused a similar decrease (31.37% ± 6.90% of baseline;  $t = 11.10$ ,  $df = 5$ ,  $P < 0.001$ ) in glutamatergic transmission (Figure 15B). Further, while the effect of UK-14,304 was absent in slices prepared from  $\alpha_{2A}$ -AR KO mice (Egli *et al*, 2005), yohimbine still caused a decrease (42.6 ± 7.5% of baseline;  $t = 5.44$ ,  $df = 5$ ,  $P < 0.003$ ) in glutamatergic transmission in the dBNST of these mice (Figure 15C). We then compared the actions of yohimbine with that of the more selective  $\alpha_2$ -AR antagonist atipamezole. A 30 min 1  $\mu$ M application of atipamezole did not reproduce the effect of yohimbine ( $P < 0.001$ ) on glutamatergic transmission (atipamezole: 75.6% ± 4.9% of baseline,  $n = 10$ ;  $t = 5.01$ ,  $df = 9$ ) (Figure 15D). This concentration of atipamezole was sufficient to antagonize effects of UK-14,304 (85.64% ± 6.82% of baseline;  $t = 2.59$ ,  $df = 2$ ,  $P > 0.12$ ) on glutamatergic transmission in the dBNST (Figure 15E). These experiments suggest that yohimbine modulates glutamatergic transmission in the dBNST independent of  $\alpha_2$ -ARs.

Figure 15

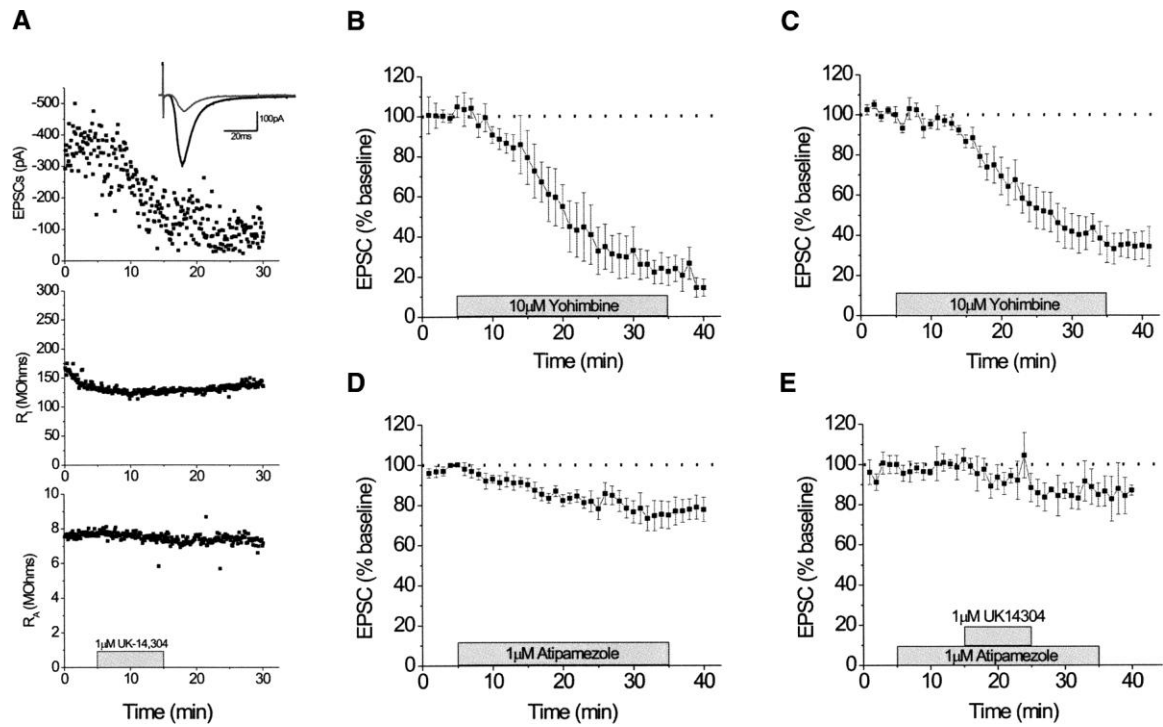


Figure 15. Yohimbine depresses glutamatergic transmission in the BNST through a non-  $\alpha_2$ -AR-dependent mechanism. EPSCs in the dIBNST: (A) 1  $\mu$ M UK-14,304 (n=1); (B) 10  $\mu$ M yohimbine (n=10); (C) 10  $\mu$ M yohimbine in  $\alpha_{2A}$ -AR KO mouse (n = 6); (D) 1  $\mu$ M atipamezole (n = 10); (E) 1  $\mu$ M UK-14,304 in the presence of 1  $\mu$ M atipamezole (n = 3). Error bars,  $\pm$  SEM.

## **Discussion**

### **Yohimbine depresses glutamatergic transmission in the dorsal BNST through an off-target action**

To address the possible mechanistic basis of the behavioral effects of yohimbine and atipamezole, we utilized whole cell patch clamp techniques and recorded from dBNST neurons. The BNST receives a dense noradrenergic input from the VNAB (Aston-Jones *et al*, 1999). When the VNAB is lesioned, there is an impairment of negative valence-learned extinction (Cole *et al*, 1987), suggesting regions innervated are essential to extinction of learned behaviors. We found atipamezole was able to block the depression in excitatory transmission caused by the  $\alpha_2$ -AR agonist UK-14,304. In contrast, yohimbine itself elicited a depression on excitatory transmission, and this effect was not replicated by the more selective  $\alpha_2$ -AR antagonist, atipamezole. Furthermore, we found that in  $\alpha_{2A}$ -AR KO mouse slices, the depression caused by yohimbine persists. One interpretation is that yohimbine is acting through a non- $\alpha_2$ -AR mechanism in this brain region, and that this may contribute to impairment of extinction in cocaine CPP.

Yohimbine is known to have a 10-fold selectivity for the  $\alpha_{2A}$ -AR over the 5-HT<sub>1A</sub>-R (Newman-Tancredi *et al*, 1998), and its effects on prepulse inhibition of acoustic startle in rats have been suggested to be mediated via the 5-HT<sub>1A</sub>-R (Powell *et al*, 2005). 5-HT causes heterogenous changes in the excitability of cells in the BNST, sometimes eliciting a hyperpolarization, hyperpolarization followed by depolarization, or a depolarization in whole cell electrophysiology recordings under current clamp conditions (Rainnie, 1999). Further studies by

this group demonstrated that a broad spectrum 5-HT<sub>1</sub>R agonist elicited a hyperpolarization in neurons of the BNST that was blocked by a 5-HT<sub>1A</sub>-R selective antagonist. *In vivo* injections of the 5-HT<sub>1</sub>R agonist attenuated the acoustic startle response, indicating a role for the 5-HT<sub>1</sub>R in the BNST for limiting anxiety-like responses to stressful stimuli (Levita *et al*, 2004). Thus, it is possible that the effects of yohimbine on anxiety are partially mediated via the 5-HT system.

## CHAPTER V

### GENERAL DISCUSSION

#### **Summary of findings**

Stress is implicated as a major driving force for relapse to drugs of abuse in humans and in animal models (Brown *et al*, 1995; Le *et al*, 2000). NE is a hormone released in response to stressors known to induce relapse in animal models of drug addiction, and is additionally released during withdrawal from drugs of abuse in both rodents and humans (Koob, 2008). The BNST is a region of the brain involved in stress and anxiety, and importantly contains the densest innervation of NE in the brain (Forray *et al*, 2004). Studies show that NE is released in the BNST in response to unconditioned and prolonged stressors (Cecchi *et al*, 2002a; Fuentealba *et al*, 2000).

$\alpha_2$ -AR agonists injected into the BNST consistently decrease measures of anxiety-like behaviors and block stress-induced relapse to drug-seeking (Delfs *et al*, 2000; Schweimer *et al*, 2005; Shaham *et al*, 2000b; Wang *et al*, 2001). Systemically,  $\alpha_2$ -AR agonists also lead to decreased anxiety-like behaviors and can block stress-induced reinstatement to drug-seeking (Erb *et al*, 2000; Highfield *et al*, 2001; Le *et al*, 2005; Morilak *et al*, 2005; Shaham *et al*, 2000a; Zislis *et al*, 2007), thus implicating either a global role of the  $\alpha_2$ -AR to limit the stress response, and/or a major role of the BNST in mediating behavioral responses to  $\alpha_2$ -AR agonists.

Studies demonstrating a potential heterosynaptic role for actions of the  $\alpha_2$ -AR at glutamatergic synapses in the brain (Delaney *et al*, 2007; Glass *et al*, 2001; Milner *et al*, 1998; Milner *et al*, 1999), in addition to previous studies in our lab demonstrating an acute depression of excitatory transmission by  $\alpha_2$ -ARs, led us to focus on the potential heterosynaptic regulation of glutamate transmission by  $\alpha_2$ -ARs. Electrophysiology studies show that  $\alpha_2$ -AR agonists depress excitatory transmission in the BNST, and this response is absent in the  $\alpha_{2A}$ -AR knockout mouse (Egli *et al*, 2005). For this reason, we focused our studies on the  $\alpha_{2A}$ -AR subtype. As noted in Figure 4 on page 65, the  $\alpha_{2A}$ -AR could potentially regulate excitatory transmission indirectly as an autoreceptor via modulation of NE release at NE terminals, directly as a presynaptic heteroreceptor on glutamate terminals regulating glutamate release, or as a postsynaptic receptor altering the response to glutamate via modulations at postsynaptic sites.

The goal of this work was to determine the role of the  $\alpha_{2A}$ -AR subtype in modulation of glutamatergic transmission, and to localize the  $\alpha_{2A}$ -AR at the light microscopy level. We additionally sought to examine the potential for the  $\alpha_{2A}$ -AR to regulate a subset a glutamatergic inputs to the BNST. Because most anatomical studies examining afferents to the BNST have been conducted in rat, we sought to confirm that relevant projections to the BNST were phylogenetically conserved in the mouse model. We also examined the specificity of the  $\alpha_2$ -AR antagonist, yohimbine, as several reports have questioned its assumed

mechanism of evoking anxiety in humans and rodent models via blockade of the  $\alpha_2$ -AR.

We have found that the  $\alpha_{2A}$ -AR functions in the BNST as a heteroreceptor presynaptically regulating glutamate release. We show that guanfacine, an  $\alpha_{2A}$ -AR selective agonist, dose-dependently depresses excitatory transmission in the BNST, and that this effect is blocked by the  $\alpha_2$ -AR antagonist atipamezole. Guanfacine causes a significant increase in the paired pulse ratio of evoked EPSCs, indicating that its effects on glutamate transmission may be mediated presynaptically. Using an HA-tagged  $\alpha_{2A}$ -AR KI mouse, we show that the distribution of the  $\alpha_{2A}$ -AR is much broader than that of the noradrenergic markers NET and TH. We show that the distribution of the  $\alpha_{2A}$ -AR partially overlaps with that of the glutamatergic terminal marker VGLUT1, and that at the light microscopy level, the  $\alpha_{2A}$ -AR appears to colocalize with VGLUT1. We additionally demonstrate that the  $\alpha_2$ -AR regulates GABAergic transmission in the dorsal BNST in a dose-dependent manner.

Our tracer studies confirm that the insular cortex, infralimbic cortex, BLA, and CeA projections to the BNST are phylogenetically conserved from the rat to the mouse model. We provide preliminary evidence that these projections are  $\alpha_{2A}$ -AR-containing, although future studies are needed to confirm our findings due to the density of the  $\alpha_{2A}$ -AR at the light microscopy level.

