α_1-ADRENERGIC RECEPTOR REGULATION OF EXCITATORY TRANSMISSION IN THE BED NUCLEUS OF THE STRIA TERMINALIS: CHARACTERIZATION, MECHANISM, AND POTENTIAL ROLE IN DISEASE

By

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In memory of Dr. Samuel Feldman, my first neuroscience professor;

To my family for their support and love of learning;

To my husband Thomas, my greatest collaborator;

and,

To all those who suffer from addiction and anxiety disorders.

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ABBREVIATIONS

γ-amino butyric acid ..... GABA

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid ..... AMPA

AMPA Receptor ..... AMPAR

Adrenergic Receptor ..... AR

Adrenocorticotrophic Hormone ..... ACTH

Basolateral Amygdala ..... BLA

Bed Nucleus of the Stria Terminalis ..... BNST

Calcium Permeable AMPARs ..... CP AMPARs

Cannabinoid Receptors ..... CBR

Central Nervous System ..... CNS

Central Nucleus of the Amygdala ..... CeA

Coefficient of Variation CV

Conditioned Place Preference ..... CPP

Corticotropin Releasing Factor/Hormone ..... CRF/CRH

Corticotropin Releasing Factor 1 Receptor CRF-1R

3,5 – Dihydroxyphenylglycine ..... DHPG

Dopamine-β-hydroxylase ..... DBH

Dopamine D1 receptor D1R

Dopamine D2 receptor D2R

Dopamine Transporter ..... DAT

Dorsal Noradrenergic Bundle ..... DNAB

Dorsolateral BNST ..... dlBNST

Excitatory Postsynaptic Current..... EPSC

Excitatory Postsynaptic Potential.....EPSP

Extracellular Signal-Regulated Kinase .....ERK

GPCR Activated Inwardly Rectifying Potassium Current..... GIRK

G-Protein Coupled Receptors..... GPCRs

GABA Receptor .....GABAR

Glucocorticoid Receptor .....GR

Glutamate decarboxylase..... GAD

High Frequency Tetanus/Stimulation.....HFT/S

5-Hydroxy Tryptophan 5-HT

Hypothalamic-Pituitary-Adrenal Axis .....HPA axis

Intracranial Self-Stimulation.....ICSS

Knock-out Mouse.....KO

Long Term Depression ..... LTD

Long Term Potentiation .....LTP

Map Kinase Kinase..... MEK

Medium Spiny Neurons .....MSNs

Metabotropic Glutamate Receptor..... mGluR

Methoxamine .....Methox

2-methyl-6-phenylethynyl-pyridine.....MPEP

Mineralcorticoid Receptor.....MR

MiniEPSC .....mEPSC

Neuropeptide Y.....NPY

NMDA Receptor ..... NMDAR

Norepinephrine ..... NE

Norepinephrine Transporter ..... NET

Nucleus of the Tractus Solitarius ..... NTS

Nucleus Accumbens ..... NAc

Paired Pulse Low Frequency Stimulation ..... PP-LFS

Paired Pulse Ratio ..... PPR

Paraventricular Nucleus of the Hypothalamus ..... PVN

Periaqueductal Grey ..... PAG

Phosphoinositide 3-Kinase PI3K

Phospholipase C ..... PLC

Post Traumatic Stress Disorder ..... PTSD

Postsynaptic Density ..... PSD

Prefrontal Cortex ..... PFC

Protein Interacting with C-kinase 1 ..... PICK 1

Protein Kinase A ..... PKA

Protein Kinase C ..... PKC

Protein Phosphatase 2B PP2B

Short Term Potentiation ..... STP

Spontaneous EPSC ..... sEPSC

Tetrodotoxin ..... TTX

Tyrosine Hydroxylase ..... TH

Ventral Noradrenergic Bundle ..... VNAB

Ventral Tegmental Area..... VTA

Ventrolateral BNST..... vlBNST

Visual Cortex ..... vCTX

Voltage Gated Calcium Channel ..... VGCC

CHAPTER I

INTRODUCTION

In this preliminary chapter I intend to introduce the reader briefly to the complex issues surrounding affective behavioral disorders and focus on why the BNST and, synaptic modulation of excitatory transmission therein, may be neural substrates underlying the pathology of these disorders. I also give a comprehensive overview of induction and maintenance of G_q family of G-protein coupled receptors (GPCRs) induced LTD in various brain regions. This chapter should serve as a conceptual framework for the hypothesis that underlies the body of work in this dissertation and the specific aims I chose to investigate. The ultimate goal of this research is to contribute to a greater understanding of the function of the brain, with the hope that the data here can positively impact those suffering from mental health disorders.

Affective Spectrum Disorders of the Human Mind

Affect, or the "pattern of observable behaviors that is the expression of a subjectively experienced" emotion or state (American Psychiatric Association, 1994), can vary widely depending on context and past experience. Although the range of affect may be broad, deviations from the normal spectrum are termed affective spectrum disorders and include over 20 recognized psychiatric disorders including but not limited to: generalized anxiety disorder, depression,

eating disorders, Post Traumatic Stress Disorder (PTSD), panic disorder and addiction to drugs of abuse. Clinicians and scientists alike tend to avoid broad classifications and, thus, the spectrum disorder model has had a difficult time coming to pass (Alarcon et al., 1987). Evidence, however, of potential similarities in pathophysiology between disorders on the affective spectrum (Hudson and Pope, 1990) and co-morbidities between the disorders, have supported the notion of examining common brain regions and neurochemical pathways.

Two particular disorders that lie on the spectrum with a very high level of comorbidity are anxiety disorders and addiction to drugs of abuse. There are several categories of anxiety disorders that all have the characteristic of a perceived danger or fear accompanied by both somatic and emotional components (American Psychiatric Association, 1994). Addiction has been described as a chronically relapsing disorder where the transition from casual user to addict is highlighted by a move from impulsivity in the earlier stages of addiction to compulsivity in the later stages. This alteration underscores the shift in motivation from taking a drug for its positive euphoric qualities to consuming the drug to prevent or alleviate the negative aspects of withdrawal, such as anxiety (Koob, 2008). The following sections will describe the neuronal substrates that appear to mediate both disorders and provide evidence for their interaction.

Canonical reward pathways and activation by drugs of abuse

The classical reward circuitry in the central nervous system encompasses the mesolimbic dopamine pathway, cortical and limbic nuclei. It would appear that the endogenous functions of these pathways are to inform the organism of environmental stimuli that promote the organism's survival. It has been found that exposure to drugs and perhaps behaviors, however, may co-opt this natural system of reward and lead to pathological dependencies.

Neuronal Systems and Molecular Targets of Drugs of Abuse

At first glance, one of the most perplexing components of substance abuse is that the molecular targets of the various classes of abused substances are all different. Psychostimulants typically target the catecholamine transporters where cocaine can block uptake and amphetamine derivatives can induce reverse transport; opiates, like heroin and morphine, activate opiate receptors; nicotine is an agonist at the nicotinic acetylcholine receptor; ethanol exerts its actions on several neuronal substrates and is best known for being a positive allosteric modulator at the $GABA_A$ receptor and for inhibiting NMDARs; hallucinogens (LSD, hallucinogenic plants and mushrooms) are partial agonists at serotonin 5-HT_{2A} receptors; caffeine is an antagonist to adenosine receptors; and the active ingredient in cannabis (marijuana and hashish) acts on cannabinoid receptors. (Cocaine can also block certain voltage gated sodium channels) Although these and other addictive substances target various receptors in the brain, they converge on common mechanisms within the reward

pathway mainly the activation of the mesolimbic dopamine pathway and inhibition of the ventral striatum or nucleus accumbens (NAc) (Nestler, 2001).

Synaptic Plasticity and Addiction

The modification and remodeling of glutamatergic synapses have long been postulated to play a role in classical learning and memory. Many studies have correlated plasticity at glutamate synapses to learning paradigms by demonstrating that interfering with or potentiating the induction/expression of the plasticity in various brain regions can disrupt or enhance several learned behaviors, as well as demonstrating that plasticity at these synapses is induced by behavioral stimulation that promotes learning (Whitlock et al., 2006).

More recently these concepts have been explored in the context of reward and substance abuse. Interfering with glutamatergic transmission alters behavioral paradigms of addiction (Wolf, 1998). Several drugs of abuse with differing pharmacological targets and stress have been shown to increase AMPA/NMDA ratios (a molecular correlate of long term potentiation – LTP) in the ventral tegmental area (VTA) (Ungless et al., 2001; Saal et al., 2003). Moreover, mice lacking the GluR1 subunit of the AMPA receptor (AMPAR) do not exhibit increases in AMPA/NMDA ratios to cocaine in the dopaminergic neurons of the VTA (Dong et al., 2004) . These findings have led to the theory that addiction is a pathological hijacking of learning-like cellular correlates in reward centers.

Behavioral Assessment of the Rewarding Properties of Abused Substances

Several behavioral paradigms are used to assess the rewarding properties of drugs of abuse. The most prevalent paradigms are behavioral sensitization, conditioned place preference (CPP) and self-administration.

Behavioral sensitization is assayed by an increasing behavioral response, often locomotor behavior, to fixed doses of an abused substance and it is thought to underlie motivational response to the drug (Kauer and Malenka, 2007). Sensitization can be observed via the administration of multiple drugs of abuse and requires the NAc for its expression. Administration of NMDAR antagonists in the VTA, however, can prevent behavioral sensitization to cocaine (Kalivas and Alesdatter, 1993) suggesting that both nuclei are important for the manifestation of this behavior. Although, much of the focus has been made on the mesolimbic-ventral striatal dopamine projection, recently there has been striking evidence that serotonergic and adrenergic mechanisms may play a role in behavioral sensitization and the gating of the increased dopaminergic tone in the NAc (Drouin et al., 2002; Auclair et al., 2004).

CPP is used to demonstrate a learned preference to a drug paired side of a divided compartment, where experimenters administer an addictive substance or saline and place the experimental animal in the appropriate compartment (with its own individual context) during the “training” phase, and then assay which compartment the animal has developed a preference for during the “test” phase (Tzschentke, 2007). Additionally, place conditioning can be used to detect

learned aversions by performing similar assay but pairing the compartments to aversive stimuli.

Self-administration examines if an animal will preferentially perform an operant task to acquire the administration of a drug of abuse. This test directly measures the animal's inherent motivation to seek the addictive substance and can be performed on either a fixed ratio or a progressive ratio of lever presses to drug infusion (Olsen and Winder, 2006).

CPP and self-administration are also used to asses other components of addiction including extinction and reinstatement to drug seeking (discussed below). Extinction is the formation of a new memory that disassociates the place-reward or lever/nosepoke-reward, which was previously learned. For example the original memory that suggested that a lever press would mean a heroin infusion, still exists; however, a new memory has formed signifying that a lever press will no longer result in the heroin infusion. The former memory can be reinstated, and behaviorally observed via lever press, by either stressful insult, a priming dose of the drug and a cue previously paired to the infusion of drug. Additionally, like behavioral sensitization, NMDAR blockade within the VTA blocks CPP and self-administration in animal models.

The “Emotional Brain”, Fear and Anxiety Pathways

Human regard for emotion appears to have always been, for lack of a better word, an “emotional” subject. A tremendous plight on those with mental disorders in society is the notion that a mental disorder is something under the control of the “will power” of the person afflicted and not a psychobiological and neurochemically mediated disease. Beginning in the late 1930’s Papez, and Kluver and Bucy identified regions within the brain, both in the limbic area and the temporal lobe that contributed to emotional systems. This work was then compiled in the 1950’s by MacLean and given the term “the limbic system” (Eichenbaum and Cohen, 2001). Although current views of central processing and integration across sensory systems make it difficult to identify the exact components of the “emotional brain” (LeDoux, 1996), the limbic system, including: the amygdalar complex, prefrontal, infralimbic, cingulate cortices, hypothalamus, hippocampus and components of the basal ganglia are regarded as major components that mediate emotive behavioral responses.

Within the limbic system, researchers have narrowed their focus on the highly interconnected amygdalar complex as the key components to the expression of learned fear and innate fear, or anxiety. Several studies have demonstrated that the basolateral nucleus of the amygdala (BLA) is directly involved in the acquisition of fear memories via fear conditioning. Intriguingly, disruption of norepinephrine (NE) signaling within the BLA can disrupt reconsolidation of fear conditioning (Debiec and Ledoux, 2004). Data from the less well characterized extended amygdala, which contains the central nucleus of

the amygdala (CeA), and the BNST has added complexity to basic amygdalar function. Both the BNST and CeA appear to be involved in mediating fear conditioning, but the CeA appears to be important for the mediating of phasic fear while the BNST is involved in the conditioning to both sustained contextual fearful stimuli and anxiety to innately fearful stimuli (Sullivan et al., 2004; Meloni et al., 2006; Walker and Davis, 2008) (This will be discussed in further detail below.)

The Bodily Stress Response: The Hypothalamic-Pituitary-Adrenal Axis

The Hypothalamic-Pituitary-Adrenal axis governs the systemic stress response within mammals. The paraventricular nucleus of the hypothalamus (PVN) is composed of parvocellular and magnocellular cells. The parvocellular cells produce the neuropeptide corticotropin releasing hormone (CRH, also called corticotropin releasing factor or CRF) which is released into the blood stream to act on the anterior pituitary gland which lies ventral and posterior to the hypothalamus. The pituitary gland in turn releases adrenocorticotrophic hormone (ACTH) into the circulatory system where it travels to the adrenal glands where it stimulates the release of glucocorticoids into the bloodstream. The glucocorticoids then can travel back to the CNS to exert their effects on either glucocorticoids receptors (GR) or mineralcorticoid receptors (MR) which are both DNA binding proteins and can affect the transcription of several genes. Additional evidence suggests that glucocorticoids can increase GABAergic and decrease glutamatergic transmission on the magnocellular cells via two different means of

retrograde signaling (Di et al., 2009). These actions can contribute to a negative feed back loop to prevent the release of CRF from the PVN.

Drugs, Alcohol and Anxiety

Data from human studies indicate that stress plays a major role in relapse to substance abuse, and there is overwhelming evidence that anxiety poses a substantial risk for relapse to drinking in abstinent alcoholics (Chick et al., 2000; Driessen et al., 2001; Willinger et al., 2002; Breese et al., 2005). In particular, abstinent addicts of various drugs of abuse often cite stressful life events occur just prior to their relapse (Sinha, 2008). Furthermore there is significant comorbidity between alcoholism and disorders of anxiety, including PTSD (Stewart, 1996), and panic disorder (George et al., 1990). Anxiety appears to be a predictor of drinking behavior in alcoholics regardless of the age of onset of adult alcoholism (Sloan et al., 2003). Furthermore, chronic ethanol consumption and withdrawal have been shown to induce long-term alterations in HPA axis function in both humans (O'Malley et al., 2002) and animal models (Rasmussen et al., 2000). In alcoholics in a laboratory setting, stress induced increases in alcohol craving as well as increases in ACTH, cortisol and plasma NE (Sinha et al., 2003; Breese et al., 2005). As described above, drugs of abuse can alter plasticity at glutamatergic synapses in the VTA (Ungless et al., 2001; Saal et al., 2003). Interestingly within these synapses an acute stressor produced a similar change which was attenuated by administration of the glucocorticoid receptor antagonist RU486. RU486 did not attenuate the cocaine effect, however,

suggesting that stress and drugs operate independently to invoke these changes (Saal et al., 2003). These data suggest that glutamatergic synapses within brain nuclei associated with reward are a site of convergence for the effects of both stress and drugs of abuse, and provides a potential means for stressful life events to lead to relapse to addictive substances or behaviors.

Synaptic Modulation and Plasticity within the Bed Nucleus of the Stria Terminalis

The ability to integrate and interpret stressful and rewarding situations is necessary for an organism's survival. Evidence suggests that maladaptive processes in brain regions associated with stress and reward may lead to pathological anxiety conditions (generalized anxiety disorder, post-traumatic stress disorder, panic disorder) and addiction. The bed nucleus of the stria terminalis (BNST) – a component of the "extended amygdala" – has been shown to play a role in contextual conditioned and unconditioned fear responses; anxiety-like behaviors; affective behaviors related to drug/alcohol dependence; and, stress-induced reinstatement of drug seeking (Walker and Davis, 1997; Shaham et al., 2000; Sullivan et al., 2004; Fendt et al., 2005; Olson et al., 2006). As many of these behaviors are postulated to involve cortical and limbic regions that provide glutamatergic inputs to the BNST, alterations in the strength of these connections within the BNST are hypothesized to play roles in the pathogenesis of addiction and anxiety disorders. In this section I will explore the current understanding of synaptic physiology in the BNST and begin to form a conceptual framework for beginning to interpret potential behavioral correlates.

BNST: early insights and anatomical positioning

Behavioral studies have highlighted the BNST as a region at the crossroads of reward and stress/anxiety networks. Although limbic and cortical projections had been shown to regulate HPA axis function, their efferents often terminate prior to the PVN, with strong evidence for the BNST to serve as a key relay between these regions (Cullinan et al., 1993). Early work demonstrated that a portion of the BNST projections to the PVN express GABAergic markers (Cullinan et al., 1993), show decreases in plasma levels of corticosterone following electrical stimulation of the lateral BNST (Dunn, 1987), and that glutamate microstimulation in the BNST induces inhibitory postsynaptic potentials in the magno- and parvocellular cells of the PVN (Boudaba et al., 1996). Moreover, swim stress increases Fos immunoreactivity in glutamate decarboxylase (GAD, the enzyme that produces GABA) containing BNST-PVN projecting neurons (Cullinan et al., 1996). These data have led researchers to infer that the BNST is a member of a collective group of nuclei that provide a strongly integrated braking mechanism controlling HPA axis induction (Cullinan et al., 2008). Infusion of an AMPA receptor antagonist into the BNST and excitotoxic lesions diminish anxiety-like behavior as measured by light enhanced startle and CRF enhanced startle respectively (Lee and Davis, 1997; Walker and Davis, 1997). Furthermore lesioning the BNST enhanced learned despair during a forced swim task (Schulz and Canbeyli, 2000), impairs fear conditioning with a prolonged stimuli, and reinstatement of conditioned fear (Waddell et al., 2006).

Additionally, BNST lesions reduce interleukin-1β induced Fos activation in the PVN and attenuate ACTH levels (Crane et al., 2003), demonstrating its critical role as a relay for stress axis activation. Single administration of ethanol (via various routes of administration) also activates Fos in dlBNST neurons (Knapp et al., 2001; Crankshaw et al., 2003) (but see (Herring et al., 2004)).Finally, blocking opiate receptors specifically within the BNST attenuates heroin self-administration (Walker et al., 2000). These studies together suggest that the BNST may serve as a relay between these limbic, cortical regions, and reward centers and the HPA axis, and may play a key role in behavioral responses to stress and substances of abuse.

Closer inspection of the anatomy reveals that the BNST receives glutamatergic inputs from several brain regions that play roles in the manifestation of various types of behavior, notably cognitive and emotional processes; and, furthermore outputs to several regions, notably regions involved with reward, feeding behavior and stress (Dong et al., 2001b; Dong and Swanson, 2004) (Figure 1). Of note, the BNST receives inputs from the central (a GABAergic projection), medial and basolateral nuclei of the amygdala, the hippocampus and the prefrontal, insular and limbic cortices (Cullinan et al., 1993; McDonald, 1998; Dong et al., 2001a). These regions have also been identified as plausible contributors to behavioral responses from processive stressors and drugs of abuse.

Ascending modulatory transmitter systems also project heavily to the BNST. The BNST receives one of the most robust noradrenergic innervations in

the CNS (Forray and Gysling, 2004). These projections arise mainly from the nucleus of the tractus solitarius (NTS) and the A1 cell groups via the ventral noradrenergic bundle (VNAB), although some of the projections also arise from the dorsal noradrenergic bundle (DNAB) stemming from the locus coeruleus (Ricardo and Koh, 1978; Woulfe et al., 1988; Banihashemi and Rinaman, 2006). The majority of these projections are made in the ventrolateral BNST (vlBNST), however, the dorsolateral BNST (dlBNST) receives innervation as well (Egli et al., 2005; Bienkowski and Rinaman, 2008). The dlBNST also receives dopaminergic innervation arising from both the ventral tegmental area (VTA) as well as the periaqueductal grey (PAG) (Hasue and Shammah-Lagnado, 2002; Meloni et al., 2006). In addition to classic neuromodulators, the BNST also receives input from neuropeptide containing neurons, for example CRF (Sakanaka et al., 1986) and neuropeptide Y (NPY) (Walter et al., 1991; Larriva-Sahd, 2006).

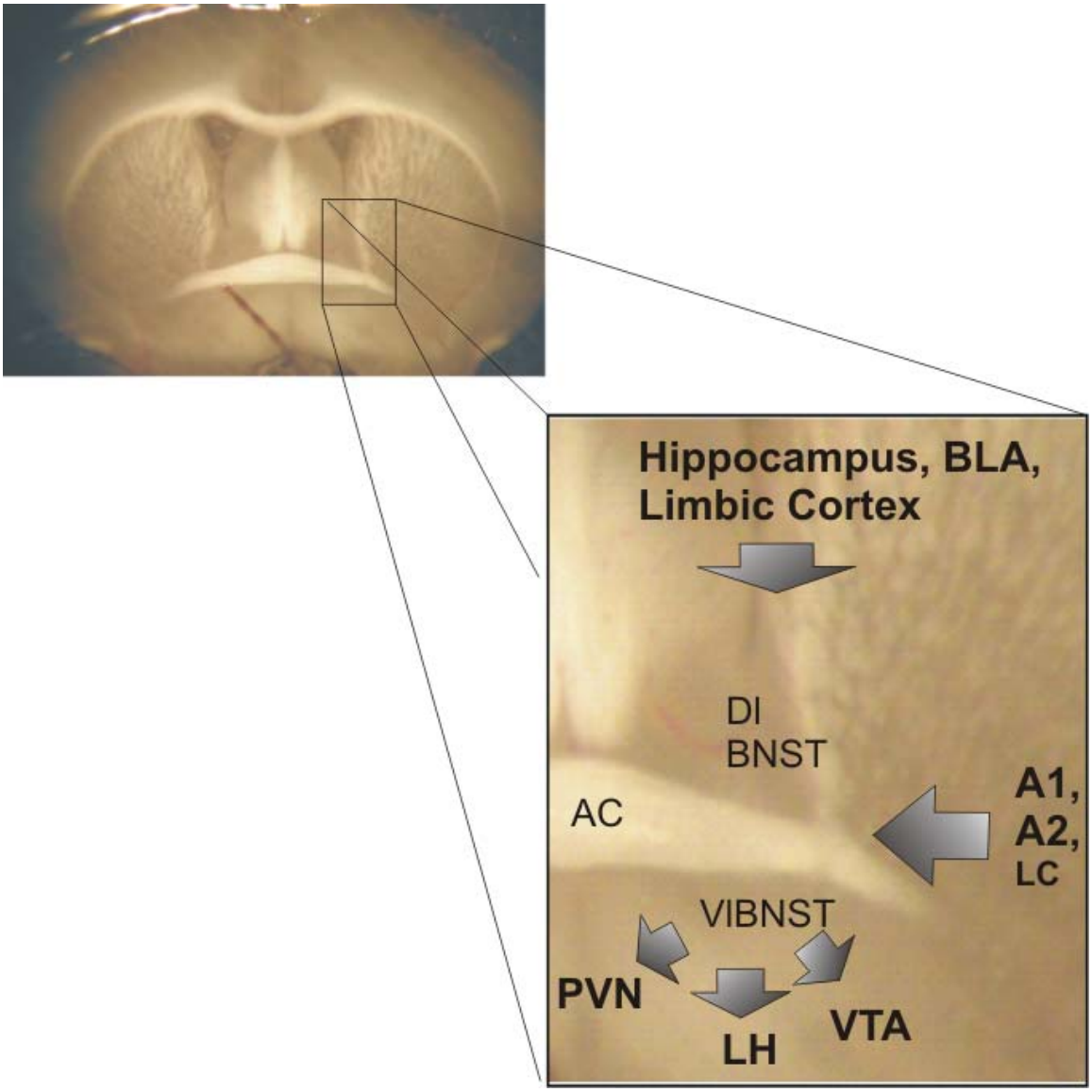


Figure 1. The Major Afferents and Efferents of the BNST
A picture of a coronal BNST slice with stimulating electrode in the left vBNST (left). The dorsolateral BNST (dlBNST) and ventrolateral (vlBNST) lie just dorsal and ventral to the anterior commissure (AC) respectively. This region receives afferents from the hippocampus, BLA and limbic cortices, and sends efferents to the PVN, LH and VTA. In addition, the BNST receives a large noradrenergic projection mainly arising from the A1 and A2 cell groups, and to a lesser extent the LC.

Properties of BNST neurons

As with the neighboring central nucleus of the amygdala and shell of the accumbens, the majority of the neurons within the BNST are GABAergic, however, there does appeart to be a distinct glutamatergic population of projection neurons as well as evidenced by functional assays and the presence of mRNA of multiple vesicular glutamate transporter genes (Georges and Aston-Jones, 2002; Allen Institute for Brain Science, 2008). In addition, they express a variety of neuropeptides. While the majority of neurons appear somewhat similar in morphology to medium spiny neurons, Golgi-impregnation studies reveal an impressive array of cellular morphologies (Larriva-Sahd, 2006). This diversity combined with strong evidence for a number of subnuclei within the BNST suggests a complex neurocircuitry.

To begin to attempt to understand the neurophysiology of this circuitry, the Winder lab and others have begun characterizing the electrical properties of neurons within the BNST. The neurons in the BNST appear very heterogeneous between the dorsal and ventral subdivisions and even within the subdivisions. Various BNST neurons have been shown to have low threshold spiking (likely mediated via T-type calcium current), I_h, I_A and inward rectifying potassium currents, and a persistent sodium current (Rainnie, 1999; Egli and Winder, 2003; Hammack et al., 2007). This suggests that synaptic input to these cells may be differentially integrated, which may have an important effect on subsequent behavior. Recently it has been shown using retrograde tracers that neurons projecting from the BNST to the VTA have distinct physiological properties.

Neurons projecting to the VTA have lower capacitance, higher input resistance, inward rectifying potassium currents and lack I_h currents (Dumont and Williams, 2004; Kash et al., 2008a). Examining the physiological properties of BNST neurons targeting other nuclei, the PVN for example, will no doubt prove useful to future studies examining synaptic integration and modulation.

Homosynaptic modulation

Long Term Potentiation

To begin to assess the ability of neurons within the BNST to undergo synaptic remodeling our group first described an extracellularly recorded synaptic response to local stimulation and demonstrated that two 100 Hz trains of stimuli (1 second each) can produce an NMDA receptor (NMDAR) dependent long term potentiation (LTP) in this region (Weitlauf et al., 2004; Weitlauf et al., 2005). Interestingly, the early portion of this LTP was found to be attenuated by acute, *in vitro* application of ethanol in a manner that was dependent on $GABA_A$ signaling and mimicked by incomplete NMDAR blockade. Furthermore it was noted that ethanol reversibly attenuates NMDAR currents by directly acting on receptors that contain the NR2B subunit (Weitlauf et al., 2004; Kash et al., 2008a). Previously, it had been proposed in the hippocampus and cortex that the NR2A subunit was responsible for the induction of NMDAR dependent LTP (Liu et al., 2004; Massey et al., 2004; Mallon et al., 2005), however, our group demonstrated that LTP in the dlBNST was intact in mice lacking NR2A subunits

and that the pharmacological blocker used to previously confirm NR2A dependence was not selective in brain slices at the concentration previously used (Weitlauf et al., 2005).

Long Term Depression

In addition to ionotropic receptors, glutamate also exerts its actions through G-protein coupled receptors (GPCRs) known as metabotropic glutamate receptors (mGluRs). Although mGluRs are not direct pharmacological targets of drugs of abuse, mGluR5 knockout mice do not self-administer cocaine nor do they exhibit locomotor responses to psychostimulants (Chiamulera et al., 2001). Moreover the mGluR5 antagonist MPEP has been shown to reduce the locomotor properties of cocaine and reduce conditioned place preference to cocaine, morphine and amphetamine (McGeehan and Olive, 2003; Herzig and Schmidt, 2004; Herzig et al., 2005). The BNST has been shown to express all three families of mGluRs and stimulation of all three mGluR families reduces glutamatergic transmission in the dlBNST (Grueter and Winder, 2005; Grueter et al., 2006). Activation of group I (specifically mGluR5) and group II mGluRs can induce long term depression (LTD) of glutamatergic synapses in the dlBNST, albeit via different mechanisms. Typically coupled to $G_{i/o}$,group II mGluRs depress synaptic transmission via a presynaptic mechanism (Grueter and Winder, 2005). mGluR5 activation, which typically couples to G_q, however, induces LTD via extracellular regulated kinase 1 (ERK1) signaling (Grueter et al., 2007; Grueter et al., 2008)). Further experiments using postsynaptic delivery of

GTP-γ-S and a dynamin inhibitory peptide suggests that the mGluR5 receptor is on the postsynaptic cell and that the LTD is maintained by postsynaptic modifications; and, requires clathrin-dependent endocytosis and actin remodeling suggesting a loss of AMPAR at the synaptic cleft by receptor internalization (Grueter et al., 2008). Furthermore, expression of this LTD, but not the early depression, is prevented by *in vivo* administration of cocaine, which can then be rescued by prior administration of the mGluR5 antagonist, MPEP (Grueter et al., 2008). This suggests that cocaine signals through mGluR5 *in vivo* to exert effects over this plasticity in the dlBNST.

