

METABOTROPIC GLUTAMATE RECEPTOR MEDIATED SYNAPTIC
PLASTICITY IN THE BED NUCLEUS OF THE STRIA TERMINALIS AS A
TARGET FOR STRESS, ANXIETY AND ADDICTION DISORDERS

By

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Dissertation

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

DOCTOR OF PHILOSOPHY

in

Molecular Physiology and Biophysics

May, 2006

Nashville, Tennessee

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**To my wonderful family; Carrie, Mom, Dad, Chad, Angie, Grandma, Chase, Cade,
Callie, Maddy**

ACKNOWLEDGMENTS

I would first, and foremost like to thank my mentor, Danny Winder. Danny has been a great mentor. He has helped steer my excitement about science and kept me in check at the same time. I will forever be grateful for his guidance as a scientist.

I would also like to thank the members of my dissertation committee, Jeff Conn, Roger Colbran, Eric Delpire, Aurelio Galli, and Greg Mathews for there excellent guidance and suggestions over the past couple years.

I would like to thank the people from the Winder lab past and present. The atmosphere in the lab has been a lot of fun. I particularly would like to acknowledge Regula Egli. Regula was a senior graduate student who taught me the ropes on the rig. And thanks to Tom Kash who has helped catalyze my interests in science during our many hours in a tiny room. I also would like to thank Adeola Pratt. Adeola has been a great resource to keep me level in the lab and remind me how big of a science geek I've become. And finally I would like to thank Julie Healey for keeping things going.

Outside of the lab I would like to thank my family. My wonderful wife has supported me and being in science herself, understands me. I would next like to thank my brother, Chad. It is Chad who has helped push me to be the best at what I do. I would like to thank my parents for being there for me and supporting me however far away from home I end up. And last but not least I would like to thank my grandma. They just don't make them like her any more!

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LIST OF ABBREVIATIONS

AMPA Receptor	AMPA
AMPA/kainate Receptor Antagonist	CNQX
Adenosine 3', 5' Cyclic Monophosphate	cAMP
Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid.....	AMPA
1S,3R-1-Amino-1,3-cyclopentanedicarboxylate	ACPD
L-(+)-2-Amino-4-phosphonobutyric Acid.....	L-AP4
DL-2-Amino-5-phosphonovaleric Acid.....	APV
Adenosine 3', 5' Cyclic Monophosphate	cAMP
Artificial Cerebro-spinal Fluid.....	ACSF
Basolateral Amygdala	BLA
Bed Nucleus of the Stria Terminalis	BNST
Calcium/Calmodulin-Dependent Protein Kinase.....	CaMK
cAMP Response Element Binding Protein	CREB
Cannabinoid Receptor	CBR
Central Nervous System	CNS
Central Nucleus of the Amygdala	CeA
(RS)-2-Chloro-5-hydroxyphenylglycine.....	CHPG
Conditioned Placed Preference	CPP
Corticotropin Releasing Factor	CRF
D1-like Dopamine Receptor	DAR
Diacylglycerol	DAG

(2S,2'R,3'R)-2-(2',3'-Dicarboxycyclopropyl)glycine.....	DCG-IV
3,5-Dihydroxyphenylglycine	DHPG
(S)-3,4-Dicarboxyphenylglycine	DCPG
Dimethyl Sulfoxide.....	DMSO
Dopamine	DA
Dopamine Transporter	DAT
Dorsolateral BNST.....	dBNST
Excitatory Postsynaptic Potential	EPSP
Extracellular Signal-regulated Kinase	ERK
G-Protein Coupled Receptors	GPCRs
Glutamate Transporters.....	GluTs
Glutamate Receptor Interacting Protein.....	GRIP
Glutamic Acid Decarboxylase	GAD
Guanosine 5'-monophosphate.....	GMP
Guanosine 5'-triphosphate.....	GTP
Guanosine Triphosphatases	GTPases
Histone Acetyltransferase.....	HAT
Hypothalamo-pituitary-adrenocortical Axis	HPA
Induced Inhibitory Postsynaptic Potentials.....	IPSPs
Inositol 1,4,5-triphosphate	IP3
Ionotropic Glutamate Receptor.....	iGluR
Intracerebroventricular	ICV
Long-term Depression	LTD

Long-term Potentiation	LTP
Low Frequency Stimulation	LFS
MAP Kinase Kinase.....	MEK
MAP Kinase Phosphatases	MKPs
MEK-binding Partner 1.....	MP1
Metabotropic Glutamate Receptor.....	mGluR
α -methyl-4-carboxyphenylglycine.....	MCPG
2-methyl-6-phenylethynyl-pyridine.....	MPEP
Mitogen-activated Protein Kinase.....	MAPK
Monoacyl-glycerol 2-arachidonoylglycerol.....	2-AG
NMDA Receptor.....	NMDAR
N-Methyl-D-Aspartate.....	NMDA
Norepinephrine Transporter.....	NET
Norepinephrine	NE
Nucleus Accumbens.....	NAc
Paired Plus Ratio.....	PPR
Paraventricular Nucleus.....	PVN
Phospholipase C.....	PLC
Prefrontal Cortex.....	PFC
Protein Kinase A.....	PKA
Protein Kinase C.....	PKC
Protein Tyrosine Phosphatases	PTP-SL
Postsynaptic Densities	PSD

Serotonin	5-HT
Serotonin-Selective Reuptake Inhibitors	SSRIs
Serotonin Transporter	SERT
Tetrodotoxin.....	TTX
Δ^9 -tetrahydrocannabinol	Δ^9 -THC
Ventral Tegmental Area	VTA

CHAPTER I

INTRODUCTION

In this introductory chapter, I primarily focus on excitatory transmission and neural networks linked to addiction and stress. I first consider addiction, the addictive process, methods to study this process, neuronal networks and their interactions, with an emphasis on long-lasting neuroadaptations (excitatory synaptic plasticity) persisting long after drug intake. Also conditions such as stress and anxiety demonstrate comorbidity with addiction and addicts cite stress as the number one reason for relapse to drug seeking. Therefore, I attempt to integrate these conditions illustrating similarities in neurocircuits between addiction and stress/anxiety by characterizing a region that is uniquely positioned as a converging point, the bed nucleus of the stria terminalis (BNST). Finally I review metabotropic glutamate receptor (mGluR) function with an emphasis on integrating mGluR-mediated synaptic plasticity and neuroadaptations in addiction and stress pathways. In addition to illuminating basic mechanisms shared between addiction, stress and synaptic plasticity, work in these areas has important implications for human health.

Addiction

Addiction is a compulsive habit maintained despite harmful consequences (Nestler, 2001). Drug addiction is the leading cause of lung cancer, and cirrhosis of the liver and costs society hundreds of billions of dollars due to loss of life and productivity (Vocci et al., 2005). It is a complex disease involving many types of social and

psychological factors. However, fundamentally it is a biological process: the effect of a chemical (drug of abuse) on a biological substrate (a brain). Consequently, advances in the drug addiction field could potentially apply to other neurophysiological disorders such as stress/anxiety and depression, as well as to the basic mechanisms involved in learning and memory. It is therefore a goal of this laboratory and many other research laboratories to study the physiological and molecular basis of drug addiction. Ultimately, these studies are designed to gain a better understanding of neurobiology and may lead to development of pharmaceutical tools to intervene in neuropathological conditions such as the process of addiction.

Addiction and Neuroplasticity

There is an abundant literature on neurobiological changes associated with chronic drug exposure. Therefore this document is focused on characterizing potential molecular substrates common to neuroadaptations mainly induced by a specific class of addictive drugs, psychostimulants. Traditionally, the focus of scientific research on drug addiction has focused on alterations in dopamine (DA) signaling. More recently, excitatory (glutamatergic) transmission has been recognized as a substrate for the neuroadaptations subsequent to drug intake. In support of this, imaging studies of human addicts have shown that stimuli associated with drug use (e.g., drug paraphernalia) trigger drug craving while simultaneously activating glutamatergic circuits (reviewed in (Wolf, 2002).

The concept of drug-induced neuroplasticity was first proposed based on studies demonstrating that glutamatergic transmission, the key component for producing and

maintaining synaptic plasticity, is required for the development of behavioral sensitization—a prominent animal model of addiction discussed in a later section (Wolf and Khansa, 1991; Vanderschuren and Kalivas, 2000). Moreover, it has been suggested that neuroadaptations leading to addiction involve the same glutamate-dependent cellular mechanisms that enable learning and memory (for reviews see (Berke and Hyman, 2000; Hyman and Malenka, 2001; Winder et al., 2002). Long-term potentiation (LTP) as well as the counter effect, long term depression (LTD), are important characteristics of glutamatergic synaptic plasticity. Phosphorylation states of key proteins, alterations in the availability of glutamate receptors at the synapse, and regulation of gene expression are thought to underlie LTP and LTD (Malinow and Malenka, 2002). Abnormal recruitment of LTP or LTD in networks related to reward may be the first step in the cascade leading to the changes that underlie neuroadaptations elicited by substances of abuse. Indeed, recent studies have begun to provide support for this hypothesis, showing that drugs of abuse can modify or induce LTP and LTD in neuronal pathways related to addiction (for review see Winder et al., 2002).

Drugs of Abuse

Drugs of abuse elicit their effects through different neurological mechanisms yet almost all of them recruit the natural reward pathways of the CNS, the mesocorticolimbic DA system. Drugs of abuse fall into three categories: depressants (e.g. heroin, barbiturates), stimulants (e.g. cocaine, crack, amphetamines) and hallucinogens (e.g. marijuana, Ecstasy, LSD). The research presented here will focus on stimulants.

Psychostimulants

Psychostimulants, as the name suggests, are agents that activate or increase motor activity. The stimulants of high abuse potential, cocaine and amphetamines, interact with monoamine transporters (Amara and Sonders, 1998). These transporters act to clear the monoamines from the extracellular space, particularly at the synapse, and therefore terminate the monoamine signal. Cocaine blocks the monoamine transporters (dopamine transporter (DAT), norepinephrine transporter (NET) and the serotonin transporter (SERT)) thereby increasing the signaling properties of these systems. Amphetamines also increase monoamine signaling but by a different mechanism. Amphetamine and its derivatives act to reverse the monoamine transporter, thus increasing levels of extracellular monoamines and ultimately enhancing monoamine signaling (Koob, 1999). A defining characteristic of psychostimulants is their positive reinforcing effects. Positive reinforcement lends to enhanced drug seeking behaviors that can be readily studied as described in a subsequent section.

Stages of Addiction

Researchers have classified the characteristics of drug addiction into different stages. It is important to note it is not necessarily easy to distinguish the behavioral indices characteristic of the stages of addiction. From the behavioral perspective I will describe the stages of addiction as: initiation, dependence (tolerance and sensitization), withdrawal, maintenance, and relapse/reinstatement. However, from the physiological perspective, I will discuss the characteristics of drug addiction in two overlapping categories, the positive reinforcing aspects of drug intake which are thought to be largely

dopamine (DA) dependent and the withdrawal/relapse states which are hypothesized to be largely dependent on glutamatergic transmission.

Initiation, as the term implies is the initial "recreational" use of a drug. *Tolerance* is defined as the reduced drug responsiveness from repeated exposure to drug. It is hypothesized that tolerance may contribute to the escalation of drug intake seen during the development of addiction. *Dependence* is an altered physiological (functional) state that develops to compensate for persistent drug exposure. If a dependent individual ceases drug intake *withdrawal* may occur. Withdrawal then may contribute to dysphoria (negative or aversive emotional state). The physiological stress and state of dysphoria is thought to be a contributor to relapse to drug seeking.