Finally, we demonstrate that yohimbine depresses glutamatergic transmission in the dorsal BNST in non- $\alpha_2$ -AR-dependent manner. The effects of yohimbine are not replicated by the more selective  $\alpha_2$ -AR antagonist,

atipamezole. Additionally, the effects of yohimbine are present in the  $\alpha_{2A}$ -AR knockout mouse. This work was done in collaboration with Adeola Davis, who demonstrated that yohimbine impairs extinction of cocaine CPP, that this effect of yohimbine is exacerbated in the  $\alpha_{2A}$ -AR KO mouse, and that the effect of yohimbine on extinction of cocaine CPP is not replicated by the more selective  $\alpha_2$ -AR agonist atipamezole (Davis *et al*, 2008).

### **Heterosynaptic $\alpha_{2A}$ -ARs in the brain serve critical roles in stress, learning, and other behaviors**

$\alpha_{2A}$ -ARs have long been known, along with  $\alpha_{2C}$ -ARs, to function as autoreceptors regulating NE release in the brain (Bucheler *et al*, 2002; Devoto *et al*, 2004; Trendelenburg *et al*, 1999; Trendelenburg *et al*, 2001). Although certainly modulation of NE release is a potential and likely mechanism of the regulation of stress and relapse behavior in rodents and humans, evidence is accumulating that  $\alpha_2$ -ARs may mediate many of their effects via heterosynaptic modulation of other neurotransmitters such as 5-HT, DA, glutamate and GABA. Gilsbach and others recently used the DBH promoter to drive expression of the  $\alpha_{2A}$ -AR exclusively in noradrenergic and adrenergic cells in  $\alpha_{2A}$ -AR and  $\alpha_{2C}$ -AR double knockout mice (Gilsbach *et al*, 2009). They found that many functions typically attributed to  $\alpha_2$ -AR autoreceptors, such as analgesia, hypothermia, sedation and anesthetic-sparing, were not rescued in mice expressing the  $\alpha_{2A}$ -AR exclusively in adrenergic neurons (curiously, they did not examine potential anxiety phenotypes in this report). They suggest that heterosynaptic  $\alpha_2$ -ARs likely play a significant role in mediating these behaviors (Gilsbach *et al*, 2009).



Studies examining the ultrastructural localization of  $\alpha_{2A}$ -AR-like immunoreactivity in the rodent brain have found  $\alpha_{2A}$ -ARs to be localized to various compartments within neurons, including presynaptically on autosynaptic and heterosynaptic terminals, as well as postsynaptically and on glial cells in regions including the hippocampus, ventrolateral medulla, and NTS (Glass *et al*, 2001; Milner *et al*, 1998; Milner *et al*, 1999). These studies also support a potential role for heterosynaptic  $\alpha_{2A}$ -ARs. Our studies demonstrate that  $\alpha_{2A}$ -ARs are distributed in a pattern similar to the glutamatergic terminal marker VGLUT1(+), and also have an overlapping albeit broader distribution than the NA and DA terminal marker TH, therefore implicating a heterosynaptic role for the  $\alpha_{2A}$ -AR in the BNST.

Recent studies have demonstrated a heterosynaptic role of the  $\alpha_2$ -AR in mediating alterations in glutamate transmission (Carey *et al*, 2009; Delaney *et al*, 2007; Yamanaka *et al*, 2006) and GABA transmission (Yamanaka *et al*, 2006) in the brain. Our work adds to this body of knowledge by demonstrating a role for the  $\alpha_{2A}$ -AR in heterosynaptic modulation of glutamatergic and GABAergic transmission in the dorsal BNST.

Studies specifically examining the role of  $\alpha_2$ -ARs in heterosynaptic modulation of synaptic transmission have revealed the importance of this receptor in synaptic plasticity and potentially learning. NE is known to be important for allowing focused attention on behaviorally-relevant external stimuli, and is also critically involved in the stress response. Delaney and others have shown that heterosynaptic  $\alpha_2$ -ARs presynaptically regulate glutamate release in a

subset of glutamatergic inputs to the central nucleus of the amygdala (Delaney *et al*, 2007). They observe a depression in excitatory transmission that is mediated through  $\alpha_2$ -AR actions to block fusion of glutamate-containing vesicles with the presynaptic terminal via the  $\beta\gamma$  subunit of the  $\alpha_2$ -AR-linked GPCR. They find that  $\alpha_2$ -ARs decrease glutamate release from the basket terminals of the PBN, but do not alter glutamate release from the BLA input (Delaney *et al*, 2007), thus implicating the  $\alpha_2$ -AR in regulation of inputs relaying information about peripheral pain.

Similarly, in the cerebellum, Carey and Regehr have found that heterosynaptic  $\alpha_2$ -ARs decrease presynaptic glutamate release at climbing fiber synapses (carrying information from the inferior olivary nucleus) to the Purkinje cells, but do not affect glutamate release at parallel fiber synapses (carrying information from the spinal cord and brainstem nuclei) with Purkinje cells (Carey *et al*, 2009). Importantly, they demonstrate an  $\alpha_2$ -AR-mediated disruption in long-term plasticity at parallel fiber-Purkinje cell synapses that is normally elicited via repeated simultaneous stimulation of these synapses and the climbing fiber-Purkinje cell synapses. Thus, they suggest that activation of  $\alpha_2$ -ARs activation can disrupt associative motor learning by selectively altering glutamate release at climbing fiber synapses (Carey *et al*, 2009).

In the BNST,  $\alpha_2$ -AR agonists also depress excitatory transmission, and this response is nearly abolished in the  $\alpha_{2A}$ -AR knockout mouse (Egli *et al*, 2005). The current studies expand upon these findings. We demonstrate a heterosynaptic  $\alpha_{2A}$ -AR-mediated presynaptic decrease of glutamate release in

the dorsal BNST. Additionally, our work has begun to demonstrate potential specificity of regulation at a subset of glutamatergic synapses in the BNST. We show that  $\alpha_{2A}$ -ARs are localized to VGLUT1(+) terminals, although our work does not allow us to rule out localization to VGLUT2(+) terminals. Using BDA-tracer injections, we were able to demonstrate that the  $\alpha_{2A}$ -AR is potentially localized to glutamatergic inputs from the BLA, insular cortex, and the infralimbic cortex. Fibers anterogradely labeled with BDA from these regions show expression of VGLUT1 and the  $\alpha_{2A}$ -AR at the light microscopy level, although future studies are needed to confirm these preliminary findings due to the density of HA-  $\alpha_{2A}$ -AR labeling.

Future studies examining whether the  $\alpha_{2A}$ -AR globally regulates glutamatergic transmission in the BNST, or is involved in regulating a subset of glutamatergic inputs, will be important in understanding how NE elicits behavioral responses to stress via its actions through the  $\alpha_{2A}$ -AR in the BNST. EM studies combining anterograde tracer work with immunolabeling of the  $\alpha_{2A}$ -AR using the HA  $\alpha_{2A}$ -AR KI mouse, is one way to potentially examine this question. Electrophysiology studies using techniques such as those employed by Delaney and others to illuminate specific inputs to the BNST are another potential way to examine this issue.

### **Activation of $\alpha_2$ -ARs in the dorsal BNST depresses GABAergic transmission**

In addition to our work demonstrating heterosynaptic  $\alpha_{2A}$ -AR-mediated alterations in glutamate release, we also show that activation of  $\alpha_2$ -ARs dose-

dependently modulates GABAergic transmission in the dorsal BNST. Our BDA tracer injections in the HA  $\alpha_{2A}$ -AR KI mouse suggests potential interaction of the  $\alpha_{2A}$ -AR with presynaptic fibers or terminals from the CeA, although these are preliminary findings in one mouse and have the same caveat as other tracer injections co-labeled with the  $\alpha_{2A}$ -AR due to the density of this label at the light microscopy level.

In contrast to this apparent presynaptic labeling of CeA fibers, our electrophysiology findings show no increase in PPR upon guanfacine application in whole-cell electrophysiology recordings. Additionally microdialysis studies examining extracellular GABA levels, and whole-cell electrophysiology studies looking at spontaneous inhibitory transmission in the BNST, lead us to suggest that actions of the  $\alpha_2$ -AR on GABAergic transmission may be mediated postsynaptically (Dumont *et al*, 2004; Forray *et al*, 1999). Further studies are needed, however, to definitely sort out the mechanism behind the depression of GABAergic transmission elicited by activation of  $\alpha_2$ -ARs.

Importantly, we must consider the possibility that the  $\alpha_{2A}$ -AR selective agonist guanfacine is eliciting changes in GABAergic transmission at higher doses due to actions at the  $\alpha_{2C}$ -AR. Guanfacine shows a 22-fold selectivity for the  $\alpha_{2A}$ -AR subtype over the  $\alpha_{2C}$ -AR (Uhlen *et al*, 1992), thus at higher concentrations of guanfacine we cannot rule out activation of the  $\alpha_{2C}$ -AR in addition to the  $\alpha_{2A}$ -AR. The potential role of the  $\alpha_{2A}$ -AR on CeA fibers in the BNST, if this localization is later confirmed, warrants further study.

Finally, our studies show that guanfacine has a higher potency on glutamatergic than GABAergic transmission in the BNST, suggesting that  $\alpha_{2A}$ -ARs may primarily mediate their effects on BNST output via modulation of glutamatergic transmission. How this difference in potency might affect BNST output is hypothesized later in this section.

### **The $\alpha_{2A}$ -AR potentially mediates some of its effects on glutamatergic and GABAergic transmission via modulation of 5HT and DA**

Many studies have demonstrated a role for  $\alpha_2$ -ARs in regulation of 5-HT and DA release via *in vitro* and *in vivo* microdialysis (Ansah *et al*, 2003; Bucheler *et al*, 2002; Gobert *et al*, 1998; Ihalainen *et al*, 2002; Maura *et al*, 1992; Numazawa *et al*, 1995; Scheibner *et al*, 2001). Additionally, work completed in forebrain synaptosomal preparations suggests that  $\alpha_2$ -ARs can modulate function of the 5-HT transporter, SERT (Ansah *et al*, 2003). Therefore, we must consider that activation of  $\alpha_2$ -ARs not only directly regulates glutamate and GABA transmission in the BNST, but that some of these effects may be mediated by altered endogenous release of 5HT and DA.

In the frontal cortex,  $\alpha_2$ -AR agonists can decrease extracellular levels of DA and NE (Gobert *et al*, 1998; Ihalainen *et al*, 2002), although  $\alpha_2$ -ARs agonists are shown to not regulate DA levels in the NAc (Ihalainen and Tanila, 2004). The BNST receives DA projections from both the VTA and PAG (Hasue *et al*, 2002; Meloni *et al*, 2006; Zahm *et al*, 2001). Our immunohistochemical studies do demonstrate the presence of the  $\alpha_{2A}$ -AR on TH(+) terminals, potentially implicating the  $\alpha_{2A}$ -AR as a potential regulator of DA transmission in the BNST.

Whole-cell electrophysiology recordings show that DA enhances excitatory transmission in the dorsal BNST via D1 and D2 receptors (Kash *et al*, 2008), and it is conceivable that activation of  $\alpha_2$ -ARs may decrease endogenous DA release, leading to a depression of excitatory transmission in the dorsal BNST. This mechanism, however, would require that DA is tonically released in the BNST under *ex vivo* slice recording conditions. Though we cannot rule this out as contributing to the depression in excitatory transmission elicited by activation of  $\alpha_2$ -ARs; the robustness of our effect, localization of the  $\alpha_{2A}$ -ARs to VGLUT1(+) glutamatergic terminals, and the increase in PPR that we see upon guanfacine application, lead us to believe this is not likely the main mechanism behind the alteration in glutamate transmission that we see upon guanfacine application.