Cocaine administration can also regulate other forms of plasticity within the BNST. Dumont and colleagues found that self-administration of cocaine or palatable food, but not yoked administration, increased AMPA/NMDA current ratios (an indirect measure of LTP) in VTA-projecting neurons in the vlBNST (Dumont et al., 2005; Dumont et al., 2008), thus suggesting a requirement for active drug seeking. Additionally it has recently been shown that chronic morphine administration can also increase AMPA/NMDA ratios in neurons projecting from the BNST to the VTA (Dumont et al., 2008). Interestingly, this was shown to be specific to the location of the stimulating electrode, suggesting that this morphine-induced plasticity may be input specific.

In addition to LTD, activation of group I mGluRs in the BNST can also induce the release of endocannabinoids from the postsynaptic cell to act on presynaptic cannabinoid type 1 receptors (CB1Rs) (Grueter et al., 2006). Activation of these receptors decreases release probability, thus reducing

glutamatergic efficacy. Recently Georges and colleagues showed that glutamatergic projections from the infralimbic cortex can stimulate BNST neurons (both dorsally and ventrally) to excite approximately 80% of the dopamine neurons in the VTA (Massi et al., 2008). The majority of this excitability was then demonstrated to be blunted by the addition of CB1R antagonists infused into the BNST which may demonstrate a mechanism for cannabinoid signaling to decrease the positive valance behaviors mediated by VTA activation.

Heterosynaptic modulation

Serotonergic Modulation

Serotonin (5-hydroxytryptophan or 5-HT) has been well established to play a role in depression and anxiety disorders. Although serotonin has not been investigated in the BNST in terms of synaptic function, a study from the Rainnie lab has investigated how serotonin can modulate the excitability properties of BNST neurons (Levita et al., 2004). Serotonin has been shown to hyperpolarize membrane potentials and reduce input resistance by means of the opening of a GPCR activated inwardly rectifying potassium current (GIRK) which is dependent on activation of 5-HT1A receptors. Furthermore, an agonist to 5-HT1 receptors decreased acoustic startle responses in the BNST.

Dopaminergic modulation

For many years, dopaminergic signaling has been the focal point of substance abuse research. Common features of addictive substances include increasing dopaminergic tone in the NAc; increasing synaptic plasticity on mesolimbic dopamine neurons in the VTA; and, animals will reliably perform intracranial self-stimulation (ICSS) of dopaminergic processes (Wise, 1998). It is important to note, however, that such dopamine transmission is not limited to the classical mesolimbic dopamine system. DiChiara and colleagues demonstrated that drugs of abuse can increase dopamine concentrations in the BNST (Carboni et al., 2000). Further, administration of addictive substances, but not non-addictive drugs activate extracellular regulated kinase (ERK) via dopaminergic signaling (Valjent et al., 2004) in the BNST. Additionally disruption of dopamine D1 receptor (D1R) signaling in the BNST can attenuate psychostimulant and ethanol reinforcement (Epping-Jordan et al., 1998; Eiler et al., 2003). These studies are additionally interesting because, as stated above, it has been shown that the BNST makes excitatory projections to VTA dopamine neurons possibly demonstrating a feed-forward loop for reinforcing drugs (Georges and Aston-Jones, 2002).

As a result of the importance of dopamine in reward, our group has begun investigations into regions with high dopaminergic innervation. Focusing on the BNST, NAc and the dorsal striatum Healey et al. (2008) examined expression levels of tyrosine hydroxylase (TH, the rate limiting enzyme in the production of dopamine) and the dopamine transporter (DAT) following either chronic exposure

or chronic intermittent exposure to ethanol vapor (Healey et al., 2008). In this study, 4-6 hours following chronic ethanol exposure there was a significant reduction in DAT, but conversely, 4-6 hours following chronic intermittent ethanol exposure there was a significant increase in DAT expression in the NAc. Interestingly, however, there was no change in DAT expression in the BNST, in either condition, in punches taken from the same exposed mice. This may be of functional significance as the BNST receives dopaminergic innervation from the PAG as well as the VTA. Competing forms of modification in the VTA and the PAG may have resulted in a lack of effect in the BNST in general.

Very recently our group investigated the possibility that dopamine may act by modulating glutamatergic transmission in the BNST (Kash et al., 2008b). Dopamine was found to increase excitability in a subset of neurons; and, in an activity dependent fashion, dopamine increased the frequency of spontaneous EPSCs (sEPSCs) in the dlBNST via signaling at D1 and D2 receptors. Due to reported anatomical and functional interactions between dopamine and CRF as well as the presence of CRF containing neurons and terminals within the BNST, Kash, Nobis and colleagues sought to determine if dopamine was acting though CRF to increase glutamatergic transmission. Consistent with a dopamine-CRF interaction blocking CRF1 receptors can prevent the effects of dopamine, and CRF or Urocortin application alone can cause an increase in the miniature EPSC (mEPSC) frequency. One possibility, therefore, is that dopamine likely acts through the D1 and D2 receptors to excite CRF containing cells within the BNST, thus releasing CRF in the BNST. In fact, dopamine can increase firing in neurons

in the BNST recorded in current clamp. Another possibility is that dopamine is acting on CRF afferents stemming from the CeA to cause the release of CRF. Additionally, Kash, Nobis et al. demonstrated that cocaine (*in vivo* and *in vitro*) and the specific dopamine transporter blocker GBR12909 could produce an NMDAR dependent enhancement of short term potentiation (STP) following tetanus. The increase in STP was prevented by both a pan-dopamine and a CRF-R1 antagonist and was absent in the D1R knockout mouse. In other brain regions, both CRF and dopamine have been demonstrated to modulate LTP (Thompson et al., 2005; Fu et al., 2007), and it has been demonstrated that CRF can modulate dopaminergic neurons in the VTA; however, these data demonstrate for the first time that dopamine can trigger a CRF dependent modulation of STP. This CRF dependent STP in the BNST may perhaps serve as a mechanism by which cognitive and limbic centers can create a feed forward loop on midbrain dopamine neurons to enhance dopaminergic tone in several brain regions.

Adrenergic Modulation

Although dopaminergic signaling has been the focal point for substance abuse research for the past three decades, NE was originally thought to be a central player in mediating reward. In the 1970's it was shown that animals could perform ICSS of noradrenergic nuclei and pathways, interfering with NE signaling disrupted ICSS, and disruption of NE signaling was shown to inhibit opiate and ethanol self-administration. (For an in-depth review see Schroeder and

Weinshenker, 2007.) Recently NE, especially the projections from the VNAB, has reemerged as a player in both reward and reinstatement to drug seeking. Olson and colleagues demonstrated that mice lacking the enzyme that produces NE (dopamine-β-hydroxylase or DBH) did not show a condition place preference to morphine, but this effect could be rescued by viral introduction of DBH to the nucleus of the tractus solitarius (NTS) (Olson et al., 2006).

Adrenergic Receptors (ARs), the receptors that NE is a ligand for, can have dramatic influence over behavior. For example, data from Raskind and colleagues has demonstrated that administering prazosin (an α_1-AR antagonist) dramatically attenuates symptoms of post traumatic stress disorder (Raskind et al., 2000; Raskind et al., 2002; Taylor and Raskind, 2002; Peskind et al., 2003) in human patients. ARs are GPCRs and are composed of 3 major families: α_1-ARs which are thought to be coupled to G_q (but see Hillman et al., 2009), α_2-ARs which are thought to be coupled to $G_{i/o}$ and β1/2 which are thought to be coupled to G_s. There are 3 members of the α_1-AR family: α_{1A}-ARs, α_{1B}-ARs and α_{1D}-ARs (see Table 1). Expression data in the BNST has demonstrated that mRNAs of both α_{1A}-ARs and α_{1B}-ARs are present in the BNST, however α_{1D}-AR was not observed (Day et al., 1997). Unfortunately, pharmacological tools are not available to fully discriminate the roles of these α_1-AR subtypes in behavior (Knepper et al., 1995; Stone et al., 2006) (but see Hillman et al. 2009), however there are KOs available for all subtypes. The α_{1B}-AR KO has interesting behavioral phenotypes including a failure to sensitize to cocaine and condition place preference to morphine (Drouin et al., 2002) and impaired spatial learning

in the Morris Water Maze (Spreng et al., 2001). The α_{1D}-AR KO on the other hand does not show impairment on the water maze task, but does appear to have deficits in working memory (Mishima et al., 2004).

Table 1. Adrenergic Receptors, Coupling, Agonists and Antagonists

	Coupling	**Agonists**	**Antagonists**
α_1-AR ($\alpha_{1A/C}$-ARs, α_{1B}-ARs, α_{1D}-ARs)	G_q (although recent data suggests coupling to $G_{i/o}$ also)	NE, EPI, Phenylephrine, methoxamine (may have selectivity at $\alpha_{1A/D}$-ARs)	Prazosin, terazosin, uripidil
α_2-AR (α_{2A}-AR, α_{2B}-AR, α_{2C}-AR)	$G_{i/o}$	NE, EPI, UK14,304, guanfacine	Yohimbine, atipamizole
β-ARs (β_1-ARs, β_2-ARs, β_3-ARs)	G_s	NE, EPI, Isoproterenol	Betaxolol (β_1-ARs), ICI 118,551 (β_2-ARs), SR 59230A (β_3-ARs)

The BNST receives one of the densest projections of NE in the CNS stemming from the ventral noradrenergic bundle that is composed of the NTS and A1 cell groups. Alteration of this projection, either by pharmacology (targeting individual ARs), or ablation, has demonstrated that this modulation can impact stress induced reinstatement to drug seeking, withdrawal aversion, anxiety-like behavior to predator stress and HPA axis regulation to a systemic stressor (yohimbine injection) (Delfs et al., 2000; Erb et al., 2000; Shaham et al., 2000; Wang et al., 2001; Fendt et al., 2005; Banihashemi and Rinaman, 2006). Furthermore blocking α_1-ARs in the BNST reduces anxiety-like behavior after a processive stressor (restraint) and decreases ACTH suggesting that NE in the BNST can regulate HPA axis output to an anxiety inducing phenomena (Cecchi et al., 2002).

The Winder lab, therefore, began a detailed investigation as to how NE modulates glutamatergic synapses in the BNST. Interestingly in different experiments in the dorsolateral BNST (dlBNST) NE could produce both an increase and decrease in glutamatergic efficacy in fEPSPs. Using pharmacology to dissect which receptors were responsible, Egli et al. showed that α_2-AR stimulation resulted in a strong, but transient, suppression of glutamatergic signaling. β-AR stimulation, however, resulted in a transient increase in glutamatergic signaling (Egli et al., 2005). Intriguingly, stimulating β-AR could not account for the entire observed increase in transmission and the increase could be subsequently blocked by an α_2-AR antagonist, suggesting a synergistic mechanism between β-ARs and α_2-ARs. This data is further complicated by

recent data examining the actions of α_2-ARs (see below (Davis et al., 2008)). In the vlBNST however, NE only produced the transient decrease in fEPSPs which was shown to be mediated via the α_{2A}-AR.

Due to the robust reinstatement data involving noradrenergic signaling, our group's previous data examining α_2-AR modulation of glutamatergic processes and the reported involvement of α_2-ARs in the facilitation of extinction behaviors following fear conditioning (Cain et al., 2004), we probed the ability for α_2-AR antagonism (with yohimbine) to facilitate extinction to the positive valence of cocaine. Surprisingly yohimbine impaired extinction to conditioned place preference to cocaine, and this impairment could not be mimicked with a more selective α_2-AR antagonist (Davis et al., 2008). Furthermore, we showed that yohimbine robustly reduced glutamatergic transmission in the BNST independently of signaling via α_{2A}-ARs. While it is well known that yohimbine is not a selective drug, it is often used for its anxiety inducing properties that are presumably evoked via enhanced adrenergic signaling via blockade of presynaptic ARs. We have demonstrated, however, that "off-target" effects of yohimbine have significant behavioral and physiological ramifications.

Recently, the BNST is gaining appreciation as a region involved in mediating the affective component of pain. Painful stimuli increase dialysis levels of NE in the BNST (Deyama et al., 2008b). Lesioning the BNST, blocking β-ARs and interfering with PKA signaling there reduces conditioned place aversion (CPA) to painful stimuli independently of nociception (Deyama et al., 2007; Deyama et al., 2008a). Interestingly, activating β-ARs and PKA in the BNST

induced CPA independently of painful stimulation. Future studies in this area may aid in the development of non-narcotic analgesics for chronic pain.

GABA and Neuropeptides

Although the focus of this dissertation is on glutamatergic transmission in the BNST, it is relevant to consider the importance of GABAergic transmission within this nucleus. The majority of neurons within the BNST are thought to be GABAergic and the BNST receives a robust GABAergic projection from the CeA which also can release CRF (Sakanaka et al., 1986). Another neuropeptide, neuropeptide Y (NPY) is expressed in adrenergic terminals and can be released upon high frequency stimulation of adrenergic neurons (Sawchenko et al., 1985; Pernow, 1988). In the vlBNST, NPY and CRF were found to respectively inhibit and increase GABAergic transmission within the BNST (Kash and Winder, 2006). NPY appeared to decrease transmission presynaptically via the Y2 receptor, while CRF increased inhibitory transmission postsynaptically via CRF-R1. The integration of CRF's effects on inhibitory transmission with the actions of CRF on glutamatergic transmission in the dlBNST (which projects to the vlBNST as well as other nuclei) will most likely shape the output to stress and reward nuclei.

Section Summary

The BNST serves as an important relay between limbic inputs and stress and reward nuclei in the brain, where synaptic modification can dramatically alter the flow of information, and can be liable to the influence of stressors and drugs

of abuse. Synaptic integration in this nucleus is undoubtedly a very complex phenomenon of which researchers have only begun to scratch the surface. Studies that have investigated the physiological properties and glutamatergic modulation within the BNST, however, have begun making progress towards reconciling animal behavior with the underlying molecular mechanism. Glutamatergic transmission is potently modified by stressors and drugs of abuse in this region. In particular, catecholamines may be released in the BNST under both stressful and rewarding conditions and they may engage alterations in glutamatergic transmission that could alter functional output behavioral responses to these experiences. Future studies that strive to discover additional links between environmental influences and synaptic modulation will broaden our understanding of the importance of such modulation in behavioral output. For example, behavioral experiments involving paradigms of stress and reward in varying strains of mice can lead to candidate mRNAs and, ultimately proteins that may be involved in synaptic modulation manifesting as changes at the behavioral level. In such a way, using genetics, bioinformatics, behavioral studies, biochemistry and physiology to address the role of the BNST the field will hopefully contribute to the long term goals of eradicating substance abuse and anxiety disorders.

Gq Coupled Long Term Depression of Excitatory Transmission

This section will focus on the mechanisms for LTD at excitatory synapses that stem from the activation of Gq G-protein coupled receptors (GPCRs) in selected brain regions where plastic changes are thought to affect learning outcome. Due to the overarching breadth of research on group 1 mGluR (mGluR1/mGluR5) induced LTD as compared to that of the other G_q coupled receptors, such as the M1 muscarinic receptor (M1AChR) and α_1 adrenergic receptors (α_1-AR) that induce LTD when activated, this section will highlight group 1 mGluR LTD in various nuclei (with the exception of the BNST which was described above) but also discuss the other receptors that mediate LTD and their relevance in the brain regions where they are known to be expressed. It will discuss mechanisms of induction, or by what means the LTD is initiated; the expression, or how the LTD manifests itself; and, maintenance, or how the LTD is actively sustained.

G_q Signaling

The G_q family of heterotrimeric G-proteins contains $G_{q\alpha}$, $G_{11\alpha}$, $G_{14\alpha}$ and $G_{15/16\alpha}$. All members of this class couple to phospholipase C-β (PLC- β) which in turn can lead to increases in intracellular Ca^{2+} and activation of protein kinase C (PKC) (Hubbard and Hepler, 2006). At first glance it remains somewhat surprising that activation of a receptor coupled to a G_q protein would result in a synaptic depression in the short term, let alone LTD. However, this GPCR family

couples to PLC- β with different affinities (Hubbard and Hepler, 2006) and can prompt a host of signaling cascades that are dependent and independent on PLC activity. This, along with the concept that various G_q GPCR subtypes couple to various G_q family members with different affinities (Hawrylyshyn et al., 2004; Wu et al., 2004), in part accounts for the promiscuity observed at the cellular level and across cell types for G_q GPCRs. As a result, the ability of a G_q GPCR family, for example α_1-ARs, to induce both increases (Gordon and Bains, 2003; Gordon et al., 2005; Gordon and Bains, 2005) and decreases (Kirkwood et al., 1999; Scheiderer et al., 2004) in excitatory synaptic efficacy not only depends on the receptors cellular location, but on the expression of various signaling components within the cellular compartment. Although multiple receptors couple to G_q, only mGluR1, mGluR5, α_1-AR (various subtypes have not been examined) and M1 AChR have been implicated to play a role in LTD mechanisms.

mGluR LTD

Cerebellar mGluR LTD

The onset of the study of LTD occurred when it was found that LTD could be induced in hippocampal slices in an NMDA dependent manner (Dudek and Bear, 1992; Mulkey and Malenka, 1992); however, prior to these seminal manuscripts, the phenomena known as LTD was described *in vivo* in the cerebellum of decerebrate rabbits (Ito et al., 1982), and was further explored in cerebellar slice preparation (Sakurai, 1990). Additional early experiments

performed in cultured Purkinje neurons demonstrated the involvement of a postsynaptic metabotropic glutamate receptor (mGluR) in the induction of LTD due to focal application of glutamate (Linden et al., 1991). Although Linden and colleagues were limited by the pharmacological agents of their time, their astute analysis demonstrated a role for depolarization of the postsynaptic cell, extra synaptic Ca^{2+} and G_q coupled mGluRs; meanwhile, demonstrating that the increase in Ca^{2+} was not via the NMDA receptor (Linden et al., 1991). Subsequent genetic experiments then confirmed that the receptor mediating the LTD is mGluR1 (Aiba et al., 1994). mGluR LTD in the cerebellum is now perhaps the best characterized G_q coupled LTD in the brain. A number of experiments have gone on to show that activation of mGluR1 couples to PLC and the subsequent activation of PKCα which then phosphorylates the AMPA receptor subunit GluR2 at serine-880 (Xia et al., 2000; Chung et al., 2003)and results in the clathrin mediated endocytosis of the AMPA receptor (Wang and Linden, 2000) in part via mediation by the PDZ containing protein Protein Interacting with C-kinase 1 (PICK 1) (Steinberg et al., 2006). Furthermore it appears that this type of LTD is necessary for certain types of motor learning like associative eyelid conditioning. (Boyden et al., 2004)

Hippocampal mGluR LTD

In the interest of brevity, the discussion of mGluR LTD in the hippocampus will be contained to the CA3 to CA1 synapse. mGluR LTD was first examined in depth in the hippocampus in 1994 when Bolshakov and Siegelbaum discovered

that pairing 5 Hz stimulation with the depolarization of the postsynaptic cell could result in NMDA independent and mGluR dependent LTD (Bolshakov and Siegelbaum, 1994). As observed in the cerebellum, they found that stimulation induced mGluR LTD in the hippocampus is dependent on the depolarization of the postsynaptic cell to allow for the requisite increase in postsynaptic Ca^{2+} by the activation of L-type voltage gated calcium channels (VGCCs). Subsequent studies, however, drew varying conclusions. Using the same stimulus protocol (however older animals and a different Ca^{2+}/Mg^{2+} ratio), Oliet et al. found that L-type VGCCs are not required for the induction of mGluR LTD (although expression was not examined) although there is a dependence on T-type VGCCs (Oliet et al., 1997). Interestingly, they also showed that mGluR LTD induction is dependent upon activation of group 1 mGluRs, a certain level of inhibition via $GABA_A$ receptors, and postsynaptic activation of PKC. Unlike NMDA dependent LTD, stimulation induced mGluR LTD was not prevented by phosphatase inhibition (Oliet et al., 1997). Additionally, mGluR dependent LTD was observed in adult animals by using paired pulse low frequency stimulation (PP-LFS) (Kemp and Bashir, 1999), however subsequent studies have implied that other G_q coupled receptor activation may contribute to LTD induced by this stimulation protocol (Volk et al., 2006). Therefore, although mGluR LTD was induced via different stimulus paradigms, it was dependent on activation of group 1 mGluRs.

More recently, the development of specific group 1 mGluR agonists has allowed for the chemical activation of mGluR LTD without utilizing a synaptic stimulation protocol. A series of experiments in adult rats from the Collingridge

group demonstrated that activation of group 1 mGluRs (by the agonist DHPG, 3,5-Dihydroxyphenylglycine) induced an LTD that was affected by the excitability of the slice showing a partial dependence on the presence or absence of Mg^{2+} in the medium (potentially implying a role for NMDA receptors), and also depending on the level of inhibition where by blocking $GABA_A$ receptors resulted in greater LTD (Palmer et al., 1997; Schnabel et al., 1999). Interestingly, unlike the LFS experiments, there did not seem to be a requirement for PKC activation, nor the release of intracellular calcium stores (Schnabel et al., 1999), further validating a role for VGCCs as determined in synaptically evoked mGluR LTD. Additionally, blockade of protein phosphatases resulted in a more potent DHPG induced LTD (Schnabel et al., 2001). Subsequent studies, however, in juvenile animals elicited mGluR LTD without altering slice excitability (Fitzjohn et al., 1999; Huber et al., 2000; Huber et al., 2001) and showed that induction of mGluR LTD occurred in synapses innervated by an unstimulated pathway (Huber et al., 2001), arguing against mGluR LTD as a Hebbian form of plasticity at least in the hippocampus. That is, sources of glutamate other than the presynaptic neuron, like an astrocyte, may potentially contribute to mGluR-LTD. While some confusion still remains on many induction mechanisms (see further discussion below), it seems like mGluR LTD is induced independently of NMDA receptor activation and is independent of concurrent stimulation.

The availability of a specific agonist allowed for more in depth investigation into the mechanism of mGluR LTD expression and maintenance. Biochemical studies demonstrated that activation of group 1 mGluRs resulted in

the increase in mRNA translation in a PLC and PKC dependent manner (Weiler and Greenough, 1993; Weiler et al., 1997). It was not confirmed that the expression of mGluR LTD was also dependent on protein synthesis until electrophysiological experiments using translation inhibitors in the whole cell recording pipette demonstrated this requirement with induction protocols for both chemical and synaptically (PP-LFS) (Kemp and Bashir, 1999) evoked mGluR LTD (Huber et al., 2000). Additional experiments highlighted that mGluR LTD signals for the regulation of translation initiation acting via extracellular regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K) – Akt/protein kinase B – mammalian target of rapamycin (mTOR) pathways (Hou and Klann, 2004; Banko et al., 2006). Further biochemical evidence linked the activation of group 1 mGluRs and requirement for protein synthesis to the maintained loss of AMPA and NMDA receptors from the postsynaptic membrane (Snyder et al., 2001). Additionally, experiments that occluded receptor internalization and actin stabilization also prevented mGluR LTD induction (Xiao et al., 2001). These experiments detailed that, at least in part, mGluR LTD is a postsynaptic mechanism maintained by the removal of ionotropic glutamate receptors from the synapse.

Although there is strong evidence for a postsynaptic locus of expression, there is evidence from stimuli and chemically induced expression of mGluR LTD for a presynaptic mechanism. An increase in the coefficient of variation (CV) after stimulus induced LTD (Bolshakov and Siegelbaum, 1994) and paired-pulse ratio (PPR) (Gereau and Conn, 1995) following DHPG

application indicates an alteration in the release probability as does similarly a decrease in the frequency of miniature/spontaneous events (Snyder et al., 2001; Xiao et al., 2001), although this can also be indicative of a silencing of synapses by loss of AMPA receptors (but see (Gereau and Conn, 1995)). Watabe and colleagues used a different approach demonstrating that inhibiting K^+ channels increases Ca^{2+} influx presynaptically and occludes expression of mGluR LTD, although manipulating only postsynaptic K^+ channels under the same extracellular ion concentrations permits expression of mGluR LTD (Watabe et al., 2002). Additionally, dye loading experiments have implicated a change in the release properties that perhaps indicates conversion from a full fusion model to "kiss-and-run" fusion (Zakharenko et al., 2002). These experiments argue for the role of a retrograde signaling molecule in the presynaptic expression and maintenance of mGluR LTD.

Additional experiments have been performed to address the possibility that the various subtypes of group 1 mGluRs have individual roles in the induction, expression and maintenance of mGluR LTD. Induction of the LTD fails to occur in mice deficient in the gene for mGluR5 (Huber et al., 2001; Volk et al., 2006) although, preincubation with both mGluR1 and mGluR5 antagonists are required to prevent induction of LTD with DHPG (Volk et al., 2006). These results imply that mGluR5 may be required for proper trafficking of mGluR1 to the cell surface or for proper mGluR1 function. Intriguingly, in the hippocampus there appears to be a receptor activation requirement for the expression, but not maintenance of mGluR LTD (Palmer et al., 1997; Fitzjohn et al., 1999; Schnabel

et al., 2001; Huang and Hsu, 2006) although results vary depending on duration of agonist application (Volk et al., 2006) and to whether mGluR1 (Volk et al., 2006) or mGluR5 (Huang and Hsu, 2006) mediate this effect. Also, although LTD is induced in mGluR1 knock out animals, it is expressed at a reduced level, however mGluR1 appears to mediate the endocytosis of GluR1 containing receptors (Volk et al., 2006) suggesting multiple maintenance mechanisms.

The induction, expression and maintenance of mGluR LTD at the CA1 to CA3 synapse in the hippocampus remains somewhat confusing. This is compounded by the age of animals in experiments, as well as varying recording and experimental conditions. It does seem clear, however, that both mGluR1 and mGluR5, and VGCC play a role in induction and that protein synthesis and pre- and postsynaptic components may contribute to the maintenance phase of mGluR LTD. This confusion, however, does not diminish the role that mGluR LTD may play within this learning and memory structure. mGluR LTD has now been postulated to play a role in the phenotype of Fragile X Syndrome, the most common form of mental retardation in humans. Fragile X mental retardation protein (FMRP), a negative regulator of translation, is itself actively translated in dendritic spines in response to activation of group 1 mGluRs (Weiler et al., 1997). Additionally, DHPG application results in the lengthening of spines in dissociated hippocampal neurons (Vanderklish and Edelman, 2002) suggesting a loss of synapses. Surprisingly, but consistent with this data, mGluR LTD in FMRP KO animals is increased under both stimulation induction and chemical induction protocols as compared to wild type (Huber et al., 2002), and the lack of FMRP

protein appears to remove the protein synthesis requirements from mGluR LTD and loss of AMPA receptors from the membrane (Nosyreva and Huber, 2006). It is theorized that group 1 mGluR activation results in an up regulation of FMRP which in turn represses translation of proteins effectively regulating the extent of mGluR LTD (Bear et al., 2004); however, in the absence of FMRP the protein components needed for LTD expression are unregulated and, thus, readily available for expression of LTD without a negative feedback loop to keep the extent of LTD in check (Nosyreva and Huber, 2006).

mGluR LTD in reward pathways

Dorsal Striatum

While the dorsal striatum is not included in what is considered classical reward pathways, increasing evidence points to an involvement in dorsal striatal plasticity in the formation of habits that often coincide with compulsive drug use (Lovinger et al., 2003). mGluR dependent LTD here was observed in the striatal medium spiny neurons (MSNs) after induction by a stimulation protocol and depolarization of the postsynaptic cell. Unlike the hippocampus, however, striatal mGluR LTD is induced by a high frequency tetanus (HFS) and, is dependent on dopaminergic transmission (via D2 receptors) (Calabresi et al., 1992; Kreitzer and Malenka, 2005) which is in turn regulated by nicotinic acetylcholine receptor activation (Partridge et al., 2002). Similar to the hippocampus and cerebellum, mGluR LTD in the striatum requires the activation of postsynaptic L-type VGCC

(specifically $Ca_{v1.3}$) and an increase in postsynaptic calcium (Kreitzer and Malenka, 2005; Wang et al., 2006). Also, contrary to the hippocampus, this LTD could be blocked by individually blocking both mGluR1 and mGluR5 receptors (Sung et al., 2001) suggesting that both are required for the induction of dorsal striatal mGluR LTD. The expression of dorsal striatal mGluR-LTD appears to be entirely dependent on presynaptic mechanisms as assayed by CV, PPR and a decrease in quantal frequency as measured by Sr^{2+} asynchronous events (Choi and Lovinger, 1997b, 1997a). It has now been shown that endocannabinoids released from the postsynaptic cell act on cannabinoid receptor 1 (CB1) in the presynaptic terminal to induce mGluR-LTD expression (Gerdeman et al., 2002; Kreitzer and Malenka, 2005; Wang et al., 2006). The LTD, therefore, is often referred to as endocannabinoid LTD or eCB-LTD. Until recently, applications of the agonist DHPG to striatal preparations failed to induce mGluR-LTD (Sung et al., 2001). This may be because MSNs exist in two distinct states, an “up-state” with more depolarized membrane potentials and a “down-state” with more hyperpolarized potentials, depending on synchronous cortical stimulation. Voltage clamping MSNs at -50 mV to mimic up-state membrane potential permitted the group 1 mGluR agonist to induce LTD in a CB1 and L-type VGCC dependent manner (Kreitzer and Malenka, 2005). Although mGluR LTD is dependent on D2 receptor activation, only a subset of MSN have D2 receptors, yet all tested express mGluR LTD. Wang et al. have recently investigated the D2 receptor activation requirement for striatal mGluR-LTD. Intriguingly, it appears that dopamine’s activation of D2 receptors on cholinergic interneurons decreases

acetylcholine output, and thus alleviates the inhibition of $Ca_{v1.3}$ VGCC by M1AChRs (Wang et al., 2006).