Maintenance of Addiction in the Absence of Continued Use

Just as *tolerance* has been offered as an explanation of escalating drug seeking (Koob, 1996), the opposite phenomena, *sensitization*, or enhanced drug responsiveness with repeated exposure to a constant dose, may contribute to the increased risk of relapse (Robinson and Berridge, 2000). Much interest lies in sensitization due to its long-lasting effects characteristic of behavioral plasticity (Shippenberg et al., 2001). Of greatest interest in addiction is the high risk of relapse. Propensity to relapse persists even in abstinent addicts long after any withdrawal symptoms have subsided and perhaps for a lifetime (Weiss, 2005). Relapse is driven by drug craving, an affective state that is hard to map onto a rodent. However, models for studying relapse or reinstatement of drug seeking behavior have been developed and will be discussed in a subsequent section.

Reinstatement

Reinstatement to drug seeking behavior can be induced by several mechanisms including re-exposure to the drug itself, drug-related cues and context or stress. Briefly, exposure to the drug, or different drugs (cross-sensitization), can reinstate drug-seeking behavior in animals in which responding had been extinguished due to removal of the reinforcer (Shaham et al., 2003). Glutamate release within the NAc increases during drug seeking behavior, and infusion of glutamate antagonist into this region blocks cocaine priming-induced reinstatement of drug-seeking behavior (Cornish and Kalivas, 2000). This suggests that glutamatergic transmission in the NAc is critical for drug-induced reinstatement.

Presentation of drug associated cues can elicit reinstatement of drug seeking in animals that have extinguished responding (Alleweireldt et al., 2001). Interestingly, excitotoxic lesioning of the basolateral amygdala (BLA) has no effect on maintenance of cocaine administration but, attenuates cue-induced reinstatement to cocaine seeking (Meil and See, 1997). Additionally, data suggests that a D1-like dopamine receptor (DAR) in the BLA is involved in cue-induced cocaine reinstatement (See et al., 2001). These findings suggest dopamine signaling in the BLA plays a critical role in cue-induced relapse to drug seeking.

While re-exposure to a drug of abuse and drug associated cues can trigger relapse to drug seeking, addicts cite stress as the most powerful instigator of relapse to drug seeking (Koob, 1999; Sinha et al., 1999; Stewart, 2000). The phenomena of stress-induced increases in susceptibility to drug-seeking is observed in animal models as well (Erb et al., 1998; Lu et al., 2003). Regions of the extended amygdala have been heavily

implicated in this aspect of relapse (Koob, 1999). For instance, work from the Stewart lab implicates noradrenergic as well as CRF signaling in the BNST and CeA in stress-induced reinstatement but not drug-induced reinstatement (Erb et al., 1998; Erb and Stewart, 1999; Erb et al., 2000; Shaham et al., 2000; Erb et al., 2001; Leri et al., 2002).

Animal Models for Studying Effects of Drugs of Abuse

One can study the influence of environmental factors, such as stress or pharmaceutical manipulations, on drug seeking behavior. Outlined below are the two most commonly used procedures assessing rewarding effects of drugs in experimental animals: intravenous drug self-administration and conditioned place preference (CPP). Additionally, behavioral sensitization, along with self-administration and CPP, is a method that is often used to assess relapse.

The basic concept of self-administration relies on the ability of natural rewards, such as food and water, and drugs of abuse to act as positive reinforcers. Animals press a lever which consequently leads to the infusion of the drug (i.e. cocaine). This can in turn lead to positive reinforcement of the lever-pressing behavior (reviewed in Le Foll and Golberg, 2005, Schramm-Sapyta, 2005). Stages of addiction (such as tolerance, desensitization) can be observed by this method.

CPP is another commonly used behavioral task that is correlated to drug addiction mostly because of its technical simplicity. More specifically, CPP is a model of drug reward among other things (Carr et al., 1988). Briefly, an animal is trained to associate one side of a chamber with sensations felt after injection of a drug and the other with a neutral stimulus, such as a saline injection. After repeated associative training, the

animal is allowed free choice between two sides of the chamber. The time spent on the drug associated side of the chamber indicates the rewarding properties of the drug. The task is more suitable to study early stages of drug addiction in which the drug is “enjoyable” (Schramm-Sapyta, 2005) yet can still be used as a model of relapse. Reinstatement of CPP in extinguished animals can be induced by drug priming (Mueller and Stewart, 2000) as well as stress (Wang et al., 2001; Lu et al., 2003).

The development of enhanced behavioral sensitivity to psychostimulants with repeated administration has been well documented (reviewed in (Kalivas et al., 1998). Behavioral sensitization is typically characterized by an augmented motor responsiveness to a cocaine or amphetamine challenge. The long-lasting changes mediating behavioral sensitization are suggested to in part underlie an increased susceptibility to relapse to drug seeking behaviors (Piazza et al., 1989; Robinson and Berridge, 1993).

Anatomy of Addiction

Brain Circuitry Involved in Reward

Over two decades ago Olds and colleagues demonstrated that rodents will work to electrically stimulate relatively discrete areas of the brain, which demonstrates the existence of so-called, brain-reward regions (Olds, 1982; Wise, 1996). Subsequently, other groups found that rodents also work to self-administer drugs of abuse (but not other drugs) and that this self-administration behavior is disrupted by lesioning these brain-reward regions (Koob, 1998; Wise, 1998). The critical regions in the reward circuitry are now taken for granted as involving the mesolimbic dopamine system, more specifically

the VTA/NAc pathways (Figure 1)(Nestler, 2004). Yet, the VTA-NAc pathways are only part of a series of parallel, integrated circuits, which involve several other key brain regions.

The VTA contains dopaminergic neurons, which are thought to convey information involved in integration of the rewarding vs. aversive properties of environmental stimuli (natural reward, drug of abuse, stress (Li et al., 2000)). As suggested above, the VTA projects to the NAc and prefrontal cortex (PFC), which together are referred to as the mesocorticolimbic dopamine system. Glutamate from various inputs such as the BLA, hippocampus, PFC and even the BNST act on dopaminergic neurons in the VTA (Christie et al., 1985; Kim and Vezina, 1999; Georges and Aston-Jones, 2002). Therefore, glutamatergic output from these regions onto the VTA neurons are likely important in behaviors associated with addiction.

The NAc, also known as the ventral striatum, is a principle target of VTA dopamine neurons and it mediates the rewarding effects of natural rewards and drugs of abuse (Koob, 1999). This region can be divided into at least two main subregions, the core and the shell. Similar to the dorsal striatum, the NAc primary output neurons are GABAergic medium spiny neurons. Neuronal activity in the NAc depends heavily on excitatory inputs from the PFC, hippocampus and the BLA. Key to the rewarding effects of psychostimulants and other drugs of abuse is the role of dopamine signaling in the

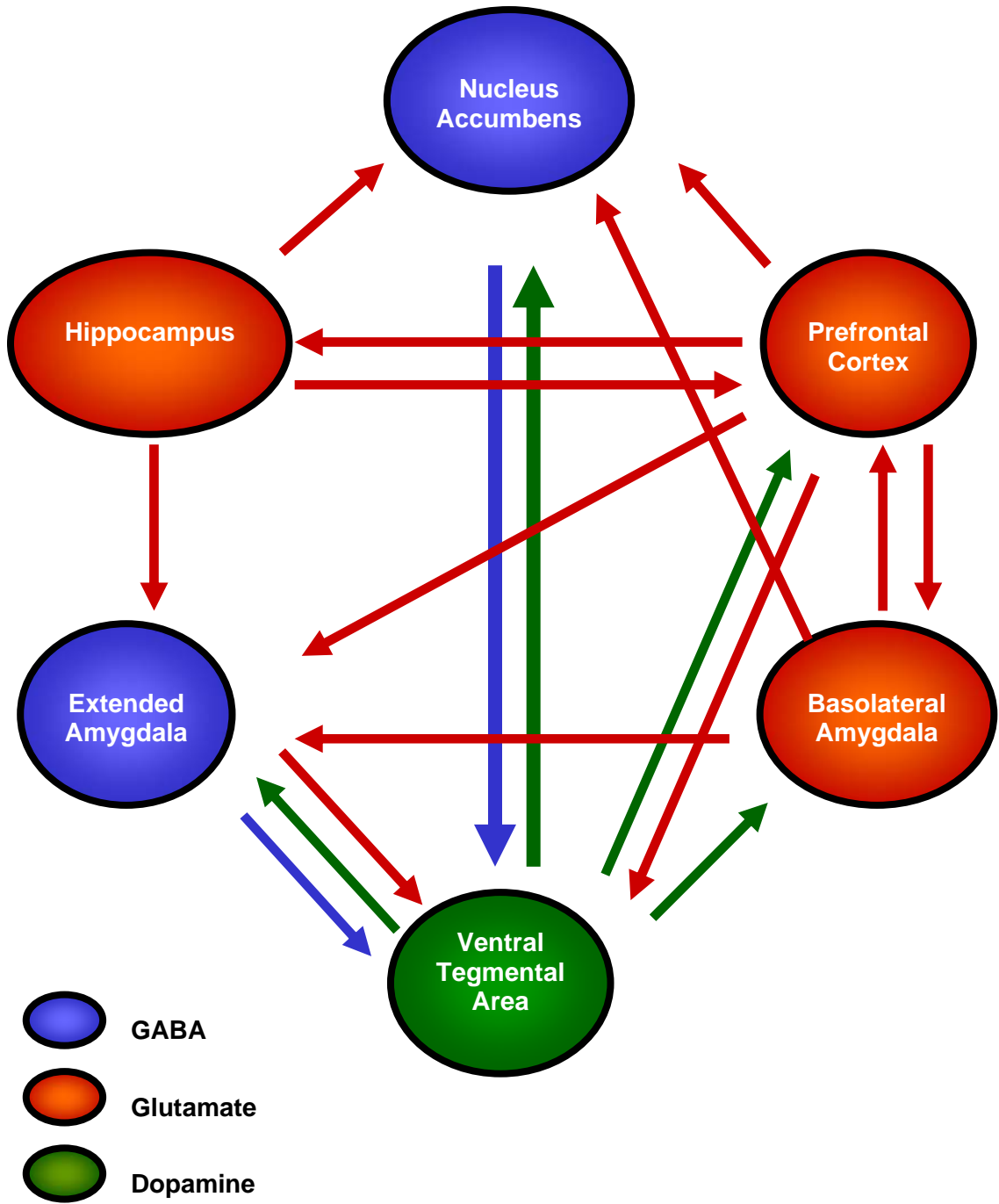


Figure 1. Schematic of reward pathways.

NAc. For instance, cocaine is self-administered directly into the rat NAc (McKinzie et al., 1999; Rodd-Henricks et al., 2002), and injection of amphetamine into the NAc can induce reinstatement (Stewart and Vezina, 1988).

The PFC is a critical part of the motivational network, it regulates the overall motivational significance and determines the intensity of behavioral responding (Goldstein and Volkow, 2002; Volkow et al., 2002). In other words, cognitive processes involved in goal-directed behaviors are thought to depend on prefrontal cortex function (Hyman, 2005). The output of the PFC is glutamatergic, and is modulated by DA among other neurotransmitters (Van Eden and Buijs, 2000). Consistent with involvement of dopaminergic afferents, the activation of the prefrontal cortex by rewarding stimuli is strongly influenced by the predictability of the reward.

The amygdala, more specifically the BLA, is particularly important for conditioned forms of learning. It is widely accepted that the amygdala is associated with fear conditioning and the processing of negative emotions. But this structure is also involved in the processing of positive emotions, and particularly in learning about the positive value of stimuli. It also interacts with the VTA-NAc pathway through excitatory inputs to these regions. The BLA therefore acts to integrate the positive or negative value of an environmental stimulus (natural reward, drug of abuse, stress).