Microdialysis studies additionally point to modulation of 5HT release by  $\alpha_2$ -ARs in regions such as the hippocampus and prefrontal cortex, (Gobert *et al*, 1998; Maura *et al*, 1992; Numazawa *et al*, 1995; Scheibner *et al*, 2001). We must therefore consider that a decrease in 5-HT release may contribute to our findings with guanfacine application on transmission in the dorsal BNST. The BNST receives a serotonergic projection from the raphe nucleus (Phelix *et al*, 1992a, b). 5-HT in the BNST is concentrated heavily in the medial BNST, although Rainnie has shown that cells in the dorsal lateral BNST show alterations in excitability in response to 5-HT application (Rainnie, 1999). Though this response is variable, cells in the lateral BNST tend to hyperpolarize in response to 5-HT application, and this is likely via activation of the 5-HT<sub>1A</sub>-R (Rainnie, 1999). More work is needed to examine the contributions of 5-HT to alterations

in glutamate transmission in the BNST. 5-HT agonists injected into the BNST, however, indicate an anxiolytic role of this neurotransmission, similar to activation of the  $\alpha_{2A}$ -AR (Levita *et al*, 2004). Thus, it is unlikely that activation of the  $\alpha_{2A}$ -AR would decrease 5-HT release and/or enhance 5-HT clearance to coordinate an anxiolytic or stress-protective effect of BNST output.

It seems more likely, that the  $\alpha_{2A}$ -AR may enhance 5-HT transmission to increase its anxiolytic effects in the BNST. Studies by Ansah and others suggest that activation of  $\alpha_2$ -AR in the forebrain can inhibit function of the serotonin transporter, SERT, thereby elevating 5-HT extracellular levels (Ansah *et al*, 2003). Therefore, we must consider that the effects of  $\alpha_2$ -AR activation on transmission in the BNST may partially be mediated by the ability of the  $\alpha_2$ -AR to inhibit SERT function. Again, the degree to which this potentially contributes to our electrophysiology findings is dependent on the endogenous levels of 5-HT in our slice preparation, something we have not yet had the opportunity to examine.

**Activation of  $\alpha_2$ -ARs in the BNST is likely critical in regulation of the stress response and relapse to drug-seeking behaviors**

Curiously, we find that activation of  $\alpha_{2A}$ -ARs depresses both glutamatergic and GABAergic transmission in the BNST under *ex vivo* whole-cell electrophysiology conditions, although the effect on glutamatergic transmission is more potent than the effect on GABAergic transmission. Regulation of both glutamatergic and GABAergic transmission by  $\alpha_2$ -ARs in the BNST complicates potential explanations for how activation of  $\alpha_2$ -ARs in the BNST results in known decreased anxiety behaviors and blockade of stress-induced relapse to drug-

seeking in animal models. Interestingly, Yamanaka and others similarly see a depression in both glutamatergic and GABAergic transmission via activation of  $\alpha_2$ -ARs in whole cell recordings of orexin-(+) neurons of the lateral hypothalamus (Yamanaka *et al*, 2006). It is likely that  $\alpha_2$ -AR-mediated depression of glutamatergic and GABAergic transmission in the BNST is coordinated to regulate BNST output to regions involved in stress and reward such as the PVN and VTA.

Excitatory input into the BNST comes from insular cortex, infralimbic cortex, the ventral subiculum and the basolateral amygdala (Dong *et al*, 2001a; Massi *et al*, 2008; Weller *et al*, 1982), other regions known to be involved in stress-reward circuitry, and regions known to express mRNA for the  $\alpha_{2A}$ -AR (Scheinin *et al*, 1994; Wang *et al*, 1996). Thus, the  $\alpha_{2A}$ -AR potentially gates these inputs, effectively decreasing excitatory drive to the BNST during events known to release NE, such as chronic stressors, withdrawal from drugs of abuse, and other social stressors (Aston-Jones *et al*, 1999; Cecchi *et al*, 2002a; Cecchi *et al*, 2002b; Delfs *et al*, 2000; Fendt *et al*, 2005).

The main inhibitory projection to the BNST is from a closely related structure, the central nucleus of the amygdala (CeA), a region also involved in fear and anxiety states. Additionally, the BNST is made up of a large network of GABAergic interneurons. Thus, modulation of inhibitory transmission by activation of  $\alpha_2$ -ARs may also influence output of the BNST by modulating GABAergic transmission at CeA-BNST synapses, or by altering activity of interneurons in the BNST.



The projection from the BNST to the PVN is known to be GABAergic (Cullinan *et al*, 1993; Herman *et al*, 1994), and activation of the BNST is thought to lead to disinhibition of this projection. Activation of  $\alpha_{2A}$ -ARs in the BNST may help limit this disinhibition during stressful circumstances. One potential mechanism is that by depressing glutamatergic transmission,  $\alpha_{2A}$ -AR activation modulates glutamate release onto inhibitory interneurons in the BNST. These interneurons may be activated under stress, therefore mediating disinhibition of the BNST. Thus  $\alpha_{2A}$ -ARs may alleviate or limit the disinhibition of the PVN under stress. Similarly, depression of GABAergic transmission on PVN projection neurons may also serve to limit the disinhibition of this projection in stressful conditions (see Figure 16 for illustration).

Figure 16.

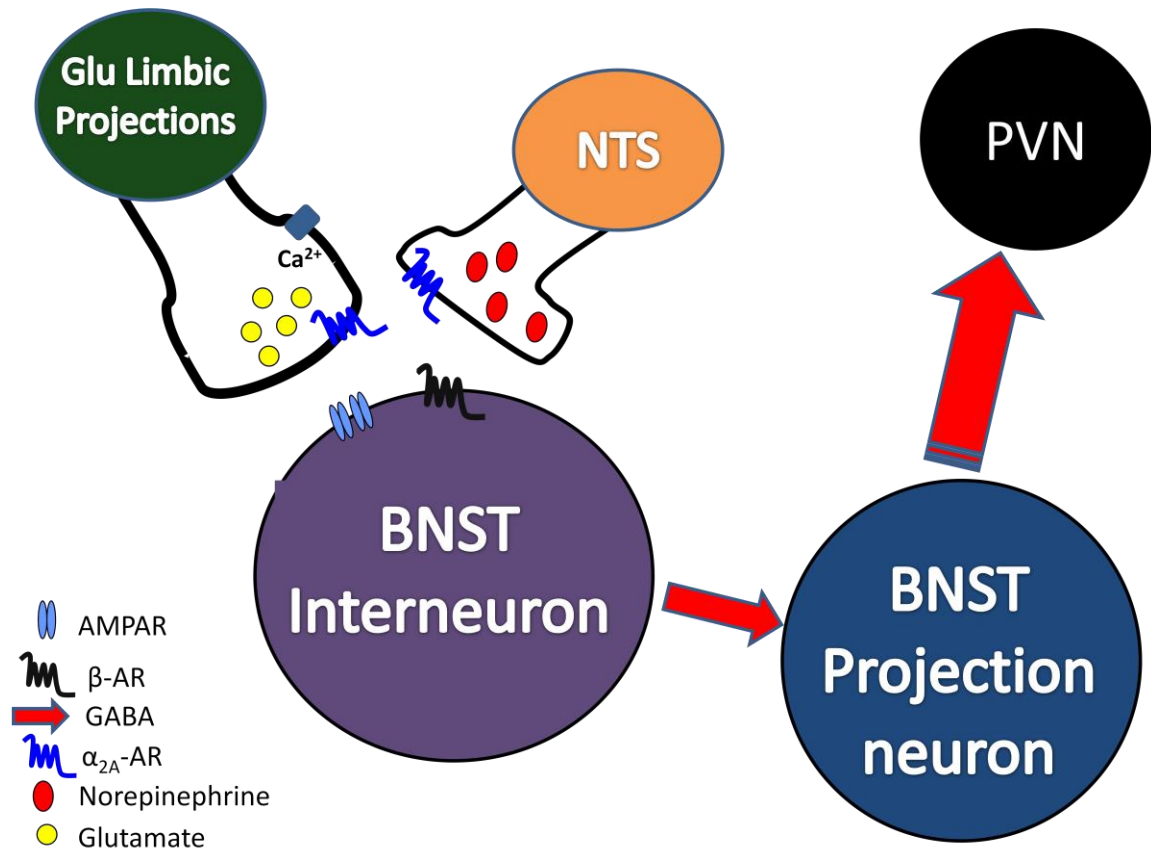


Figure 16. Illustration of one way activation of  $\alpha_{2A}$ -ARs could lead to decreased inhibitory and excitatory transmission in the dorsal BNST, and thereby limit disinhibition of the PVN when glutamatergic inputs are activated under stress.

The makeup of the BNST projection to the VTA is controversial. While output of the BNST is typically considered to be GABAergic (McDonald, 1983; Sun *et al*, 1993), recent studies have led researchers to conclude that this projection is excitatory (Dumont *et al*, 2004; Georges *et al*, 2002), and is CRF(+) (Rodaros *et al*, 2007). In general though, activation of the BNST is thought to lead to activation of the VTA. For example, Massi and others have demonstrated that cannabinoid receptors in the BNST control infralimbic cortex-mediated excitation of the VTA (Massi *et al*, 2008). Studies demonstrate that  $\alpha_2$ -AR agonists in the BNST block stress-induced reward-seeking behaviors, and this data suggests that activation of the  $\alpha_2$ -AR receptor serves to limit the stress response (Delfs *et al*, 2000; Wang *et al*, 2001). Thus, we can imagine that  $\alpha_{2A}$ -ARs in the BNST regulate projections to the VTA by decreasing or limiting activation of excitatory BNST projections to the VTA, or augmenting activation of inhibitory BNST projections to the VTA.

Importantly, understanding the physiologic role of the  $\alpha_{2A}$ -AR in the BNST will be critical for interpreting the findings of this thesis work. While we demonstrate presence of the  $\alpha_{2A}$ -AR in the BNST, and show that  $\alpha_{2A}$ -AR selective agonist guanfacine can heterosynaptically regulate excitatory and inhibitory transmission under *ex vivo* whole-cell electrophysiology conditions, the extent to which these receptors are activated by NE under normal physiological conditions is unknown. Our findings demonstrate a widespread distribution of the  $\alpha_{2A}$ -AR outside that of NET and TH, raising the possibility that NE may not reach a significant portion of the heteroreceptor  $\alpha_{2A}$ -AR population when low levels of

NE are present in the BNST (such as when an animal is at rest or under low-stress conditions). It is conceivable that these receptors are activated only in response to prolonged or intense stress (and therefore prolonged or greater increases in NE levels in the BNST), or that these receptors are rarely activated. Future studies examining the extent and result of endogenous activation of  $\alpha_{2A}$ -ARs in the BNST under varying stress-conditions would help elucidate the physiological function of  $\alpha_{2A}$ -ARs in the BNST. The presence of  $\alpha_{2A}$ -ARs on glutamatergic terminals in the BNST as well as the ability of the  $\alpha_{2A}$ -AR selective agonist guanfacine to depress excitatory transmission in this region, make  $\alpha_{2A}$ -ARs an intriguing target for anti-anxiety therapeutics.