Ventral Striatum (Nucleus Accumbens)

As opposed to the dorsal striatum the ventral striatum appears to play a role in the sensitization to psychostimulants (Winder et al., 2002). In the Nucleus Accumbens (NAc) mGluR LTD is induced in cortical-MSN synapses both by application of DHPG and a physiologically relevant stimulus protocol (13 Hz 10 min) (Robbe et al., 2002). While this LTD is also mediated by CB1 receptors presynaptically (Robbe et al., 2001), unlike the dorsal striatum it appears to be mediated entirely by mGluR5 on the postsynaptic cell and the release of Ca^{2+} from intracellular stores as opposed to VGCCs (Robbe et al., 2002) as seen in the dorsal striatum and hippocampus. Interestingly a single administration of the active component of cannabis (Δ^9-THC) in mice prevents the expression of the stimulus induced mGluR-LTD in the NAc (Mato et al., 2004), however, continued exposure (7 days) results in a rescue of stimulus induced mGluR-LTD but, fascinatingly by presynaptic group 2 mGluRs that normally fail to contribute to LTD under this induction mechanism (Mato et al., 2005).

Ventral Tegmental Area

The VTA comprises the mesolimbic dopamine system that consists of dopaminergic neurons that synapse in the NAc and prefrontal cortex where they

are though to play a key role in reward circuitry; however, this nucleus is regulated by glutamatergic inputs from the cortex and other reward nuclei (including the BNST) (Georges and Aston-Jones, 2002; Winder et al., 2002). Initial studies of LTD in the VTA demonstrated that LFS (1 Hz 7 min) induces LTD in a NMDA and mGluR independent mode, but may require non-L-Type VGGC activation (Thomas et al., 2000)(but see (Bellone and Luscher, 2005, , 2006)). Subsequent studies revealed that stimulating the VTA with burst like firing protocols (5 stimuli at 66 Hz) yields mGluR LTD that is expressed postsynaptically, can be mimicked with DHPG (voltage clamped at -50 mV), is dependent on mGluR1 activation, increases in intracellular Ca^{2+}, and PKC activation (Bellone and Luscher, 2005). Bellone and Lüscher also found that a change of some of the AMPA receptor subunit composition at the synapse, from those lacking RNA edited GluR2 subunits (thus Ca^{2+} permeable and rectifying and here after called Ca^{2+} permeable AMPARs or CP AMPARs) to those only containing subunits with RNA edited GluR2 within synapse, is required for expression of mGluR LTD in the VTA (Bellone and Luscher, 2005). In a compelling set of experiments it was also shown that a single injection of cocaine results in a physical switch of some receptor subunits at the synapse from edited GluR2 containing, to CP AMPARs and that this is dependent on functional PICK 1 and could be reversed by activating mGluR1 *in vivo* (Bellone and Luscher, 2006). In the VTA, unlike cerebellar mGluR LTD that is also induced by mGluR1 and dependent on PICK1, mGluR LTD appears to be mediated by removing CP AMPARs (Bellone and Luscher, 2006).

One of the most fascinating aspects of mGluR LTD in various regions of the brain remains the variety of induction, expression and maintenance mechanisms across regions. This undoubtedly impacts the way both drugs of abuse and potential pharmacological therapies could affect plasticity within the above nuclei, and underscores the flexibility of LTD induction via a G_q coupled mechanism.

M1AChR and α1-AR induced LTD

Although far fewer experiments have investigated the possibility of G_q GPCR induced LTD by other G_q coupled receptors, it was recognized early on that disrupting both cholinergic and adrenergic inputs into the visual cortex (vCTX) interfered with synaptic depression induced by ocular deprivation (Bear and Singer, 1986). Subsequently it was shown that LTD could be induced at glutamatergic synapses in the vCTX by the activation of M1 AChRs or α_1-AR (with a paired pulse protocol) in an input specific and NMDA dependent manner (Kirkwood et al., 1999). More recently in the same region it has been established that LFS (1 Hz 15 minutes) induces LTD that can only be blocked by simultaneous co-application of antagonist to mGluR5, M1 AChRs and α_1-AR during the induction protocol (Choi et al., 2005).

Both cholinergic, and adrenergic innervation have been implicated in hippocampal dependent memory formation. LTD induced by the activation of M1 AChRs and α_1-AR has now been described in the hippocampus where, like the vCTX, it is dependent on NMDA receptors and input specificity (Scheiderer et al.,

2004; Scheiderer et al., 2006) unlike mGluR LTD in the same region. Intriguingly, septal lesions that disrupt cholinergic innervation in the hippocampus prevent the induction of M1 AChR LTD, however subsequent sympathetic sprouting rescues this LTD (Scheiderer et al., 2006) suggesting that in the face of neurodegeneration the brain attempts to maintain the capacity for this plasticity in the hippocampus. M1 AChR LTD can also be expressed in the vCTX where it appears to be dependent on ERK1/2 and protein synthesis, but is independent of PKC signaling (McCoy and McMahon, 2007).

Although LTD can be induced in these brain regions by stimulating either M1 receptors or α_1-ARs, the stimulation of these receptors have other effects over excitatory transmission as well. M1 activation in the perirhinal cortex induces a long-lasting depression (although not LTD as it can be reversed by subsequent antagonist application) that is independent of NMDA activation and concurrent stimulation (Massey et al., 2001). α_1-ARs have been shown to have varying effects on glutamatergic synaptic transmission depending on the region examined. In the caudal NTS α_1-ARs mediate a transient depression in excitatory transmission (Zhang and Mifflin, 2006). In contrast, in the paraventricular nucleus of the hypothalamus, α_1-AR activation results in an increase in synaptic efficacy at excitatory synapses via both a pre- and postsynaptic mechanism (Gordon and Bains, 2003; Gordon et al., 2005; Gordon and Bains, 2005).

Section Summary

Research into roles that G_q induced LTD may play in various neural systems is still in its infancy, however it provides interesting insight into understanding various behavioral phenomena and, perhaps more exciting, may provide potential therapeutic targets for neural disease states. Targeting group 1 mGluRs with an antagonist may prove to be a therapeutic treatment for the multitude of symptoms that are exacerbated by Fragile X Syndrome (Bear et al., 2004; Bear, 2005). Conversely, G_q coupled LTD has been demonstrated to be attenuated in aged rats who show age related cognitive deficits, but not their equally old unimpaired counterparts (Lee et al., 2005). Together this data suggests that this type of plasticity may play a striking role in the balance of learning mechanisms throughout life. Additional data demonstrates that targeting α_1-AR may alleviate other neurological/psychiatric disorders. An unintentional result occurred when two combat veterans who were actively seeking treatment for post traumatic stress disorder (PSTD) began taking prazosin (a selective α_1-AR antagonist) as part of a regiment for benign prostatic hypertrophy. In both cases the patients reported an elimination of combat trauma nightmares (Raskind et al., 2000) which in turn prompted a several studies in combat and civilian trauma victims who experienced both day and night PTSD symptoms. In all cases over the course of weeks with antagonist treatment PTSD suffers saw a dramatic alleviation of both daytime and nighttime symptoms (Raskind et al., 2002; Taylor and Raskind, 2002; Peskind et al., 2003; Taylor et al., 2006). It will

be interesting to see in the future if other neurological conditions are linked with improper plasticity via G_q coupled receptors.

Hypothesis and Specific Aims

Hypothesis

NE induces a long term depression of excitatory transmission in the Bed Nucleus of the Stria Terminalis via signaling by the α_1-AR and this plasticity may be manipulated by behavioral paradigms of affective disorders.

Specific Aims

1. Characterize LTD induction mechanisms in the BNST.
2. Test the hypothesis the α_1-AR LTD is maintained via similar synaptic mechanism as mGluR5 LTD in the BNST
3. Test the hypothesis that α_1-AR LTD is disrupted in two genetic models of affective disorder, the α_2-AR and NE transporter (NET) knock out mice; as well as, in two stress inducing behavioral paradigms, chronic ethanol exposure, and chronic restraint stress.

CHAPTER II

CHARACTERIZATION OF THE INDUCTION PROPERTIES OF α1-ADRENERGIC RECEPTOR INDUCED LONG TERM DEPRESSION OF EXCITATORY TRANSMISSION IN THE BED NUCLEUS OF THE STRIA TERMINALIS AND ITS ABSENCE IN GENETIC MOUSE MODELS OF AFFECTIVE DISORDERS

Introduction

Relapse to drug use after extended abstinence remains a troublesome aspect of addiction. Clinical studies have implicated psychological stress as a major factor that induces relapse behavior. Although the noradrenergic system has long been implicated in withdrawal related-behaviors (Aston-Jones and Harris, 2004), recent evidence has highlighted its role in addiction (Weinshenker and Schroeder, 2007), particularly with the stress-induced relapse response (Shaham et al., 2000). For example, administration of an α_2-AR agonist or lesioning of the ventral noradrenergic bundle (VNAB) attenuates stress-induced reinstatement to opiate seeking (Erb et al., 2000; Wang et al., 2001) and viral restoration of dopamine beta-hydroxylase (DBH, the enzyme that produces NE) in the nucleus tractus solitarius (NTS) of mice lacking DBH restores conditioned place preference (CPP) to morphine (Olson et al., 2006).

The BNST, a component of the extended amygdala, receives input from the VNAB, as well as glutamatergic inputs from cortical/limbic areas and sends outputs to stress and reward centers (Forray and Gysling, 2004). The lateral BNST (lBNST), and α_2- and β-AR signaling therein, regulates stress-induced relapse behaviors (Wang et al., 2001; Leri et al., 2002). Moreover, blockade of excitatory transmission within this same region also disrupts anxiety-related behavior (Walker and Davis, 1997). Due to these observations, our group characterized NE modulation of glutamatergic signaling in the dorsal and ventral lBNST (dlBNST, vlBNST), focusing on α_2- and β-AR signaling (Egli et al., 2005). Studies, however, have also suggested a role for α_1-ARs in the BNST, demonstrating that blocking α_1-ARs decreases anxiety responses concurrent with reductions in hypothalamic-pituitary-adrenal (HPA) axis activation (Cecchi et al., 2002). Further, activation of α_1-ARs also increases spontaneous inhibitory postsynaptic current (IPSC) frequency in the vlBNST of animals exposed to morphine (Dumont and Williams, 2004).

α_1-ARs have been reported to modulate glutamatergic transmission in other brain regions. α_1-AR activation leads to depression of excitatory transmission that is long-lasting in hippocampus and visual cortex (Kirkwood et al., 1999; Scheiderer et al., 2004) and transient in the caudal NTS (Zhang and Mifflin, 2006); For review see Grueter et al., 2007.) In contrast, in the paraventricular nucleus of the hypothalamus (PVN), α_1-AR signaling enhances excitatory transmission through both pre- and postsynaptic mechanisms (Gordon and Bains, 2003; Gordon et al., 2005; Gordon and Bains, 2005).

Here we investigate the impact of α_1-AR signaling on excitatory transmission in the lBNST. We find an extended application of NE results in robust LTD that is dependent on α_1-AR activation and that can be mimicked by α_1-AR agonists. Intriguingly, the LTD described here differs from the previously described α_1-AR LTD in the hippocampus and visual cortex in its induction characteristics. Additionally, because of the relative importance of the BNST in relapse and anxiety paradigms, and adrenergic signaling therein, we sought to determine if chronic alterations in adrenergic signaling would interfere with the expression of α_1-AR-LTD. We found that BNST α_1-AR-LTD is intact in animals acutely treated with cocaine, yet, is disrupted in both the α_{2A}-AR KO and the NET KO lines, suggesting that chronic, but not transient, alterations in adrenergic signaling modulate the expression of α_1-AR-LTD

Methods

Animal Care

Male C57BL/6j mice 5-10 weeks old (The Jackson Laboratories, Bar Harbor, ME) and α_{2A}-AR KOs and NET KOs which were both generated from in-house breeding on a C57BL/6 background were used in experiments. All animals were provided with food and water *ad libitum* and housed in groups within the Vanderbilt Animal Care Facilities. Experiments were performed under Vanderbilt Animal Care and Use committee approved guidelines. Animals receiving cocaine were pre-handled for 5 days, receiving saline injections for 4 days.

Brain slice preparations

Slicing methods were as previously described in Grueter et al. (2006). Briefly, animals 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. 300 µm coronal slices were made on a Leica VT1000S vibratome (Leica Microsystems, Bannockburn, IL) in a 1-4° C, oxygenated (95% O_2, 5% CO_2), high-sucrose low Na^+ artificial cerebral spinal fluid (ACSF in mM: 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$).

Field potential recordings

After slicing, whole or hemisected slices were transferred immediately to interface chambers where they rested for at least 30 minutes in a humidified and oxygenated environment while continuously being perfused with oxygenated and heated (approx. 28-30° C) ACSF (in mM: 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$) at a rate of 2ml/min. Following this initial incubation, 25 µM picrotoxin was added to the bath to block $GABA_A$ receptors and slices were allowed to rest at least another 30 minutes prior to recording, picrotoxin was included during the entirety of all experiments to isolate excitatory transmission. This concentration has been shown by our group to sufficiently block all inhibitory transmission via the $GABA_A$ receptor (Egli and Winder, 2003). Recording electrodes of approximately 1 MΩ resistance were

pulled on a Flaming/Brown microelectrode puller (Sutter Instruments, Novato, CA) and filled with ACSF. A bipolar Nichrome (A-M Systems, Carlsborg, WA) stimulating electrode was placed dorsally to the recording electrode within the dlBNST 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 (Weitlauf et al., 2004; Egli et al., 2005; Grueter and Winder, 2005). The amplitude (voltage) of the N2 was measured at a stimulation intensity that resulted in a voltage approx. 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.

Whole cell recordings

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 minutes. 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 as with the field recordings) to isolate currents evoked by glutamate receptor activation at a rate of 2 ml/min. Stimulating electrodes were the same as for field recordings in dorsal recordings and medial to the lBNST in ventral recordings. Patch electrodes (3-6 MΩ) were pulled on a Flaming/Brown microelectrode puller (Sutter Instruments, Novato, CA) and filled with either Cs- or K-gluconate

intracellular solution (in mM: Cs- or K-gluconate 135, NaCl 5, $MgCl_2$ 2, HEPES 10, EGTA 0.6, Na_2ATP 4, Na_2GTP 0.4; there was no observable difference with either intracellular solution on the LTD effect). In all whole cell experiments, cells were clamped at -70 mV throughout and excitatory postsynaptic currents (EPSCs) were recorded using Clampex 9.2 (Molecular Devices, Sunnyvale, CA). 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.

Analysis of field recordings

All recorded data were analyzed via Clampfit 9.2 (Molecular Devices, Sunnyvale, CA). All field recordings contain a 20 minute baseline recording prior to agonist application and all data points were normalized to the baseline 5 minutes prior to agonist application. Plotted time courses for field experiments are represented as 1 min averages. For the majority of LTD experimental measurements, the LTD measurement was taken 55-60 min post agonist application. The exceptions are the experiments with prazosin (fig 1C and 1D) which the LTD measurement was the final 5 minutes of each recording.

Analysis of whole cell recordings

A 5 minute baseline prior was acquired prior to agonist application and all points were normalized to minutes 3-5 within each experiment (with the exception of the low concentration methoxamine experiments where a 10 minute baseline was acquired). Points are 30s averages on plotted time course. For the majority of LTD experiments the LTD measurement is taken at 58-60 min within the experimental time course. The exceptions to this rule are the dual agonist application experiments, where the first LTD measurement is at 28-30 min within the time course and the second measurement is at min 55-57 within the time course. The experiments with the $GABA_B$ antagonist and the lower concentration of methoxamine had measurements taken in the last 2 minutes of recording due to the shorter duration of wash.

Statistics

All data points were reported as the mean $\pm$ S.E.M. and significance (determined by paired and unpaired Students t-test) is reported in the text and figure legends. Significant differences were defined as having a $P<0.05$.

Reagents

(2S)-3-[[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydro xypropyl] (phenylmethyl) phosphinic acid (CGP 55845, Tocris, Ellisville, MO), Cocaine (Sigma, St. Louis, MO), (RS)-3,5-Dihydroxyphenylglycine (DHPG, Tocris, Ellisville, MO), DL-2-Amino-5-phosphonopentanoic acid (DL-APV, Sigma, St.

Louis, MO), Methoxamine-HCl (Sigma, St. Louis, MO), 2-Methyl-6-(phenylethynyl) pyridine hydrochloride (MPEP, Tocris, Ellisville, MO), nimodipine (Tocris, Ellisville, MO), picrotoxin (Tocris, Ellisville, MO), prazosin (Tocris, Ellisville, MO). Dimethylsulfoxide (DMSO) was the solvent used for stock solutions of CGP 55845, MPEP, nimodipine, picrotoxin and prazosin where the maximum final concentration of DMSO was .02% by volume.

Results

α_1-AR Activation Produces LTD of Excitatory Responses in the BNST

Both the dlBNST and the vlBNST are activated in response to various stressors (footshock, yohimbine, restraint, etc.) (Funk et al., 2006). Additionally electrical stimulation of the dlBNST and vlBNST produces behavior similar to that observed after experiencing a stressor (Casada and Dafny, 1991). We began our investigation into the modulation of excitatory synapses via the α_1-AR in the BNST by recording extracellular excitatory potentials in the dlBNST (Fig 2A). We first applied the α_1-AR selective agonist methoxamine (100 μM) for 20 minutes and observed a long lasting depression in excitatory transmission (79.5% $\pm$ 4.7%, $p<0.01$, N=6; Fig 2B) that was absent in the presence of the α_1-AR antagonist 10 μM prazosin (101.9 $\pm$ 5.6%, N=6; Fig 2C). To verify that this phenomenon was LTD and not the result of a constitutively activated α_1-AR we applied prazosin (10 μM) to slices 60 minutes after a 20 minute application of methoxamine (100 μM) was terminated. This treatment failed to reverse the

depression observed after agonist application (67.3% ± 3.0%, $p<0.001$, N=5; Fig 2D) suggesting that the 20 minute activation of α_1-ARs results in an LTD of excitatory inputs. The BNST, however, is composed of a heterogeneous population of dendrites and cell bodies and therefore extracellular recordings may potentially reflect effects on excitability of the postsynaptic dendrites/cell. We therefore utilized whole-cell voltage clamp recordings to measure isolated EPSCs in dlBNST and vlBNST neurons. Application of methoxamine (10 μM or 100μM) for 15 minutes produced a robust depression of the evoked EPSC that persisted for at least 40 minutes after washout of agonist (dlBNST: 10 μM, 65.6 ± 6.3%, N=5, $p<0.001$, Fig 3A; 100 μM, 46.1%± 9.8%, N=6, $p<0.001$, Fig 3B; 10 μM vs. 100 μM was not statistically different $p>.15$; vlBNST 100 μM: 63.9% ± 8.3%, N=9, $p<0.001$, Fig 3C; representative experiment from the dlBNST 100 μM methoxamine recording Fig 3D). We did not see changes in the input resistance (IR) following application of methoxamine in either the dlBNST (10 and 100 μM) nor the vlBNST (100 μM) (97.3 ± 5.5%, $p>0.7$, N=19, Fig 3D representative trace) Additionally, we did not observe a significant change to a second 15 minute application of methoxamine in the dlBNST (second methoxamine application vs. first methoxamine application $p>0.05$, N=6; Fig 3E). This, along with the extent of the depression suggests that the initial induction of α_1-LTD was saturated under our conditions although we cannot rule out that receptors may have become desensitized to the first agonist application.

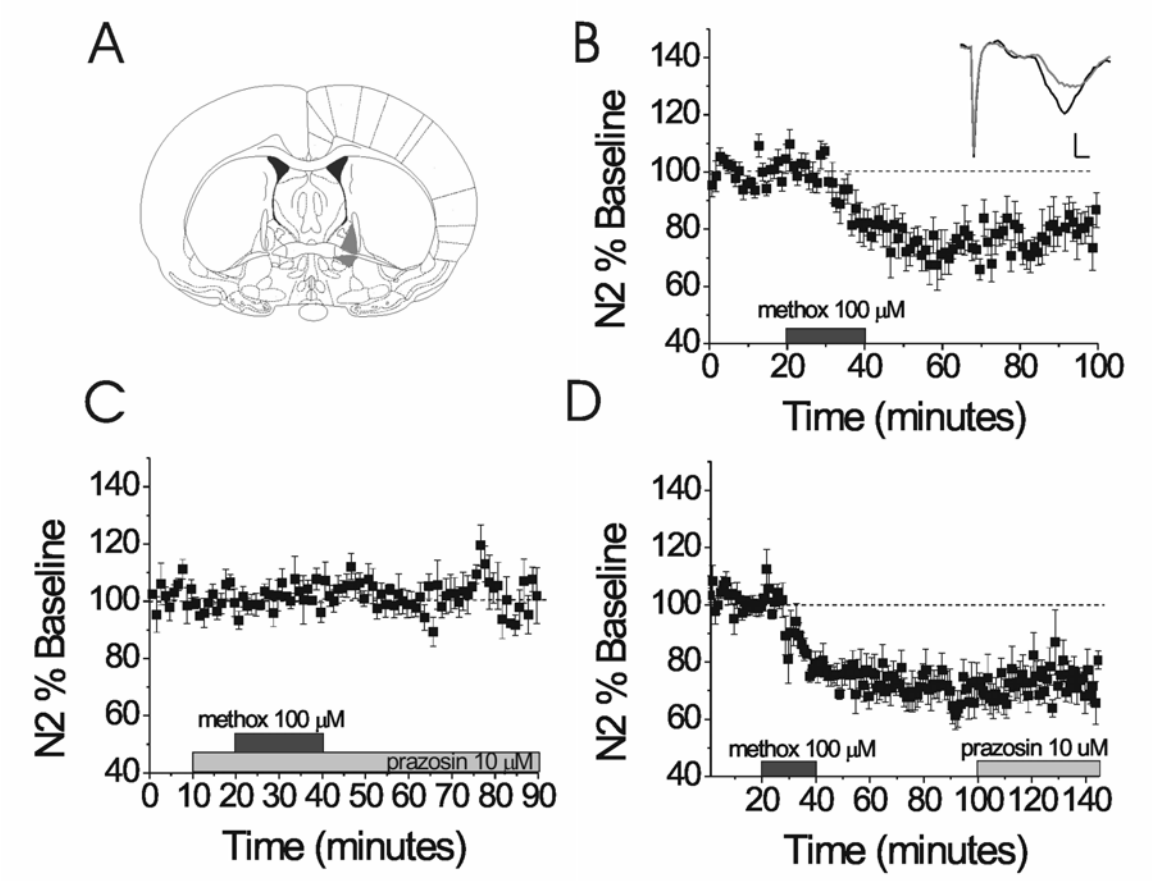


Figure 2 α_1-AR Activation Induces LTD in the BNST
(A) BNST schematic adapted from Paxinos and Franklin (2001). Gray shading represents the lateral BNST, above anterior commissure dorsal lateral BNST, below anterior commissure ventral lateral BNST. (B) Application of the α_1-AR selective agonist methoxamine (100 µM) induces a depression of extracellularly recorded excitatory responses that persists for over 60 min post wash (N = 6). (Inset) Representative traces of N1 and N2, 5 min average of baseline (black) and LTD (gray) (scale bars 0.2mV by 0.5 ms). (C) The α_1-AR antagonist prazosin (10 µM) blocks induction of the depression by methoxamine (100 µM; (N = 6). (D) Prazosin (10 µM) cannot reverse the depression induced by methoxamine (100 µM) when applied in wash (N = 5).

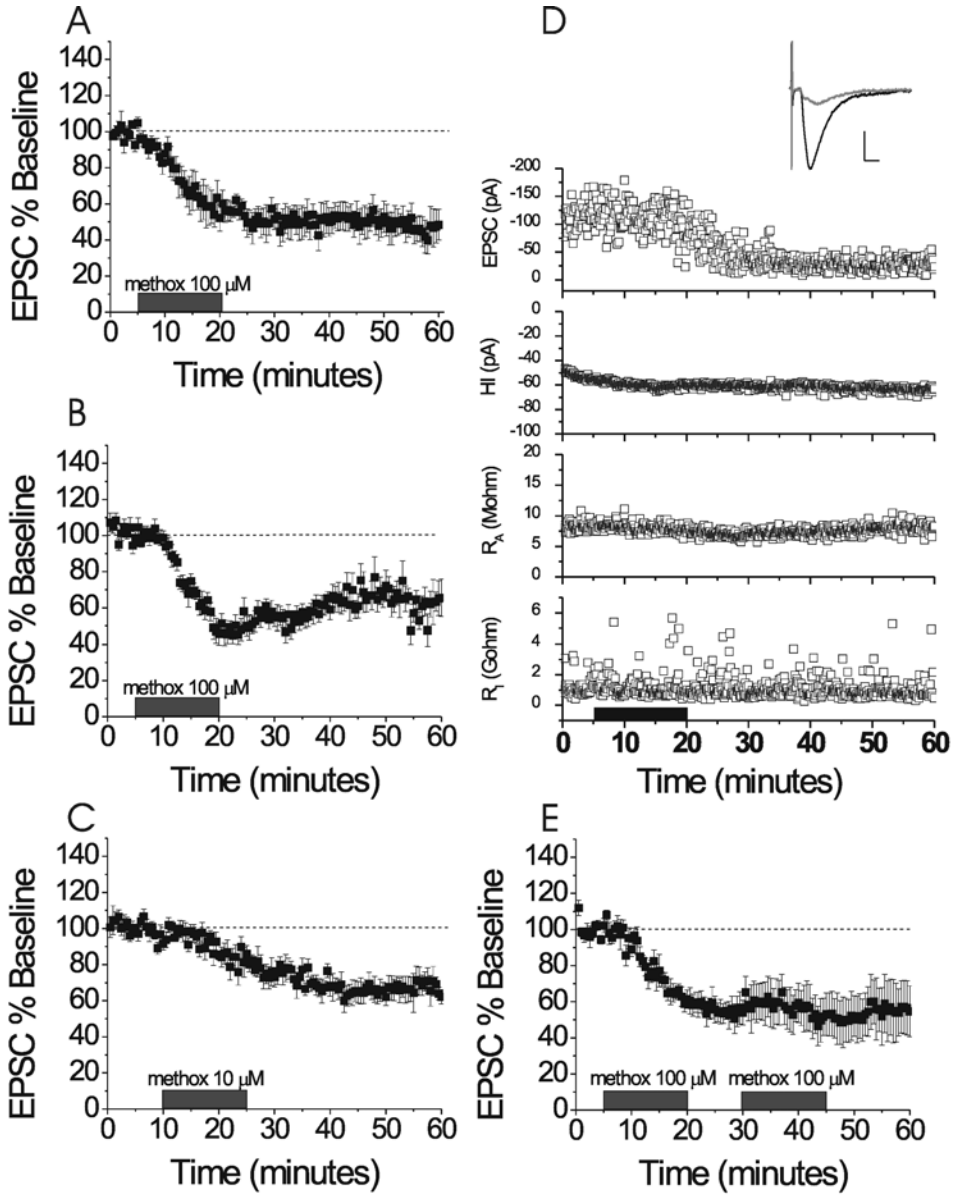


Figure 3 EPSCs are Depressed by α_1-AR Agonist Application.
(A) Whole cell voltage clamp (-70mV holding potential) experiments were performed to assess long-term depression (LTD) in the dorsal lateral BNST (dlBNST). Methoxamine (100 µM) was applied for 15 min and washed out for 40 min resulting in LTD (N = 6). (B) Under the same conditions 100 µM methoxamine also induced LTD in the ventral lBNST (vlBNST). (N = 8) (C) A lower concentration of methoxamine (10 µM) applied for the same duration also can induce significant LTD in the dlBNST. (D) A representative experiment in the dlBNST: EPSC (pA) = EPSC amplitude, HI (pA) = holding current, RA (MΩ) = Access Resistance, and RI (GΩ) = Input resistance. (D inset) Representative traces of EPSCs, each a 2 min average of baseline (black line) and LTD (gray line; scale bar 40 pA by 5 ms). (E) Applying 100 µM methoxamine after previous induction of LTD fails to further depress EPSCs (N = 6).