The extended amygdala is connected with the VTA and several other brain structures hypothesized to be involved in the reinforcing effects of abused drugs (Koob, 1999, 2003). The extended amygdala is composed of several basal forebrain regions that have similar morphology, immunoreactivity and connectivity. Along with the NAc shell, the BNST and the central nucleus of the amygdala (CeA) make up the extended

amygdala. The extended amygdala is the aforementioned area implicated as a key mediator of stress-induced relapse (Stewart, 2000, 2003).

Molecular Targets in Drug Addiction

Dopamine and Drug Addiction

Much research in the NAc has illuminated an intracellular signaling cascade downstream of DA which ultimately leads to changes in gene expression through which exposure to cocaine modifies subsequent responsiveness to the drug. Dopamine transmission is upregulated during maintenance of the reinforcing properties of cocaine. Depletion of dopamine in the NAc decreases reinforcement behaviors (Roberts et al., 1980; Pettit et al., 1984; Caine and Koob, 1994). Consistent with a role of increased DA in the NAc, D1-like and D2-like DA receptors are implicated in drug addiction. The D1-like DA receptors consists of D1 and D5 which are positively coupled to the cAMP signaling cascade, whereas D2-like receptors consist of D2, D3 and D4 and are linked to inhibition of adenylyl cyclase activity. Antagonists of these receptors decrease the reinforcing effects of cocaine (Caine and Koob, 1994) for review see (Everitt and Wolf, 2002). More specifically D1-like and D2 DA receptor activation in the NAc reduces the reinforcing effects of cocaine (Caine et al., 1995; Bari and Pierce, 2005). Furthermore, downstream mechanisms of DA receptor signaling have also been implicated in the reinforcing effects of cocaine and amphetamines. For instance, chronic cocaine use resulted in decreased levels of the G-protein linked to inhibition of adenylyl cyclase activity, $G_{\alpha i}$, which coincided with an increase in adenylyl cyclase and PKA activity

(Striplin and Kalivas, 1993; Schoffelmeer et al., 1996; Unterwald et al., 1996).

Consistent with the effects of increased PKA activity following chronic cocaine use, it was found that injection of the nonhydrolyzable cAMP analogue Sp-cAMPS into the NAc increased cocaine self-administration (Self et al., 1998). Another target downstream of D1-like DA receptors is the extracellular signal regulated kinase pathway. Recent discoveries show D1-like DA receptor dependent enhancement of ERK activation in a number of brain regions including the BNST following exposure to addictive but not non-addictive drugs (Valjent et al., 2004).

Cocaine has been shown to alter gene expression downstream of DA receptor and cAMP signaling in the NAc (Nestler, 2004). For instance overexpression of CREB in this region decreases the rewarding effects of cocaine, while reducing CREB signaling by overexpression of a dominant-negative mutant CREB increases the rewarding effects of cocaine (Carlezon et al., 1998). A potential downstream effect of DA receptor and cAMP signaling cascades in the NAc following cocaine or amphetamine exposure is alterations in synapse structure. For instance, it was found that chronic amphetamine administration causes an increase in dendritic spine density and number of branched spines in the NAc (Robinson and Kolb, 1997).

Glutamatergic Transmission and Drug Addiction

A common feature of the brain regions of the reward circuitry studied to date (i.e. the NAc, VTA, BLA, and PFC) is that they possess high levels of glutamate receptors and are subject to multiple forms of synaptic plasticity. It is therefore not surprising that drugs of abuse alter glutamatergic activity in these complex networks which leads to

fundamental changes in the output of the reward circuitry and ultimately the behavior of the organism. Changes in glutamatergic transmission following exposure to drugs of abuse will be discussed in greater detail in later sections.

Stress and Anxiety

As is the case for addiction, stress-related disorders such as anxiety and depression are a major health concern (Wong and Licinio, 2004). Stress is the imposition or perception of environmental or physical change, either positive or negative, that elicits a spectrum of physiological homeostatic alterations in the organism (Herman and Cullinan, 1997). Anxiety can be defined as a state of apprehension, uncertainty, and fear resulting from the anticipation of a realistic or imagined threatening event or situation. In general, anxiety- and stress-related illnesses are thought of as a collection of disorders that have in common excessive or inappropriate brain excitability within crucial brain circuits, which leads to the expression of a spectrum of psychic (for example, excessive worry) or somatic (for example, disruptions of sleep, cardiovascular and gastrointestinal functions) symptoms. Anxiety disorders represent a range of conditions that include generalized anxiety, panic attacks, post-traumatic stress disorder, obsessive–compulsive syndrome and social phobias.

Adaptations to stressors result in complex responses involving changes in mood, cognition, behavior, autonomic function and endocrine output (Dayas et al., 2001). A key component in the neurocircuitry of stress is the hypothalamo-pituitary-adrenocortical axis (HPA). The hypothalamus is the major integrating link between the nervous system and the endocrine systems. Briefly, painful, stressful, and emotional stimuli can lead to

activation of the paraventricular nucleus of the hypothalamus (PVN). Hormones released from the hypothalamus in turn stimulate or suppress the pituitary gland by secretion of releasing hormones or inhibiting hormones, respectively. The pituitary is then capable of secreting corticotrophs which leads to secretion of adrenocorticotrophic hormone (ACTH) which then can stimulate the adrenal cortex to secrete glucocorticoids.

Ultimately, the secretion of corticotrophin-releasing hormone, a key neuropeptide involved in integrating hormonal, autonomic and behavioral responses to stress is a critical mediator of the anxiety state (Koob, 1999). However, much work has demonstrated that dopamine is one of the transmitters most potently modulating the mechanisms underlying states of fear and anxiety (Millan, 2003). The limbic system has been linked to emotionality, emotional evaluation of sensory stimuli, emotional learning and memory, and affective disorders (anxiety and depression). Additional components in the stress response are the afferent regions that modulate the HPA axis including parts of the limbic system, cerebral cortex and thalamus (Figure2).

In addition to the neurotransmitters traditionally known to be involved in stress/anxiety disorders, the monoamines, γ -amino-butyric acid (GABA), and many other modulators have been targeted for therapeutic intervention of stress/anxiety disorders. These include but are not limited to adenosine, cannabinoids, neuropeptides, hormones and glutamate. Accordingly, though benzodiazepines (which reinforce transmission at GABA_A receptors), serotonin (5-HT)_{1A} receptor agonists and 5-HT reuptake inhibitors are currently the main drugs used for treatment of anxiety disorders, there is considerable scope for the development of alternatives (Millan, 2003). However, targeting these systems has proven to have either limited efficacy or unwanted side effects. For instance,

dysregulation of monoaminergic function cannot fully explain psychiatric disorders. The modulation of glutamatergic transmission by mGluRs has promising therapeutic implications for treating stress/anxiety disorders and will be the focus of later sections.

Processive vs. Systemic stress

Stressors can be separated into at least two categories “processive” and “systemic” (Herman and Cullinan, 1997). Processive stressors recruit brain regions involved in higher order decision making to determine if a perceived stimulus is “stressful”. Systemic stressors are innate responses to a stimulus that poses a direct immediate threat to survival such as a hemorrhage or hypoxia. Systemic stressors do not require higher level processing to elicit a response (Herman and Cullinan, 1997).

A key anatomical and functional converging point of the processive and systemic stress pathways prior to reaching the PVN is the BNST. As the BNST is composed of multiple subnuclei, the effects of activation of this region by specific stimuli are complex. For instance, electrical stimulation of the lateral BNST resulted in a decrease in plasma corticosterone levels, an output of the HPA axis, while stimulation of the medial BNST had the opposite effect (Dunn, 1987). Additionally, lesioning the anterolateral BNST results in a decrease in CRF mRNA in the PVN whereas lesioning the posterior medial BNST results in an increase in CRF mRNA in the PVN (Herman et al., 1994).

Bed Nucleus of the Stria Terminalis (BNST)

The BNST is a key anatomical bridge uniquely positioned to integrate responses to stress as well as drugs of abuse (Figure 3). It is located along a rostral-caudal gradient

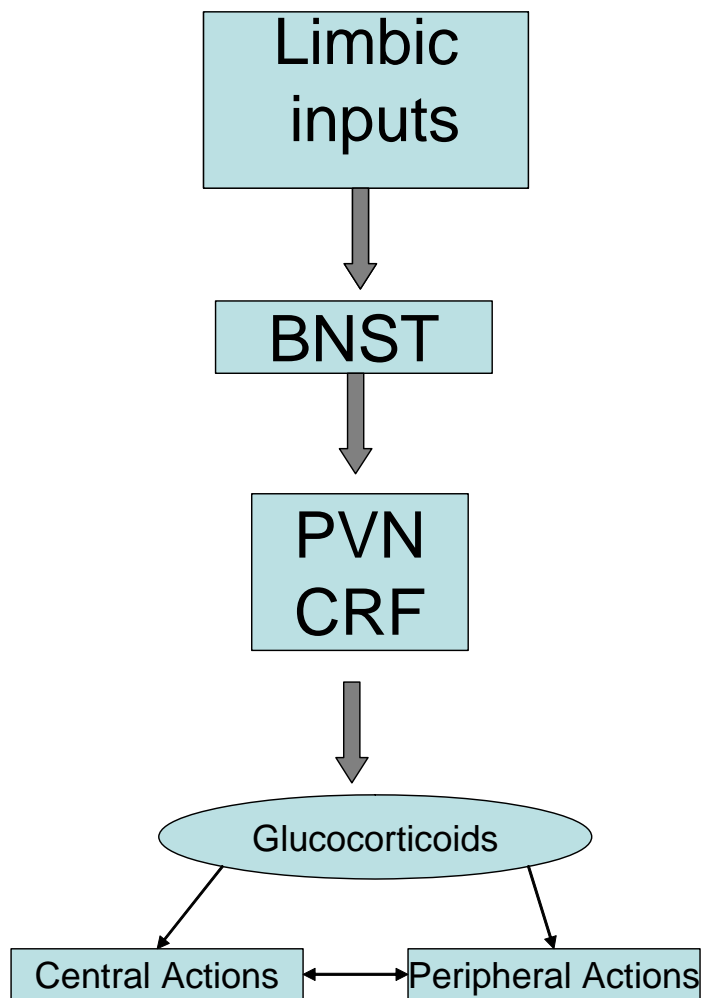


Figure 2. Schematic of stress circuitry.

in the rostral portion of the basal forebrain. The BNST consists of many nuclei which have been grouped into divisions. Original data presented in this thesis were obtained from neurons in the anterior division of the BNST which can be divided into the anterodorsal, anterolateral and anteroventral areas (which will sometimes be referred to as the dorsolateral BNST or dBNST). More specifically, the dBNST can be divided into separate nuclei; the antolateral nuclei, consisting of the juxtacapsular, oval rhomboid, and fusiform nuclei (Dong et al., 2001). Physiologically, the BNST has multiple functions which include but are not limited to the integration of information to the stress and reward pathways. A majority of BNST neurons stain positive for glutamic acid decarboxylase (GAD), the enzyme responsible for the conversion of glutamate to GABA (Sun and Cassell, 1993). This would suggest the primary output of the BNST is GABAergic; however, evidence for other neuropeptides and glutamatergic outputs exist (Ju and Swanson, 1989; Georges and Aston-Jones, 2002).

Characterization of membrane properties of BNST neurons was done by the Winder lab as well as preliminary work reported by Donald Rainnie (Rainnie, 1999; Egli and Winder, 2003). Briefly, neurons in the BNST have a resting membrane potential of -68 to -69 mV and a reported input resistance of approximately 115-128 M Ω when measured with sharp microelectrodes (Egli and Winder, 2003). An important feature of these neurons is that they are under tonic GABAergic inhibition, possibly keeping these cells in a quiescent state.