**$\alpha_{2A}$ -AR selective agonists may be important therapeutic agents for treating anxiety disorders and in preventing stress-induced relapse to drug-seeking**

Effective pharmacological tools for helping addicts with anxiety and craving are extremely limited at this time. Guanfacine, an  $\alpha_{2A}$ -AR selective agonist, is FDA-approved for treatment of some anxiety disorders including post-traumatic stress disorder (PTSD) and chronic Tic disorder. Guanfacine is also prescribed as a centrally-acting hypertensive agent to reduce blood pressure in patients struggling with hypertension (Kisicki *et al*, 2007), and is used for treatment of patients suffering from schizophrenia (McClure *et al*, 2007). Guanfacine is currently undergoing clinical trial for its role in preventing stress-induced relapse to cocaine, smoking, and alcohol use (Clinicaltrials.gov). Additional clinical trials looking at its efficacy in treating PTSD, ADHD, and schizophrenia are also underway (Posey *et al*, 2007).

Guanfacine is a partial agonist at the  $\alpha_{2A}$ -AR, and data suggest that partial agonism at this receptor subtype results in fewer unwanted side effects such as sedation in the potential treatment of anxiety disorders (Tan *et al*, 2002). Additionally, in disorders such as addiction, PTSD, or ADHD, where chronic therapeutics are needed, an agonist that does not readily induce desensitization of its target may be beneficial. Guanfacine desensitizes  $\alpha_{2A}$ -ARs much less readily than clonidine, likely explaining the longer duration of action by guanfacine clinically despite the two partial agonists having a similar half-life (Lu *et al*, 2009). Thus, guanfacine is a promising potential therapy for anxiety disorders and relapse in addiction. We theorize that the effects of guanfacine on  $\alpha_{2A}$ -ARs in the BNST are partially responsible for its effectiveness as a therapeutic agent in anxiety- and addiction-related behaviors.

**The presumed  $\alpha_2$ -AR-mediated mechanism of action of yohimbine in evoking anxiety-like behaviors in humans and rodents should be reconsidered**

As discussed in chapter IV, yohimbine is a putative  $\alpha_2$ -AR antagonist that has been widely used to evoke anxiety in human and rodents. Studies conducted by Adeola Davis and others demonstrate that the effects of yohimbine on fear-extinction are not likely mediated via the actions of yohimbine on  $\alpha_2$ -ARs (Davis *et al*, 2008). Our electrophysiology work also demonstrates that at the slice level, yohimbine elicits alterations in glutamatergic transmission that are not due to its actions at the  $\alpha_2$ -AR. Whereas short-term application in electrophysiology recordings shows no effect of yohimbine in the dorsal BNST (Egli *et al*, 2005), longer application demonstrates an off-target mechanism of

action that we theorize to be mediated through the 5-HT<sub>1A</sub>-R. We show that the more selective  $\alpha_2$ -AR antagonist, atipamezole, does not replicate effects of yohimbine in whole-cell voltage clamp recordings in the dorsal BNST. Additionally, we demonstrate no loss of effectiveness of yohimbine to depress glutamatergic transmission in the  $\alpha_{2A}$ -AR KO mouse (Figure 15), a model in which  $\alpha_2$ -ARs are thought to be almost fully desensitized (McElligott *et al*, 2008). Yohimbine has only a 10-fold selectivity for the  $\alpha_2$ -ARs over the 5-HT<sub>1A</sub>-R (Newman-Tancredi *et al*, 1998). Others have shown that the effects of yohimbine on acoustic startle are mediated via the 5-HT<sub>1A</sub>-R, as the effects of yohimbine were blocked by a 5-HT<sub>1A</sub>-R antagonist, but not an  $\alpha_2$ -AR agonist (Powell *et al*, 2005).

This work demonstrates the need for reevaluation of results utilizing yohimbine as an  $\alpha_2$ -AR antagonist, and elicits further investigation into the role of the 5-HT<sub>1A</sub>-R in mediating anxiety-related behaviors.

## **Conclusions**

In summary, my thesis work demonstrates an important heterosynaptic role for the  $\alpha_{2A}$ -AR in mediating glutamatergic and GABAergic transmission in the BNST. We show that the  $\alpha_{2A}$ -AR-mediated depression of glutamatergic transmission occurs via alterations in presynaptic glutamate release, and that  $\alpha_{2A}$ -ARs are localized to VGLUT1(+) and TH(+) terminals at the light microscopy level. These findings add to the growing body of evidence implicating the  $\alpha_{2A}$ -AR as mediating many of its behavioral actions via heterosynaptic regulation of non-

NE neurotransmitter release, in addition to its role as an autoreceptor regulating NE release.

We additionally have confirmed that several regions involved in stress and anxiety, including the infralimbic cortex, insular cortex, BLA, and CeA, show phylogenetically conserved projections to the BNST in the rat and mouse model.

Finally we demonstrate that the reported  $\alpha_2$ -AR antagonist yohimbine has significant  $\alpha_2$ -AR-independent effects on behavior and synaptic transmission. These findings demand more cautious interpretations regarding the mechanism of yohimbine in evoking anxiety-like behaviors in human and rodent models.

## APPENDIX

### APPENDIX A

#### FURTHER CHARACTERIZATION OF THE $\alpha_2$ -ADRENERGIC RECEPTOR AND RELATED MARKERS IN THE BNST

Please see Chapter II Experimental Procedures for review of methods.

##### **Immunohistochemistry**

In the course of my research, I have examined various proteins of interest, and their potential co-distribution in the dorsal BNST. Findings are presented below and will be discussed after presentation of the data.

All labeling was conducted on tissue from the HA  $\alpha_{2A}$ -AR KI mouse. Primary antibodies used include: guinea pig anti-VGLUT2 from Chemicon (1:1000), guinea pig anti-VGLUT1 (1:1000-1:8000); rabbit anti-VGLUT2 from Synaptic Systems (1:1000); rabbit anti-GAD65 from Chemicon (1:1000), and mouse anti-HA from Covance (1:500-1:1000). Secondary antibodies are all from JacksonImmuno Research and include cy5 donkey anti-guinea pig (1:1000), cy5 and cy2 donkey anti-rabbit (1:1000), and cy2 donkey anti-mouse (1:1000). All methods for fluorescent immunohistochemistry are as described in chapter II.



Figure 17. Distribution of the vesicular glutamate transporters in the dBNST.

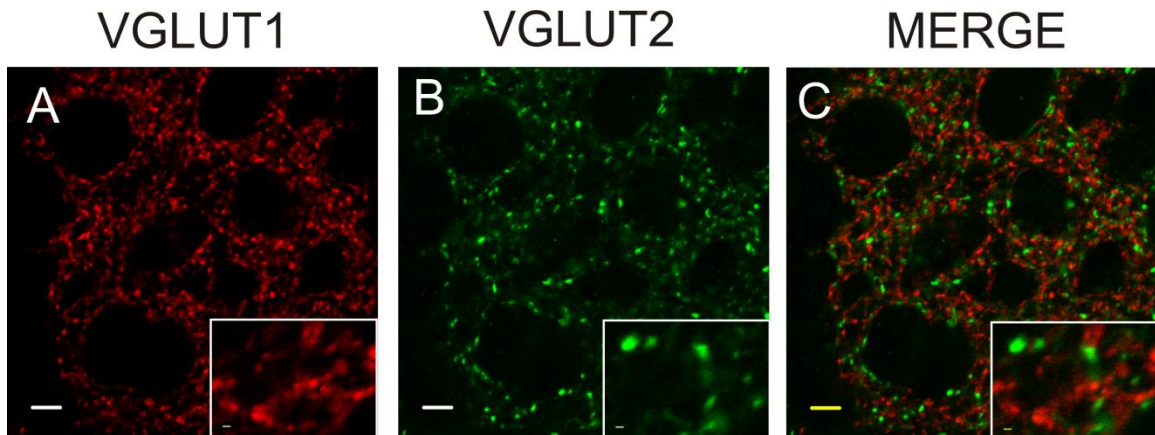


Figure 17. VGLUT1 and VGLUT2 do not appear to colocalize in the dorsal lateral BNST. A-C) Scale bar equals 5  $\mu\text{m}$ , inset scale bars are 0.5  $\mu\text{m}$ . A) VGLUT1 B) VGLUT2 (Synaptic systems). C) Merge. \*\*Note, VGLUT2 antibodies from Chemicon and Synaptic Systems show complete overlap in labeling.

As described earlier, the vesicular glutamate transporters (VGLUTs) are proteins responsible for packaging of glutamate into synaptic vesicles. VGLUT1 and VGLUT2 mRNA are known to have a primarily complementary distribution in glutamatergic output regions of the brain (Herzog *et al*, 2001), whereas VGLUT3 is found in populations of neurons expression the vesicular monoamine transporter (VMAT), the vesicular acetylcholine transporter (VACHT), and the vesicular gamma-aminobutyric acid transporter (VGAT) (Boulland *et al*, 2004). For this reason, VGLUT1 and VGLUT2 are considered glutamatergic markers, whereas VGLUT3 is not (Herzog *et al*, 2004). VGLUT1 is expressed by cortical neurons, neurons in the hippocampus, and olfactory bulb, as well as other nuclei, whereas VGLUT2 mRNA is found primarily in the thalamus, hypothalamus, and brainstem. Most glutamatergic inputs to the BNST are from cortical or cortical amygdalar regions such as the insular cortex, infralimbic cortex, hippocampus, and basolateral amygdala, and our data shows potential colocalization of the  $\alpha_{2A}$ -AR

(see chapter III) with projections from these regions. Inputs to the BNST such as the parabrachial nucleus of the hypothalamus, however, would be expected to express primarily VGLUT2 based on previous findings (Herzog *et al*, 2001). As might be expected based on afferents to the BNST, VGLUT2 distribution is less dense than VGLUT1 distribution in the BNST (see Figures 8-9 from Chapter II for comparison). At the light microscopy level, we find little colocalization between VGLUT1 and VGLUT2 qualitatively, as expected based on their reported complementary distribution (Figure 17).

Figure 18. Distribution of the  $\alpha_{2A}$ -AR and VGLUT2 in the dBNST.