Prolonged Exposure to NE Results in α_1-AR-Dependent LTD

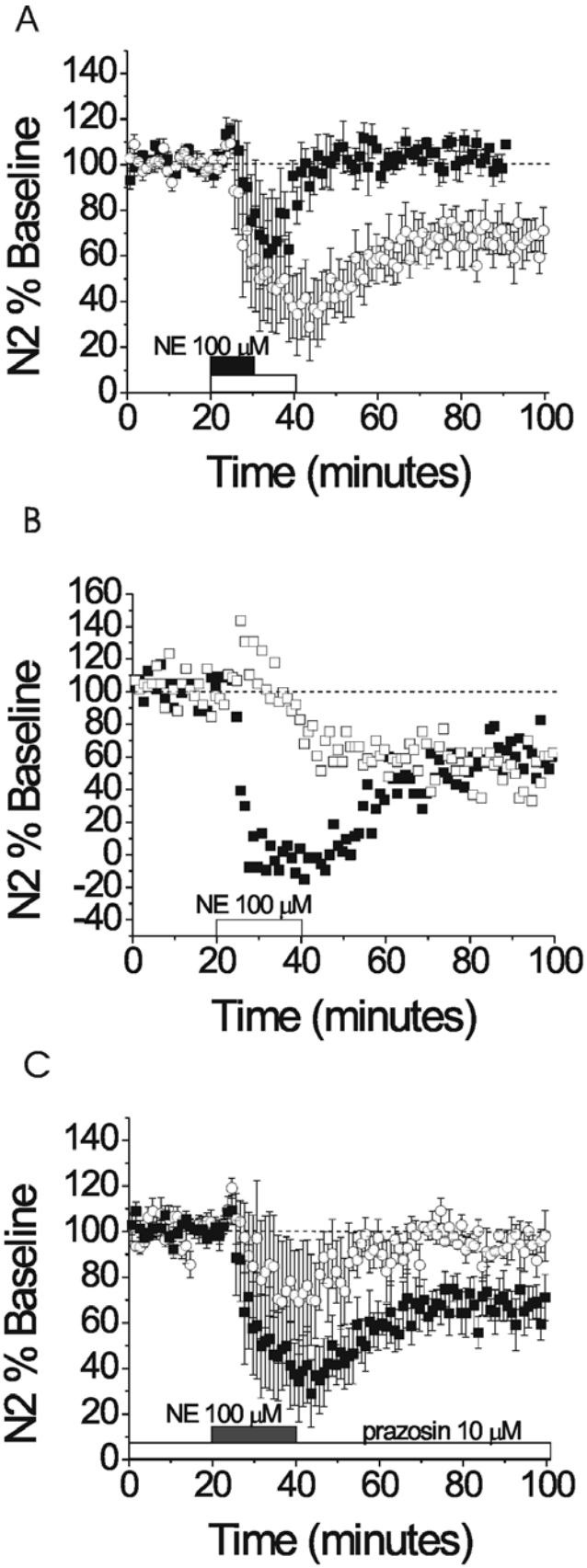
NE application can elicit LTD of excitatory inputs in the visual cortex and the hippocampus (Kirkwood et al., 1999; Scheiderer et al., 2004). Thus, we decided to test whether NE induces similar LTD in the BNST, a nucleus heavily innervated by adrenergic fibers. Our group has previously reported that a 10 minute application of 100 μM NE results in a transient bimodal response (an increase or decrease) in the extracellular field potential that is mediated by α_2- and β-ARs (Egli et al., 2005). This application, however, was insufficient to produce a sustained depression in excitatory transmission (98.4% $\pm$ 3.4%; Fig 4A, N=6, Egli et al., 2005) in field recordings. In the BNST and other regions, chronic stressors are thought to promote lasting increases in extracellular NE levels by shifting the firing patterns of noradrenergic cells from phasic firing to burst firing (Forray and Gysling, 2004). We found that increasing the duration of application of NE to 20 minutes at the same concentration produced a robust LTD of excitatory responses (67.1% $\pm$ 7.3%, $p<0.01$, N=6) that persisted for over 60 minutes after agonist application (Fig 4A). Moreover, the experiments where we applied 100 μM NE for 20 minutes were significantly different from the experiments where we applied 100 μM NE for 10 minutes ($P<0.05$) during the LTD phase of the recording. With the extended application time we still observed the same bimodal effect to NE in our transient responses. (2 of 6 of the experiments resulted in an initial increase in synaptic efficacy and 4 of 6 in an initial decrease in synaptic efficacy.) The sustained depression, however, was observed irrespective of the polarity of the initial response to NE (Fig 4B). To

test whether the persistent depression induced by 100 μM NE was due to activation of α1-ARs we applied the α1-AR specific antagonist prazosin (10 μM) to the slice prior to NE application and throughout the experiment. The application of prazosin completely ablated the ability of NE (20 minutes at 100 μM) to induce a long-lasting depression in excitatory responses (94.6% ± 4.5%, N=5; Fig 4C); however, we still observed the initial bimodal response (3/5 increases in synaptic efficacy, 2/5 decreases in synaptic efficacy).

α_1-AR-LTD in the BNST Is Not Dependent on NMDAR Activation or Concurrent Stimulation of Presynaptic Fibers but Is Dependent on L-type VGCCs

α_1-AR-LTD has been previously described in the visual cortex and most recently in the hippocampus where it has been shown to require concurrent activation of presynaptic inputs and NMDARs (Kirkwood et al., 1999; Scheiderer et al., 2004). We found, however, that applying methoxamine in the absence of stimulation resulted in significant LTD (82.3% ± 2.4%, $p<0.01$, N=6; Fig 5A) that was indistinguishable from that observed with concurrent stimulation. The absence of concurrent stimulation itself, however, had no effect on the amplitude of subsequent field potentials (97.4% ± 9.5%, N=5; inset, Fig 5A). To examine the influence of NMDAR activation on α_1-AR-LTD in the BNST we recorded EPSCs at -70 mV in the presence of 100 μM DL-APV. Again under these conditions, a 15 minute application of methoxamine still produced robust LTD (66.7%±13.3%, N=5, $p<0.05$, not significantly different from single methoxamine application $p>0.05$; Fig 5B). LTD induced by group 1 mGluR receptors (that are

Figure 4 NE Induces α_1-AR LTD Via a Time-Dependent Mechanism.
(A) Expressed as percent of baseline, a 20 min application of NE (100 μM) results in a sustained depression of extracellularly recorded excitatory response that lasts for over 60 min post drug application (open symbols; N = 6). However, responses following a 10 min application of NE (100 μM) fail to induce a sustained depression of the excitatory response (closed symbols; N = 6). (B) Two representative experiments with 20 min applications of NE (100 μM) demonstrate the transient bimodal effect, open symbols: increase in EPSP response followed by LTD, closed symbols: decrease in EPSP response followed by LTD. (C) 10 μM of the α_1-AR specific antagonist prazosin blocks the sustained depression of the excitatory field potential (open symbols; N = 5).


A
N2 % Baseline
140
120
100
80
60
40
20
0
NE 100 µM
0
20
40
60
80
100
Time (minutes)
B
N2 % Baseline
160
140
120
100
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40
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NE 100 µM
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Time (minutes)
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NE 100 µM
prazosin 10 µM
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20
40
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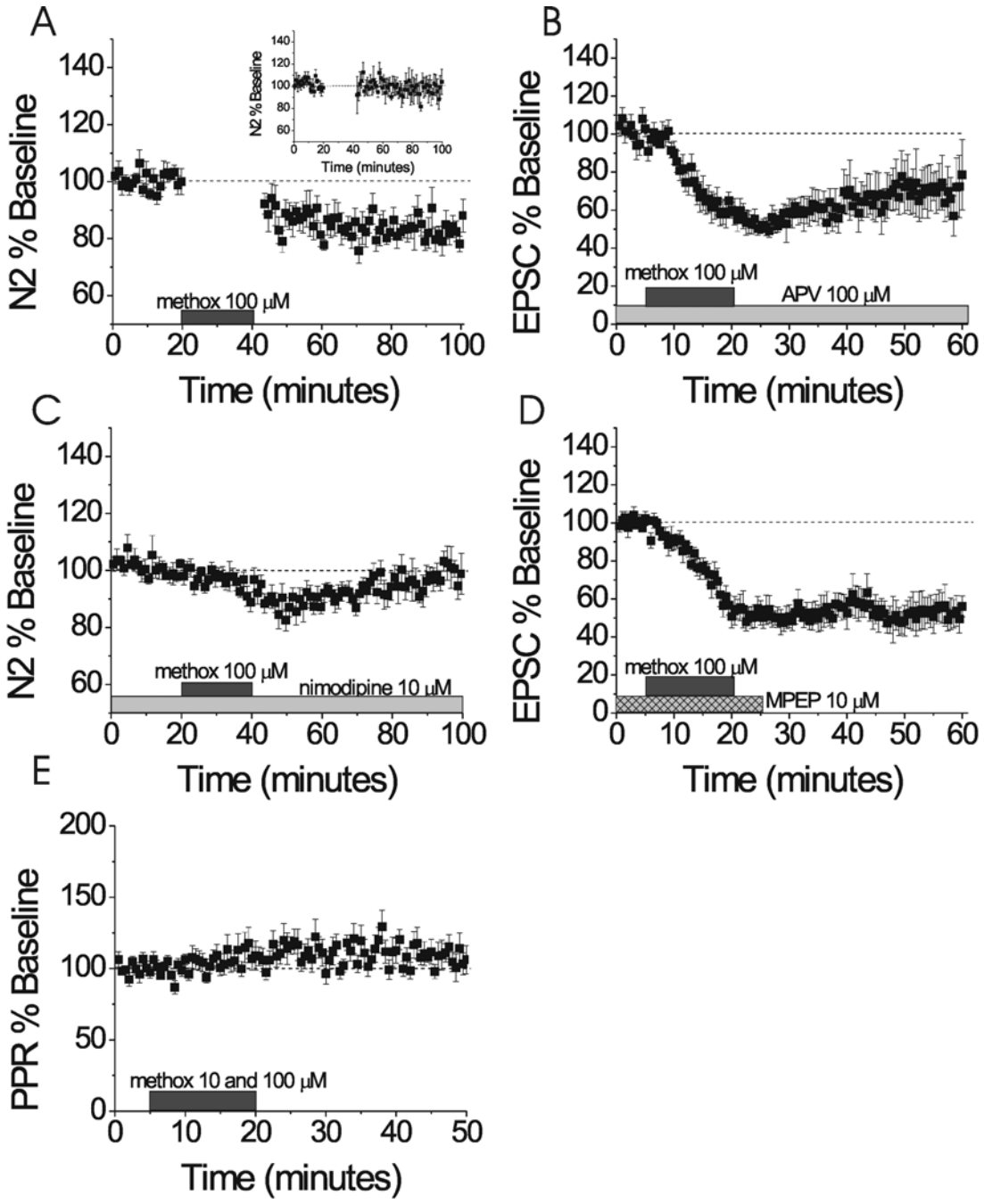
80
100
Time (minutes)

coupled to G_q) in other brain regions has been shown to involve L-type VGCCs (For review see Grueter et al, 2007). Thus, we hypothesized that α_1-AR–LTD might also require L-type calcium signals. We applied methoxamine (100 μM) in the presence of the L-type VGCC blocker nimodipine (10 μM) and found that, although there was a small early depression (min 47-49, 86.6 $\pm$ 3.4%, $p<0.01$), LTD was blocked (99.5 $\pm$ 4.8%, N=7; Fig 5C). Our group has previously described LTD in the dlBNST that can be induced by activating the group 1 mGluR, mGluR5 (Grueter et al., 2006). To ensure that α_1-LTD was not the result of increased glutamate inducing mGluR5-LTD, we applied methoxamine in the presence of the mGluR5 antagonist MPEP (10 μM) at a concentration that prevents the induction of mGluR-LTD (Grueter et al., 2006). We found that MPEP had no effect on α_1-AR LTD (54.3% $\pm$ 6.7%, $p<0.005$, N=5; Fig 5D). These data thus suggest that α_1-ARs heterosynaptically induce LTD via a non-Hebbian mechanism in the dlBNST in an L-type VGCC dependent manner.

LTD can be maintained at synapses via either a pre- or postsynaptic mechanism. To begin to address questions of the synaptic locus of α_1-AR LTD we conducted paired pulse ratio (PPR) analysis. Increases observed in the PPR associated with a decrease in EPSC amplitude are suggestive of a presynaptic mechanism. Evoked EPSCs to two paired stimuli with a 50 ms inter-stimulus interval were acquired during whole cell recordings and we analyzed the ratio of the second response to the first response. In the dlBNST we did not observe a change in the PPR upon application of methoxamine (10 and 100 μM) nor at the LTD time point (N=11, $p>0.15$; Fig 5E). In contrast, in the vlBNST we observed a

significant transient increase in PPR (166.1 ± 19.2 % of baseline, N=7, $p>0.05$; Fig 6A) that was dependent on $GABA_BR$ signaling (N = 5; Fig 6B). $GABA_BR$ blockade, however, did not prevent the induction of LTD in the vlBNST (Fig 6B inset.)

Figure 5 α_1-AR LTD (as measured in the dlBNST) is induced independently of evoked glutamatergic synaptic activity but dependent on L-type voltage gated calcium channels (VGCCs).
(A) To address the involvement of presynaptic stimulation the stimulus was turned off prior to 100 μM methoxamine application and turned back on 2 min post α_1-AR agonist removal. This did not disrupt LTD expression. (N = 7). (A inset) To control for the lack of stimulation interleaved experiments were run without the presence of agonist (N = 5). (B) To assess the role of N-methyl-D-aspartate receptor (NMDAR) activation α_1-AR LTD experiments were performed in whole cell voltage clamp (-70mV holding potential) and DL-APV (100 μM) was included throughout the duration of the experiment (N = 5). (C) The L-type calcium channel blocker nimodipine (10 μM) prevented the induction of α_1-AR LTD by
100 μM methoxamine, however it did not prevent a significant transient depression. (N = 7) (D) To verify that α_1-AR LTD does not require mGluR5 signaling we applied 100 μM methoxamine in the mGluR5 antagonist MPEP (10 μM; N = 5). (E) Paired pulse ratios do not change in response to methoxamine (10 or 100 μM) in dlBNST (N = 11).


A
N2 % Baseline
Time (minutes)
methox 100 µM
B
EPSC % Baseline
methox 100 µM
APV 100 µM
Time (minutes)
C
N2 % Baseline
methox 100 µM
nimodipine 10 µM
Time (minutes)
D
EPSC % Baseline
methox 100 µM
MPEP 10 µM
Time (minutes)
E
PPR % Baseline
methox 10 and 100 µM
Time (minutes)

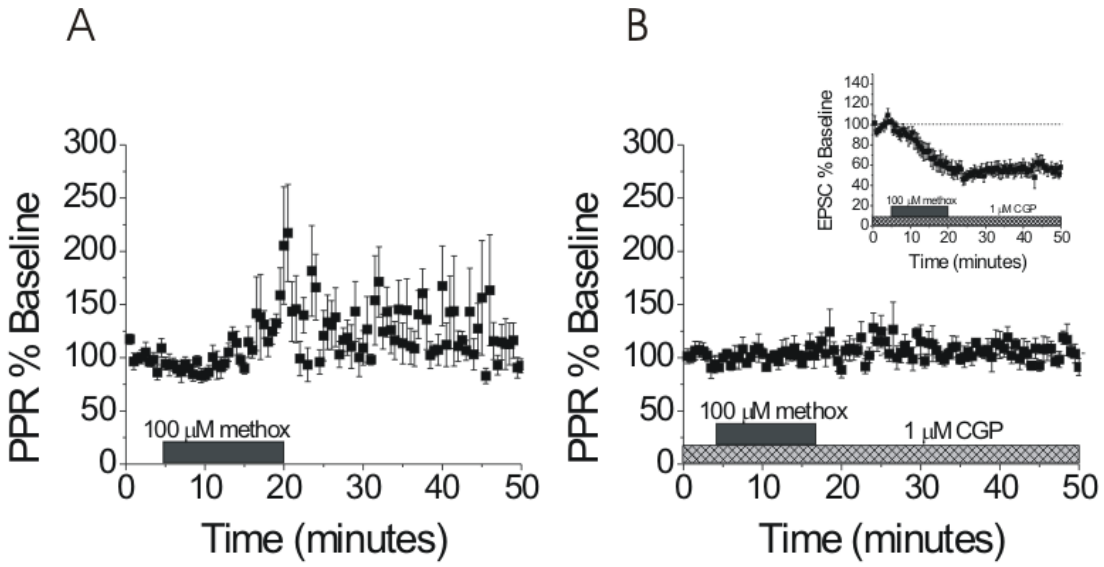


Figure 6 Methoxamine increases PPR in the vlBNST via a $GABA_BR$ dependent mechanism A.
100 µM methoxamine significantly increased PPR just following removal of drug (N = 7, $p<0.05$) B. The $GABA_BR$ antagonist CGP (1 µM) prevented the transient increase, but did not affect LTD induction by 100 µM methoxamine (N=5).

α_1-AR-LTD Is Disrupted in Mice with Aberrant Noradrenergic Signaling

Alterations in noradrenergic signaling may underlie several affective disease states (Raskind et al., 2000; Stone et al., 2006). A single administration of cocaine elicits a transient increase in extracellular NE, while depression, anxiety and alcoholism are thought to involve more chronic alterations in adrenergic tone. Noradrenergic signaling in the BNST has been implicated in anxiety, depression and drug abuse (Shaham et al., 2000; Forray and Gysling, 2004; Morilak et al., 2005) and synaptic plasticity is altered by multiple substances of abuse and stress in reward nuclei (Saal et al., 2003). We therefore chose to examine α_1-AR-LTD in the BNST in animals treated with cocaine (20 mg/kg) 30 minutes prior to slicing, and two animal models of affective disorders, α_{2A}-AR and NET KOs. Both of these KO lines of mice have altered adrenergic systems and behavior (Bohn et al., 2000; Xu et al., 2000; Schramm et al., 2001; Lahdesmaki et al., 2002; Dziedzicka-Wasylewska et al., 2006; Keller et al., 2006). Animals receiving cocaine 30 minutes prior to slicing still showed robust α_1-AR-LTD (54.1 $\pm$ 9.4%, $p<0.005$, N=5; Fig 7A.) In both KO animal models, α_1-AR-LTD was not observed upon application of methoxamine (α_{2A}-AR KO: 96.0% $\pm$ 2.8%, $p>0.05$, N=5; NET KO: 104.2% $\pm$ 6.0%, $p>0.05$, N=7; Fig 7B, 7D) however, application of the mGluR5 agonist DHPG still resulted in robust depression in the α_{2A}-AR knock out mouse indicating that LTD via $G_{\alpha q}$ coupled mechanisms is still intact (57.6% $\pm$ 3.7%, $p<0.001$, N=5; Fig 7C). This is additionally intriguing because induction of α_1-AR-LTD occludes further

depression of EPSCs in response to subsequent application of DHPG (100 μM) ($p>0.05$, N=5; Fig 7C inset).

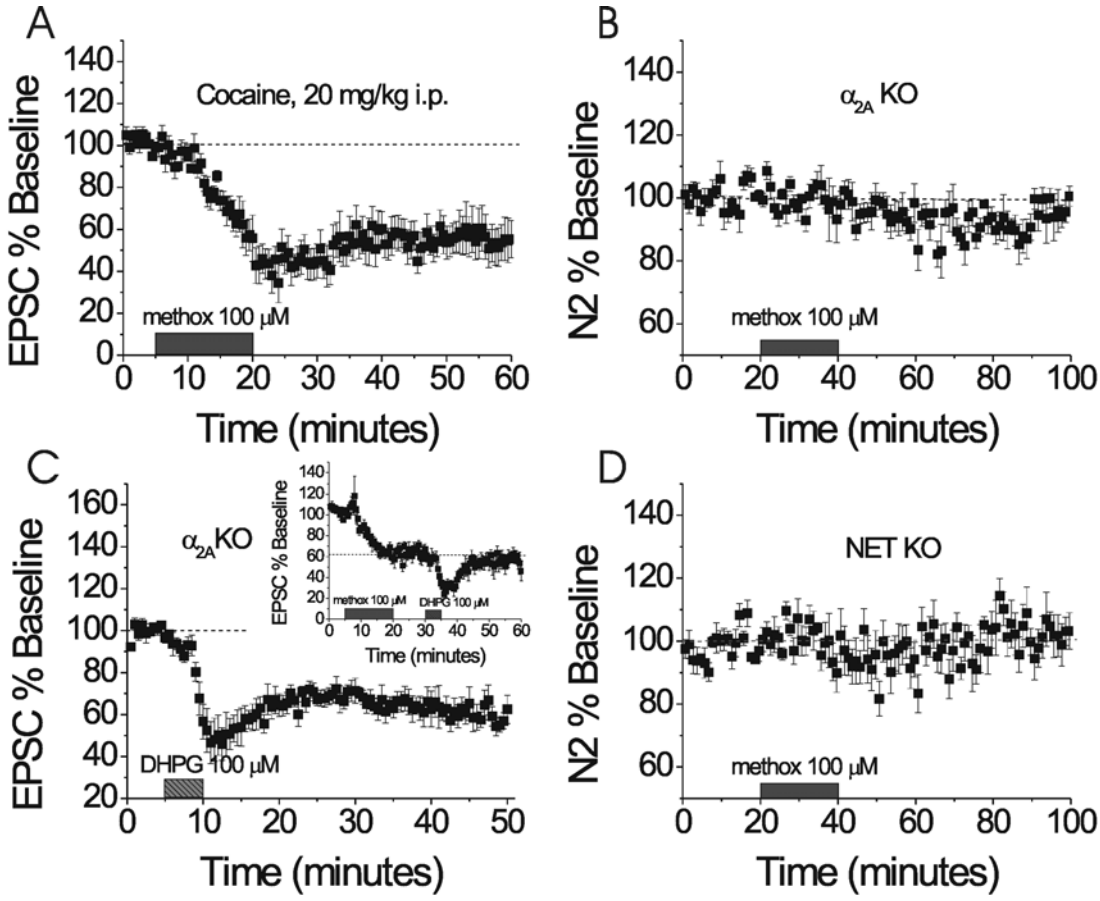


Figure 7 α_1-AR Is Disrupted in Animal Models of Affective Disorders.
(A) Cocaine (20 mg/kg) injected 30 min prior to slicing does not prevent the induction/expression of α_1-AR LTD (N = 5). (B) A 20 min application of methoxamine (100 µM) fails to induce α_1-AR LTD in α2A-AR KO mice (N = 5). (c) DHPG (100 µM) induces mGluR5-LTD in α2A-AR KO mice (N = 5). (d) Methoxamine (100 µM) fails to induce α_1-AR LTD in NET KO mice (N = 7).

Discussion

Previously, our group reported on noradrenergic modulation of excitatory synapses within the dlBNST, finding that both α_2- and β-ARs contributed to effects observed with a 10 minute application of 100 μM NE and that α_2-ARs contributed to effects in the vlBNST (Egli et al., 2005). Egli et al. found that in the dlBNST, 100 μM NE resulted in either a transient increase or decrease in excitatory transmission, while in the vlBNST it resulted in a transient decrease in excitatory transmission. Here we investigated the possibility that α_1-ARs modulate glutamatergic synapses in this region as well. We found that a 20 minute, but not a 10 minute, 100 μM NE application resulted in an LTD of excitatory transmission that was mediated by the α_1-AR and L-type VGCCs, however, this LTD was independent of NMDAR, and mGluR5 activation, or concurrent stimulation. Finally, we found that α_1-AR-LTD in the dlBNST was disrupted in two KO mice that have genetically manipulated adrenergic systems, and exhibit altered anxiety, depression and reward phenotypes; but, not in animals that received a transient alteration of their adrenergic system – a single i.p. injection of cocaine 30 minutes prior to slicing.

NE Induces LTD in a Time-Dependent Manner

Our group has shown that while a 10 minute application of NE in the dlBNST results in a bimodal transient regulation of the glutamatergic field potential (Egli et al., 2005) it fails to induce LTD via the α_1-AR. Doubling the

duration of NE application, however, results in α_1-AR-LTD. This duration-dependent result was an intriguing finding. It is clear that in the 10 minute NE application experiments agonist wash-in has occurred and GPCRs are being activated based on data from our group (Egli et al., 2005) and the data shown here. Additionally, Dumont and Williams (2004) demonstrated that a brief (< 2 min) application of 100 μM NE (in a submerged recording chamber) activated α_1-ARs in the vlBNST to transiently increase spontaneous IPSCs, thus, the α_1-AR is also presumably activated in our recordings, but at a level below threshold for LTD. The duration-dependence may play a role physiologically, as it may be disadvantageous to induce this plasticity with transient NE increases in the BNST. Therefore this LTD may be activated when the animal experiences a lasting stressor. Microdialysis studies have demonstrated that NE levels remain elevated above baseline in the BNST over an hour after restraint stress and blocking α_1-ARs attenuates stress induced rises in ACTH (Pacak et al., 1995; Cecchi et al., 2002). Moreover, Banihashemi and Rinaman (2006) showed that ablating BNST NE inputs prevents increases in corticosterone to i.p. yohimbine 90 minutes post injection. Therefore, under stress-producing conditions, α_1-AR-LTD may be recruited to alter the engagement of the HPA stress axis by the BNST over an hour after the initial stress insult. Thus, this α_1-AR LTD may specifically participate in stress responses generated by long term increases in NE in normal subjects and may be dysregulated in addictive states such as alcoholism and anxiety disorders like post traumatic stress disorder (PTSD).

α_1-AR-LTD in the BNST Is a Heterosynaptic Form of Plasticity

LTD mediated via group I mGluRs remains the best characterized $G_{\alpha q}$-coupled receptor LTD and has been described in cerebellum, hippocampus, cortex, dorsal and ventral striatum, ventral tegmental area and the BNST (Ito, 2001; Robbe et al., 2002; Malenka and Bear, 2004; Bellone and Luscher, 2005; Grueter et al., 2006). An interesting aspect exhibited by group I mGluR-LTD is a degree of promiscuity of mechanism depending on the synapse where the LTD is expressed. This notion can now be extended to the less characterized α_1-AR-LTD. Unlike in the hippocampus and visual cortex (Kirkwood et al., 1999; Scheiderer et al., 2004), α_1-AR-LTD within the BNST is independent of the activation of NMDARs (in both the induction phase and the maintenance phase of the LTD) and concurrent presynaptic stimulation. Intriguingly, we found that α_1-AR-LTD expression in the BNST is dependent on L-type VGCC activity. L-type VGCC activity (particularly $Ca_{V1.3}$) is required in the dorsal striatum to induce corticostriatal group I mGluR-LTD (Wang et al., 2006). Furthermore, it has recently been demonstrated in the hippocampus that signaling via PLC can increase the conductance of $Ca_{V1.3}$ at negative potentials (Gao et al., 2006). Consistent with the role of the L-type VGCC in the induction of group 1 mGluR-LTD in the dorsal striatum, postsynaptic cells must be depolarized to -50 mV (Adermark and Lovinger, 2007). In contrast, however, we were able to induce α_1-AR-LTD holding the cell at -70 mV and in the absence of concurrent stimulation; although, subsequent stimulation, following the activation of α_1-AR, may activate L-type channels in the maintenance phase of the LTD. NE can modulate cell

excitability within the BNST, causing depolarization especially in non-projection cells (Dumont and Williams, 2004) and, therefore, it is possible that the application of methoxamine directly depolarized the cell sufficiently to activate L-type VGCCs. Additionally, the concentrations of our extracellular and intracellular recording solutions may have produced sufficiently depolarizing conditions. These seem unlikely given previous sharp microelectrode studies under identical conditions that indicated resting membrane potentials of -64-66 mV (Egli and Winder, 2003) coupled with a lack of change in the input resistance. Another possibility was that α_1-AR-LTD may be dependent on signaling via mGluR5 downstream of α_1-AR activation. We found, however, that blockade of mGluR5 during the induction of α_1-AR LTD does not impact the expression of this LTD. This suggests that in this region adrenergic afferents may influence plasticity independently of descending glutamatergic inputs to the BNST from areas like the limbic cortex, hippocampus and amygdala.

The postulated heterosynaptic mechanism of this α_1-AR LTD could be of behavioral significance. A hallmark of some affective disorders is the inherent inability to overcome the disorder by reasoning (i.e. feelings/urges are beyond the cognitive control of the patient) (American Psychiatric Association, 1994). α_1-ARs in the BNST modulate ACTH levels in animals who have been exposed to a stressor (Cecchi et al., 2002). Moreover it has recently been shown that the same axon collaterals from the nucleus tractus solitarius that project to the BNST also may project to the PVN (Banihashemi and Rinaman, 2006). NE may therefore modulate this circuitry regardless of input from cortical structures. It is

intriguing to think that this dissociation of induction of α_1-AR LTD from the glutamatergic input of cognitive centers innervating the BNST potentially contributes to alterations in behavior in a disease state such as generalized anxiety or addiction. Clearly, however, additional work would be needed to provide support for this notion.

α_1-AR-LTD Is Not Observed in Mice with Chronically Altered Adrenergic Signaling

Previously our group reported that a 10 minute application of 100 μM NE failed to alter glutamatergic transmission in α_{2A}-AR KOs, mice with altered anxiety phenotypes. It was concluded that the α_{2A}-AR may gate responses to NE within the BNST via its interactions with other receptors (Egli et al., 2005). Due to the shorter duration of agonist application, however, contributions by the α_1-AR in the α_{2A}-AR KOs may not have been observed. Therefore, we decided to examine α_1-AR-LTD in the α_{2A}-AR KOs. Surprisingly, α_1-AR-LTD could not be induced via a 20 minute application of methoxamine. This implied that perhaps the lack of LTD was due to functional desensitization of the α_1-AR or *in vivo* induction of α_1-AR-LTD as a result of increased extracellular concentration of NE. These ideas are supported by increased metabolite/transmitter ratios within several brain regions in the α_{2A}-AR KOs (Lahdesmaki et al., 2002), although these ratios can also be interpreted as increases in catabolism and therefore have caveats (Commissiong, 1985). To support our data with the α_{2A}-AR KOs we next used the NET KOs, another mouse model with altered behavioral phenotypes and

adrenergic transmission. Fast cyclic voltammetry experiments in the BNST in the NET KOs have demonstrated that NE clearance rates are over six times slower in the KOs as compared to wild-type controls (Xu et al., 2000). As was observed with the α_{2A}-AR KOs, the NET KOs also failed to express LTD after a 20 minute exposure to methoxamine. Interestingly, the α_{2A}-AR KOs express mGluR5-LTD after an application of DHPG, demonstrating that signaling via GPCRs, and more specifically those coupled to Gq, are still intact. Autoradiography data in the NET KOs shows a reduction in the cell surface expression of α_1-ARs in several brain regions (Bohn et al., 2000; Xu et al., 2000; Dziedzicka-Wasylewska et al., 2006) but an up-regulation of $\alpha_{2A/C}$-AR within the BNST (Gilsbach et al., 2006). One possibility of our results, taken together with this data, suggest that within these mouse models α_1-ARs and/or their signaling pathways are desensitized, preventing induction of α_1-AR-LTD. An intriguing observation was that α_1-AR-LTD can occlude mGluR5-LTD in the BNST. This suggests that the two LTDs share a common mechanism as they do in the visual cortex (Choi et al., 2005) There is evidence, however, in dopaminergic cells in the VTA that α_1-ARs desensitize group I mGluRs (Paladini et al., 2001). The L-type VGCC experiments provide indirect support for the desensitized receptor hypothesis as well. In these experiments there is a significant transient depression in response to the application of methoxamine that is not observed in either of the mouse models. One possibility for this transient depression would be $GABA_B$ receptor activation due to the activation of α_1-ARs on interneurons (Dumont and Williams, 2004). This transient depression is not observed in either KO animal suggesting that

there may be a tonic reduction in α_1-ARs. Additional experiments will need to be conducted to confirm these hypotheses.