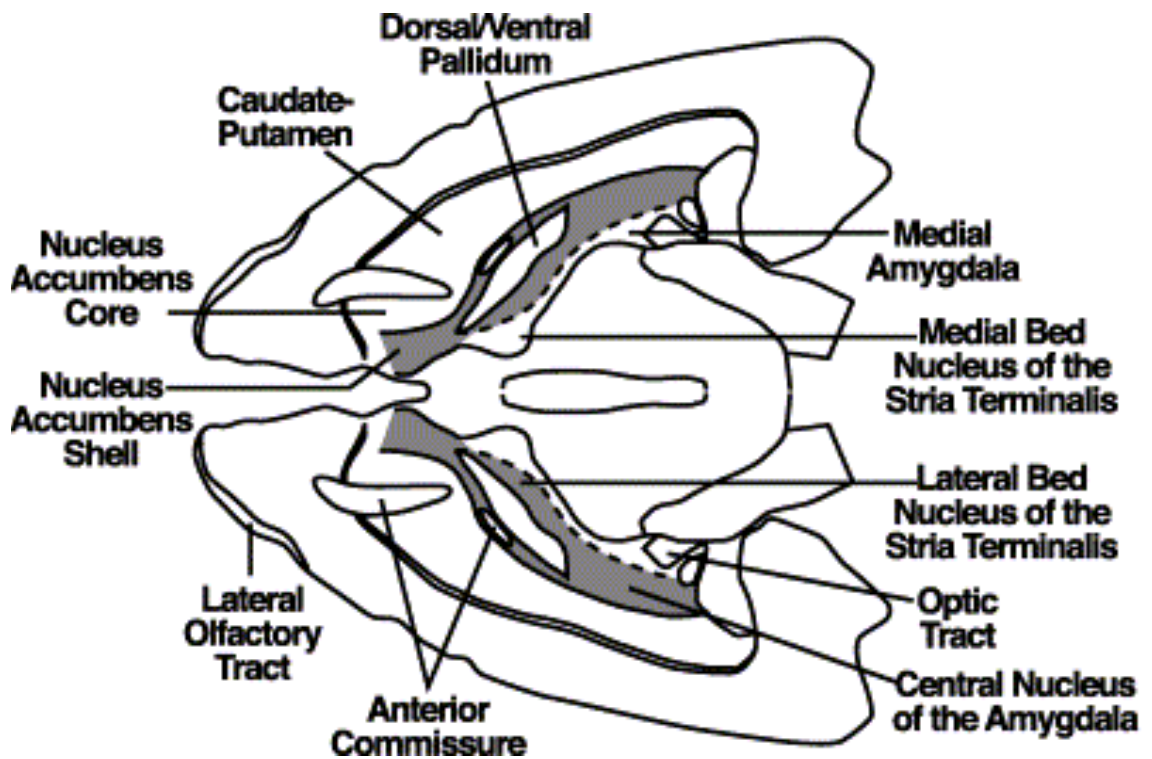


Figure 4. Diagram illustrating the extended amygdala, its neuropharmacological components, afferent and efferent connections and functional attributes (Adapted from Koob, 2004).

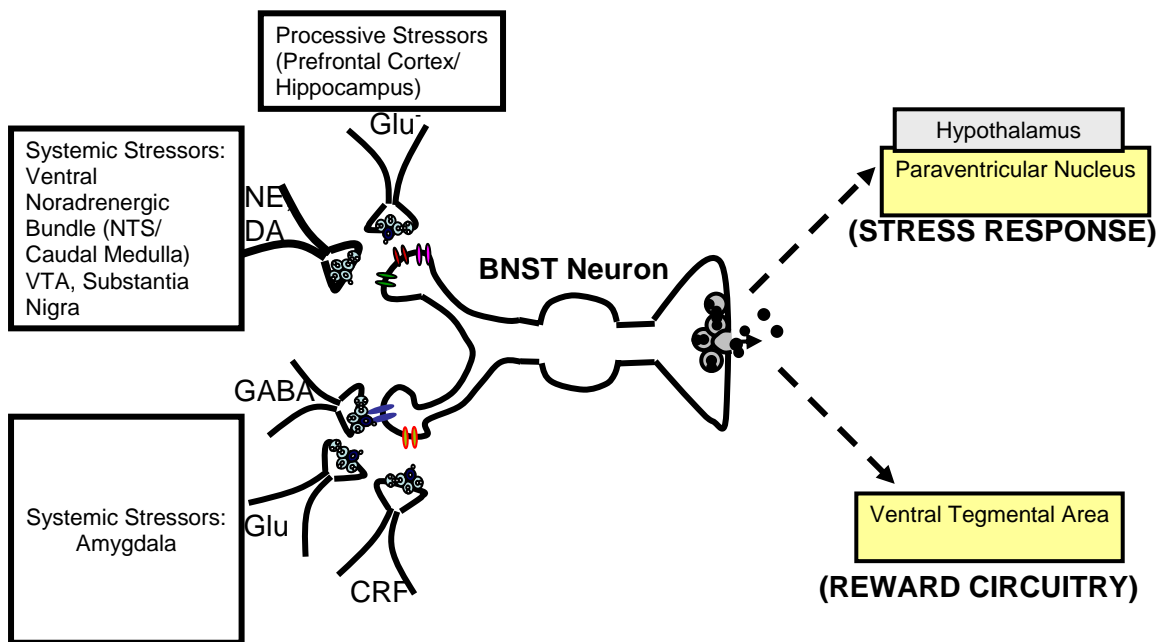


Figure 3. Schematic representing the key afferents and efferents of the BNST.

BNST Afferents

The BNST receives excitatory inputs primarily from the ventral subiculum of the hippocampus, limbic cortical regions and BLA inputs (Cullinan et al., 1993; Snyder et al., 2001). The glutamatergic inputs to the BNST are variable. For instance, infralimbic cortical inputs are lighter to the dBNST in respect to the vBNST. Additionally, there are no direct inputs from the prelimbic cortex to the dBNST with only light innervation to the vBNST (McDonald et al., 1999).

There is also functional and behavioral evidence suggesting glutamatergic inputs to the BNST are important in the output of this region. Of behavioral relevance, blockade of non-NMDA ionotropic glutamate receptors (iGluRs) transmission in the BNST resulted in an attenuation of fear-potentiated startle (Walker and Davis, 1997). Functionally, Boudaba and colleagues were able to elicit IPSPs in the PVN in response to glutamate puffing in the posterior BNST (Boudaba et al., 1996). Additionally, recent work in the Winder lab has shown local stimulation of the BNST in an *in vitro* slice preparation elicits excitatory AMPA receptor mediated currents that are sensitive to noradrenergic modulation (Egli and Winder, 2003; Egli et al., 2005). Further work from the Winder lab demonstrated that NMDAR-dependent LTP of excitatory transmission can be elicited in this region and that this LTP is sensitive to ethanol (Weitlauf et al., 2004).

In addition to glutamate, the BNST also receives noradrenergic, dopaminergic, serotonergic and CRF inputs. NE inputs arise primarily from the ventral noradrenergic bundle (Roder and Ciriello, 1994). Consistent with NE innervation of the BNST, NE can

modulate the activity of BNST neurons as well as synaptic transmission (Egli and Winder, 2003; Egli et al., 2005).

There is a strong dopaminergic input to the dorsal BNST coming from various brain regions including the substantia nigra, VTA, dorsal raphe nucleus, periaqueductal gray and the brain stem through the medial forebrain bundle (Sawada and Yamamoto, 1981; Phelix et al., 1992; Hasue and Shammah-Lagnado, 2002). Consistent with other brain regions which receive a vast dopaminergic innervation, drugs of abuse such as nicotine, morphine, cocaine, amphetamine, and ethanol increase dialysate DA levels in the BNST. It is notable that the magnitude of the effect and the sensitivity to the drug is higher in this area as compared to the NAc shell (Di Chiara et al., 1999).

Serotonergic input to the BNST is from the midbrain raphe nuclei (Phelix et al., 1992, 1992). Functional studies have shown that c-fos expression in the BNST is increased by *in vivo* manipulations that either enhance, or deplete, 5-HT levels (Li and Rowland, 1996; Chung et al., 1999; Morelli and Pinna, 1999). Additionally activation of serotonergic system in the BNST, most likely through 5-HT_{1A}, leads to an inhibition of BNST neurons (Rainnie, 1999; Levita et al., 2004).

BNST Efferents

As has been suggested by the arguments above, the BNST acts as a key intermediary by receiving stressor inputs from cortical regions and sending its own projections to the stress response regions of the brain, more specifically the PVN, to regulate hormonal CRF release and pituitary activation and ultimately the stress response. Several regions are reciprocally connected to the BNST. For instance, the BNST sends

projections to the VTA, raphe nucleus and the CeA, all of which send projections back to the BNST. Of great importance, the BNST is one of a discrete set of regions innervating the parvocellular CRF-containing PVN neurons including the other hypothalamic subnuclei, brainstem nuclei such as the raphe and the nucleus of the solitary tract (Marsicano et al., 2003).

Consistent with interconnectivity to the stress pathways, inhibition of glutamatergic transmission in the BNST by AMPA receptor antagonists blocks stress responses to some stressors. Specifically, glutamatergic inhibition in the BNST leads to blockade of light enhanced startle and CRH-mediated startle, but not fear-potentiated startle (Walker et al., 2003). Therefore, excitatory, glutamatergic transmission in the BNST plays a critical role in the response to stress and anxiety.

In addition to the BNST being interconnected to the stress axis, it is a brain region uniquely positioned to regulate reward centers. For example, the dBNST sends projections to the NAc (Dong et al., 2001). Furthermore, as mentioned above, the vBNST projects to and regulates the firing of dopaminergic cells within the VTA (Georges and Aston-Jones, 2002). There is evidence from microinfusion of ionotropic glutamate receptor antagonists indicating glutamatergic influence on VTA cells from efferents of the vBNST (Georges and Aston-Jones, 2002). Thus the BNST is uniquely positioned to receive stress axis information and integrate it into reward/motivation circuitry.

The BNST and Stress-Induced Relapse to Drug Seeking

Consistent with the anatomical interconnections mentioned above, recent data demonstrate that the BNST plays a key role in mediating stress-induced relapse to cocaine-seeking behavior (Erb and Stewart, 1999; Sinha et al., 1999; Shaham et al., 2000; Erb et al., 2001), as well as in stress-induced maintenance and reinstatement of morphine-conditioned place preference (Wang et al., 2001). Furthermore, Delfs et al. (2000) showed that the BNST plays a key role in morphine withdrawal-induced conditioned place aversion (Delfs et al., 2000). Additional work in the vBNST suggested that excitatory synaptic transmission is enhanced following cocaine self-administration (Dumont et al., 2005). The background present here makes synaptic plasticity in the BNST an attractive substrate for mediating drug-related behaviors.

Synaptic Transmission

Cells of the central nervous system consist of neurons and glia. Most information transfer in the brain is via an electrical and chemical process referred to as synaptic transmission (Kandel et al., 2000). Neurons exhibit specific organization of their membrane components that allow for electrical signaling. A typical neuronal response involves the depolarization of a cell body that leads to the propagation of an electrical signal down the axon. At the end of the axon are specialized structures called presynaptic terminals. Depolarization of the presynaptic terminals results in calcium entry and transiently enhances the probability of release of neurotransmitter from synaptic terminals. Neurotransmitters, the chemical component of the electro-chemical signal, act at receptors on postsynaptic membranes. Dendrites are the specialized region of neurons

and are the primary target for synaptic input. As such they act to receive and integrate information from other neurons. The postsynaptic membrane contains ligand-gated ion channels that, when activated flux sodium and potassium (sometimes calcium) which results in the depolarization of the local membrane. If the membrane is sufficiently depolarized, an action potential is propagated down the dendrites to the cell body and thus the process can repeat down the axon to the next synapse in the circuit. Synaptic transmission can be grouped into three forms: fast excitatory, fast inhibitory and neuromodulatory. The focus of this thesis is on excitatory transmission and its modulation.