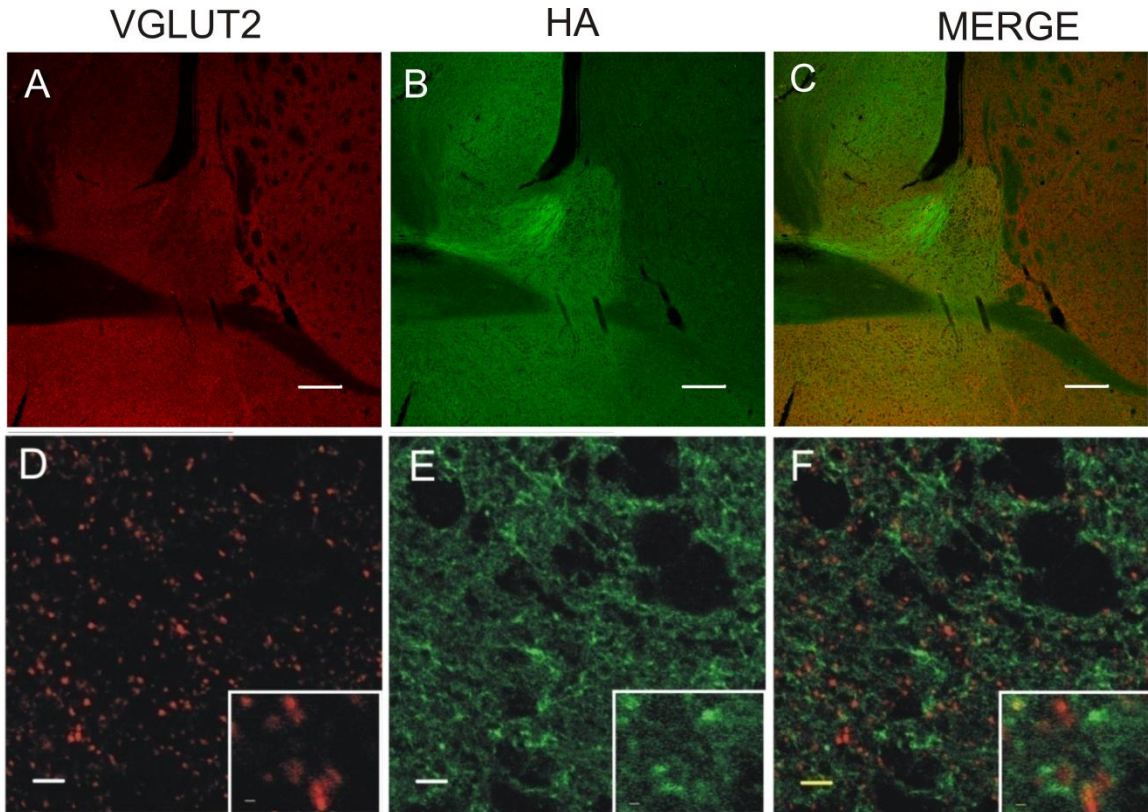


Figure 18. VGLUT2 distribution is similar to, but less dense than HA distribution in the dorsal lateral BNST. A-C) Scale bar equals 200  $\mu\text{m}$ . A) VGLUT2 (Chemicon). B) HA. C) Merge. D-F) Scale bar equals 5  $\mu\text{m}$ , inset scale bars are 0.5  $\mu\text{m}$ . D) VGLUT2 (Chemicon). E) HA. F) Merge.

Interestingly, we see very low levels of colocalization of the  $\alpha_{2A}$ -AR with VGLUT2 qualitatively (see Figure 18 above), whereas we saw more extensive colocalization with VGLUT1 (see Figure 9). Although additional studies are needed to confirm our qualitative light microscopy level findings, it is possible that only a subset of glutamatergic inputs to the BNST are regulated by the  $\alpha_{2A}$ -AR, and we hypothesize that the  $\alpha_{2A}$ -AR might regulate glutamate release in the VGLUT1(+), but not VGLUT2(+), subset of inputs to the BNST.

Figure 19. Distribution of the  $\alpha_{2A}$ -AR and GABAergic marker GAD65 in the dBNST.

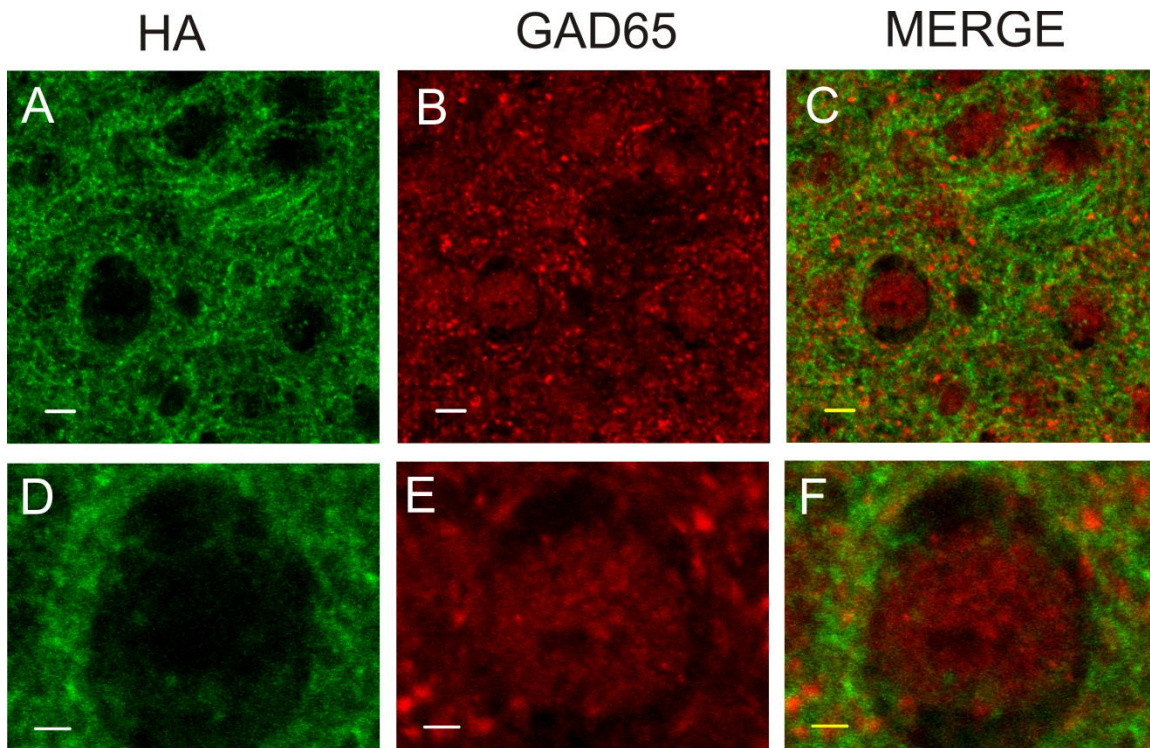


Figure 19. HA surrounds GAD65(+) cell bodies in the dorsal lateral BNST. A-C) Scale bar = 5  $\mu$ m. A) HA. B) GAD65. C) HA-GAD65. D-F) Scale Bar = 2  $\mu$ m. D) HA. E) GAD65. F) HA-GAD65.

Although the density of GAD65 and the  $\alpha_{2A}$ -AR make it difficult to assess the degree of potential colocalization of these two proteins (and due to variability in qualitative colocalization across slices), it is very clear that the  $\alpha_{2A}$ -AR surrounds GAD65(+) cell bodies of the dorsal BNST. Because the  $\alpha_{2A}$ -AR and VGLUT1 are both heavily distributed around these cell bodies of the dBNST, it is likely that much of the  $\alpha_{2A}$ -AR noted here is presynaptic and potentially localized to glutamatergic terminals (see Figure 9). However, these findings also lend support to our electrophysiology findings demonstrating  $\alpha_{2A}$ -AR-mediated

modulation of GABAergic transmission in the dorsal BNST, and confirm studies that report a high density of GABAergic interneurons and projection neurons (McDonald, 1983).



Figure 20. Distribution of the  $\alpha_{2A}$ -AR and CRF in the dorsal BNST.

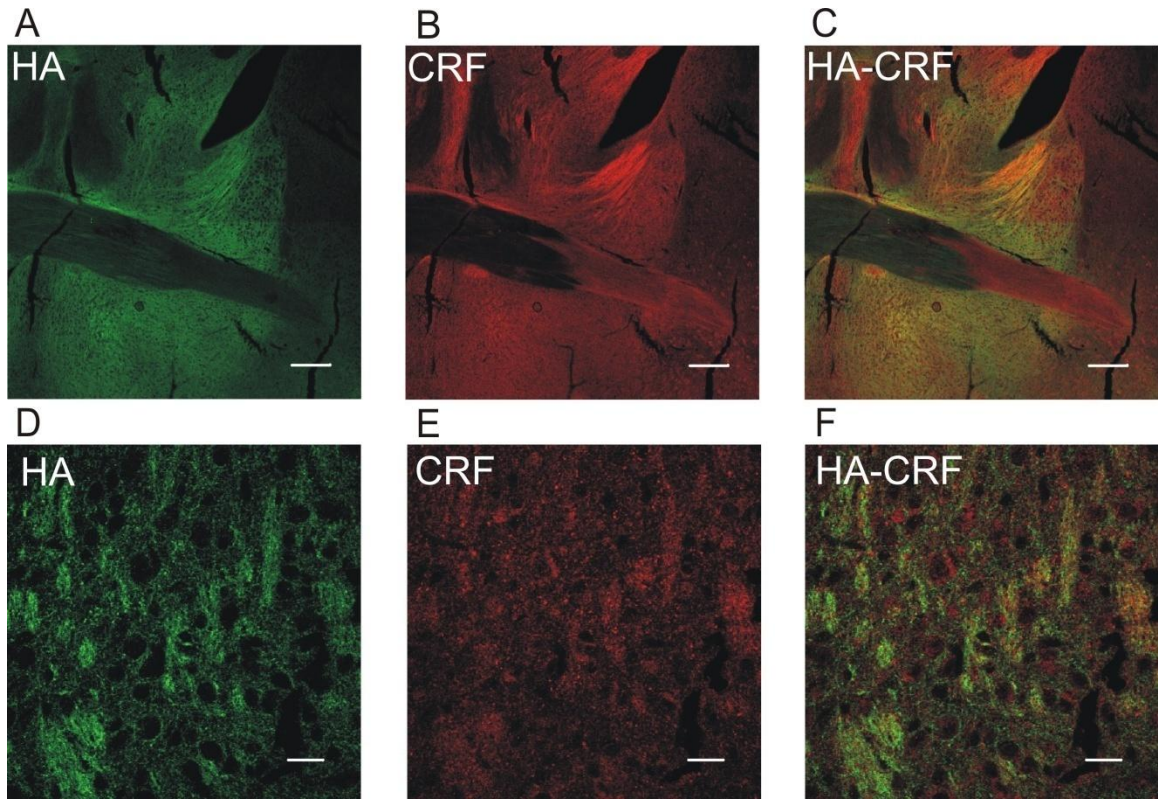


Figure 20. CRF distribution is similar to HA distribution in the medial BNST. A-C) Scale bar equals 200  $\mu\text{m}$ . A) HA. B) CRF. C) Merge. D-F) Scale bar equals 5  $\mu\text{m}$ , inset scale bars are 0.5  $\mu\text{m}$ . D) HA. E) CRF. F) Merge.

As discussed in the introduction, much data exists to suggest a functional interaction between NE and CRF. We have done extensive work at the light microscopy level to examine a potential colocalization of CRF and the  $\alpha_{2A}$ -AR in fibers of the stria terminalis, as shown in the figure above. Unfortunately, due to inconsistency of antibodies and even different lots of the same antibody (goat anti-CRF S-19 from Santa Cruz; lot #3108 and #1208 do not show stria labeling and show a dissimilar pattern of labeling to the lot presented in the images above), we cannot yet make any conclusions from the work above. This particular lot of antibody shown above, gave us consistent fiber labeling in the

stria that qualitatively colocalizes with the  $\alpha_{2A}$ -AR, but we did not have access to a CRF-KO animal to examine antibody specificity. More experiments and analysis using an antibody with proper controls are needed to determine a potential interaction or co-distribution of CRF and the  $\alpha_{2A}$ -AR. If CRF and the  $\alpha_{2A}$ -AR are co-localized on terminals in the BNST, this would suggest that activation of the  $\alpha_{2A}$ -AR may regulate CRF release from these sites.

Figure 21. Distribution of the noradrenergic markers NET and TH in the locus coeruleus and BNST.