Interestingly, both of these knockout mice have altered anxiety/depression phenotypes (Schramm et al., 2001; Lahdesmaki et al., 2002; Dziedzicka-Wasylewska et al., 2006; Keller et al., 2006) including increased anxiety-like behavior in the elevated plus maze (α_2-AR KOs), increased response to injection stress (α_2-AR KOs), decreased struggling/mobility in the forced swim and tail suspension tests (NET KOs) and bradycardia to stressful stimuli (NET KOs). In addition the NET KO animals have heightened sensitivity to psychostimulants, enhanced conditioned place preference to cocaine, and increased analgesia to opiates(Bohn et al., 2000; Xu et al., 2000; Hall et al., 2002). Furthermore it has been shown that the BNST sends monosynaptic projections to dopaminergic VTA neurons to modulate reward (Georges and Aston-Jones, 2002). During withdrawal from morphine there is an inhibition of the firing of these dopaminergic cells that can not only be reversed with the α_2-AR agonist clonidine, but potentiated by its administration (Georges and Aston-Jones, 2003). It is an interesting notion that the NET KO animals lack a mechanism (α_1-AR LTD) that may contribute to the inhibition of the dopaminergic cells in the withdrawal state while simultaneously demonstrating increased behavioral sensitization and reward mediated behaviors to drugs of abuse. A lack of functioning α_1-ARs and/or their signaling pathways may impact such behavior. Although α_1-AR activation can affect the excitability of cells in various ways, the

lack of a long term change in cell function, like synaptic plasticity induced by the α_1-ARs, in these animals could have implications for their exhibited behavior.

Clinical data have highlighted the α_1-AR as a therapeutic target for anxiety disorders. Raskind and colleagues reported that α_1-AR antagonists are efficacious for patients with combat/non-combat induced post-traumatic stress disorder (Raskind et al., 2000; Raskind et al., 2002; Taylor and Raskind, 2002; Peskind et al., 2003; Taylor et al., 2006). Additionally, α_1-AR antagonists are used to treat ailments like benign prostatic hypertrophy and hypertension and thus specific pharmacological agents are available for further investigation into their benefits in the treatment of affective disorders. Furthermore, the notion that alterations in adrenergic mediated synaptic plasticity within nuclei like the BNST, and others implicated in stress and anxiety, may contribute to the pathological learning (learned fear) that may mediate PTSD and similar disorders remains an interesting possibility.

Previous work has highlighted the role of adrenergic modulation in the BNST in behavioral paradigms of stress induced relapse to drug seeking and anxiety. Our findings showed that NE modulates excitatory synapses in the dlBNST by inducing an LTD that is dependent on the α_1-AR and L-type VGCC activation and independent of the NMDAR and stimulation from presynaptic inputs. A crucial element to α_1-AR -LTD is that the induction depends on the length of exposure to NE, preventing transient increases in NE to elicit plasticity. Furthermore, a lack of this plasticity in animal models of affective disorders may impact their behavioral phenotypes. Together, our results demonstrate a

mechanism by which NE may modulate BNST functional output under conditions of psychological stress.

CHAPTER III

α_1-ADRENERGIC RECEPTOR LONG TERM DEPRESSION IS MAINTAINED VIA DIFFERENT MECHANISMS FROM mGluR5 LTD IN THE BED NUCLEUS OF THE STRIA TERMINALIS AND IS ATTENUATED IN MODELS OF CHRONIC STRESS

Introduction

The BNST is a relay nucleus that receives inputs from cognitive and emotion processing areas, like the prefrontal and limbic cortices, the hippocampus, and the amygdala, is integrated, and outputs to reward and stress nuclei such as the paraventricular and lateral nuclei of the hypothalamus (the PVN and LH respectively) and the ventral tegmental area (VTA) (Cullinan et al., 1993; McDonald, 1998; Dong et al., 2001a; Dong et al., 2001b; Dong and Swanson, 2004). In addition, the BNST receives a strong noradrenergic projection arising mainly from the A1 and A2 (nucleus of the tractus solitarius – NTS) cell groups (Forray and Gysling, 2004). These anatomical connections strongly suggest that noradrenergic signaling in the BNST may play a role in both anxiety disorders and substance abuse. Supporting this hypothesis, lesioning the BNST alters anxiety responses to light enhanced startle (Walker and Davis, 1997), and disrupting noradrenergic signaling can alter reward and stress induced reinstatement to drug seeking (Erb et al., 2000; Shaham et al., 2000; Wang et al., 2001; Olson et al., 2006).

α_1-AR signaling within the BNST can potently regulate the function of the hypothalamic-pituitary-adrenal stress axis and mediates the anxiety response after a stressor (Cecchi et al., 2002).The α_1-AR antagonist prazosin has been shown to attenuate self-administration in ethanol dependent rats (Walker et al., 2008a) and reduces opiate self administration (Greenwell et al., 2009). Compelling data from clinical trials has demonstrated that prazosin can effectively be used to alleviate symptoms of post-traumatic stress disorder (PTSD) (Raskind et al., 2000; Raskind et al., 2002; Taylor and Raskind, 2002; Peskind et al., 2003; Taylor et al., 2006) and can reduce drinking behavior in alcoholics (Simpson et al., 2008). These data strongly suggests that the noradrenergic system in the BNST, and specifically the α_1-AR, may be excellent targets for the treatment of anxiety and substance abuse disorders.

Previously we described that NE can induce a time dependent long term depression (LTD) in the BNST (McElligott and Winder, 2008). Although α_1-AR LTDs have only been described in a handful of brain regions (Kirkwood et al., 1999; Scheiderer et al., 2004; Scheiderer et al., 2008), there are already stark contrasts between the signaling mechanisms that result in LTD. For example, in the hippocampus α_1-AR LTD is dependent on the activation of NMDARs (Scheiderer et al., 2004) while in the BNST it is independent of NMDARs but dependent on L-type VGCC (McElligott and Winder, 2008). Such differences are not surprising when taken in context with other forms of LTD that are resulting from signaling via $G_{\alpha q}$ coupled GPCRS, such as the group I metabatropic glutamate receptor (mGluR) LTD (for review see (Grueter et al., 2007). Because

of the dramatic differences between the various group I mGluR LTDs and because very little is known about the mechanism underlying α_1-AR LTD we decided to probe the maintenance mechanism underlying the expression of α_1-AR LTD in the BNST. Here we confirm our hypothesis that α_1-AR LTD is maintained by postsynaptic mechanisms. We further demonstrate that the LTD results in the loss of calcium permeable AMPA receptors (CP-AMPAR) from the synapse via clathrin dependent endocytosis. Surprisingly, this mechanism differs from mGluR5 LTD in the BNST, although α_1-AR LTD can occlude the induction of mGluR5 LTD (McElligott and Winder, 2008). Additionally we demonstrate that the LTD is attenuated in mice undergoing withdrawal from chronic ethanol exposure and cannot be expressed in mice that have experienced chronic restraint stress; however, this may be due to a desensitization process rather than induction of the LTD *in vivo*.

Methods

Animal Care

All mice used in experiments were male C57BL/6j mice 5-10 weeks old (The Jackson Laboratories, Bar Harbor, ME). All animals were provided with food and water *ad libitum*, with the exception of the 2 hours of stress (see below) experiments, and housed in groups within the Vanderbilt Animal Care Facilities. Approved guidelines from the Vanderbilt Animal Care and Use committee were used for all experiments.

Brain Slice Preparation

Upon retrieval from the Vanderbilt Animal Facility, mice were allowed to rest for a minimum of one hour in sound and light attenuating chambers. Mice were then anesthetized (isoflurane) and moved to a separate room for decapitation and slicing. Brains were hemisected and slices (300 μM) were made using a Leica VT1000S vibratome (Leica Microsystems, Bannockburn, IL) in an oxygenated (95% O_2, 5% CO_2) high sucrose, low Na^+ artificial cerebral spinal fluid maintained at 1-4° C. (sucrose ACSF in mM: 194 sucrose, 20 NaCl, 4.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.2 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$).

Whole Cell Recordings

Slices were allowed to rest for 30 minutes following slicing in heated (28° C), oxygenated (95% O_2, 5% CO_2) ACSF or low Ca^{2+} ACSF (only for the low Ca^{2+} ACSF experiments). (ACSF in mM: 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$; Low Ca^{2+} ACSF: 124 NaCl, 2.5 KCl, 1 $CaCl_2$, 2.8 $MgCl_2$, 1 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$). Following this incubation period, slices were moved to the recording chamber where they were continuously perfused (2 ml/min) with the appropriate heated and oxygenated ACSF. 25 μM picrotoxin was then added to the ACSF in all experiments to isolate glutamatergic currents and allowed to equilibrate for an additional 30 minutes. Utilizing a Flaming/Brown microelectrode puller (Sutter Instruments) patch

electrodes (3-6 MΩ) were pulled and filled with a Cs-gluconate intracellular solution (Intracellular in mM: 135 Cs-gluconate, 5 NaCl, 2 $MgCl_2$, 10 HEPES, 0.6 EGTA, 4 Na_2ATP, 0.4 Na_2GTP). In some experiments intracellular peptides were used at 2 mM. These peptides were allowed to infuse into the cell for at least 30 minutes prior to recording. In evoked stimulation experiments, cells were clamped at – 70 mV throughout the recording period and excitatory postsynaptic currents (EPSCs) were recorded. In miniature and spontaneous excitatory postsynaptic currents (mEPSCs and sEPSCs) release experiments cells were clamped at – 90 mV throughout to increase the driving force of ions through the glutamate receptors. mEPSC recordings were made in the presence of 1 μM tetrodotoxin (TTX) to block sodium channel activity. All data was acquired using Clampex 9.2 (Molecular Devices). To insure quality of recordings, series resistance was continuously monitored and data was excluded with a change greater than 20%. In evoked experiments, a bipolar Ni-chrome stimulating electrode was placed dorsal (dBNST recordings) or medial (vBNST recordings) to the recording area. EPSCs were evoked at a frequency of 0.167 Hz and 50-400 pA EPSCs were recorded. All drugs were bath applied at final concentrations.

Analysis of Whole Cell Recordings

All evoked experiments were analyzed with ClampFit 9.2 (Molecular Devices.) Prior to all drug bath applications, a 10 min or 5 min (GluR peptide experiments) baseline was recorded with and all points were normalized to the two minutes

preceding the drug application. 30 second averages were plotted on the time courses in the figures. LTD measurements were taken the last two minutes of each recording for evoked EPSCs. mEPSC and sEPSC recordings were analyzed with Mini Analysis (Synaptisoft.) Detection parameters were set for amplitudes > 5 pA and rise times < 3 ms. All events were checked and verified by eye. Baseline frequency and amplitude are composed of two, 2 minute blocks and LTD frequency and amplitude time points are composed of 2, two minute blocks 36-40 minutes after removal of drug.

Ethanol and Stress Procedures

Ethanol chamber experiments were performed in accordance with the INIA-Stress standard operating procedure (Healey et al., 2008). 95% ethanol is made volatile by passing air through an air stone and then pumped into the Plexiglas chambers at a rate of 5 liters (vapor)/min which maintains the air in the chamber at 19-22 mg/l air. At the onset of cycles 1.6 g/kg ethanol will be administered to the CIE mice and an equal volume of saline to controls, both groups received 1mmol/kg pyrazole. In the CCE mice, the 1mmol/kg pyrazole was received every 24 hours. These conditions allow for stable blood ethanol concentrations (BECs, taken from sentinel mice run in the same chamber decapitated post cervical dislocation) in the range of 150-185 mg/dl in our lab (Healey et al., 2008). Intermittent air samples were taken from chambers to assess vapor levels and insure the function of the apparatus. Sham mice received the same treatments but were exposed to air vapor chambers as opposed to ethanol.

Mice were stressed for 2 hours for 10 consecutive days and recorded from on the 11th day. Restraint devices were 50 mL conical tubes with several (approximately 15 holes) in the front and rear (cap) to maintain air flow. While in restraint devices animals were placed inside separate sound and light attenuating boxes, and they were returned to their home cage immediately following restraint.

Statistics

All points are reported as the mean ± SEM. In most cases significance is determined by either a paired or unpaired Student's T-test, with the exception being the bar graph in figure 4 and 8 where significance was determined via a one way ANOVA across 3 groups.

Reagents

(RS)-3,5-Dihydroxyphenylglycine (DHPG, Ascent Scientific, Bristol, UK), Dynamin inhibitory peptide (Tocris, Ellisville, MO), 95% ethanol, Methoxamine-HCl (Sigma, St. Louis, MO), 1-Naphthylacetyl spermine trihydrochloride (Naspm, Sigma, St. Louis, MO), 5-Chloro-N-(cyclopropylmethyl)-2-methyl-N-propyl-N'-(2, 4,6-trichlorophenyl)-4,6-pyrimidinediamine hydrochloride (NBI 27914, Tocris, Ellisville, MO), Pep1-TGL (Tocris, Ellisville, MO), Pep2-SKVI (Tocris, Ellisville, MO), Picrotoxin (Ascent Scientific, Bristol, UK), pyrazole (Sigma, St. Louis, MO), U0126 (Tocris, Ellisville, MO) Dimethylsulfoxide (DMSO) was the solvent used

for stock solutions of picrotoxin, and maximum final concentration of DMSO was 0.02% by volume.

Results

α_1-AR LTD is Maintained Via a Postsynaptic Mechanism

NE can induce a LTD in the BNST that is dependent on signaling via the α_1-AR. The α_1-AR agonist methoxamine (100 μM) can mimic the effects of NE and does not confer an alteration in the paired pulse ratio (PPR) after the induction of LTD suggesting a postsynaptic mechanism (Fig 8A,B) (McElligott and Winder, 2008). To further explore this possibility we performed the LTD experiment in ACSF with reduced calcium concentrations which can reveal presynaptic mechanisms. Following the equation where the instantaneous flux (F) of Ca^{2+} in the presynaptic terminal equals the probability of opening (P_O) multiplied by the number (N) of presynaptic voltage gated calcium channels (VGCCs) and the current through a single channel (i)

$$F = P_O * N * i;$$

if the actions of α_1-AR stimulation resulted in either a reduction in P_O or N then lowering the concentration of calcium [Ca^{2+}], which would reduce *i,* would in turn increase the effect of α_1-AR, and, thus result in a more robust LTD (Wheeler et al., 1996; Watabe et al., 2002). Previously we have shown that alternating the ACSF divalent cation concentrations to 1 mM Ca^{2+} and 2.8 Mg^{2+}(thus reducing the [Ca^{2+}] from 2.5 mM) significantly increases basal PPR values in the dlBNST

(Grueter et al., 2008). This alteration, however, does not enhance the α_1-AR LTD in the BNST (LTD in normal Ca^{2+}: N = 6, 55.7 $\pm$ 3.6 % of baseline, LTD in low Ca^{2+}: N = 5, 70.5 $\pm$ 3.7% of baseline, Fig 1A) nor does it have any effect on the PPR following induction of the α_1-AR LTD (Fig. 8C).

To further assess the maintenance mechanisms for α_1-AR LTD, we next analyzed the effect of 100 μM methoxamine on miniature excitatory postsynaptic currents (mEPSCs) in the presence of 1 μM tetrodotoxin (TTX). Classically a reduction in mEPSC frequency is interpreted as being indicative of a decrease in glutamate release, while a decrease in amplitude is considered an indication of a decrease in the sensitivity of the postsynaptic receptors. We predicted that the observed LTD of EPSCs produced by methoxamine would be paralleled by a decrease in either the amplitude or the frequency of mEPSCs. Surprisingly, the application of methoxamine failed to cause an alteration in either the frequency (N = 4, baseline: 0.70 $\pm$ 0.3 Hz; LTD: 0.68 $\pm$ 0.3 Hz) or the amplitude (N=4, baseline 16.3 $\pm$ .8 pA; LTD: 15.8 $\pm$ 1.0 pA) of the mEPSCs at the LTD time point (Fig. 9A, B).

Our lab has recently identified an activity dependent process where by dopamine can regulate glutamatergic transmission (Kash et al., 2008b). We thus tested whether the lack of effect of methoxamine on mEPSCs was due to the blockade of sodium channels with TTX. To do this, we sampled spontaneous EPSCs (sEPSCs) in the absence of TTX before and after methoxamine application (Fig. 10). Surprisingly we found that α_1-AR activation robustly increased, rather than decreased, the frequency of sEPSCs in the BNST 40

minutes following methoxamine application (N=10, baseline: 1.48 ± 0.4 Hz; LTD: 4.30 ± 1.3 Hz; $p<0.03$; Fig. 10B). α_1-AR signaling has been shown to increase spontaneous inhibitory postsynaptic currents (IPSCs) in the BNST (Dumont and Williams, 2004). Furthermore, these GABA IPSCs can originate either from intrinsic neurons or from extrinsic afferents from the central nucleus of the amygdala (CeA), both of which can contain corticotrophin releasing factor (CRF) (Sakanaka et al., 1986; Day et al., 2002). We recently showed that the increase of frequency of sEPSCs induced by dopamine requires the CRF 1 receptor (CRF-R1) (Kash et al., 2008b). Activity dependent release of CRF by methoxamine therefore may mask depression of glutamatergic transmission by α_1-AR LTD mechanisms. We therefore determined whether the increase in sEPSCs following methoxamine was due to signaling through the CRF-R1. The CRF-R1 antagonist NBI 27914 (1 μM) blocked the methoxamine induced increase in sEPSC frequency (N = 5; baseline: 3.23 ± 1.2; LTD: 2.48 ± 1.0 Hz; $p>0.19$; Fig. 10D) and revealed a significant reduction in sEPSC amplitude (N=5; baseline: 21.1 ± 1.6 pA; LTD: 17.8 ± 1.2 pA; $p<0.01$; Fig. 10C) indicative of a postsynaptic mechanism for LTD. Furthermore, NBI 27914 (1 μM) did not prevent the expression of α_1-AR LTD (N = 5; 64.9 ± 8.0 % of baseline; Fig. 10E) suggesting the increase in sEPSC frequency (Fig. 10A, B) is mechanistically independent of the LTD.

Figure 8. LTD Is Expressed Normally in Low Calcium ACSF
A. LTD is expressed in both low calcium (black squares) and normal ACSF (open circles) however the LTD is less robust in low calcium ($p<0.02$). B. Representative traces of normal and low calcium experiments. Baseline = black line, LTD timepoint = gray line. Error bars are 50 pA by 20 ms. C. and D.100 μM methoxamine does not alter PPR in either normal or low calcium ACSF.

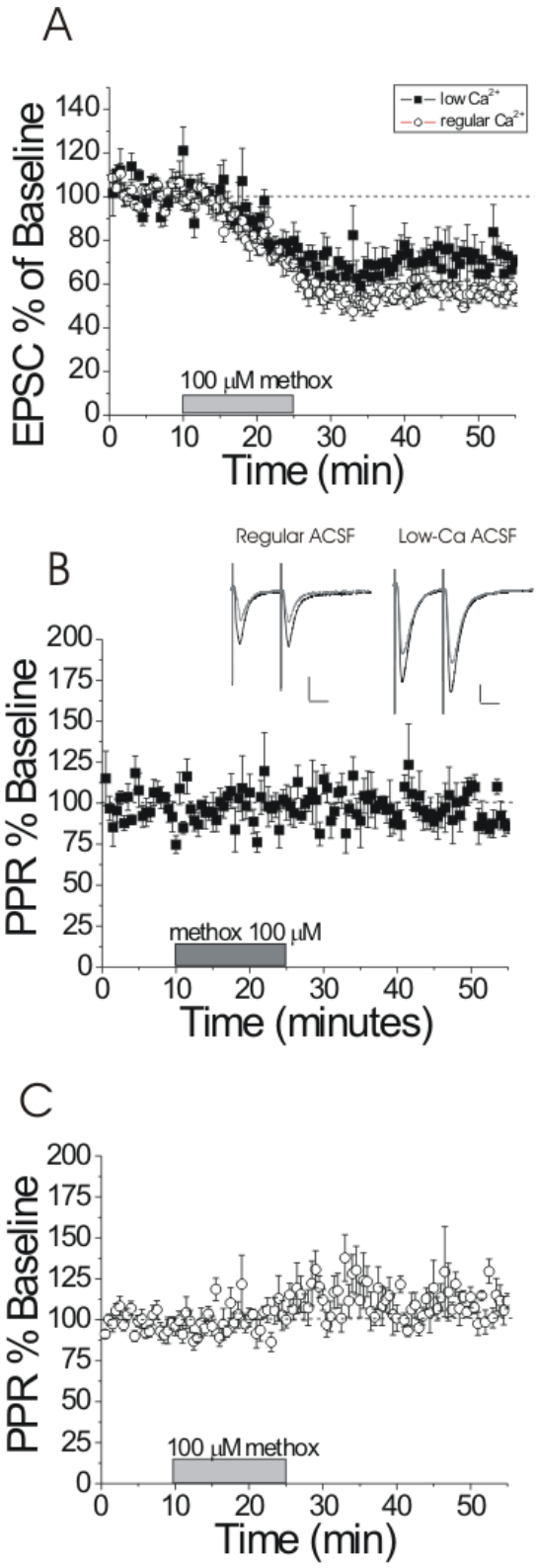

A
low Ca2+
regular Ca2+
EPSC % of Baseline
100 µM methox
Time (min)
B
Regular ACSF
Low-Ca ACSF
PPR % Baseline
methox 100 µM
Time (minutes)
C
PPR % Baseline
100 µM methox
Time (min)

Fig. 8

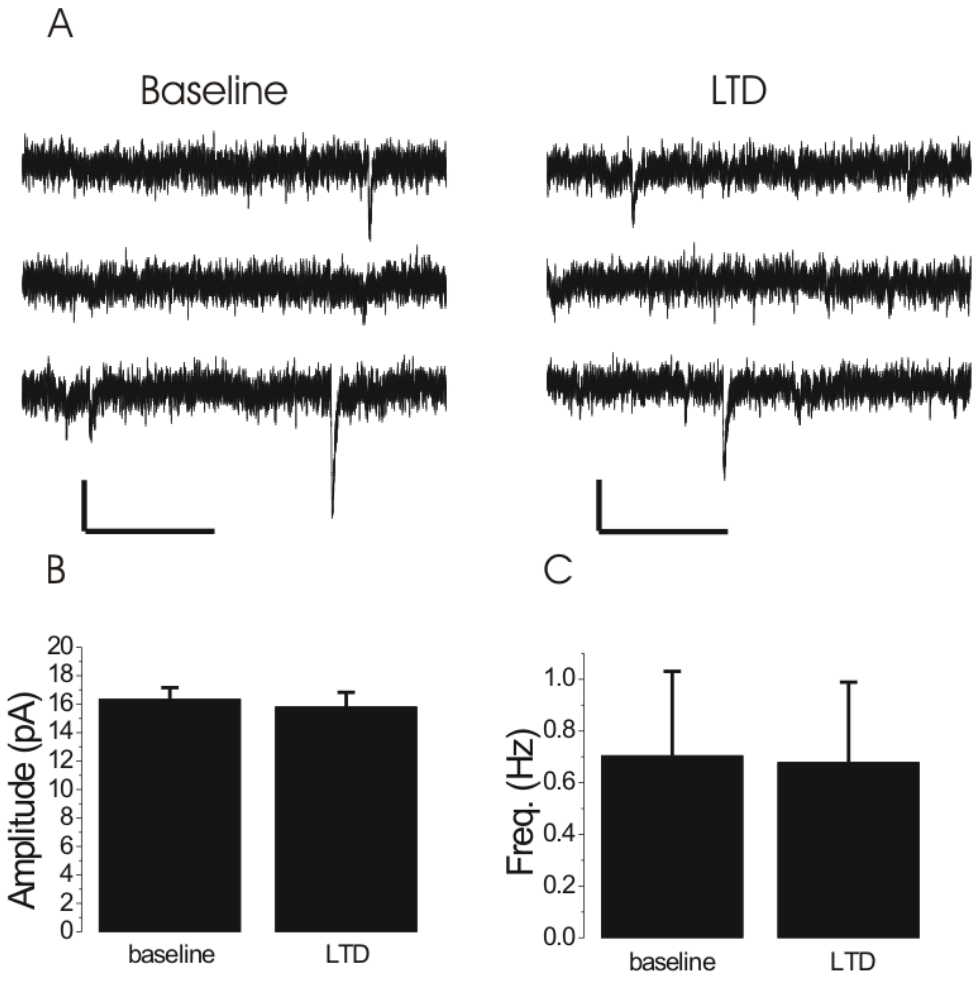


Figure 9 Methoxamine Does Not Reduce mEPSC Amplitude or Frequency
A. Representative traces of mEPSC currents, scale: 20 pA by 200 ms. B. Amplitudes at the baseline and LTD time point are not significantly different (N = 4). C. Frequencies at the baseline and LTD time point are not significantly different (N = 4)

Figure 10 Methoxamine Effects on sEPSC
A and B. 100 μM methoxamine increases the frequency of sEPSCs at the LTD time point but not the amplitude of the events. (N = 10, $p<0.03$) C and D. 100 μM methoxamine in 1 μM NBI 27914 prevented the increase in frequency, and revealed a decrease in the amplitude of sEPSCs (N = 5, $p<0.01$). E. 1 μM NBI 27914 did not prevent 100 μM methoxamine's induction of LTD in evoked EPSCs

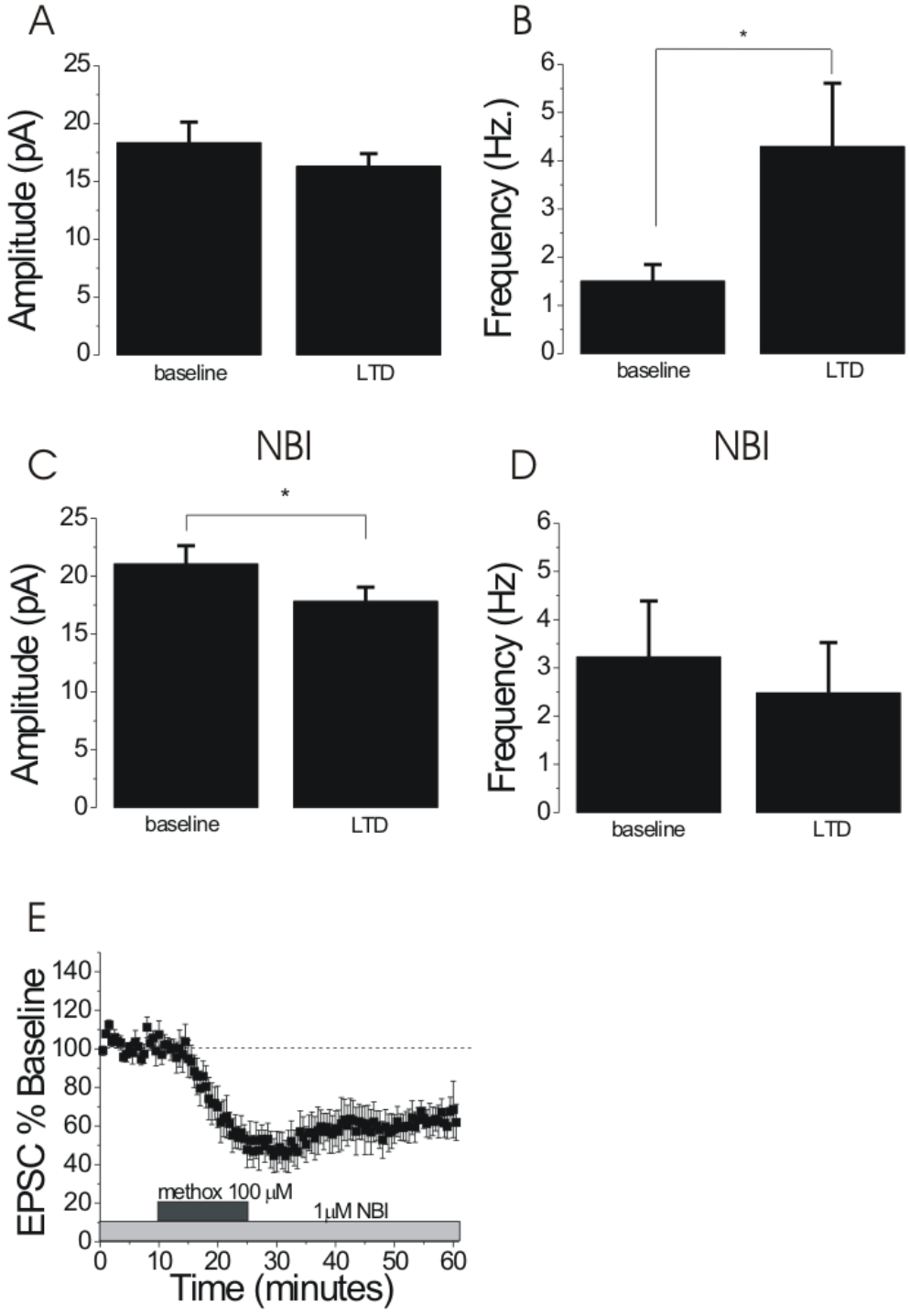

A
Amplitude (pA)
25
20
15
10
5
0
baseline
LTD
B
Frequency (Hz.)
*
6
5
4
3
2
1
0
baseline
LTD
C
NBI
*
Amplitude (pA)
25
20
15
10
5
0
baseline
LTD
D
NBI
Frequency (Hz)
6
5
4
3
2
1
0
baseline
LTD
E
EPSC % Baseline
140
120
100
80
60
40
20
0
methox 100 µM
1µM NBI
0
10
20
30
40
50
60
Time (minutes)

Fig. 10

α_1-AR LTD Results in the Loss of Functional Calcium Permeable AMPARs

The lack of PPR change, lack of a more robust LTD in low [Ca^{2+}] and a reduction in sEPSC amplitude all support the possibility that a postsynaptic maintenance mechanism underlies α_1-AR LTD. Two plausible postsynaptic mechanisms include alteration in function of AMPARs and the removal of these receptors from the synapse. To begin to address the latter, we infused a dynamin inhibitory peptide (2mM) into the postsynaptic cell via the patch pipette 30 minutes prior to recording to prevent clathrin-dependent endocytosis (Grueter et al., 2008). In the presence of the dynamin inhibitory peptide, 100 μM methoxamine caused a transient depression that returned to baseline during the LTD time point suggesting a requirement for clathrin dependent endocytosis (N = 5; 95.7 $\pm$ 10.1 % of baseline; $p>0.63$; Fig 11A, B.). The LTD time point was also significantly different from experiments lacking the dynamin inhibitory peptide ($p<0.03$, Fig 11A, B; experiments reprinted from Fig 8).