Excitatory Transmission

Glutamate is the major excitatory neurotransmitter in the mammalian brain. Since its discovery as a neurotransmitter in 1954 (Hayashi, 1954), glutamate has been investigated for its function in various processes including but not limited to neuronal plasticity and more recently neuromodulation. Due to the immense literature, the aim of this introduction on glutamatergic transmission is to provide an overview of excitatory transmission and neuromodulation of synaptic transmission by mGluRs. An emphasis is placed on how synaptic plasticity, mGluRs and their potential downstream targets play a role in drug addiction and stress/anxiety.

Different classes of glutamate receptors in the postsynaptic membrane transduce the glutamate signal released from the presynaptic terminal into electrical and biochemical events in the postsynaptic neuron. Glutamate release from synapses activates ion channel-forming receptors, the iGluRs, at postsynaptic cells and consequently

mediates fast excitatory postsynaptic transmission. The subsequent discovery of metabotropic glutamate receptors (mGluRs) revealed that glutamate can also mediate slow synaptic potentials, modulate ion channels and activate GTP binding proteins. mGluRs will be discussed in detail later.

The iGluRs are classified as NMDA (NR1, NR2A–D, NR3), AMPA (GluR1–4) and kainate (GluR5–7, KA1–2) receptors, based on their pharmacological characteristics and sequence information (Hollmann and Heinemann, 1994; Borges and Dingledine, 1998; Dingledine et al., 1999). Whereas AMPA and kainate receptors contribute to fast neurotransmission, all three ionotropic subtypes are thought to play roles in synaptic plasticity (Bortolotto et al., 1999). AMPA receptors (AMPA receptors) are hetero-oligomeric proteins that form a tetrameric receptor complex (Rosenmund et al., 1998). In the adult hippocampus two species of AMPAR appear to predominate: receptors made of GluR1 and GluR2 or those composed of GluR3 and GluR2 (Wenthold et al., 1996). The AMPAR complexes form ligand-gated ion channels that when bound by glutamate, flux sodium and potassium (sometimes calcium) consequently depolarizing the cell membrane. The resulting effect can be recorded as a potential (excitatory postsynaptic potential, EPSP) or a current (excitatory postsynaptic current). The NMDA receptor is an ion channel that is inactive at resting membrane potentials due to a voltage sensitive magnesium block. Upon ligand binding (glutamate and glycine) and a sufficient depolarization, NMDA receptors flux sodium, potassium and calcium. Regulation of AMPA receptors will be covered in more detail in a later section.

Synaptic plasticity

Bliss and Lomo (1973) obtained the first solid experimental evidence supporting the theory that information storage in the brain involves alteration in the strength of synaptic communication between mammalian neurons. A lasting alteration in synaptic strength is referred to as synaptic plasticity. Synaptic plasticity lasts for many hours in brain slices and even for weeks in intact animals (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973; Malenka and Nicoll, 1999). In long term potentiation (LTP), brief repetitive stimulation of excitatory glutamate containing pathways (produced by high frequency stimulation, referred to as a tetanus) leads to an increase in synaptic strength. Different (usually lower frequency) patterns of synaptic activation produce an opposite change, termed long term depression (LTD) (Dudek and Bear, 1992; Mulkey and Malenka, 1992; Kirkwood et al., 1993).

Changes in synaptic efficacy can occur by presynaptic mechanisms such as altered neurotransmitter release as well postsynaptic mechanisms. One primary means of persistently modulating synaptic transmission is by changing the activity or abundance of postsynaptic AMPAR's (Carroll et al., 1999). A more detailed description of synaptic locus is developed in the mGluR section.

AMPA regulation

The expression of excitatory synaptic plasticity is largely thought to be mediated by alterations in AMPA receptor expression and function in the postsynaptic cell. Additionally, changes in subunit composition can influence AMPA receptor function as

this composition influences the functional properties of iGluRs (Borges and Dingledine, 1998; Dingledine et al., 1999).

The regulation of AMPAR function during synaptic plasticity occurs via two related mechanisms. First, there is evidence for covalent modification (phosphorylation and dephosphorylation) of synaptic AMPARs resulting in an enhanced ion flux. For instance, CaMKII phosphorylation of GluR1 leads to an increase in single channel conductance of AMPARs (Derkach et al., 1999). Changes in phosphorylation of GluR1 at S831 (Esteban, 2003; Esteban et al., 2003), a CaMKII/PKC site and at S845 (Roche et al., 1996; Barria et al., 1997; Mammen et al., 1997), a PKA site also occur during LTP and LTD.

Second, like all integral membrane proteins, glutamate receptors turn over in the membrane and importantly phosphorylation is implicated in this process as well. Activity can drive AMPARs into the synapse resulting in LTP (Liao et al., 2001; Esteban, 2003; Esteban et al., 2003) or remove them from the synapse resulting in LTD (Lissin et al., 1999). Along with lateral movement from extrasynaptic sites, increasing or decreasing AMPARs levels at the synapse occurs via vesicular exocytosis and endocytosis (Carroll et al., 1999).

AMPARs are delivered to synapses through two different pathways, depending on their subunit composition. As mentioned above, they contain tetrameric combinations of subunits GluR1 to GluR4 (Keinanen et al., 1990), each interacting with a specific set of intracellular proteins (Scannevin and Huganir, 2000). An important distinction among AMPAR subunits is the nature of their C-terminal tails, which are either long or short. GluR1 always has a long C-terminal tail and GluR4 a short C-terminal tail. The C-

terminal tails of GluR 2 and 3 are alternatively spliced such that these subunits can have either long or short C-terminal tails.

Cycling of GluR1 (long form) to the postsynaptic membrane requires synaptic activity whereas GluR2 (short form) membrane expression is constitutively turning over (Passafaro et al., 2001; Piccini and Malinow, 2002). There are also constitutive and regulated pathways for removal of AMPA receptors from the synapse as well (Carroll et al., 1999; Carroll et al., 2001; Luscher and Frerking, 2001; Brecht and Nicoll, 2003). Regulated endocytosis of postsynaptic AMPA receptors mediates many types of LTD, including NMDA receptor-dependent LTD, mGluR-dependent LTD, and endocannabinoid-dependent LTD (Malenka and Bear, 2004). LTD appears to involve the internalization of GluR1 containing AMPA receptors as well as AMPA receptors that overlap with the constitutively recycling pool, GluR2 (Collingridge et al., 2004). Therefore, molecules involved in membrane trafficking and structural integrity of the postsynaptic signaling complexes play an important role in the initiation and maintenance of synaptic plasticity.

Psychostimulants and Glutamatergic Synaptic Plasticity

As mentioned earlier, although psychostimulants such as cocaine directly interact with monoaminergic signaling (e.g. DA, NE and 5-HT), there is a strong body of evidence suggesting alterations in excitatory synaptic plasticity play an important role in behaviors related to addiction (Thomas et al., 2001; Beurrier and Malenka, 2002; Fourgeaud et al., 2004).

As mentioned above, dopamine neurons in the VTA receive dense glutamatergic innervation (Christie et al., 1985; Kim and Vezina, 1999; Georges and Aston-Jones, 2002), interestingly, these synapses have been shown to undergo synaptic plasticity subsequent to *in vivo* treatment with several drugs of abuse. More specifically drugs of abuse elicit increased excitatory input (LTP as defined by increases in AMPA/NMDA ratios) while attenuating LTD mechanisms in the VTA (Jones et al., 2000; Ungless et al., 2001; Saal et al., 2003; Borgland et al., 2004). Further studies revealed that amphetamine blocks LTD in the VTA via release of DA and activation of D2 receptors. Another potential mechanism for increased synaptic efficacy of VTA dopamine neurons is an increase in AMPA receptor expression following cocaine administration (Fitzgerald et al., 1996; Lu et al., 2002; Lu et al., 2004). Similarly, in humans, cocaine overdose is associated with VTA up-regulation of the GluR2 AMPA receptor subunit (Tang et al., 2003).

Downstream of the VTA, NMDAR-dependent LTD in the NAc in naïve animals is unaltered by DA or DA receptor antagonists (Thomas et al., 2000) and is associated with internalization of AMPA receptors and decreases in synaptic AMPA receptor expression (Heynen et al., 2000; Malinow and Malenka, 2002). However, to complicate matters, variances in molecular targets of drugs of abuse in the NAc during different stages of addiction have been observed. For instance, in contrast to drug-induced regulation of synaptic plasticity in the NAc, extinction training during withdrawal from chronic cocaine self-administration leads to an up-regulation in both GluR1 and GluR2 AMPA subunits in the NAc (Lu et al., 2003; Sutton et al., 2003), the same subregion exhibiting LTD in cocaine withdrawal (Thomas et al., 2001). Extinction training

diminishes cocaine-seeking behavior as animals learn that cocaine reinforcement is no longer available. This leads to a strong correlation between AMPA receptor expression in the NAc and drug seeking behavior. Furthermore, either GluR1 or GluR2 over-expression during extinction training leads to prolonged attenuation of stress-induced relapse to cocaine seeking (Self and Choi, 2004).

Neuromodulation

Neuromodulation is a subtle influence on synaptic efficacy or neuronal excitability. It is this “fine tuning” by neuromodulators that make them attractive therapeutic targets for disorders such as addiction and stress/anxiety. Neuromodulation occurs through signaling of neurotransmitters, such as monoamines, glutamate or neuropeptides working primarily through G-protein coupled receptors. Neuromodulation can also involve longer-lasting changes such as alterations in gene expression. As mentioned above, and as the major focus of this thesis, there are mGluRs that function to modulate neuronal transmission. Other neuromodulatory systems include metabotropic GABA receptors, monoamine neurotransmitter receptors and multiple neuropeptide systems such as CRF.

G-Protein Coupled Receptors

G-protein coupled receptors (GPCRs) are 7 transmembrane domain proteins that interact with G-proteins. Receptor activation leads to associated G-protein hydrolase activity and ultimately to activation or inhibition of an effector protein (Pierce et al., 2002). G-protein regulation of effectors occurs through two broad mechanisms. First

there is direct G-protein binding to ion channels. More traditionally, G-proteins function to activate or inhibit second messenger-forming enzymes leading to: direct second messenger binding to ion channels, second messenger activation of kinases or phosphatases that phosphorylate/dephosphorylate ion channels, or alterations in protein-protein interactions involving an ion channel.

There are multiple potential consequences of neuromodulation. Neuromodulation can lead to changes in probability of neurotransmitter release, small depolarization of membrane potentials accompanied by small conductance changes, small hyperpolarization of membrane potential accompanied by a conductance that resists depolarization, closing of K⁺ channels that prevent neurons from reaching AP threshold (e.g. M-type K⁺ current inhibition) and alterations in repolarization of the action potential. There are many channels that are potential targets of neuromodulation that lead to the aforementioned changes in membrane excitability. For example: voltage-gated channels (Na⁺, K⁺, Ca²⁺), inwardly rectifying K⁺ channels, channels that are active at RMP (i.e. “leak conductance” channels), ligand-gated channels (such as AMPARs) and cyclic nucleotide-gated channels.

Metabotropic Glutamate Receptors

In the mid-80's, glutamate was shown to stimulate IP₃ production indicating the potential existence of a “metabotropic” or non-ion-channel-forming glutamate receptor (reviewed in (Coutinho and Knopfel, 2002). This led to the distinction between ionotropic and metabotropic glutamate receptors and the search for the receptors responsible for the IP₃ production. In 1991, two groups isolated and cloned cDNA of the

first mGluR (Houamed et al., 1991; Masu et al., 1991). Today there are eight subclasses of mGluRs identified and cloned. mGluRs belong to family C of the G-protein coupled receptor superfamily and act to modulate neuronal excitability in many brain regions. The subclasses are classified into three groups based on pharmacology, similarities in coupling mechanisms and sequence homology (Table 1). mGluRs of the same group show about 70% sequence identity, whereas between groups this percentage decreases to about 45%. Group I receptors (mGlu1 and mGlu5) are linked to $G\alpha_q$, whereas, group II and group III mGluRs are coupled to $G\alpha_i$ (Gereau and Conn, 1994; Anwyl, 1999). Intracellularly, the large C-terminus of mGluRs can interact with a variety of signaling systems.