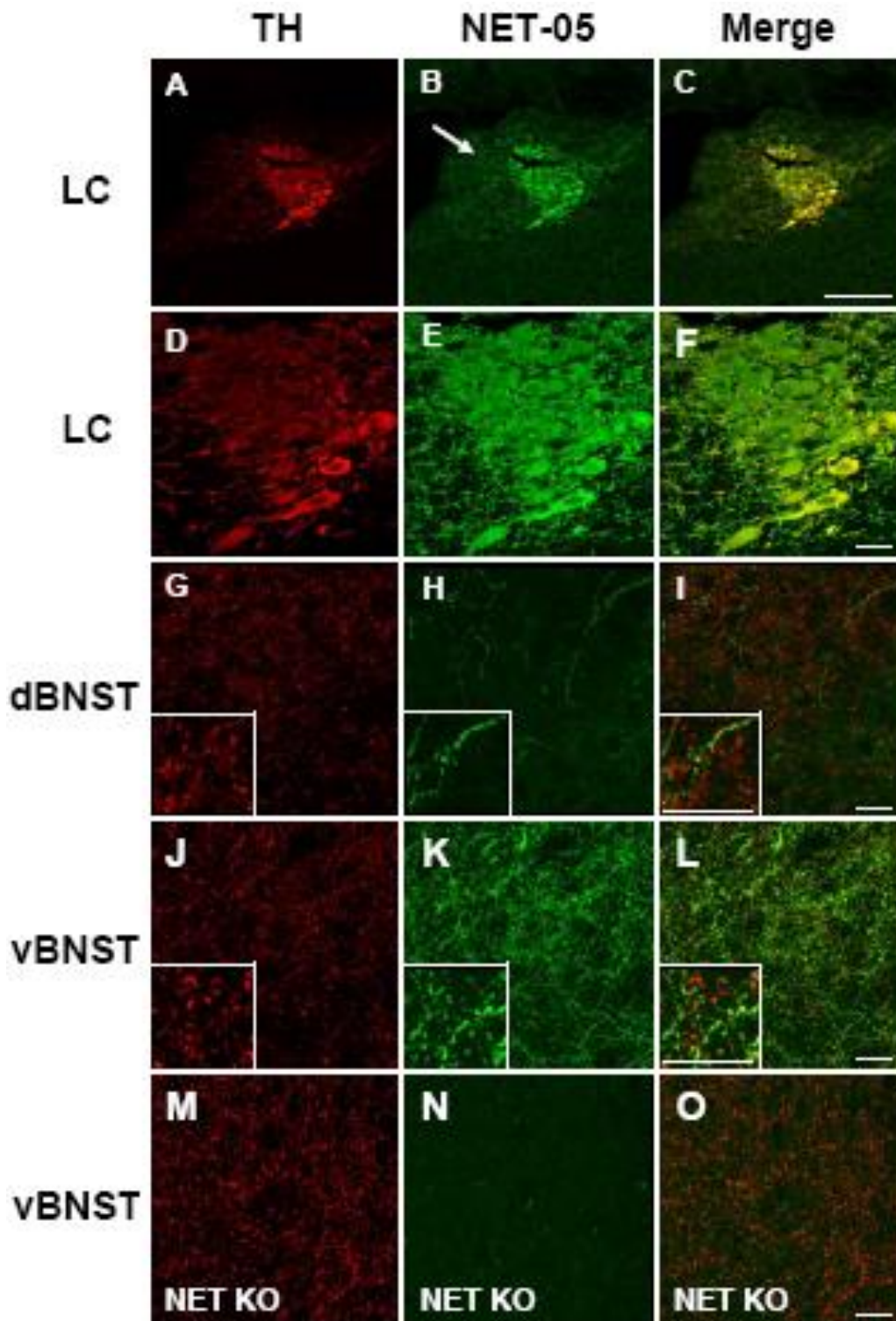




Figure 21. NET and TH are colocalized in the LC and BNST. Accepted to *BMC Neuroscience*. Heinrich Matthies et al. 2009

Mouse brain sections were processed for confocal-assisted imaging of NET immunoreactivity as described in Materials and Methods. A-C. Coronal sections encompassing the locus coeruleus (LC) were doubled-labeled using antibodies against both tyrosine hydroxylase (TH, red) (A, D) and NET (B, E, green). Panels C and F are merged images of A/B and D/E, respectively. Arrow in B identifies a dual labeled LC dendrite. Scale bar in A-C = 200  $\mu\text{m}$ ; scale bar in D-F = 20  $\mu\text{m}$ . G-I. Projections of six confocal sections (63X) taken from the dorsal BNST (dBNST). TH and NET immunoreactivities are shown in panel G (red) and panel H (green), respectively. Panel I is the merge of G and H. Insets of G-I show a digital zoom to illustrate individual fibers. J-L: Projections of six confocal sections (63X) taken from the ventral BNST (vBNST). TH immunoreactivity is shown in panel J (red) and NET immunoreactivity is shown in panel K and the overlap in panel L. All scale bar = 20  $\mu\text{m}$ .

These data demonstrate that the noradrenergic markers NET and TH are heavily colocalized in the locus coeruleus (LC), as would be expected since the LC is known to primarily contain noradrenergic cell bodies. We additionally show here that while the NET distribution is much heavier in the ventral BNST than the dorsal BNST, there still exists a large amount of NET immunoreactivity in the dorsal BNST. This main source of this NE in the BNST comes from the NTS via the VNAB. NET and TH also colocalize in the dorsal and ventral BNST, however, the broader distribution of TH likely demonstrates DA terminals innervating the BNST. Both the PAG and VTA provide dopaminergic innervation to the BNST.

## Whole cell electrophysiology

Figure 22. The  $\alpha_2$ -AR agonist UK-14,304 depresses excitatory and inhibitory transmission in Swiss-Webster mice.

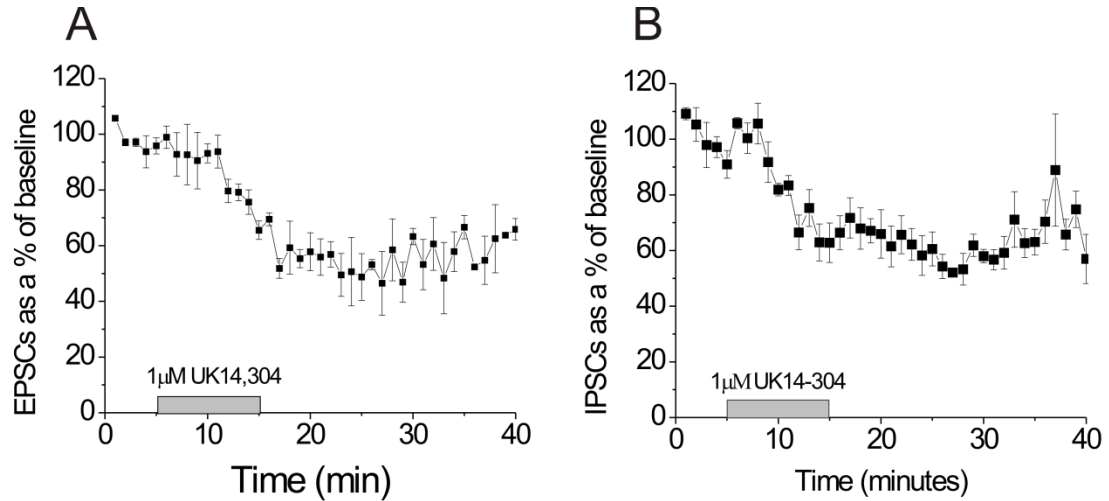


Figure 22. Activation of  $\alpha_2$ -ARs causes a depression in transmission in the dorsal BNST in male Swiss-Webster mice. A) Excitatory transmission (n = 2-3). B) Inhibitory transmission (n = 3-4).

In Swiss Webster mice, activation of  $\alpha_2$ -ARs in the dorsal BNST leads to a depression in both excitatory and inhibitory transmission. These findings are similar to what we find in C57BL/6J mice (see Figure 10 for comparison).

Figure 23. Reboxetine does not alter excitatory transmission in the dorsal BNST of WT mice.

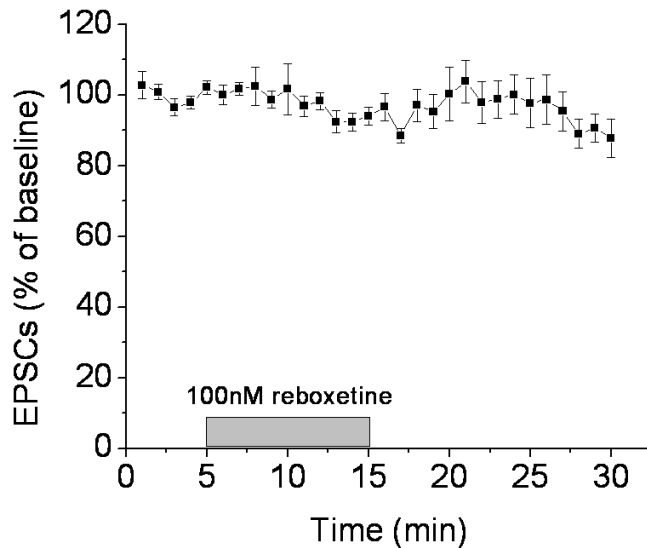


Figure 23. The NET blocker reboxetine (100 nM) does not alter excitatory transmission in the dorsal BNST of C57BL/6J mice (n = 6).

Our studies show that the NET blocker reboxetine does not alter excitatory transmission in the dorsal BNST, suggesting that there is minimal endogenous NE release elicited by our stimulation protocol. Additionally, it suggests that there is little endogenous NE in our *ex vivo* slice preparation. Previous studies examining the effectiveness of NET blockers to alter excitatory transmission in an *ex vivo* slice preparation, however, typically see loss of their effect within 1 second (Delaney *et al*, 2007). Therefore, it is highly likely that we are missing any potential effect of endogenous NE release on excitatory transmission in our protocol.

Figure 24. UK-14,304 effects on excitatory transmission are unaltered in NET KO mice.

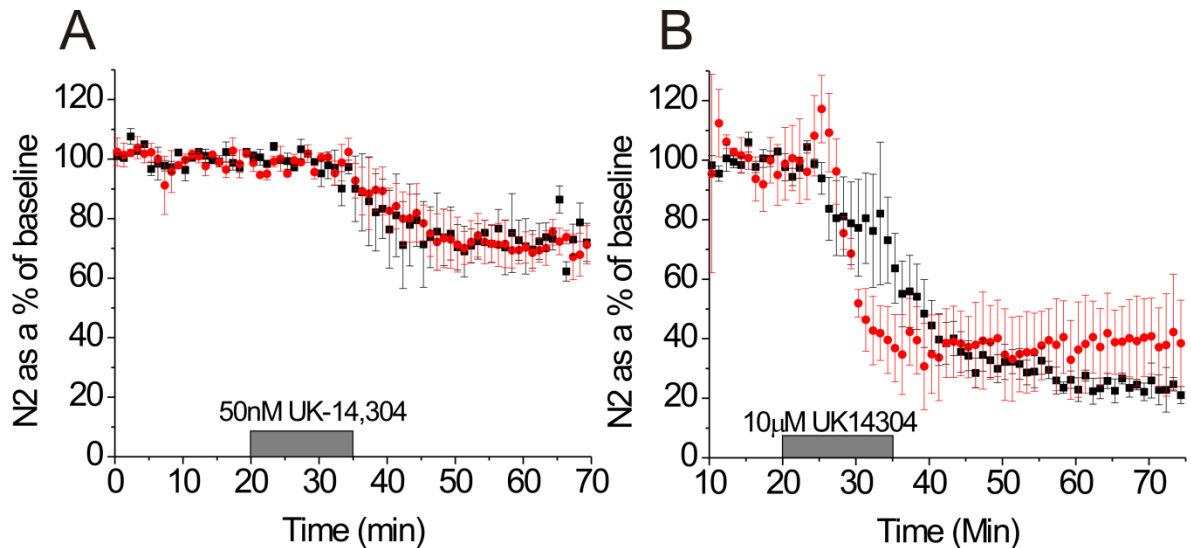


Figure 24. In field electrophysiology recordings, UK-14,304 causes a depression of excitatory transmission in the dorsal BNST of WT C57BL/6J mice, and this effect is unaltered in NET KO mice. A) 50 nM UK-14,304, C57BL/6J (red, n = 3), NET KO (black, n = 2). B) 10 µM UK-14,304, C57BL/6J (red, n = 4), NET KO (black, n = 4).