In addition to α_1-AR LTD, BNST glutamate synapses also express another G_q-GPCR LTD, induced by mGluR5 (Grueter et al., 2006). We previously reported the engagement of α_1-AR LTD at synapses on BNST neurons blocked subsequent mGluR5 LTD (McElligott and Winder, 2008), which is typically consistent with their presence on a common population of synapses. The dynamin inhibitory peptide also disrupts the expression of mGluR5 LTD (Grueter et al., 2008). This result and our previous occlusion data suggest that there are similarities between the mechanisms required for expression of both α_1-AR and mGluR5 LTD in the BNST. To further explore this hypothesis, we examined the

involvement of the mitogen-activated protein kinase kinase 1/2 (MEK1/2) in the induction of α_1-AR LTD. Unlike mGluR5 LTD (Grueter et al., 2006), co-application of U0126 (20 μM) did not alter the expression of α_1-AR LTD (N = 5, 49.0 $\pm$ 5.9 % of baseline, Fig 11C)

The dynamin inhibitory protein data suggested that certain populations of AMPAR may be internalized following α_1-AR LTD. G_q coupled LTDs in other brain regions, like the VTA, have been shown to target various populations of AMPA receptors, particularly calcium permeable AMPA receptors (CP AMPARs) that lack a RNA edited GluR2 subunit (Bellone and Luscher, 2005). We took a pharmacological approach to investigate if CP AMPARs are in the synapses of BNST neurons. Utilizing the selective CP AMPAR antagonist Naspm (a synthetic analog of Joro Spider Toxin) (Koike et al., 1997), we found that 100 μM Naspm application reduced EPSCs in the BNST (N = 6, 70.2 $\pm$ 4.9 % of baseline; $p<0.002$; Fig.12A,D). NMDARs also contain an external polyamine site which could be potentially sensitive to Naspm application (Mueller et al., 1991). Additionally, NMDARs have been localized to presynaptic afferents in the BNST, as well as postsynaptic neurons (Gracy and Pickel, 1995), suggesting they may play a role in the modulation of presynaptic cells. To account for an effect of Naspm on glutamate release we examined the PPR before and following Naspm application and found no change, suggesting that our observed decrease was due to blockade of postsynaptic receptors. Following induction of α_1-AR LTD, Naspm (100 μM) failed to further reduce EPSC amplitude suggesting that α_1-AR LTD confers a loss of Naspm sensitivity at CP AMPARs (N = 5; $p>0.83$; Fig.

12B,D). When 100 μM Naspm was applied following the induction of mGluR5 LTD (via 100 μM DHPG) however, there was a similar reduction in EPSC size as compared to basal condition (N = 8; $p>0.001$ to pre-Naspm baseline: $p>0.05$/N.S. one way ANOVA vs. Naspm alone ; Fig. 12C,D). Furthermore the reduction was statistically different from time matched control experiments (N = 7; $p<0.03$, Fig. 12C). This data suggests that α_1-AR LTD is maintained, at least in part, by a loss of functioning CP AMPARs but that mGluR5 LTD is maintained by different mechanisms.

Figure 11 α_1-AR LTD Requires Clathrin Dependent Endocytosis
A. 100 μM methoxamine produces a robust depression in control cells (N = 6, closed squares) but not in cells infused with 2 μM (in patch solution) of the dynamin inhibitory protein (open circles, N = 5, $p>0.63$). B. Histogram comparing the LTD time point of the two conditions in A. Black bar is control and white bar is the dynamin inhibitory peptide ($p<0.03$). C. 20 μM U0126 did not prevent the induction of LTD by 100 μM methoxamine (N = 5, $p>0.0001$).

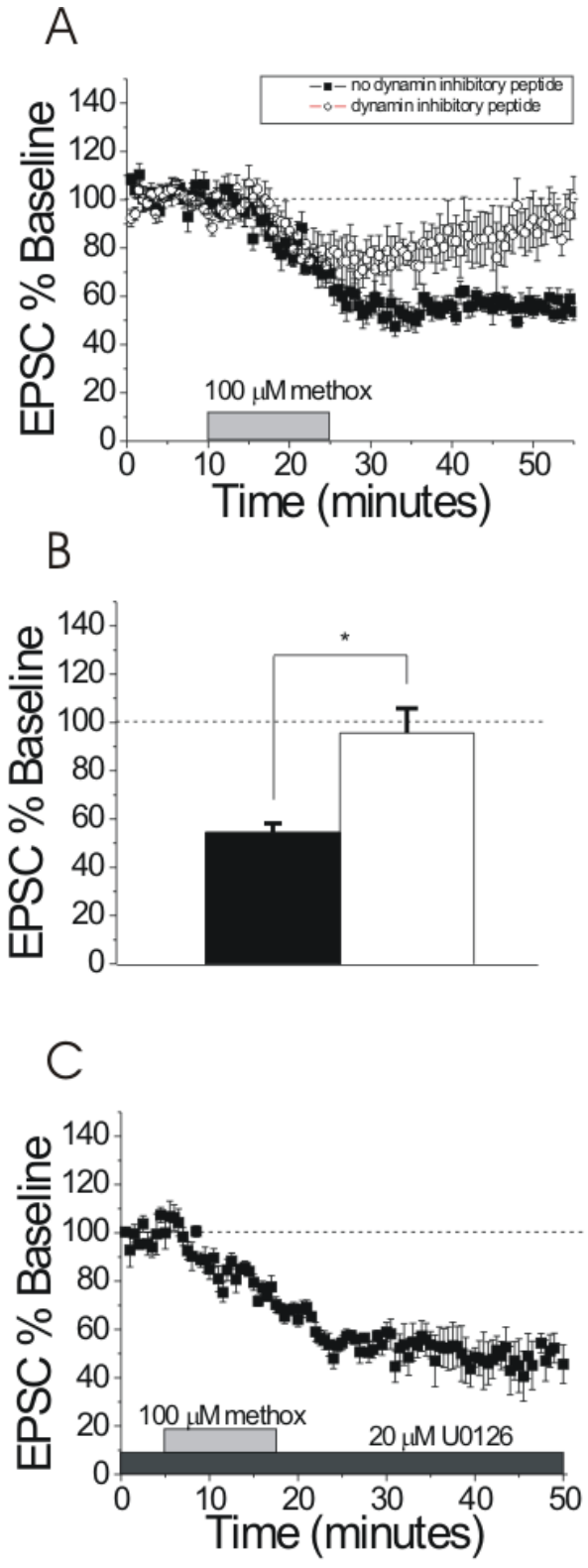

A
no dynamin inhibitory peptide
dynamin inhibitory peptide
EPSC % Baseline
140
120
100
80
60
40
20
0
100 μM methox
0
10
20
30
40
50
Time (minutes)
B
EPSC % Baseline
*
C
EPSC % Baseline
100 μM methox
20 μM U0126
Time (minutes)

Fig. 11

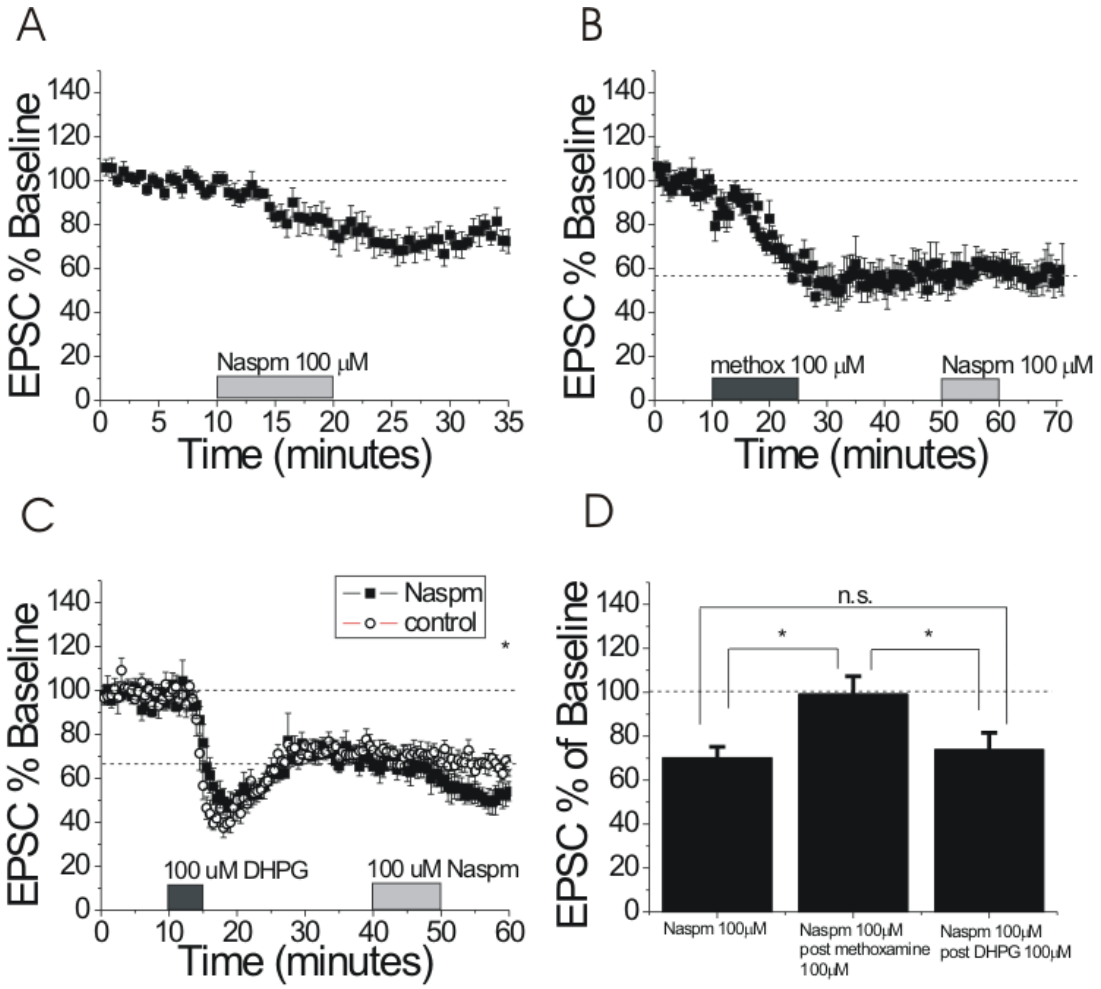


Figure 12 α_1-AR LTD Confers a Loss of Sensitivity to 100 μM Naspm in the BNST

A. 10 minutes of 100 μM Naspm produced a depression of EPSCs in naïve slices (N = 6, $p<0.002$). B. Following induction of LTD by 100 μM methoxamine, 100 μM Naspm failed to further reduce EPSCs (N=5, $p>0.83$). C. Following induction of mGluR 5 LTD by 100 μM DHPG, 100 μM Naspm significantly reduced EPSCs (N = 8, $p<0.001$) and this depression was significantly different from time matched control experiments without Naspm (N = 7, $p<0.03$). D. By one-way ANOVA, the depression produced by 100 μM Naspm in naïve slices and slices expressing mGluR 5 LTD did not have significantly different sensitivity to Naspm, yet they were significantly different from the lack of depression to 100 μM Naspm following α_1-AR LTD. ($p<0.05$ for ANOVA)

α_1-AR LTD Is Partially Maintained by the Loss of Proper GluR1 Subunit Trafficking

CP AMPARs lack an edited GluR2 (Q → R, second transmembrane loop) subunit in the AMPAR tetramer. It is hypothesized, however, that nearly all GluR2 receptors are edited in the mammalian brain (Cull-Candy et al., 2006) and, thus, most CP AMPARs are thought to be comprised of GluR1 homomers, GluR3 homomers and GluR1/3 heteromers. We therefore further probed the potential involvement of AMPAR function/trafficking in α_1-AR LTD. By infusing a peptide (via the patch pipette) corresponding to the C-terminus of either the GluR1 protein (pep1-TGL, Tocris) or the GluR2 protein (pep2-SKVI, Tocris) into the cell for 30 minutes prior to recording we could disrupt the trafficking of these subunits. After this initial incubation time, 100 μM methoxamine was applied to the cell for 15 minutes. The cells that were infused with the GluR1 peptide demonstrated significantly attenuated LTD (N = 6; 82.5 $\pm$ 6.4 % of baseline; Fig 13A) as compared to the cells that were infused with the GluR2 peptide (N = 5; 59.8 $\pm$ 7.6% of baseline; Fig 13A; GluR1 peptide vs. GluR2 peptide $p<0.05$). Furthermore, when the same peptides were used to probe mGluR5 LTD in the BNST, neither peptide was able to attenuate the LTD (GluR1 peptide: N = 7, 63.7 $\pm$ 8.0 % of baseline; GluR 2 peptide: N =4, 68.7 $\pm$ 7.2 % of baseline; Fig 13B).

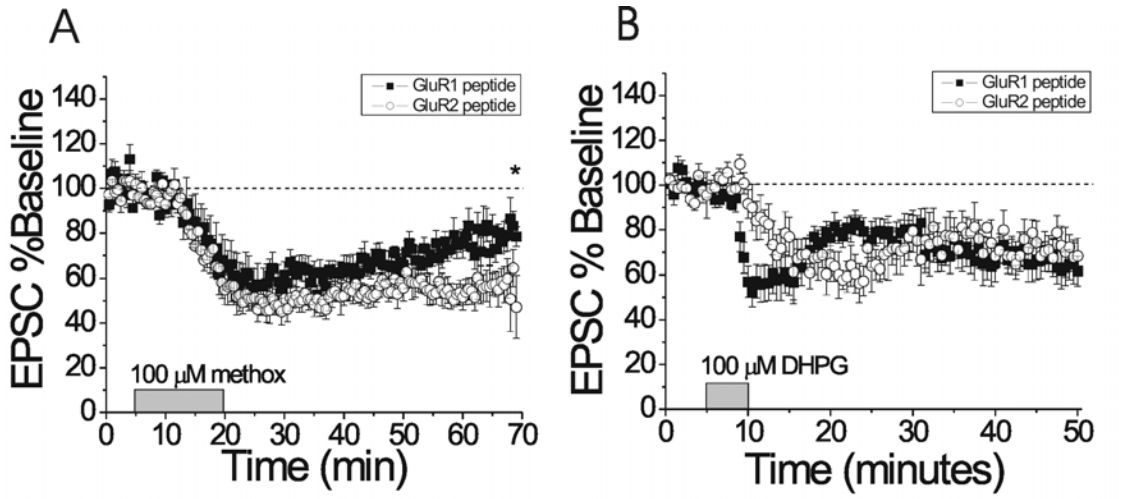


Figure 13 GluR1 C-terminal Peptide Attenuates α_1-AR LTD but not mGluR5 LTD

A. Infusion of a peptide from the c-terminus of GluR1 (N = 6) but not GluR2 (N = 5) attenuates the LTD induced by application of 100 μM methoxamine ($p<0.05$, GluR1 vs. GluR2). B. Neither the GluR1 (N = 7) nor GluR2 peptide (N = 4) attenuated the LTD induced by 100 μM DHPG.

α_1-AR LTD Is Disrupted by Withdrawal from Chronic Ethanol Exposure and Chronic Restraint Stress

Data from human studies suggests that NE is increased in the CNS of alcoholics (Borg et al., 1981; Hawley et al., 1985) where it may play a role in the pathogenesis of alcoholism (Breese et al., 2005). Additionally the adrenergic system remains an attractive target for intervention in alcoholism (Nutt et al., 1988; Simpson et al., 2008). Recently α_1-AR signaling has been linked to drinking behavior in withdrawn dependent animals (Walker, 2006). We therefore wanted to examine the persistence of α_1-AR LTD in ethanol exposed mice. Mice that received Chronic Continuous Ethanol (CCE) were exposed to 64 hours of continuous vapor, while mice that received Chronic Intermittent Ethanol (CIE) were exposed to 4 days of 16 hours of ethanol exposure with 8 hour withdrawal periods interspersed. Animals were sacrificed 4-6 hours in the final withdrawal under each condition. Both the CCE and CIE conditions resulted in significantly attenuated α_1-AR LTD from sham mice, however, the LTD was not fully occluded by these treatments (Sham mice: N = 6, 58.4 $\pm$ 6.7 % of baseline; CCE mice: N = 7, 81.4 $\pm$ 7.6 % of baseline; CIE mice: N = 5, 77.8 $\pm$ 6.8 % of baseline; Fig 14). Surprisingly, there was not a significant difference between CCE and CIE conditions (Fig. 14D).

We hypothesized that the ethanol treatments were not strenuous enough to fully engage mechanisms for occlusion of the LTD and thus we sought a more robust stressor. Restraint stress is known to increase NE in the BNST which in turn signals through BNST α_1-ARs to increase anxiety like behaviors and ACTH

in the plasma (Cecchi et al., 2002). We sought to determine if similar restraint stress would induce α_1-AR LTD *in vivo*. Mice were restrained in 50 mL conical tubes punctured with air holes for 2 hours over a 10 day period and decapitated and recorded from 24 hours later. Recording from the ventralateral BNST (vlBNST), that receives the most robust NE projection (Egli et al., 2005), we found that the 15 minute 100 μM methoxamine application produced a large transient depression but failed to induce LTD in the restrained animals (N = 6; 96.9 $\pm$ 8.8 % of baseline; $p>0.78$; Figure 15B). Naïve control animals demonstrated pronounced LTD in the vlBNST (N = 4; 62.5 $\pm$ 9.9 % of baseline) as has been reported previously (McElligott and Winder, 2008). The occlusion of α_1-AR LTD is a very intriguing result, but insufficient to demonstrate an *in vivo* induction of LTD. To address this issue, we used the presence or absence of CP-AMPARs as a molecular marker to assess whether α_1-AR LTD was induced in the stressed mice. In both naïve mice and mice that had experienced restraint stress, 100 μM of Naspm produced a depression of glutamatergic transmission in the vlBNST, although there was a trend for attenuation of the response in stressed mice. (Naïve mice: N =6, 75.6 $\pm$ 2.8 % of baseline $p<0.01$; Stressed mice: N = 6, 85.0 $\pm$ 4.6 % of baseline, $p<0.03$; Naïve vs. Stressed $p>.11$) This suggests that in the restrained animals LTD had not been induced *in vivo* and the lack of LTD may have been due to desensitization of a signaling mechanism.

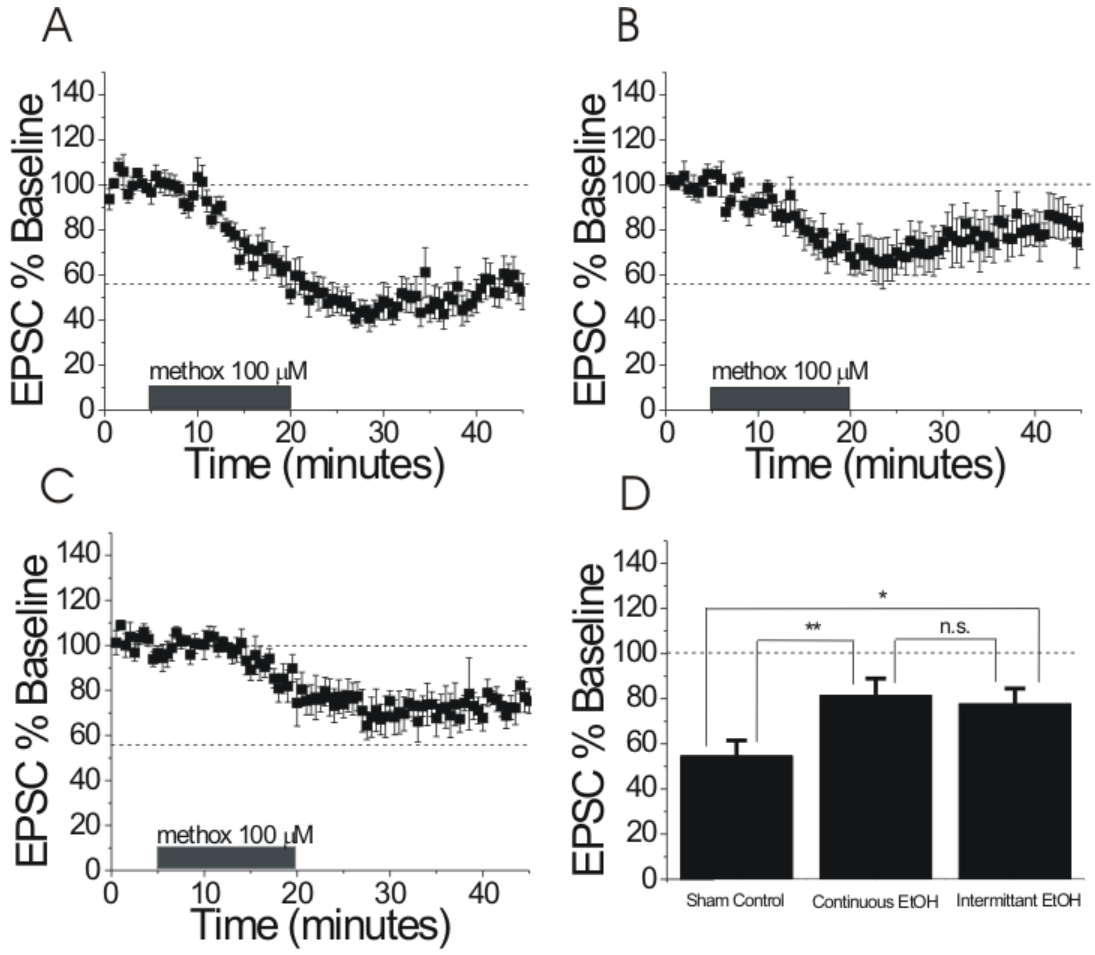
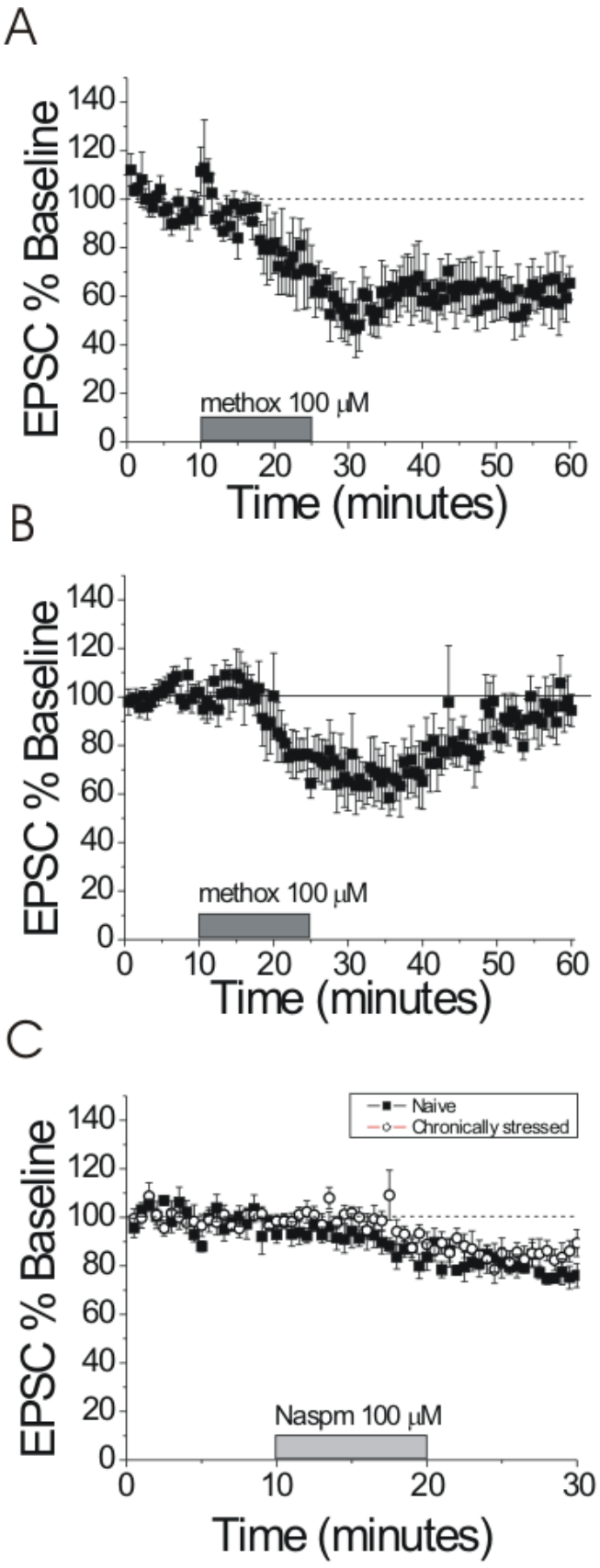


Figure 14 Chronic Exposure to Ethanol Attenuates α_1-AR LTD in the BNST A. Sham mice demonstrated robust LTD similar to naïve mice. B. LTD was attenuated in the BNST in mice that had experienced one withdrawal following Chronic Continuous Exposure (CCE). C. 100 μM methoxamine resulted in attenuated LTD following a Chronic Intermittent Exposure (CIE) paradigm. D. Both CCE and CIE conditions are significantly attenuated from control conditions by one way ANOVA ($p<0.001$ for ANOVA, CIE vs. Sham $p<0.05$, CCE vs. Sham $p<0.05$).

Figure 15 Chronic Restraint Stress Occludes LTD in the vlBNST but Does Not Confer a Loss of Sensitivity to Naspm.
A. Naïve mice have robust LTD when stimulated with 100 μM methoxamine ($N = 4$, $p<0.03$), while B. in cells mice that have experienced 10 days of 2 hour chronic restraint stress 100 μM methoxamine does not induce LTD ($N = 6$, $p>0.78$).


A
EPSC % Baseline
140
120
100
80
60
40
20
0
methox 100 μM
0
10
20
30
40
50
60
Time (minutes)
B
EPSC % Baseline
140
120
100
80
60
40
20
0
methox 100 μM
0
10
20
30
40
50
60
Time (minutes)
C
Naive
Chronically stressed
EPSC % Baseline
140
120
100
80
60
40
20
0
Naspm 100 μM
0
10
20
30
Time (minutes)

Discussion

Previously we have characterized the means by which α_1-AR LTD is induced within the BNST (McElligott and Winder, 2008). In this manuscript we have extended our findings to examine the mechanism by which α_1-AR LTD is maintained and examined its expression and maintenance *in vitro* when challenged by a stressor *in vivo*. We found that α_1-AR LTD is maintained postsynaptically and may involve the removal of CP AMPARs from the postsynaptic membrane, unlike mGluR5 LTD in the same cells. Furthermore, through the course of this study we found that α_1-AR stimulation enhanced sEPSCs in a CRF1R dependent fashion; however, this manipulates excitatory transmissions through a mechanism independent of α_1-AR LTD. Finally we found that the α_1-AR LTD is attenuated or occluded by stressful challenges, however, it appears that the loss of LTD expression is not due to maintained expression *in vivo*, but perhaps may be due to desensitization in the signaling mechanism required for induction of LTD.

α_1-AR LTD Is Maintained by a Different Postsynaptic Mechanism than mGluR5 LTD in the BNST

The connection between plasticity and behavior/learning and memory, while appealing, lacks rigorous evidence to demonstrating exactly how plasticity may affect behavior and/or if plasticity is a result of behavior. One way we may gain insight into this problem is by investigating the mechanism of how plasticity is expressed and maintained, so that it may be further used to investigate behavior.