Functions of mGluRs

mGluRs largely extended the classical role of glutamate as a fast excitatory synaptic transmitter, acting as a neuromodulator and even as an activator of inhibitory mechanisms at certain synapses. They are widely distributed throughout the CNS, where they play important roles in regulating cell excitability and synaptic transmission (Gereau and Conn, 1994). More specifically, they can activate enzymes such as kinases which result in modifications in physiological functions such as the modulation of synaptic transmission, synaptic integration, and plasticity. In addition to activation of intracellular signaling cascades, direct and indirect modulation of various voltage sensitive ion channels by mGluRs results in a faster action on neuronal physiology. This impact on physiological activity is of principal interest as the firing pattern of neuronal networks, and changes in synaptic plasticity in these networks as a consequence of a behavioral

experience may provide a basis for understanding the role of mGluRs in pathophysiological conditions such as stress/anxiety and addiction.

Table 1. Classification of mGluRs

Family receptor	Coupling	Group/subtype-selective pharmacological agents
Group I		
mGluR1	Gq-coupled	Agonists: DHPG, 1S,3R-ACPD, quisqualate Antagonist: LY393675 Inverse agonist (allosteric antagonist): LY367385
mGluR5	Gq-coupled	agonists: DHPG, 1S,3R-ACPD, quisqualate, CHPG Inverse agonist (allosteric antagonist): MPEP
Group II		
mGluR2	Gi/o-coupled	Agonists: DCG-IV, LY354730, 1S,3R-ACPD Antagonist: LY341495
mGluR3	Gi/o-coupled	Agonists: DCG-IV, LY354730, 1S,3R-ACPD Antagonist: LY341495
Group III		
mGluR4	Gi/o-coupled	Agonists: L-AP4, L-SOP Antagonist: MSOP, MAP4
mGluR6	Gi/o-coupled	Agonists: L-AP4, L-SOP Antagonist: MSOP, MAP4
mGluR7	Gi/o-coupled	Agonists: L-AP4, L-SOP Antagonist: MSOP, MAP4
mGluR8	Gi/o-coupled	Agonists: L-AP4, L-SOP, 3,4-DCPG Antagonist: MSOP, MAP4

Modified from Swanson et al., 2005.

Glutamate Sources from which mGluRs can be Activated

Overall mGluRs have been shown to be primarily located perisynaptically. That is, mGluRs are often located in the areas adjacent to the synapse (Baude et al., 1993; Lujan et al., 1997). Functionally, their location suggests that they become activated in situations of repeated stimulation of afferents which results in considerable glutamate accumulation in the synaptic cleft coupled to spill-over to extra-synaptic sites. Therefore,

in an attempt to increase the amount of glutamate released at the synapse and to mimic physiological glutamate release, low frequency stimulation (LFS) is a commonly incorporated method to activate mGluRs (Charpak and Gahwiler, 1991; Batchelor and Garthwaite, 1997). This indicates that detectable receptor activation in many cells requires pooling of glutamate in order to reach sufficiently high levels at the extrasynaptic mGluR sites (Baude et al., 1993; Nusser et al., 1994).

In addition to the frequency of release, the extracellular concentration of glutamate also depends on the efficacy of neuronal and glial uptake, which is dictated by the number of glutamate transporters (for review see (Baker et al., 2002)). Glutamate transporters (GluTs) along with passive diffusion are the primary means of glutamate removal from the extracellular space. They function to prevent the accumulation of glutamate and influence the occupancy of receptors at synapses. The ability of metabotropic glutamate receptors to participate in signaling is tightly regulated by GluT activity (Kawasaki et al., 2004).

Another contributor to extrasynaptic glutamate is the cystine-glutamate exchanger. The non-synaptic cystine—glutamate antiporter exchanges extracellular cystine for intracellular glutamate, with a minimal contribution of synaptic glutamate release (Baker et al., 2002). Data from Kalivas' group suggests that one form of cocaine-induced neuroadaptation may be the decrease in basal levels of extracellular glutamate in the NAc after several weeks of withdrawal from cocaine, an effect attributed to a reduced function of the cystine—glutamate transporter (Baker et al., 2003; Moran et al., 2005).

Group I mGluRs

Group I receptors (mGluR1 and mGluR5) are linked to activation of phospholipase C leading to phosphoinositide hydrolysis, calcium release and protein kinase C activation. Activation of group I mGluRs can also indirectly regulate adenylyl cyclase leading to increased cyclic AMP formation, as well as cyclic GMP accumulation, PLA and ERK activation (Gereau and Conn, 1994; Anwyl, 1999).

To date, no one has looked at the expression of group I mGluRs in the BNST; however, they have been shown to be expressed in other brain regions belonging to the stress axis as well as the reward circuitry. Group I mGluR function has been reported at many of these synapses as well. For instance, both mGluR1 and mGluR5 function in the NAc and the PFC. However, although highly homologous, studies suggest these receptors have distinct functions in regulating synaptic transmission (Gereau and Conn, 1994; Attucci et al., 2001). This implies differential regulation of these receptors and potential divergence in effector systems. Conversely, at some synapses only one type of group I mGluR is functionally important: for instance only mGluR1 has been shown to function in the VTA and cerebellum (Shigemoto et al., 1992; Batchelor et al., 1997; Bellone and Luscher, 2005).

Group I mGluR expression at the plasma membrane and its signaling properties are dependent upon scaffolding proteins, more specifically, Homer proteins. There are three genes encoding Homer proteins. Homer 1a is an immediate-early gene product while the remainder (Homer 1b/c, Homer 2 and Homer 3) are constitutively expressed (Brakeman et al., 1997). Homer proteins link group I mGluRs through the C-terminal tail (Xiao et al., 2001). They are part of the scaffolding at postsynaptic densities (PSD) and

therefore link group I mGluRs with the proteins in the PSD. Homers also link these receptors to intracellular calcium stores via IP3 receptors and other scaffolding proteins such as Shank (Brakeman et al., 1997). It is suggested that Homer proteins may regulate the expression and function of group I mGluRs at multiple levels including targeting, surface expression, clustering, physical linkage to other synaptic and subsynaptic complexes and modulation of constitutive activity. Interestingly, biochemical evidence suggests the C-terminus of Homer 2a interacts with the Rho family of small guanosine triphosphatases (GTPases), in a GTP-dependent manner (Shiraishi et al., 1999). Homer proteins have been also been shown to form complexes with Shank proteins which act as scaffolding proteins and link group I mGluRs with other proteins in the postsynaptic densities (PSD) such as Dynamin2, an important molecule indicated in endocytosis and AMPAR-glutamate receptor interacting protein (GRIP) complexes (Tu et al., 1999).

Pharmacology of Group I mGluRs

The recent development of mGluR selective ligands (e.g. phenylglycine derivatives acting either as agonists or as antagonists) has helped begin the elucidation of the functional roles of mGluRs in brain and behavior. The pharmacological profile of group I mGluRs has been determined in mammalian heterologous expression systems on cloned mGluRs (Gereau and Conn, 1994). The rank order of potency of the most common agonists is: quisqualate > 3,5-dihydroxyphenylglycine (DHPG) = glutamate > 1S,3R-1-amino-1,3-cyclopentanedicarboxylate (ACPD) (Gereau and Conn, 1994; Pin and Duvoisin, 1995). The specific agonist for group I mGluRs used in the studies presented below is DHPG, which is devoid of activity at other mGluRs (Ito et

al., 1992; Fitzjohn et al., 1998). An agonist for mGluR5 has been reported (RS)-2-chloro-5-hydroxyphenylglycine (CHPG), although it is not very potent (Doherty et al., 1997).

The first antagonist described for group I mGluRs was α -methyl-4-carboxyphenylglycine (MCPG). However, MCPG also antagonizes group II mGluRs (reviewed in (Bordi and Ugolini, 1999). There are several drugs that selectively inhibit mGluR5; the most widely used is the noncompetitive antagonist MPEP (2-methyl-6-phenylethynyl-pyridine), with the caveat that at high concentrations it blocks NMDA receptors (Spooren et al., 2001).

Synaptic Locus of Group I mGluRs

Mechanisms underlying group I mGluR function vary across brain regions and synapses. Much information on function of these receptors can be gained by determining the synaptic locus of action of these receptors. Immunohistochemical and electrophysiological results reveal group I mGluRs localize and function presynaptically, postsynaptically and on glia (Shigemoto et al., 1993; Romano et al., 1995; Balazs et al., 1997; Conn and Pin, 1997; Lujan et al., 1997; Manzoni et al., 1997; Lopez-Bendito et al., 2002). However, group I mGluRs are predominantly located at the periphery of the postsynaptic density (perisynaptically) (Baude et al., 1993; Lujan et al., 1997).

Activation of group I mGluRs cause postsynaptic effects such as neuronal depolarization, excitation and spike frequency adaptation in a number of brain regions. For instance, selective activation of group I mGluRs with DHPG increases postsynaptic membrane excitability in hippocampus (Muda et al., 1996), cortex (Libri et al., 1997; Attucci et al.,

2001), striatum (Pisani et al., 1997), amygdala (Keele et al., 1997), subthalamic nucleus (Abbott et al., 1997) and hypothalamic nuclei (Schrader and Tasker, 1997).

Increase in neuronal excitability via activation of group I mGluRs is primarily through modulation of K^+ channels. Specific activation of group I mGluRs induces net inward currents by inhibiting K^+ channel conductance in neurons in the hippocampus (Gereau and Conn, 1994) and hypothalamus (Schrader and Tasker, 1997). For instance, K^+ channels are a major target of mGluRs, and many diverse types of K^+ channels are inhibited following activation of mGluRs. Activation of mGluR has been shown to block the I_{AHP} , (I_{AHP} is outward K^+ current with slowly activating and inactivating (~2s kinetics) and thereby reduce accommodation of spike firing in hippocampal CA1 pyramidal cells, CA3 inhibitory cells, the dentate gyrus, cultured cerebellar Purkinje cells and basolateral amygdaloid nucleus. The mGluR-mediated inhibition of I_{AHP} was found to be G-protein dependent.

Activation of group I mGluRs have also been shown to modulate voltage-gated calcium channels (VGCCs)(Lester and Jahr, 1990; Swartz and Bean, 1992; Sahara and Westbrook, 1993; Swartz et al., 1993; Chavis et al., 1995; Sung et al., 2001). Alterations in VGCCs can occur either presynaptically (Gereau and Conn, 1994), consequently changing vesicular release probability, or postsynaptically (Lester and Jahr, 1990), leading to changes in intracellular calcium levels. For instance, Chavis et al found that activation of mGluRs leads to facilitation of the L-type Ca^{2+} channels (Chavis et al., 1995; Chavis et al., 1996). Although mGluR dependent modulation of channels has been shown to be a consequence of direct G-protein linked action, for example, inhibition of Ca^{2+} channels, many other effects occur as a result of activation of intracellular messenger

pathways. Group I in contrast to group II mGluRs can use several distinct signal transduction pathways to inhibit Ca^{2+} channels, both Ca^{2+} intracellular-independent and -dependent mechanisms (McCool et al., 1998).