Using field recordings, we demonstrate that even low doses of UK-14,304 (50 nM) elicit a depression in excitatory transmission of C57BL/6J mice (Figure 24A). Moreover, these effects are unaltered in the NET KO mouse at both 50nM and 10 µM UK-14,304 (Figure 24A-B). The NET KO mouse is known to have increased NE levels throughout the brain (Xu *et al*, 2000), and also shows upregulation of  $\alpha_2$ -ARs (Gilsbach *et al*, 2006). NET KO mice have been shown to behave like anti-depressant treated mice in rodent models of depression, and are known to be supersensitive to psychostimulants such as cocaine (Xu *et al*, 2000). These findings demonstrate an important role for NET in maintaining NE levels throughout the brain, as well as important behavioral consequences of losing this tight regulation of NE in the brain. Interestingly,  $\alpha_1$ -AR-mediated LTD,

but not mGluR5-mediated LTD, is absent in the NET KO mouse (McElligott *et al*, 2008), indicating a disruption of noradrenergic signaling in these mice. We find here, however, that  $\alpha_2$ -ARs function normally in these mice, suggesting they are not subject to desensitization as easily as  $\alpha_1$ -ARs. In fact, the upregulation of  $\alpha_2$ -ARs throughout the brain of the NET KO mouse suggests that the role of the  $\alpha_2$ -AR as an autoreceptor to decrease NE release may be upregulated in these mice in attempt to control NE levels throughout the brain and compensate for the lack of NET.

## APPENDIX B

### THE CEA PROJECTS TO THE BNST IN THE C57BL/6J

This section examines the projection from the CeA to the BNST. While this projection has been shown numerous times in the rat model, no one has anatomically demonstrated whether the projection is phylogenetically conserved in the mouse model.

#### **Introduction**

Previous studies in rat and other models indicate a very dense GABAergic projection from the central nucleus of the amygdala (CeA) to the BNST (Dong *et al*, 2001a; Krettek and Price, 1978; Price *et al*, 1981; Shin *et al*, 2008; Sun *et al*, 1991; Weller *et al*, 1982) that is CRF(+) (Sun *et al*, 1991). While the BNST and CeA are very homologous structures with similar afferents and efferents, it is hypothesized that the BNST is involved in unconditioned prolonged anxiety states (Walker *et al*, 2003) whereas the CeA is involved in conditioned fear to distinct sensory cues (Campeau *et al*, 1995; Kim *et al*, 1993; Wilensky *et al*, 2006). Work by Erb and others have demonstrated that the projection from the CeA to the BNST is critical in stress-induced reinstatement to cocaine seeking behaviors (Erb *et al*, 2001).

Therefore, we used BDA injections to determine if the projection from the CeA to the BNST was upheld in the mouse model.

## **Experimental Procedures**

Methods used are the same as those described in Chapter III Experimental Procedures. BDA injections were made in the CeA (AP -1.34, L 2.4, D 4.53).

## **Results**

### **BDA injections in the CeA**

One HA  $\alpha_{2A}$ -AR KI mouse on a C57BL/6J background was injected with BDA into the CeA. The CeA is known to contain a heavy reciprocal connection with the BNST in rat (Dong *et al*, 2001a; Erb *et al*, 2001; McDonald, 1991; McDonald *et al*, 1999; Price *et al*, 1981; Shin *et al*, 2008; Sun *et al*, 1991; Weller *et al*, 1982; Zahm *et al*, 1999), and a similar pattern of labeling from the CeA to the BNST is noted here (Figure 6A-C). Figure 25A contains the Franklin Paxinos atlas image corresponding to the injection site shown in Figure 25B. A coronal section containing BNST from this mouse is shown in Figure 25C. The projection from the CeA to the BNST was the densest projection noted in the study of four regions (BLA, CeA, infralimbic cortex, insular cortex). Fibers from the CeA were thin and beaded in appearance, as shown in Figure 25B. Interestingly, the projection to the ventral BNST appeared to be denser than the projection to the dorsal BNST in all BNST sections examined. We were not able to differentiate injections into various subcompartments of the CeA, as our injection site covered several subcompartments. Therefore, we cannot compare our results with the more comprehensive analyses of CeA projections to the BNST done previously

by Dong and others in rat (Dong *et al*, 2001a). Five mice were injected just medial to the CeA, in the globus pallidus, and sparse fibers (likely from minimal overlap with the CeA) were noted in the BNST in two of these mice. The other three mice showed no projection to the BNST (data not shown).



Figure 25.

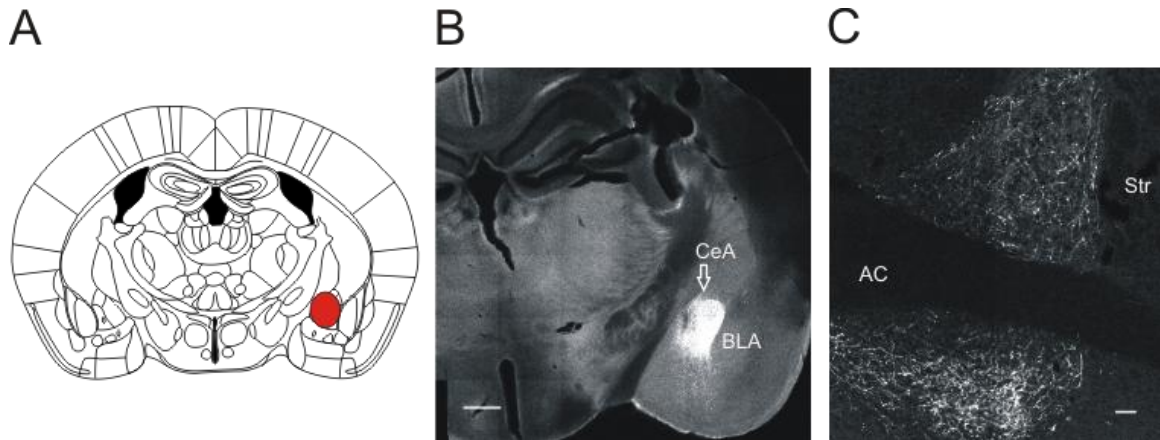


Figure 25. The central nucleus of the amygdala (CeA) projects to the BNST. A) Franklin Paxinos image coronal plane 40, corresponding to Figure 25B. Red sphere indicates CeA. B) 10x tile coronal image demonstrating injection of BDA into the CeA. Scale bar = 500  $\mu$ m. C) 10x tile illustrating BDA(+) fibers in the BNST projecting from the CeA. Scale bar = 50  $\mu$ m.

### **Projections from the CeA to the BNST are GAD65(+)**

The CeA projection to the BNST has been shown in multiple studies to be GABAergic, and we sought to confirm that this is phylogenetically conserved in the mouse model. Both in the dorsal (Figure 26A-C) and ventral (Figure 26D-F) BNST, we observe colocalization of BDA-filled projections from the CeA with the GABAergic marker GAD65. These results suggest that as in the rat model, the projection from the CeA to the BNST is GABAergic in nature. Due to the density of GAD65 labeling, however, future studies are needed to confirm findings noted here.

Figure 26.

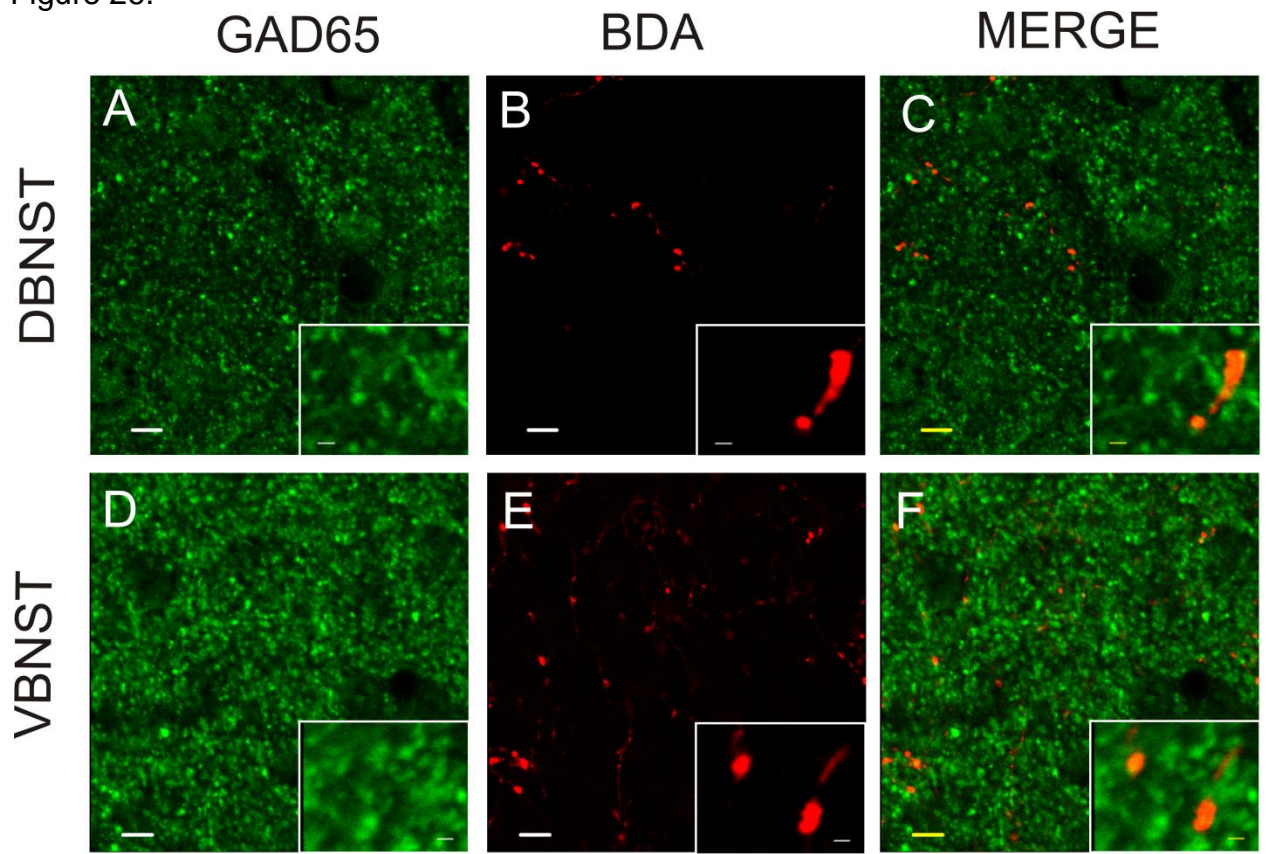


Figure 26. The central nucleus of the amygdala projection to the dorsal and ventral BNST is GAD65 containing. A-C) BDA projection to the dBNST. A) GAD65. B) BDA fibers. C) Merge. D-F) BDA projection to the vBNST. D) GAD65 E) BDA fibers. F) Merge. Scale bars = 5  $\mu$ m. Insets, scale bars = 1  $\mu$ m.