Previously, we found that α_1-AR LTD induction occluded the further induction of mGluR5 LTD in the BNST (McElligott and Winder, 2008) leading us to hypothesize that both LTDs activated similar signaling cascades and mechanistic pathways (Choi et al., 2005). Recently we have published evidence that mGluR5 LTD in the BNST is maintained via postsynaptic mechanisms involving endocytosis mechanisms and rearrangement of the actin cytoskeleton (Grueter et al., 2008). We now show that α_1-AR LTD also requires clathrin dependent endocytosis. Unlike mGluR5 LTD, a lack of MEK1/2 involvement (Figure 4C) suggests that a different signaling pathway, and potentially a different mechanism, underlie the expression and maintenance of both LTDs. Here we present data suggesting that in contrast to mGluR5 LTD, it appears that the AMPAR targeted for endocytosis in α_1-AR LTD are CP AMPARs. Additionally, the mEPSC/sEPSC profile is different between mGluR5 LTD, where a decrease in the frequency of events is observed with mEPSCs (Grueter et al., 2008), and α_1-AR LTD, where a difference in the amplitude of events is observed with sEPSCs, suggesting that α_1-AR LTD is dependent on the activation of voltage gated sodium channels. These results open up the possibility that the respective LTDs may be occurring on different synapses. Moreover, there is evidence that α_1-AR signaling can actively desensitize mGluR5 signaling at IP_3 receptors (Paladini et al., 2001) which may explain why we observed occlusion of mGluR5 LTD in our previous manuscript (McElligott and Winder, 2008).

α_1-AR LTD, but not mGluR5 LTD, Results in the Functional Loss of CP AMPARs

CP AMPARs are less abundant in the CNS than the more conventional Ca^{2+} impermeable receptors (Cull-Candy et al., 2006). Their role in CNS function, and particularly plasticity, however, has begun to be appreciated in recent years. It is now apparent that CP AMPARs are a third venue (other than NMDARs and VGCC) for Ca^{2+} entry into a cell to induce plastic changes and can activate LTP and LTD in various regions on various cell types (Cull-Candy et al., 2006). Recently it was described that another Gq linked LTD, mGluR1 mediated LTD in the VTA, can induce the removal of CP AMPARs from the membrane (Bellone and Luscher, 2005). To investigate CP AMPARs many physiologists examine their unique property of rectification at depolarized potentials with the inclusion of poly amines in the patch pipette. However, recent data suggests that post synaptic proteins, in particular the transmembrane AMPA receptor regulatory protein (TARP) stargazin, can interfere with the polyamine block at depolarized potentials and thus confounds the interpretation of the result (Soto et al., 2007). Additionally, rectification experiments require precise voltage clamp over synapses in dendritic spines which may be increasingly difficult the more distal the location of the spines on which the synapses are made (Williams and Mitchell, 2008). Unpublished data from our lab, furthermore, failed to demonstrate much if any rectification of AMPA currents in the BNST (Grueter and Winder) perhaps due to these confounds. Utilizing the polyamine Naspm as a pharmacological blocker of CP AMPARs we noted that approximately 30% of

the evoked EPSC response was sensitive to Naspm. Furthermore, this sensitivity was lost following induction of α_1-AR LTD and a peptide inhibitor of the C-terminus of the GluR1 subunit significantly attenuated LTD. These converging lines of evidence suggest that the loss of CP AMPARs comprises at least a portion of the mechanism underlying α_1-AR LTD. Although, additional postsynaptic elements may contribute to the LTD as stated above we found only approximately 30% of our EPSCs contained CP AMPARs while we observe a more robust LTD. A loss of CP AMPARs at the cell membrane may have profound effects on signaling in the postsynaptic cell. Although it is not known if signaling via CP AMPARs may alter plasticity within the BNST, if such modulation is possible, then a loss of CP AMPARs following α_1-AR LTD may result in a metaplastic shift within the BNST that could have profound changes over subsequent flow of information.

Additionally, we demonstrated that mGluR5 LTD in the BNST is neither maintained by the functional loss of CP AMPA receptors from the postsynaptic density, nor is it sensitive to the inclusion of the GluR1 peptide in the intracellular solution. These results in conjunction with the differing mEPSC/sEPSC profile strongly suggest that while the same cell possesses the required elements for the induction of either α_1-AR or mGluR5 LTD, they occur by distinctly different mechanisms. Furthermore, the induction of one form of G_q coupled LTD manipulates the plasticity of the other LTD (McElligott and Winder, 2008), which differs from observations in other brain regions where dual G_q coupled LTDs have been studied (Choi et al., 2005; Lee et al., 2005; Scheiderer et al., 2008). It

is possible to envision that the occlusion of mGluR LTD in the BNST by α_1-AR LTD could influence subsequent behavior, and perhaps a lack of proper α_1-AR induction/expression (see below) may result in pathological mGluR LTD expression.

Expression of α_1-AR LTD is Manipulated by Chronic Stressors

Previously we demonstrated that α_1-AR LTD cannot be induced in two animal models of affective disorders, the α_{2A}-AR knockout (KO) mouse and the NE transporter KO mouse (McElligott and Winder, 2008). Both KOs have altered adrenergic tone (Xu et al., 2000; Lahdesmaki et al., 2002) and studies from the NET KO suggested that the lack of expression of LTD (in both models) was probably the result of chronically desensitized α_1-ARs (Bohn et al., 2000; Xu et al., 2000; Dziedzicka-Wasylewska et al., 2006). We thus wanted to examine if stressful manipulations, that presumably would increase adrenergic tone *in vivo*, could alter the expression of α_1-AR LTD *in vitro*. Withdrawal from alcohol intoxication has been shown to increase anxiety (George et al., 1990), and patients experiencing withdrawal have elevated levels of NE and its metabolites (Borg et al., 1981; Hawley et al., 1985; Manhem et al., 1985; Nutt et al., 1988). Furthermore, CIE can increase anxiety-like behavior (Kliethermes et al., 2004). We utilized a CCE protocol with a single withdrawal and a CIE protocol with 4 withdrawals. We originally expected to only see differences after the CIE repeated withdrawal paradigm, however, both protocols significantly reduced the expression of LTD and were not significantly different from each other. This

suggests that the exposure paradigm itself may have been stressful enough to induce these changes. Neither paradigm, however, completely occluded the expression of α_1-AR LTD. We therefore used a more prolonged chronic stressor to investigate the induction of α_1-AR LTD. Restraint stress has been shown to increase NE levels within the BNST and α_1-AR signaling therein increases anxiety-like behavior and activates the HPA axis (Cecchi et al., 2002). Mice that received 10 days of 2 hour restraint stress failed to express α_1-AR LTD under our induction paradigm. Both the CCE/CIE and stress experiments suggested that the LTD had already been expressed or that the mechanisms for inducing α_1-AR LTD were not functioning. To determine if α_1-AR LTD had been induced *in vivo*, we probed the cells in the vlBNST for functional synaptic CP AMPARs using Naspm and found no difference in either the stressed mice or the naïve controls. Unlike the α_{2A}-AR and NET KOs, however, application of methoxamine produced a significant early effect despite lacking LTD. This result was similar to what was observed when clathrin dependent endocytosis was prevented (Fig. 4) and when L-type VGCC were blocked (McElligott and Winder, 2008). These results suggest that in the stressed animals, the blockade of LTD may lie downstream of the α_1-AR. Although α_1-AR signaling modulates HPA axis function to increase the stress response in naïve animals (Cecchi et al., 2002), the lack of signaling in an animal that experiences a chronic stressor is consistent with the HPA axis profiles of humans and animals receiving chronic stress. Patients with a variety of stress and anxiety disorders experience hypocortisolism and lack appropriate stress responses to HPA axis challenges (Heim et al., 2000). Furthermore,

ethanol dependent mice and mice in withdrawal have persistent decreases in HPA axis function (Rasmussen et al., 2000; Richardson et al., 2008). We speculate that induction of α_1-AR LTD may occur (to some degree) in the normal response to a stressor, but that the lack of plasticity in the pathological/chronic state may lead to a challenged stress axis.

An intriguing result of this investigation was that α_1-AR activation leads to lasting increases sEPSC frequency presumably via the release of CRF acting on the CRF1 receptor, a kin to dopamine signaling within the BNST (Kash et al., 2008b). Patients with PTSD have been shown to have over engaged central CRF systems (Bremner et al., 1997). It would therefore be interesting to investigate whether this signaling is intact in animals that have experienced the chronic restraint stress paradigm.

Earlier investigations found that NE can modulate stress and anxiety responses via activation of the α_1-AR within the BNST (Cecchi et al., 2002) and we further showed that the stimulation of this receptor resulted in a robust LTD of excitatory signaling (McElligott and Winder, 2008). Our current results investigated the maintenance mechanisms underlying α_1-AR LTD. We found that the LTD is maintained postsynaptically specifically through the loss of CP AMPA receptors, in contrast to mGluR5 LTD. Furthermore we demonstrated that α_1-AR LTD is attenuated or occluded in mice undergoing withdrawal from ethanol exposure and mice that were chronically stressed respectively, although, presumably not via induction of the LTD *in vivo*. This data together with our previous results in mouse models of affective disorder strongly correlate the loss

of α_1-AR LTD with a number of psychiatric ailments, and lends credence to clinical trials that have demonstrated α_1-AR antagonists as potential therapeutics for the alleviation of PTSD and alcoholism (Raskind et al., 2000; Raskind et al., 2002; Taylor and Raskind, 2002; Peskind et al., 2003; Taylor et al., 2006; Simpson et al., 2008).

CHAPTER IV

GENERAL DISCUSSION

Summary of α_1-AR LTD Induction, Expression and Maintenance

The BNST is uniquely positioned in the central nervous system to relay information between cognitive, memory and emotional nuclei, and, stress, reward and feeding centers. One of the most striking features of the innervation of the BNST is that it receives perhaps the densest projection from adrenergic nuclei in the brain. This innervation is has been shown to manipulate aspects of reward, the aversion to withdrawal, stress induced reinstatement of drug seeking, the affective component of pain, and anxiety (Delfs et al., 2000; Erb et al., 2000; Shaham et al., 2000; Wang et al., 2001; Cecchi et al., 2002; Olson et al., 2006; Deyama et al., 2007; Deyama et al., 2008a). Although drugs of abuse and stress have several molecular targets in the CNS, experiments have shown that the glutamatergic synapse is a common target of several reinforcing drugs and acute stress (Saal et al., 2003).

The Winder lab therefore sought to determine how NE modulated glutamatergic synapses within the BNST. Initial experiments demonstrated that NE could increase and decrease glutamatergic signaling within the region in a manner dependent on signaling via β-ARs (increase) and α_2-ARs (increase and decrease) (Egli et al., 2005). α_1-AR signaling, however, did not appear to play a role in these affects. α_1-ARs are known to modulate glutamatergic transmission

in other brain regions, however, depending on the location of the receptor and signaling components, stimulation of α_1-ARs has a varied effect. In the vCTX and the hippocampus α_1-AR activation results in a LTD (Kirkwood et al., 1999; Scheiderer et al., 2004; Choi et al., 2005; Scheiderer et al., 2008) and in the NTS a long lasting depression (Zhang and Mifflin, 2006); however, in the PVN α_1-ARs increase glutamatergic efficacy via presynaptic and postsynaptic glial interactions (Gordon and Bains, 2003; Gordon et al., 2005; Gordon and Bains, 2005). α_1-ARs are expressed in the BNST (Day et al., 1997), and their activation there modulates the frequency of sIPSCs (Dumont and Williams, 2004). **As a result of these data, I hypothesized that α_1-ARs in the BNST could induce a LTD of glutamatergic transmission and that this LTD may play a role in the pathology of various affective disorders.**

Summary of α_1-AR LTD Induction and Maintenance

NE Induces α_1-AR LTD Via a Time Dependent Mechanism

The lack of an accounted for α_1-AR component in the preliminary study (Egli et al., 2005) performed by the Winder lab to assess the effect of NE on excitatory transmission in the BNST appeared conclusive at first glance. In these experiments 100 μM NE was applied to slices for 10 minutes and robust effects due to the stimulation of α_2-ARs and β-ARs were observed. Although α_1-AR have lower affinity for NE than α_2-AR, they have greater affinity than β-ARs (Hieble et al., 1995; MacDonald et al., 1997). Furthermore, a 2 minute bath application of NE at the same concentration or an order of magnitude lower was able to induce

increases in sIPSCs by α_1-AR stimulation, suggesting that slower pharmacokinetics were not the cause for a lack of α_1-AR effect on excitatory transmission. I found, however, that a 20 minute bath application of the α_1-AR agonist methoxamine (100 μM) could induce robust LTD of excitatory transmission in the dorsal and ventral BNST (see Fig 2,3) (McElligott and Winder, 2008). Fascinatingly, a similar 20 minute application of 100 μM NE, but, as observed previously, not a 10 minute exposure, could mimic this LTD induction (in the N2 of field potential recordings) in a manner independent of the immediate effect and entirely dependent on signaling via the α_1-AR (see Fig 4) (McElligott and Winder, 2008). The time dependent nature of the induction of this LTD has not been observed in other brain regions where a 10 minute exposure of NE can induce LTD (Scheiderer et al., 2004). This time dependence, thus, may be a unique feature of α_1-AR LTD in the BNST and may have physiological consequences. When mice are exposed to restraint stress, NE levels in the BNST remain high for prolonged periods of time, even after termination of the stressor (Pacak et al., 1995; Cecchi et al., 2002). Furthermore evidence suggests that the BNST may be responsible for sustained fear, rather than phasic fear, which may account for the need for a longer environmental signal (on the order of several minutes) for conditioning and to thus initiate plasticity within this region (Sullivan et al., 2004; Waddell et al., 2006; Walker et al., 2008b; Walker and Davis, 2008).

α_1-AR LTD in the BNST Is a Novel Form of LTD

α_1-AR LTD has been previously described in the CA1 region of the hippocampus (Scheiderer et al., 2004; Scheiderer et al., 2008) and the vCTX (Kirkwood et al., 1999; Choi et al., 2005). In both these regions the LTD may be better described as a noradrenergic modulation of homosynaptic LTD. This is due to the data that demonstrates that both concurrent glutamatergic stimulation and NMDARs, thus the presumed depolarization of the postsynaptic cell, are required for expression of the LTD. α_1-AR LTD in the BNST, however, requires neither presynaptic input from glutamatergic fibers nor the activation of postsynaptic NMDA receptors for expression of the plasticity (see Fig 5) (McElligott and Winder, 2008). Additionally, α_1-AR LTD in the hippocampus appears to be mediated in part by signaling by ERK (Scheiderer et al., 2008), where as α_1-AR LTD in the BNST is independent of MEK signaling (the kinase that phosphorylates ERK, see Fig 11) An interesting difference between the LTD in the vCTX/hippocampus and BNST may lie in the variance of cell types between these areas. Although the hippocampus is considered a sub-cortical structure, the glutamatergic cells within CA1 are pyramidal in nature. The BNST, however, is comprised of cells that more closely resemble the medium spiny neurons of neighboring NAc (Egli and Winder, 2003). Also unlike the LTD in the hippocampus, in the BNST α_1-AR LTD is saturating after a single exposure (Fig 3), where the hippocampus requires several exposures to fully saturate (Scheiderer et al., 2004). Additionally, I demonstrated that α_1-AR LTD in the BNST requires signaling via L-type VGCCs. It is not clear, however, where within

the microcircuit these channels may lie. It is possible that the L-type channels that contribute to α_1-AR LTD are on a different cell than the cells experiencing LTD for two reasons: chelating calcium in the postsynaptic cell with an intracellular supply of BAPTA did not result in the preventing the induction of LTD (data not shown), also, L-type VGCC are usually closed at potentials of -70 or -90 mV which is where the cells are clamped for all whole cell experiments. The distinct possibility remains, however, that the spines are not as well clamped as the cell bodies within my recordings (Williams and Mitchell, 2008). Additionally, release of Ca^{2+} from IP_3 sensitive stores can shift the activation range for L-type VGCC to more depolarized thresholds (Gao et al., 2006). These data suggest that the induction of α_1-AR LTD in the BNST demonstrates a novel form of heterosynaptic LTD within the CNS.

A fascinating component of affective disorders is that often the afflicted person or patient has full knowledge and awareness of the malady. This knowledge has often led to the stigma behind many affective disorders, where it is perceived often that those afflicted should be able to mentally overcome their problems and return to a normal baseline. A key component to affective disorders, however, is that mere will-power alone cannot induce recovery, and patients often require behavioral therapy, psychoanalysis and often some form of pharmacotherapy to recover because these disorders are due to improper neurochemical functioning. An interesting aspect of α_1-AR LTD in the BNST is that a lack of dependence on concurrent glutamatergic input or NMDARs suggests that this LTD may be induced without “authorization” by the presynaptic

cell, thus excluding the Hebbian notion of “cell’s that fire together, wire together” from this type of plasticity. When one takes into account that the presynaptic components are carrying information from cognitive and emotional neural centers to relay in the BNST, these data provide a means for more primitive brain regions (like the adrenergic nuclei) to functionally disconnect the “thinking/feeling” parts of the brain from the stress and reward centers. Although these notions are highly speculative, it will be interesting to see if α_1-AR LTD in the BNST affects synapses stemming only from a single or a few afferent regions, such as the infralimbic cortex and not the BLA (or vice versa).

α_1-AR LTD Has a Distinct Maintenance Mechanism from mGluR5 LTD in the BNST

In the second aim of my dissertation, I hypothesized that α_1-AR LTD in the BNST was expressed via the same maintenance mechanisms as mGluR5 LTD in the BNST. Although G_q coupled LTDs are very promiscuous across brain regions, it stands to reason that if both receptors are located on the same postsynaptic cell and if both receptors couple to the same canonical pathway, then the mechanism for LTD may be one and the same. Data from the vCTX and hippocampus provide strong evidence for these assumptions, where multiple receptors appear to be signaling via the same pathways to induce the same LTD (Choi et al., 2005; Scheiderer et al., 2008). My preliminary evidence in the BNST suggested that this concept also held true for α_1-AR and mGluR5 LTDs as prior induction of α_1-AR LTD prevented the further induction of mGluR5 LTD, without desensitization of the mGluR5 receptor (Fig 7) (McElligott and Winder, 2008). It

has been demonstrated, however, that in the dopaminergic neurons of the VTA α_1-AR signalling can desensitize mGluR5 signalling at the level of the IP_3 receptor on the endoplasmic reticulum (Paladini et al., 2004). In addition, as discussed above, α_1-AR LTD induction was independent of MEK 1/2 signaling (see Fig 11) unlike mGluR5 LTD in the BNST that is dependent on MEK 1/2 signaling and ERK 1 (Grueter et al., 2006).

To further investigate these findings I examined how α_1-AR LTD is maintained in the BNST. Similarly to mGluR5 LTD, α_1-AR LTD appears to be maintained by postsynaptic mechanisms (Grueter et al., 2006; Grueter et al., 2008). α_1-AR activation did not confer an alteration in PPR (McElligott and Winder, 2008) nor was the LTD it elicited more robust in a depleted calcium ACSF (see Fig 8). Furthermore, both α_1-AR LTD and mGluR5 LTD appeared to be maintained via clathrin dependent endocytosis (see Fig 11), presumably via a loss of AMPAR at the postsynaptic membrane. Also, α_1-AR LTD did not demonstrate a change in the coefficient of variation, which is another means of examining a presynaptic mechanism (data not reported). When mini-analysis (in the presence of 1 μM TTX) was examined in the context of α_1-AR LTD, however, there was a dramatic shift in the profile from mGluR5 LTD. Examining mEPSCs before and after induction of LTD by 100 μM methoxamine failed to result in a change in either the frequency or the amplitude of the events, however, after induction of mGluR5 LTD there is a significant reduction in mini frequency (Grueter et al., 2008). These data suggested that α_1-AR LTD may be dependent on voltage gated sodium channels. To verify this, I next omitted TTX from my

recording ACSF to record sEPSCs before and after the induction of α_1-AR LTD. Under this recording configuration, I observed a decrease in the amplitude of the sEPSCs but not in their frequency (see Fig 10); however, these results were obtained only when the CRF1 antagonist was present in the ACSF (see below for detailed discussion).

The above results suggested the possibility that while both α_1-AR LTD and mGluR5 LTD are maintained postsynaptically and seemingly via the loss of AMPARs at the cell surface, they may be targeting different populations of AMPARs. Unpublished results by Dr. Brad Grueter in the lab demonstrated that following mGluR5 LTD in the BNST there was greater rectification at synapses suggesting that mGluR5 LTD was maintained by a functional loss of calcium impermeable (thus GluR2 containing) receptors at the synapse. In the VTA, however, group I mGluR LTD results in the loss of CP AMPARs. One way to examine if a given cell contains CP AMPARs is to look for rectification at depolarized potentials when polyamines, which block the pores of CP AMPARs, are included in the patch pipet. Preliminary data demonstrated little to no rectification of glutamatergic synapses in the BNST (unpublished data Brad Grueter) suggesting a lack of CP AMPARs in naïve cells. Rectification studies, however, have two significant caveats. One issue is that precise voltage control must be maintained over the cell from which one is recording. This type of voltage control is increasingly hard when the synapses one is investigating are located distally from the recording pipet (Williams and Mitchell, 2008). Another issue surrounding rectification experiments is that when CP AMPARs associate

with the TARP stargazin the rectification properties are attenuated (Soto et al., 2007). Because of these confounds and due to the lack of rectification in the BNST neurons, I decided to see if an external polyamine Naspm, that is a synthetic analogue of the CP AMPAR blocker Joro Spider Toxin, would depress synapses within the BNST. Bath application of 100 μM Naspm resulted in a depression of approximately 30% of the evoked EPSC (see Fig 12A). Following the induction of α_1-AR LTD, however, the same application of Naspm failed to further reduce EPSCs suggesting that the α_1-AR LTD functionally desensitized the CP AMPARs (see Fig 12B,D). In contrast, however, following mGluR LTD, Naspm reduced EPSCs to the same extent as in naïve cells (see Fig 12C,D). Moreover this depression was significantly different from time matched control LTD experiments (see Fig 12C). CP AMPARs can be comprised of either GluR1 or GluR3 homomers or GluR1/3 heteromers. To expand on the results obtained using Naspm, I next examined the ability of peptide analogues (delivered intracellularly) of the c-terminus of the GluR1 subunit or the c-terminus of the GluR2 to interfere with α_1-AR and mGluR5 LTD. These peptide subunits have been shown to interfere with the trafficking of these receptors (Shi et al., 2001) as well as some of the protein players in the postsynaptic density (Li et al., 1999; Daw et al., 2000). The GluR1 c-terminal peptide attenuates α_1-AR LTD, but does not completely occlude LTD expression. This peptide has no effect on the expression of mGluR5 LTD. Additionally, the GluR2 c-terminal peptide does not have an effect on either α_1-AR LTD or mGluR5 LTD expression (see Fig 13). Although the c-terminal peptide to GluR1 does not completely occlude the

maintenance of α_1-AR LTD, this does not suggest that functional CP AMPARs are still available for signaling. GluR3 is also robustly expressed in the BNST (Allen Institute for Brain Science, 2008) and potential GluR3 homomers may be expressed in the postsynaptic densities. Additionally, α_1-AR LTD is more robust than the depression observed by Naspm. This may suggest that additional mechanisms, like receptor dephosphorylation for example, may contribute to the maintenance of α_1-AR LTD.

Although the data suggest that a functional loss of CP AMPARs underlies α_1-AR LTD, it does not provide information as to how this is mediated. The experiments that demonstrated that clathrin dependent endocytosis is required for the maintenance of α_1-AR LTD suggest that one mechanism may be the removal of CP AMPARs from the cell membrane into intracellular compartments. It has been previously shown, however, that group I mGluRs internalize upon ligand binding and that this internalization may play a role in their proper signaling (Mundell et al., 2001). There remains the possibility this may be a common mechanism by which GPCRs signal and internalization of α_1-AR is required for induction of LTD. Biochemical studies, such as BS3-crosslinking or biotinylation assays could determine if α_1-AR LTD in the BNST involves the removal of CP AMPARs from the synapse to intracellular compartments.

CP AMPARs are an additional means by which cellular signaling processes, especially those that are calcium dependent, can induce plastic change (Cull-Candy et al., 2006). At this time it is unknown if calcium entry via CP AMPARs can induce plasticity within the BNST. If such plasticity could take

place, however, it is probable that the removal of CP AMPARs from the synapse by α_1-AR LTD may modulate CP AMPAR dependent plasticity to be induced. This suggests that α_1-AR LTD may not only be a form of synaptic plasticity, but also, metaplasticity.

Table 2. A comparison of mGluR5-LTD vs. α_1-AR LTD in the BNST (Grueter et al., 2006; Grueter et al., 2008; McElligott and Winder, 2008)

	α_1-AR LTD	**mGluR5-LTD**
Paired Pulse Ratio	Early increase in vlBNST, No change in dlBNST	Early transient increase, CB1 dependent
Low Ca^{2+} ACSF	Small significant decrease, suggests postsynaptic mechanism	No significant change, suggests postsynaptic mechanism
mEPSC/sEPSC profile	Decrease in sEPSC amplitude, no change in mEPSCs	Decrease in mEPSC frequency
MEK1/2 dependence	Independent	Dependent
L-type VGCC dependence	Dependent	--
Occlusion	--	Occluded by induction of α_1-AR LTD
clathrin mediated endocytosis dependent	Dependent	Dependent
CP AMPAR involvement	Insensitive to CP AMPAR antagonist following LTD	Sensitive to CP AMPAR antagonist following LTD
GluR1 involvement	Sensitive to pep1-TGL	Insensitive to pep1-TGL, expressed in GluR1 KO mouse

α_1-AR LTD: Implications for the Pathophysiology of Disease

α_1-AR Blockade as a Treatment for Affective Disorders

A role for α_1-AR signaling in affective disease states has been enforced in recent years with exciting results from clinical studies in humans. In 2000 Raskind and colleagues reported that two Vietnam veterans with the most severe score of PTSD on the Clinician Administered PTSD Scale and Clinical Global-Impressions-Change suddenly reported alleviation of the majority of their symptoms (night terrors, hyper vigilance, low anger threshold, etc). Neither of these patients had previously responded well to either behavioral therapy or pharmacotherapy. The only recent alteration in care that either veteran had received was a diagnosis of benign prostatic hypertrophy (BPH) or an enlarging prostate for which the α_1-AR antagonist prazosin was prescribed (Raskind et al., 2000). These case studies led to additional larger clinical trials where combat and non-combat sufferers of PTSD were examined and prazosin was demonstrated to be effective in the alleviation of both day and nighttime symptoms of PTSD in the majority of cases (Raskind et al., 2000; Raskind et al., 2002; Taylor and Raskind, 2002; Peskind et al., 2003; Taylor et al., 2006). These studies have now led to the more routine prescription of prazosin by psychiatrists in the United States (including psychiatrists at Vanderbilt University and the VA hospital here, personal communication Dr. Sachin Patel) as a pharmacotherapy for PTSD. Sufferers of PTSD often display a high co-morbidity with alcoholism (Stewart, 1996) and coincidently Raskind's team found that persons on prazosin

therapy for PTSD self reported less consumption of alcohol than prior to therapy (personal communication Dr. Andrew Saxon at RSA, 2007). Simpson and colleagues recently expanded on this observation with a small clinical trial for the use of prazosin to treat alcohol dependence in alcoholics without co-morbid PTSD. The results of this preliminary study demonstrate that prazosin treatment reduces both days drinking and drinks per day in non-abstinent alcoholics (Simpson et al., 2008). Both the results of the PTSD and the preliminary alcoholism clinical trials are compelling due to the very few pharmaceutical compounds that have proven reliable in the past to alleviate symptoms of either affective disorder. Because of these compelling data, in my third aim I decided to investigate the persistence of α_1-AR LTD in both genetic and behavioral models affective disorder.

α_1-AR LTD Is Occluded in Genetic Models of Affective Disorder

Two animal models with altered noradrenergic systems have multiple affective disorder phenotypes. The α_{2A}-AR KO mouse has altered metabolite (3-Methoxy-4-hydroxyphenylglycol or MHPG) to transmitter (NE) ratios, and disrupted depression and anxiety phenotypes (Schramm et al., 2001; Lahdesmaki et al., 2002), while the NET KO demonstrates slower clearance of NE, altered reward capacity, heightened analgesia to opiates and alteration in anxiety and depression like behaviors (Bohn et al., 2000; Xu et al., 2000; Hall et al., 2002; Dziedzicka-Wasylewska et al., 2006; Keller et al., 2006) (see Table 3). A previous study from the Winder Lab demonstrated that the α_{2A}-AR KO did not

demonstrate an alteration in glutamatergic efficacy to a 10 minute application of 100 μM NE (Egli et al., 2005). I further demonstrated that the neither the α_{2A}-AR KO nor the NET KO express α_1-AR LTD in the BNST (McElligott and Winder, 2008) (see Fig 7). Although it is possible that the occlusion of LTD was the result of a previous induction of LTD *in vivo*, the NET KO has demonstrated significant desensitization of α_1-ARs across several brain regions (Bohn et al., 2000; Xu et al., 2000; Dziedzicka-Wasylewska et al., 2006). These data may suggest that the lack of LTD expression is due to an inability of proper signaling to result in LTD rather than an *in vivo* induction, however, additional evidence would be required for this conclusion.