However, in addition to the postsynaptic modulation of synaptic transmission by alteration of neuronal excitability, group I mGluRs have been shown to function presynaptically. One example is in the hippocampus, at the level of the synapses between Schaffer collaterals and CA1 pyramidal cells. Two groups report an increase in paired pulse facilitation, suggested a decrease in vesicular release probability (Gereau and Conn, 1994; Fourgeaud et al., 2004). Consistent with the reported presynaptic function, immunochemical data shows that mGluR5 is on axon terminals at these synapses (Balazs et al., 1997).

Group I mGluRs and Plasticity

Group I mGluRs have been shown to potentiate either NMDAR (Fitzjohn et al., 1996; Pisani et al., 1997; Attucci et al., 2001) or AMPAR mediated responses (O'Connor et al., 1995; Ugolini et al., 1999). However, a major function of group I mGluR activation is to produce a persistent weakening of glutamatergic transmission at synapses in the CNS. There appear to be at least two mechanisms through which this is accomplished. One involves the recruitment of endocannabinoid signaling and presynaptic alterations, and has been observed in the dorsal and ventral striatum (Gerdeman and Lovinger, 2001; Gerdeman et al., 2002; Robbe et al., 2002; Robbe et al., 2003). A second major mechanism is through endocannabinoid-independent signaling mechanisms (Rouach and Nicoll, 2003), and has been heavily studied in the hippocampus

and cortical regions (Huber et al., 2000; Hannan et al., 2001; Huber et al., 2001). Both presynaptic (Gereau and Conn, 1994) and postsynaptic mechanisms (Snyder et al., 2001) have been described for endocannabinoid independent forms of group I mGluR LTD.

The mitogen-activated protein kinase pathway is another downstream effector of group I mGluRs that is activated in brain regions known to be involved in drug addiction following administration of abused drugs (Huber et al., 2000; Valjent et al., 2001; Valjent et al., 2004). Both the cannabinoid and ERK pathways will be discussed in a later section.

Group I mGluRs can alter iGluR Function

Group I mGluRs also act to modulate iGluRs. The potentiation of excitatory responses can be explained by an mGluR-mediated membrane depolarization that increases cell excitability (Xiao et al., 2001). PKC, which is triggered by activation of Group I mGluRs, has been proposed to mediate the potentiation of iGluR responses (Ugolini et al., 1999; Skeberdis et al., 2001; Liang et al., 2005). PKC phosphorylates NMDA and AMPA receptors (Roche et al., 1996). The activation of PKC by mGluRs might therefore modulate the phosphorylation state of these two ionotropic receptors, resulting in a potentiation of the response.

As mentioned in the AMPA receptor section above, activation of group I mGluRs can lead to a removal of AMPA receptors from the membrane resulting in a depression of synaptic transmission (Snyder et al., 2001). More specifically, following activation of group I mGluRs there is a reduction in the density of postsynaptic AMPARs and the

number of synapses (Tanaka et al., 2005). Redistribution of AMPARs has also been reported following group I mGluR activation (Xiao et al., 2001).

Group II mGluRs

Group II mGluRs (mGluR2/3) are widely distributed throughout the CNS, where they exhibit moderate to high expression in brain regions that are commonly associated with anxiety disorders, including the hippocampus, PFC and amygdala (Swanson et al., 2005). mGluR2 is generally localized at the periphery of the presynaptic terminal suggesting a role for these receptors at instances of spillover of glutamate beyond the synaptic cleft in response to repetitive stimulation. By contrast, mGluR3 is more diversely localized including both pre- and postsynaptic localization on neurons, as well as localization on certain glial cells (Ohishi et al., 1995; Shigemoto et al., 1996; Mannaioni et al., 2001; Poisik et al., 2003). Immunohistochemical data shows mGluR2 and mGluR3 are expressed in neuropil of the BNST (Tamaru et al., 2001).

Group II mGluRs most commonly function presynaptically to decrease the probability of synaptic vesicle release (Gereau and Conn, 1994), but at multiple synapses within the CNS postsynaptic mechanisms have been reported (Heinbockel and Pape, 2000; Otani et al., 2002). Voltage-dependent Ca^{2+} channels are likely to be involved in the presynaptic inhibition mediated by mGluRs (Anwyl, 1999). Additionally, it has been suggested that K^{+} channels mediate some of the effects induced by activation of group II or III mGluRs (Anwyl, 1999).

LY354740 is a potent and very selective agonist (up to 1 μM) at mGluR2 and 3 with an EC_{50} of 10–50 nM in the rat cortex, hippocampus and striatum, and 10 or 30 nM

in cells expressing recombinant mGluR2 or mGluR3, respectively (Fitzjohn et al., 1998). LY341495 is an antagonist of mGluRs, with nanomolar potency against group II mGluRs and micromolar potency against group III mGluRs (Fitzjohn et al., 1998).

LTD can be induced by activation of group II mGluRs in various brain regions (Otani et al., 1999; Man et al., 2000; Kahn et al., 2001; Kawasaki et al., 2004). For example, group II mGluR LTD has been shown presynaptically in the BLA (Man et al., 2000), the nucleus accumbens (Robbe et al., 2002) and the striatum (Lovinger and McCool, 1995; Kahn et al., 2001). In contrast, it was shown that stimulation of thalamic inputs to the lateral nucleus of the amygdala induces a group II mGluR mediated postsynaptic LTD (Heinbockel and Pape, 2000). Additionally, in the dentate gyrus and the medial PFC it was found that group II mGluR activation induced a postsynaptic LTD that was PKA and PKC dependent (Otani et al., 2002; Kawasaki et al., 2004).

Group III mGluRs

Like group II mGluRs, group III mGluRs are coupled to inhibition of cAMP production, and modulation of ion channels (Gereau and Conn, 1994). However, in contrast to the other groups, group III mGluRs are primarily located at presynaptic active zones at the axon terminal (Shigemoto et al., 1996; Lujan et al., 1997).

As with the group II mGluRs, the group III mGluRs inhibit neurotransmitter release. Unfortunately, there are few pharmacological tools with the desired receptor subtype selectivity ideal for testing the significance of the subtypes of group III mGlu receptors. 1-AP4, the most commonly used selective group III agonist has a high affinity for mGluR4/6/8 and a low affinity for mGluR7 (O'Hara et al., 1993; Cartmell and

Schoepp, 2000). DCPG, a more recently described group III mGluR agonist, is a relatively specific mGluR8 agonist (Linden et al., 2003).

While there is no functional data on the role of the group III mGluRs in the BNST mGluR7 but not mGluR4 mRNA has been detected in the BNST (Ohishi et al., 1995).

MGluRs; Implications in Drug Addiction and Stress/Anxiety

Group I MGluRs in Stress/Anxiety

Agonists and antagonists of mGluRs show anxiolytic-like properties in animal models (Chojnacka-Wojcik et al., 1996; Bond et al., 1997; Chojnacka-Wojcik et al., 1997; Monn et al., 1997; Klodzinska et al., 1999). Important to the stress response is the activation of the HPA axis by group I mGluRs. Specifically, group I mGluRs agonists and antagonists given intracerebroventricularly have been shown to cause a long lasting enhancement of serum corticosterone levels in rats (Johnson et al., 2001). Several studies within the last 5 years examined the anxiolytic potential of the mGluR5 antagonists, MPEP and MTEP, and found them to be effective in attenuating anxiolytic-like behaviors in a range of anxiety tests (Carlezon et al., 1998; Klodzinska et al., 2000; Chojnacka-Wojcik et al., 2001; Spooren et al., 2001; Tatarczynska et al., 2001; Pilc et al., 2002; Busse et al., 2004; Wieronska et al., 2004). For example, oral administration of MPEP has anxiolytic-like effects on behavior in response to fear potentiated startle (Schulz et al., 2001). Spooren et al (2000) showed that MPEP significantly increased open arm time in the elevated plus maze and increased the time of social contact in the social exploration test in rats (Spooren et al., 2001). Activation of group I mGluRs by

intracerebroventricular (icv) administration of DHPG has also been shown to attenuate baclofen-mediated anxiety behaviors in rats (Car et al., 2000).

The behavioral effects of mGluR activation/inhibition on stress related behaviors in brain regions previously shown to be involved in the stress pathway have been studied. For instance, it was found that intrahippocampal administration of a group I mGluR antagonist or a group II mGluR agonist results in anxiolytic-like behaviors (Tatarczynska et al., 2001). Additionally, it was found that ICV administration group I mGluR agonists or antagonists cause a long-lasting enhancement of serum corticosterone levels in rats (Johnson et al., 2001). This suggests group I mGluRs play a role in modulation of the stress pathway.

Group I mGluRs and Addiction

mGluR5 has been implicated in cocaine-related behaviors, most profoundly mGluR5-null mutant mice are insensitive to the locomotor-stimulating and rewarding properties of cocaine (Chiamulera et al., 2001). Consistent with this effect, the mGluR5 specific antagonist, MPEP, has been shown to attenuate cocaine mediated behavioral effects, including cue induced relapse to cocaine seeking (Chiamulera et al., 2001; Herzig and Schmidt, 2004; Backstrom and Hyytia, 2005). Additionally, Homer proteins, as well as another scaffolding protein, PSD95, which are involved in linking mGluRs to the PSD are modified by *in vivo* cocaine administration (Hu et al., 1999; Yao et al., 2004; Swanson et al., 2005). More specifically, deletion of either Homer1 or Homer2 results in an increase in sensitivity to cocaine similar to animals withdrawn from cocaine (Szumlinski et al., 2004). The authors suggest the similarities in cocaine related

behaviors in Homer KO mice and the effects of chronic cocaine treatment implicate Homer in regulating addiction to cocaine.

mGluR alterations in drug related paradigms could be due to interactions with the monoaminergic signaling systems. Page et al., (2005) performed an interesting set of experiments in which they looked at the effects of MPEP on the levels of NE in the frontal cortex during a behavioral stress test (Page et al., 2005). Interestingly, they suggest that blockade of mGluR5 attenuates stress-induced NE efflux. As NE signaling in the BNST is critical for stress induced reinstatement (Leri et al., 2002) this begs the question as to whether there is an interaction between the noradrenergic system and perhaps the dopaminergic system with group I mGluRs in the BNST.

At the level of synaptic transmission endocannabinoid-dependent group I mGluR-induced LTD is disrupted in the ventral striatum in animals previously exposed to cocaine, suggesting that alterations in this form of plasticity may play a role in cocaine-induced alterations in animal behavior (Swanson et al., 2001; Fourgeaud et al., 2004).

Group II and III mGluRs in Stress/Anxiety

Excessive glutamatergic transmission has been implicated in neurological disorders such as stress and anxiety. It is therefore not surprising that activation of $G_{i/o}$ -linked group II and III metabotropic glutamate receptors (mGluRs) produce anxiolytic-like effects in behavioral paradigms designed to test stress/anxiety (Fitzjohn et al., 1998; Klodzinska et al., 1999; Pile et al., 2000). A broad spectrum of behavioral tests demonstrates the anxiolytic-like effects after oral administration of a group II mGluR agonist LY 354740 (Helton et al., 1998). More specifically, LY364740 produced a

lasting, dose-dependent blockade of fear-potentiated startle response in rats, with a near complete blockade in fear-enhanced startle with no effects on basal startle (Helton et al., 1998). Activation of group II mGluRs was shown to have anxiolytic-like properties in another behavioral paradigm used to study stress, the elevated plus maze. LY354740 increased the time spent in the open arm of the elevated plus maze (Helton et al., 1998). Collectively, these findings indicate that activation of group II mGluRs might function to maintain brain excitability in anxiety states by modulating excitatory neurotransmission at specific synapses within the stress pathway, including the BNST.