### **Projections from the CeA to the BNST contain $\alpha_{2A}$ -ARs**

Recent data suggests that the  $\alpha_{2A}$ -AR plays an important role in modulation of GABAergic transmission in the BNST (Chapter II). Using the HA tagged  $\alpha_{2A}$ -AR KI mouse, we have begun to examine the potential distribution of  $\alpha_{2A}$ -ARs on fibers from a main GABAergic input to the BNST, the CeA. Qualitatively, the  $\alpha_{2A}$ -AR appears to colocalize on CeA inputs to both the dorsal (Figure 27 A-C) and ventral BNST (Figure 27D-F). Future studies examining localization of the  $\alpha_{2A}$ -AR on these projections should be conducted to confirm current findings, as the density of the  $\alpha_{2A}$ -AR precludes us from conducting reliable quantification of our data set. Data obtained using electrophysiology and microdialysis studies indicate that regulation of GABAergic transmission by the  $\alpha_{2A}$ -AR might be primarily post-synaptic [Chapter II, (Forray *et al*, 1999) (Dumont *et al*, 2004)]. These localization data, however, provide evidence for potential presynaptic regulation of GABAergic transmission in the BNST by  $\alpha_{2A}$ -ARs.

Figure 27.

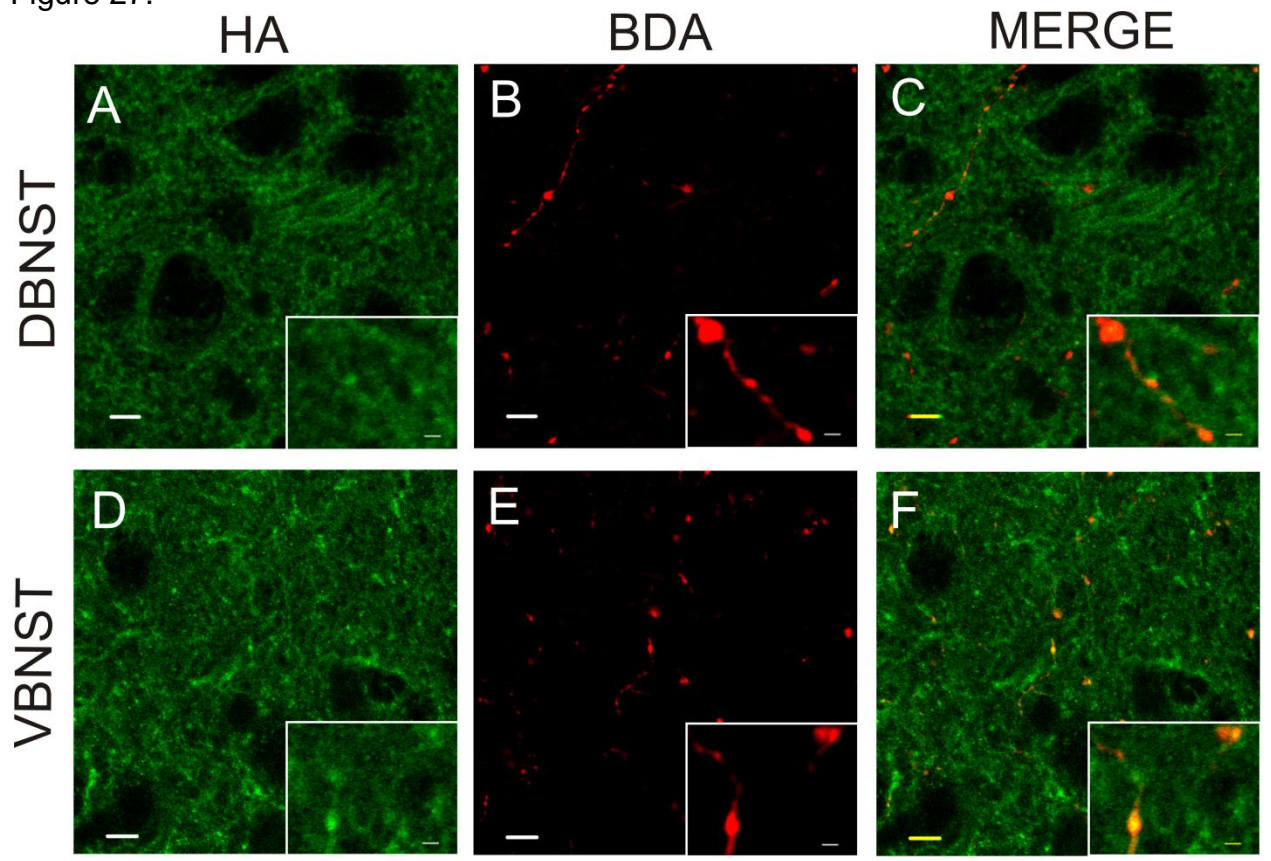


Figure 27. The central nucleus of the amygdala projection to the dorsal and ventral BNST is  $\alpha$ 2A-AR containing. A-C) BDA projection to the dBNST. A) HA. B) BDA fibers. C) Merge. D-F) BDA projection to the vBNST. D) HA. E) BDA fibers. F) Merge. Scale bars = 5  $\mu$ m. Insets, scale bars = 1  $\mu$ m.

## **General Discussion**

The GABAergic projection from the CeA to the BNST appears to be phylogenetically conserved between rat and mouse, supporting behavioral data implicating this projection as critical in mediating stress-induced behaviors to drug-seeking (Erb *et al*, 2001). Further studies (larger N) should be conducted to confirm findings in this mouse.

## APPENDIX C

### QUANTIFICATION OF BDA LABEL WITH HA-TAGGED $\alpha_{2A}$ AR, VGLUT1, AND VGLUT2

Please refer to Chapter III for background and sample confocal images.

We attempted to quantify the degree of colocalization of BDA tracer containing VGLUT1, VGLUT2, and the HA-tagged  $\alpha_{2A}$ AR to determine whether a subset of projections to the BNST contained the  $\alpha_{2A}$ AR, and to confirm studies demonstrating that cortical projections to the BNST are primarily VGLUT1-containing. All findings, however, need to be examined at the EM level due to diffuse labeling and background issues as explained below.

Using Metamorph, one 0.5  $\mu$ M section of a z-stacked image of the dorsal lateral BNST per mouse was separated by channel. Background was subtracted manually. The pixel by pixel colocalization was then measured as described in the tables below. Results from each mouse receiving a projection in a particular region were then averaged and the standard error was calculated.

VGLUT1 and VGLUT2 are shown to have an overall complementary distribution throughout the brain (Hayashi *et al*, 2001). Cortical regions are shown to have high levels of VGLUT1 mRNA, but express low levels of VGLUT2 (Fremeau *et al*, 2001; Herzog *et al*, 2001). Therefore, we expected to see moderate-high levels of VGLUT1 colocalization with BDA, and our results are as expected. We expected little to no colocalization of VGLUT2 with BDA, however, we find that about 13-16% (on average) of BDA(+) pixels from cortical and BLA

injections sites also contain VGLUT2(+) pixels. We theorize that this represents false-positive colocalization, or that there is some VGLUT2 protein expressed in these primarily VGLUT1-expressing regions.

We find a consistently high level of  $\alpha_{2A}$ AR colocalization with all BDA tracer injections from the BLA, insular cortex, and infralimbic cortex. We additionally found that 83.3 +/- 5.7% of BDA contained the  $\alpha_{2A}$ AR in the one CeA mouse we injected with BDA. These data imply that the  $\alpha_{2A}$ AR may modulate glutamatergic transmission in all three glutamatergic input regions studied. Data from the CeA mouse suggest that the  $\alpha_{2A}$ AR may also modulate GABAergic transmission in this mouse presynaptically, although more work is needed to examine and confirm this finding both with anatomical and functional studies.

Results are displayed in the Tables below. Preliminary findings confirm our hypothesis, but due to the diffuse  $\alpha_{2A}$ AR and VGLUT1 labeling, and the moderate background seen with the VGLUT2 antibody, all data below need to be confirmed and expanded upon at the EM level. Additional studies examining distribution of the  $\alpha_{2A}$ AR at the EM level are currently underway.



Table 6. IHC quantifications in the Insular Cortex

**Insular Cortex**

<b>BDA containing:</b>	<b>N</b>	<b>Range (%)</b>	<b>Average % +/- std err</b>
HA	4	69.2-88.7	79.5 +/- 4.7
VGLUT1	4	54.3-80.0	71.8 +/- 6.8
VGLUT2	4	6.5-20.2	13.4 +/- 3.3

Table 7. IHC quantifications in the infralimbic cortex.

### Infralimbic Cortex

<b>BDA containing:</b>	<b>N</b>	<b>Range (%)</b>	<b>Average % +/- std err</b>
HA	3	67.3-84.3	73.9 +/- 6.4
VGLUT1	3	24.7-52.8	35.8 +/- 10.6
VGLUT2	3	11.7-20.0	14.9 +/- 3.0

Table 8. IHC quantifications in the BLA

**BLA**

<b>BDA containing:</b>	<b>N</b>	<b>Range (%)</b>	<b>Average % +/- std err</b>
HA	3	67.0-86.2	77.9 +/- 7.0
VGLUT1	3	29.7-59.2	47.2 +/- 11.0
VGLUT2	2	16.3-17.1	16.7 +/- 0.3

## APPENDIX D

### YOHIMBINE DEPRESSES EXCITATORY TRANSMISSION IN SWISS- WEBSTER MICE

Methods used are as those described in Chapter IV Experimental Procedures.

Figure 28. Yohimbine depresses excitatory transmission in Swiss-Webster Mice.

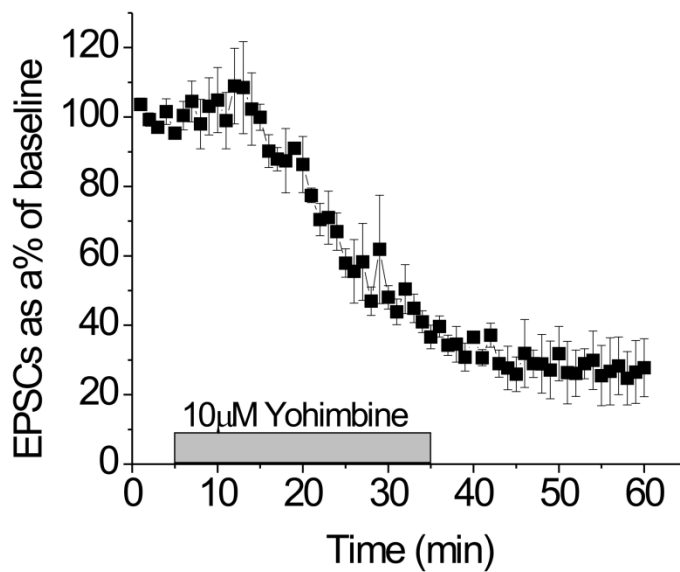


Figure 28. Yohimbine causes a significant depression in the dorsal BNST of Swiss-Webster mice (n = 3).

These findings further confirm that our results are similar in both C57BL/6J mice and Swiss Webster mice (compare Figure 28 to Figure 15). As in the C57BL/6J mouse, we see a dramatic depression of excitatory transmission upon application of the reported  $\alpha_2$ -AR antagonist yohimbine.

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