Table 3. Comparison of α_{2A}-AR and NE Transporter Knockout Mice

	α_{2A}-AR KO	NET KO
Extracellular NE	Increased metabolite/transmitter (Lahdesmaki et al., 2002)	Reduced clearance (BNST, cyclic voltammetry) and increased concentration (cerebellum, microdialysis) (Xu et al., 2000)
Anxiety Phenotype	Increased anxiety-like behavior in the elevated-plus maze (reduced closed arm entries and head-dips) (Lahdesmaki et al., 2002) and increased anxiety due to injection stress (light-dark box) (Schramm et al., 2001)	Increased tachycardia to fearful stimuli and bradycardia to chamber (Keller et al., 2006)
Learned Despair/Depression Phenotype	--	Decreased mobility in tail suspension test (Dziedzicka-Wasylewska et al., 2006)
Reward Phenotype	--	Increased locomotor response (Xu et al., 2000) and CPP (Hall et al., 2002) to cocaine
Analgesia Phenotype	--	Increased analgesia to opiates (Bohn et al., 2000)
Additional Behavioral Phenotype	Disrupted circadian rhythm (decreased locomotor responses in the dark) (Lahdesmaki et al., 2002)	--
α_1-AR Surface Expression	--	Blunted [^{3}H]prazosin binding in cortex (Dziedzicka-Wasylewska et al., 2006)

Georges and colleagues found that morphine withdrawal results in an inhibition of VTA dopamine neurons that is not merely reversed by administration of an α_2-AR agonist, but enhanced (Georges and Aston-Jones, 2003). The NET KO animals demonstrated increased rewarding properties to drugs of abuse and heightened analgesia to opiates and failed to express α_1-AR LTD, a mechanism that may decrease excitatory drive to the VTA and other efferent projections during withdrawal. Because of this, it would be interesting to see if the NET KOs experience attenuated aversion to withdrawal from opiates, which has been shown to require adrenergic modulation in the BNST (Delfs et al., 2000).

α_1-AR LTD is Attenuated in Mice Experiencing Withdrawal from Chronic Ethanol Exposure

A prominent theory in the development of alcohol dependence is that a kindling process occurs (Ballenger and Post, 1978) and this process may be supported by increases in anxiety during withdrawal (George et al., 1990). Chronic intermittent ethanol exposure mitigates handling induced convulsions in laboratory mice, and increases drinking behavior (Becker et al., 1997; Veatch and Becker, 2002; Duka et al., 2004). NE and its metabolite have been observed to be elevated in patients undergoing withdrawal from alcohol intoxication (Borg et al., 1981; Hawley et al., 1985; Manhem et al., 1985; Nutt et al., 1988). Fluctuations in NE, similar to those observed in alcoholics, have also been shown to induce kindling within animals, and clonidine (α_2-AR agonist) has been shown to retard kindling development while yohimbine (α_2-AR antagonist)

promotes kindling (Gellman et al., 1987). Furthermore α_2-AR agonists have been shown to reduce both processive (anxiety) and systemic (sympathetic tone) attributes to withdrawal from ethanol (Wilkins et al., 1983; Manhem et al., 1985; Nutt et al., 1988) and opiates (Gold et al., 1979b; Gold et al., 1979a), and attenuates stress-induced relapse to alcohol self administration in rat (Le et al., 2005). Additionally, prazosin attenuates self administration in ethanol-dependent rats (Walker, 2006). This suggests that alcohol withdrawal, leading to increases in adrenergic tone and decreases in GABAergic tone, promotes anxiety which leads to further alcohol intake and subsequent withdrawal thus promoting the kindling pattern.

Thus I examined two paradigms of withdrawal and the ability to induce α_1-AR LTD in each condition. The first paradigm consists of a 64 hour continuous exposure to ethanol vapor followed by a single 4-6 hour withdrawal, while the second paradigm consists of an intermittent 64 hour exposure (16 hours exposed to vapor during the dark cycle, followed by 8 hours withdrawal) with a final 4-6 hour withdrawal. I hypothesized that LTD would be affected in the intermittent exposure paradigm (CIE) verses the continuous paradigm (CCE) because the repeated withdrawal cycles have been suggested to lead to the increased kindling phenomena. Surprisingly, both paradigms resulted in significantly attenuated LTD, but not occluded LTD. This may suggest that the systemic stress of intoxication may have sufficiently inhibited the expression of LTD.

α_1-AR LTD Is Occluded in Mice that Experienced Chronic Restraint Stress

Following the attenuation data in the chronic ethanol paradigms, I chose to examine the availability of α_1-AR LTD induction in mice that had experienced a more strenuous stressor and a processive stressor rather than a stressor with unknown systemic actions (ethanol). Animals were stressed for 2 hours daily for 10 days and recorded from on the 11^{th} day. Restraint has been shown to increase NE concentrations in the BNST (Cecchi et al., 2002). To insure that the cells assayed received the majority of NE release, I only recorded from cells in the vlBNST. Naïve animals demonstrated robust LTD to bath application of 100 μM methoxamine, however restrained animals showed a significant early effect but did not express maintained LTD (see Fig 15). This suggested the possibility that α_1-AR LTD may have been induced in the restrained animals *in vivo*. To assess this, I used the function of CP AMPARs in these cells as a molecular marker for the prior induction of LTD. Interestingly, cells from restrained animals demonstrated the same depression to 100 μM Naspm as cells from naïve animals (see Fig 15). These results strongly suggest that the LTD was not induced in these animals; however, there is a possibility that the CP AMPARs were redistributed to other synapses. Furthermore, the profile of the response to 100 μM methoxamine in the restrained animals is different from the α_{2A}-AR and NET KOs. In the KO animals, stimulation with the α_1-AR agonist failed to alter the glutamatergic efficacy throughout the entire experiment (see Fig 7), but in the restrained animals agonist application produced a significant early effect (see Fig 15). A similar early effect was observed in the experiments demonstrating LTD

dependence on the L-type VGCCs (see Fig 5) and in the experiments demonstrating the dependence on clathrin dependent endocytosis (see Fig 11). One can infer from these results that in the restrained animals (and perhaps the ethanol exposed animals) the α_1-AR is functionally available for signaling, but that a component downstream from the α_1-AR is functionally desensitized or previously induced. It is not known what accounts for the early depression observed in the restrained mice, however, in the vlBNST, α_1-AR stimulation transiently increased PPRs that returned to baseline during the LTD time point (see Fig 4). α_1-AR stimulation enhances the release of GABA in the BNST (Dumont and Williams, 2004). Although picrotoxin is included in all experiments to isolate glutamatergic signaling by blocking ionotropic $GABA_A$ receptors, GABA can still be signaling in these slices to stimulate $GABA_B$ receptors. Blocking metabotropic $GABA_B$ receptors prevented the observed increase in PPR without disrupting the expression of α_1-AR LTD. This blockade, surprisingly, did not seem to attenuate the early component following α_1-AR stimulation (Fig 6). Several other transmitters could be acting to induce the observed early effect. The BNST contains dopamine, serotonin, and several neuropeptide neurotransmitters which may be mediating this effect. An interesting observation is that the amount of attenuation in the animals undergoing ethanol withdrawal is strikingly similar to the amount of attenuation by the intracellular GluR1 peptide. It would be interesting to see if cells in the BNST were sensitive to Naspm following these ethanol paradigms.

Potential Significance of α_1-AR LTD in the BNST

Hypocortisolemia in Anxiety Disorders

At first glance, the lack of *in vivo* activation of α_1-AR LTD following restraint stress is a disappointing result and contrary to my hypothesis. A functional desensitization, however, either via loss of α_1-ARs on the cell membrane or another component required for signaling or maintenance of α_1-AR LTD, may be more consistent with the data from human and animal models. As stated previously, alcoholics have higher tonic levels of NE and kindling (repeated exposure and withdrawal) is thought to increase adrenergic tone in the CNS (Breese et al., 2005). Patients with PTSD and other affective disorders, including addiction, have demonstrated hypocortisolemia (Schuder, 2005) which can be express as a reduced baseline cortisol level or a reduced HPA axis response to a stressful challenge (Heim et al., 2000). Interestingly, rats exposed to both chronic foot shock or restraint stress demonstrate both a decrease in the activation of the HPA axis and a diminished CRF binding in the anterior pituitary; and, moreover this effect was independent of feedback as it was observed in adrenalectomized animals (Rivier and Vale, 1987; Hauger et al., 1988). Although corticosterone is often viewed as the “end point” in the stress response, daily fluctuations (circadian rhythms) of corticosterone are important in the maintenance of homeostasis by the organism. Not surprisingly, in the rodent model, corticosterone and NE have the same circadian rhythm. Since α_1-ARs in

the BNST have been shown to have direct modulation over HPA axis function to a stressor, a lack of ability to induce LTD, which may result from chronically high levels of NE, could perhaps lead to a depressed HPA axis response. Thus, the ability to induce α_1-AR LTD in the BNST may actually serve as a protective mechanism to the organism. Additionally, all of our experiments are performed on animals under a normal light/dark cycle where lights on" is at 7:00 AM CST and "lights off" is at 7:00 PM CST. Slices are made in the morning at the relative beginning of the light cycle when NE levels should be the most reduced. It would be intriguing to see if α_1-ARs LTD could be induced in naïve mice under my protocol when NE release should be most robust at the onset of the dark cycle.

α_1-ARs: CRF Effect Versus LTD

While investigating the mechanism of α_1-AR LTD in the BNST, there was an intriguing, but unexpected finding. I found that the same application of 100 μM methoxamine for 15 minutes that induced LTD also resulted in an increase in sEPSC frequency (see Fig 10), indicating an increase rather than a decrease in synaptic efficacy. Although this result was unanticipated, it is not surprising given data that has demonstrated that α_1-AR stimulation can increase sIPSCs in the BNST (Dumont and Williams, 2004) and that the GABAergic projection from the CeA is also known to contain CRF neurons (Sakanaka et al., 1986) which is also expressed in intrinsic neurons within the BNST (unpublished observations Bob Mathews). Further experimentation demonstrated that this mechanism was independent of inducing LTD (see Fig 10) suggesting that α_1-AR stimulation can

result in multiple effects within the BNST. Stimulation of β-AR can also increase glutamatergic efficacy (Egli et al., 2005) presumably also via a CRF dependent mechanism (unpublished data, Kash and Winder). These data are interesting due to the fact that restraint stress can elevate NE in the BNST, induce anxiety like behavior and produce increases in ACTH in the bloodstream; however, the anxiety like behavior is attributed to both α_1-AR and β-AR signaling while the activation of the HPA axis (i.e. the increase in ACTH) is mediated solely by the α_1-AR. Moreover, i.c.v. injection of CRF can enhance acoustic startle (a measure of anxiety) in adrenalectomized animals and lesioning the BNST reduces CRF enhanced startle (Lee and Davis, 1997). These data disconnect the anxiety-like behavioral response from HPA axis function (Lee et al., 1994). My data thus demonstrates a plausible physiological mechanism for the difference between these two phenomena. Furthermore, it would be intriguing to observe if the CRF effect is intact, or enhanced in animals that have undergone withdrawal from chronic ethanol exposure or chronic stress and if these sorts of stressors would augment CRF enhanced acoustic startle. These experiments would be particularly interesting in light of evidence which suggests increased activity of the central CRF systems in persons with PTSD (Bremner et al., 1997).

Confounds with Prazosin Therapy

There remain several potential problems with the use of prazosin as a treatment for affective disorders. A major issue is that the half-life of prazosin is very short, requiring patients to consume several doses *per diem*. Additionally

α_1-AR stimulation increases blood pressure and thus high doses of prazosin can lead to dangerously low blood pressure in some patients. Additionally recent data from the LeDoux lab has demonstrated that systemic administration of prazosin (at 1 mg/kg and .5 mg/kg) actually enhances auditory fear conditioning and retards extinction of conditioned fear (Cain et al., SFN Annual Meeting, 2006). These studies are currently being expanded upon to investigate where in the amygdala α_1-AR signaling prevents the acquisition of fear conditioning (personal communication from Dr. Christopher Cain). Depending on efferent pathways and circuitry (for example the projection from the Oval Nucleus to the CeA), the BNST would be a probable nucleus where this action may be occurring.

α_1-AR LTD in the BNST: Additional Questions

The experiments outlined in this dissertation have made strides to characterize the induction, mechanism and significance of α_1-AR LTD in the BNST (see Fig 16); however, many questions regarding α_1-AR LTD in the BNST still remain unanswered. Although my data demonstrates that this form of plasticity is independent of signaling via MEK1/2 pathways (see Fig 11), there remains a disconnect as to how activation of the receptor is signaling to induce the functional desensitization of CP AMPARs. Canonically, increases in calcium and PKC, which may be a potential downstream kinase of α_1-AR activation, result in phosphorylation of GluR1 subunits at serine-831 which leads to an increase in conductance of the AMPAR. This sort of phosphorylation would lead to an increase rather than a decrease in postsynaptic glutamatergic efficacy.

PKC, however, has been previously shown to mediate group I mGluR LTD in several brain regions, where it may interact with PICK-1 to modulate AMPA receptor surface population, leaving it an attractive candidate kinase for the induction of α_1-AR LTD (Oliet et al., 1997; Bellone and Luscher, 2005; Steinberg et al., 2006). Additionally, it is possible that phosphatases may play a role in the induction of α_1-AR LTD. Protein phosphatase 2B (PP2B), also known as calcineurin, has been shown to interact with dynamin for clathrin dependent endocytosis of AMPARs (Winder and Sweatt, 2001). Another unknown facet of α_1-AR LTD in the BNST is a potential dependence on protein synthesis which is required for group I mGluR LTD in the hippocampus in wild-type mice (Huber et al., 2000; Huber et al., 2001). Finally, it would be interesting to see if the same population of α_1-AR is involved in the increase in sEPSC frequency as well as the LTD. Very recently, it has been shown that α_{1A}-AR activation in the hippocampus increases GABAergic tone in a pertussis toxin sensitive manner, suggesting that the α_{1A}-AR in this interneuronal population is coupled to $G_{i/o}$ GPCRs (Hillman et al., 2009). It would be interesting to see if either the increase in sEPSC frequency or the LTD would be sensitive to pertussis toxin.

Figure 16 Two Hypothesized Models of α_1-AR LTD Induction and Maintenance in the BNST

A. The first model postulates that the α_1-AR is situated on the postsynaptic BNST neuron and that all signaling events transpire within that postsynaptic cell. B. The second model suggests the possibility that a third neuron contains the α_1-AR and L-type VGCC and mediates the release of an unknown factor to induce α_1-AR LTD on the post synaptic neuron.

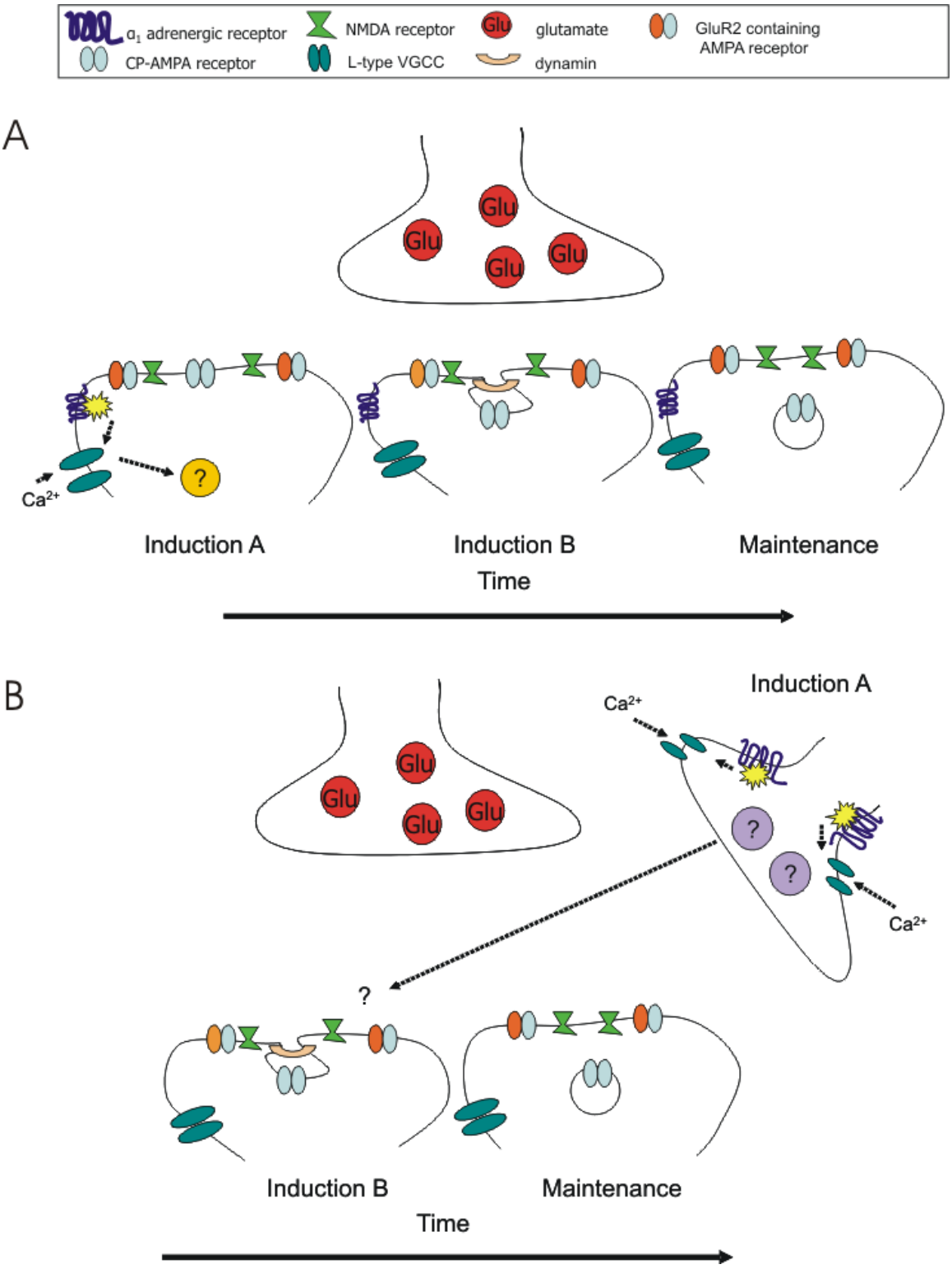
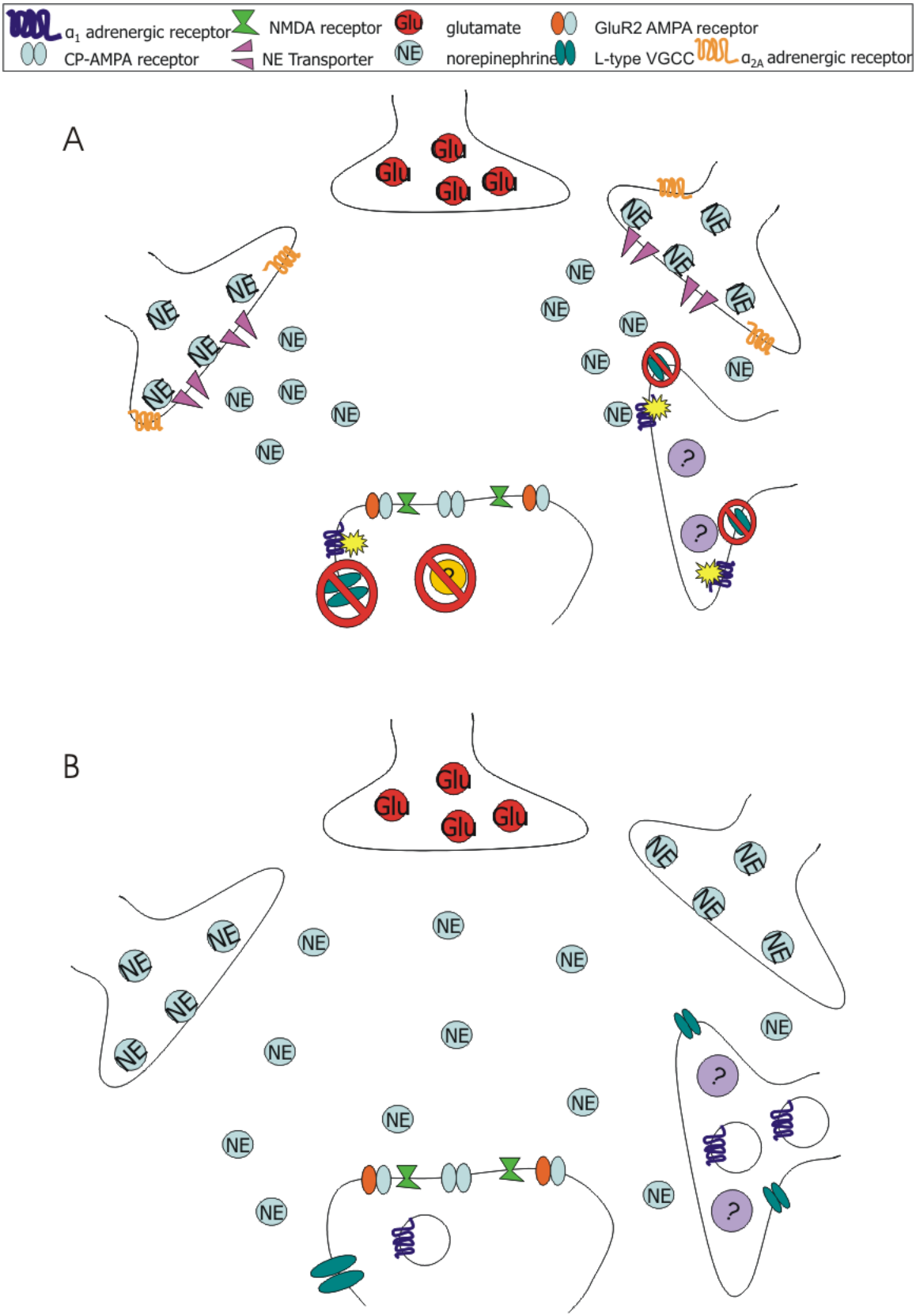

α1 adrenergic receptor
CP-AMPA receptor
NMDA receptor
L-type VGCC
Glu
glutamate
dynamin
GluR2 containing AMPA receptor
A
Glu
Glu
Glu
Glu
Ca2+
?
Induction A
Induction B
Maintenance
Time
B
Induction A
Ca2+
Glu
Glu
Glu
Glu
?
?
Ca2+
?
Induction B
Maintenance
Time

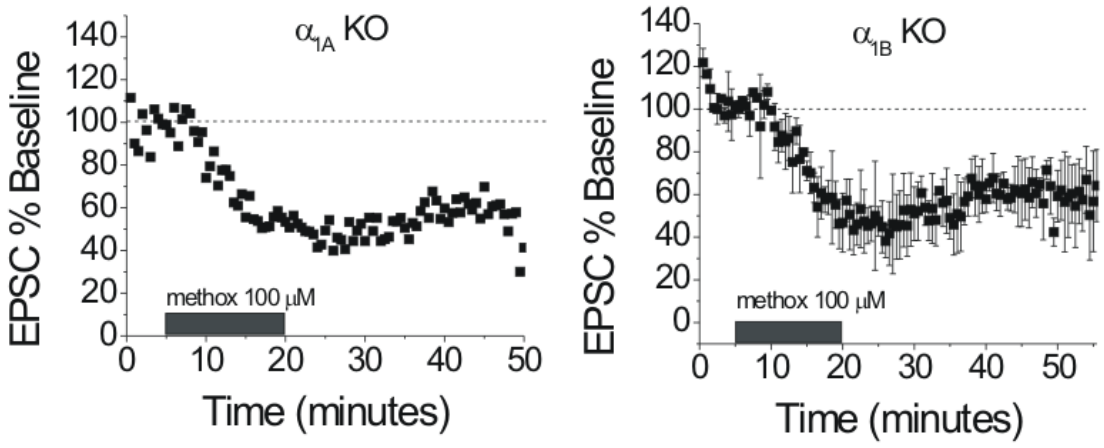
Figure 17 Hypotheses for Occlusion of α_1-AR LTD in Models of Affective Disorders

A. In mice experiencing chronic stress, activation of α_1-AR led to a significant early depression but an occlusion of LTD. This occlusion did not result in the removal of CP AMPARs from the cell surface, suggesting a desensitization of the LTD rather than an *in vivo* induction. These data are similar to those where L-type VGCC have been blocked or dynamin inhibitory peptides were used which may suggest a desensitization of either induction or maintenance mechanisms. B. In genetic models of affective disorder (α_{2A}-AR KO and NET KO), α_1-AR activation did not have any functional consequence over excitatory synaptic transmission. This, along with data from other groups suggesting a lack of α_1-AR binding in these mice, suggests that the α_1-AR itself may be desensitized in these animals.


α1 adrenergic receptor
CP-AMPA receptor
NMDA receptor
NE Transporter
Glu
glutamate
NE
norepinephrine
GluR2 AMPA receptor
L-type VGCC
α2A adrenergic receptor
A
B
?

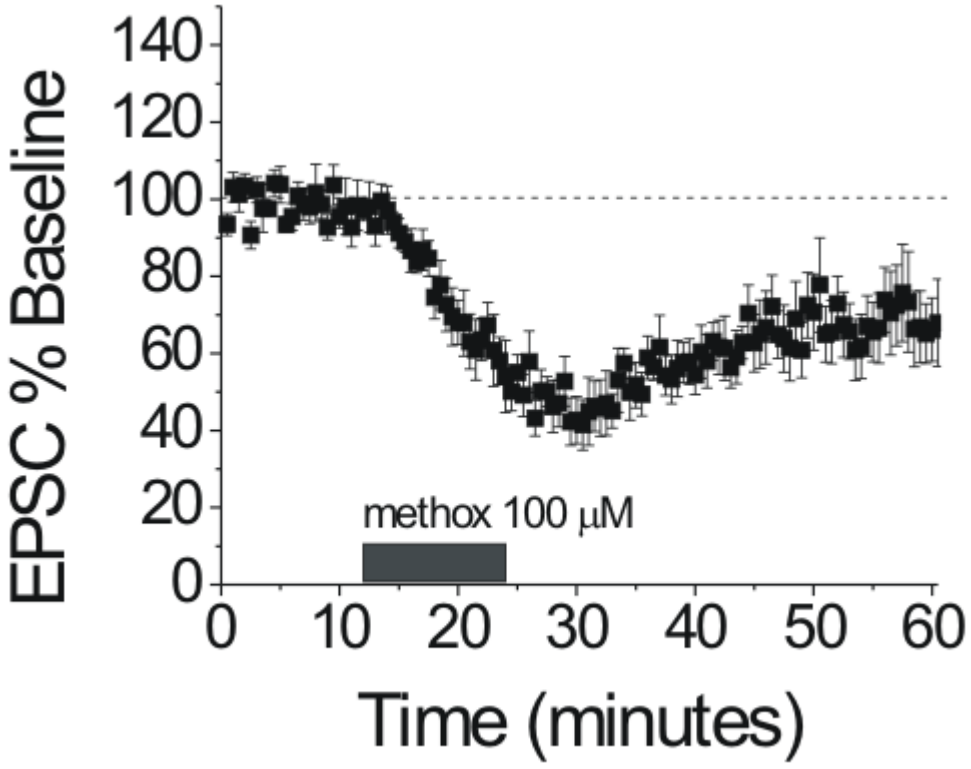
APPENDIX A

α_1-AR LTD is expressed in either the α_{1A}-AR KO or the α_{1B}-AR KO



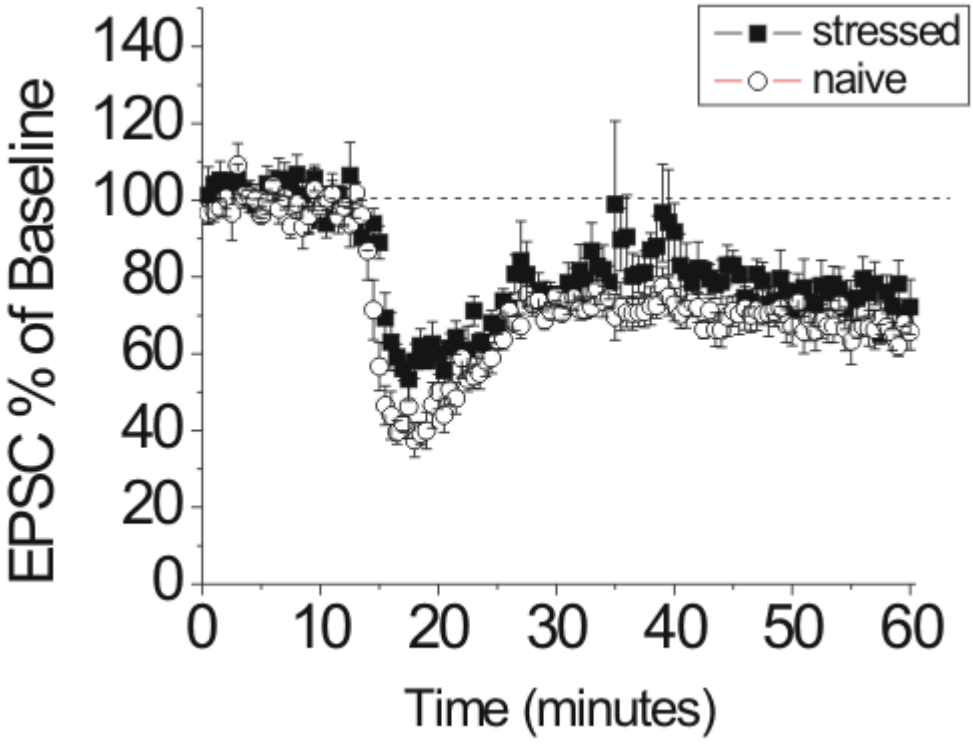
APPENDIX B

Intracellular BAPTA does not prevent expression of α_1-AR LTD



APPENDIX C

10 days of 2 hours restraint stress does not prevent the expression of mGluR5 LTD in the BNST



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