Determining the roles of group III mGluRs in stress/anxiety has proven more difficult due to lack of brain permeable pharmacological agents acting at these receptors. To begin to study the roles of group III mGluRs by pharmacological manipulation researchers have had to inject these compounds into the CNS. Injection of the group III agonist L-SOP resulted in an anxiolytic-like effect in the Vogel conflict test (Tatarczynska et al., 2001). However, genetic manipulations have given us insight into the functions of these receptors in these disorders. For instance, mGluR7 is abundant in brain regions known to be critical for manifestations of the stress response, such as the hippocampus, amygdala, locus coeruleus and the BNST (Kinoshita et al., 1998). Studies on the mGluR7 KO mice suggest this receptor may play a role in the integration of the stress response. mGluR7 KO mice have been reported to show anxiolytic-like activity in the elevated plus maze, staircase test, light–dark box and stress-induced hyperthermia models (Cryan et al., 2003). Interestingly, in contrast to the mGluR7 KO animals, mGluR8 KO mice have an increase anxiolytic-like behavior in the elevated plus maze (Linden et al., 2002).

Group II and III mGluRs in Addiction

Changes in glutamatergic transmission mediated by group II mGluRs have also been observed in the NAc of rats previously exposed two weeks earlier to repeated systemic injections of amphetamine, indicating that these receptors may potentially contribute to the expression of sensitization by amphetamine as well as other psychomotor stimulants (Kim and Vezina, 1999; Kim et al., 2005). Additionally, injection of a group II mGluR agonist during the preexposure to VTA amphetamine completely blocked sensitization (Kim and Vezina, 1998). In other words, in combination with the well demonstrated role of group II mGluRs in synaptic plasticity, the Vezina group suggests the induction and expression of locomotor plasticity (a form of behavioral sensitization) involves group II mGluR-DA interactions. By injecting the group III mGluR agonist L-AP4 into the striatum it has also been shown that activation of these receptors can inhibit the acute locomotor enhancement by cocaine (Mao and Wang, 2000).

Systemic injections of LY379268 or other mGluR2/3 agonists attenuate several behavioral effects of abused drugs (Kenny and Markou, 2004), including opiate withdrawal symptoms (Vandergriff and Rasmussen, 1999), amphetamine-induced locomotor sensitization (Vezina, 2004), discriminative cue-induced reinstatement of cocaine seeking (Baptista et al., 2004) and heroin seeking (Bossert et al., 2005). Consistent with a decrease in levels of $G_{\alpha i}$ in NAc following administration of drugs of abuse discussed earlier, group II mGluR LTD in the NAc is attenuated in morphine

withdrawn animals (Robbe et al., 2002). Another G_{ai} -linked receptor implicated in drug addiction is the cannabinoid receptor 1 (CB1R).

Cannabinoids

Marijuana (*cannabis sativa*) is one of the oldest and most widely used drugs in the world. Δ^9 -tetrahydrocannabinol (Δ^9 -THC) is the primary psychoactive ingredient of cannabis. Δ^9 -THC along with other naturally occurring and synthetic cannabinoids, binds to two separate GPCRs: cannabinoid receptor 1 (CB1) and CB2 (Matsuda et al., 1990; Gerard et al., 1991; Munro et al., 1993). CB1 receptors are found predominantly in the CNS and the periphery while CB2 receptors are located mostly within the immune system (Zimmer et al., 1999). Two candidate endocannabinoids, monoacyl-glycerol 2-arachidonoylglycerol (2-AG) and the fatty acid amide N-arachidonoyl ethanolamin (anandamide) along with these receptors make up the endogenous cannabinoid (endocannabinoid) system. 2-AG is thought to be produced by a largely calcium independent manner downstream of PLC activity, while anandamide production and release appears to be more calcium dependent.

This cannabinoid system has been proposed to play a role in pain (Calignano et al., 1998), cognition (Marsicano et al., 2002), drug dependence (Varma et al., 2001) and excitotoxicity (Marsicano et al., 2003). The CB1R is one of the most highly expressed neuromodulatory receptors in the brain and is activated by the endogenous lipid signaling molecules, anandamide and 2-AG (Wilson and Nicoll, 2002). Cannabinoid signaling differs from traditional synaptic signaling in that endogenous cannabinoids can function as retrograde synaptic messengers. They are released from postsynaptic neurons and

travel in a retrograde manner across synapses, activating CB1 receptors at presynaptic terminals. Consequently, CB1 receptors, which are coupled to inhibitory G-proteins, act to suppress neurotransmitter release. CB1 activation suppresses neurotransmitter release by decreasing the local release probability of synaptic vesicles (Wilson and Nicoll, 2002).

Of great interest in the mGluR field was the finding that endocannabinoid synthesis can be triggered by activation of group I mGluRs. Retrograde signaling through the cannabinoid system helped explain biophysical evidence suggesting a presynaptic change in probability of neurotransmitter release with immunochemical data suggesting group I mGluRs are localized almost exclusively to postsynaptic structures (Baude et al., 1993; Lujan et al., 1997). Interestingly, it was shown that cannabinoid receptor activation leads to a cascade involving downregulation of PKA activity that leads to an increase in ERK phosphorylation (Walker et al., 2003). The ERK pathway is also implicated as a downstream target of group I mGluR activation.

Extracellular Signal-Regulated Kinase (ERK)

The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway is a signaling cascade that plays a crucial role in a variety of cell regulatory events (Chen et al., 2001). The role of ERK signaling in cell proliferation, differentiation, and survival is well established, but emerging evidence has suggested an involvement of ERK signaling in long-term synaptic changes and behavior (Orban et al., 1999; Mazzucchelli and Brambilla, 2000; Adams and Sweatt, 2002). In neurons, ERK activation has been shown to be involved in processes associated with synaptic remodeling and long-term changes in synaptic efficacy. These processes include protein

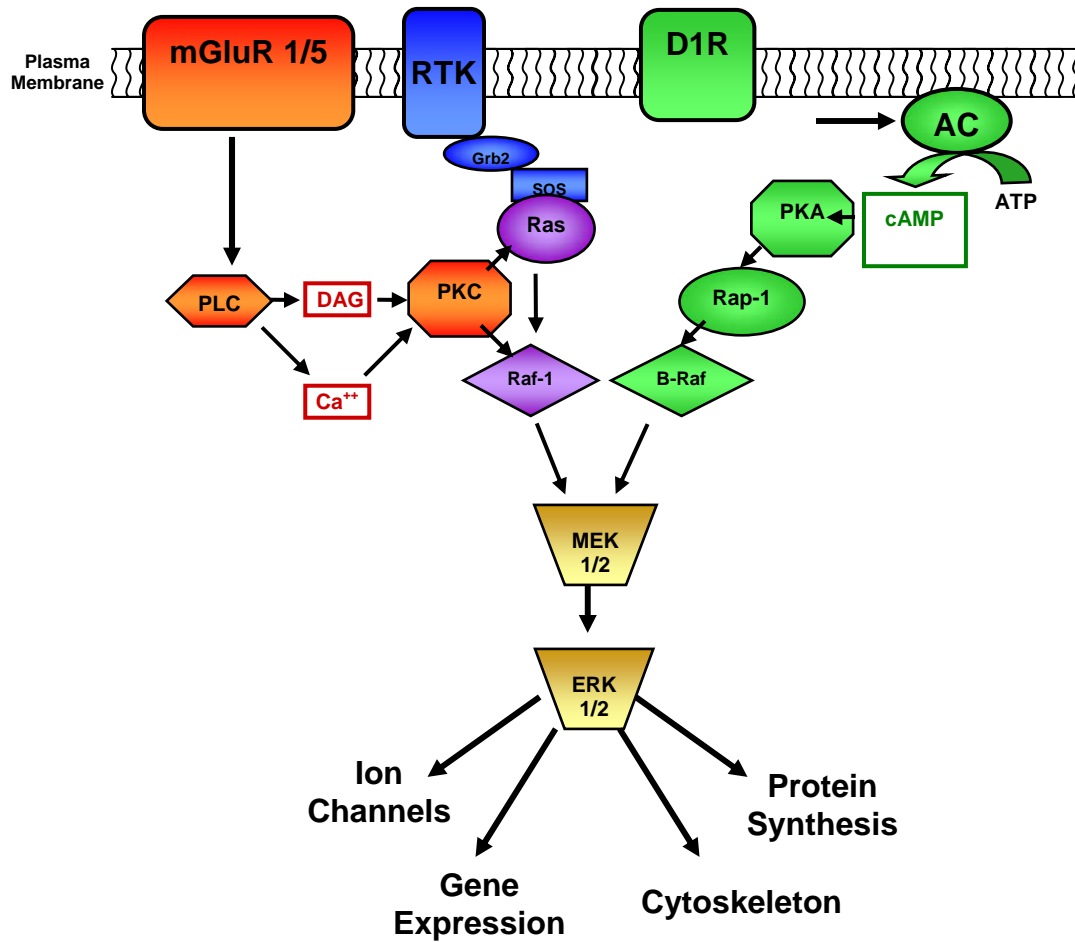


Figure 5. Schematic of ERK 1/2 neuronal signaling pathways.

synthesis, changes in gene expression, dendritic spine stabilization, modulation of ion channels, and receptor insertion (Figure 5; (Sweatt, 2004).

The ERK cascade is activated by a variety of extracellular agents, which include growth factors, hormones, and neurotransmitters (Chen et al., 2001). The best described pathway of ERK activation is stimulation in response to activators of tyrosine kinase family receptors. In brief, tyrosine kinase receptors are bound by activators and stimulate the GTPase Ras via the scaffolding protein Grb2 and the guanine nucleotide exchange factor Sos. Ras then activates the ubiquitous Raf-1 pathway, the first of a series of three kinases that characterize signaling in the MAPK superfamily of enzymes. The classic MAPK signaling pathway begins with a MAP kinase kinase kinase (Raf-1 or B-Raf). Serine/threonine phosphorylation by either of these kinases leads to activation of MAP kinase kinase (MEK). MEK, in turn, is a dual specificity kinase that phosphorylates both threonine and tyrosine residues on MAPK (ERK1/ERK2).

ERK1/2 activities are primarily regulated at two levels, one at phosphorylation, and the other at translocation from the cytoplasm to the nucleus. The former is controlled by the balance of kinase and phosphatase activities. Two phosphatases, MAP kinase phosphatases (MKPs) with dual specificities for tyrosine and serine/threonine (Muda et al., 1996) and protein tyrosine phosphatases (PTP-SL) with a specialty for threonine (Pulido et al., 1998; Zuniga et al., 1999), have been reported to dephosphorylate and therefore inactivate ERK1/2. When ERK1/2 is activated, the phosphorylation promotes ERK1/2 to dimerize and then ERK1/2 is rapidly translocated from the cytoplasm to the nucleus (Robinson and Cobb, 1997; Khokhlatchev et al., 1998). However, in some cases, ERK1/2 can still be detected in the cytoplasm. Cytoplasmic retention of the ERK1/2 is

controlled by cytoplasmic anchor proteins such as MEK1/2 (Fukuda et al., 1997), β -arrestin (Dale et al., 2002), or by the dephosphorylation with MKP3 (Muda et al., 1996; Muda et al., 1996) and PTP-SL (Zuniga et al., 1999)

ERK serves as an important point of convergence for the PKC and PKA pathways. PKC regulates ERK activity through an interaction with either Ras or Raf-1 leading to activation of MEK and consequently ERK (Sweatt, 2001; Adams and Sweatt, 2002). Additionally, it was discovered that DAG, another second messenger product of PLC activity, is capable of activating ERK independent of PKC activation (Ebinu et al., 1998; Kawasaki et al., 1998). Phosphorylation of both tyrosine and threonine residues in MAP kinases, which are found in the activation segment of the kinase domain, is essential for full kinase activity of ERK. In turn, ERK targets only serine and threonine residues that are closely followed by one or more prolines in the ERK recognition motif.

To date, evidence has suggested that ERK2 is the primary ERK isoform (Sweatt, 2004). First, ERK2 is the predominant isoform expressed in the hippocampus and amygdala. Second, NMDAR-dependent synaptic plasticity in the hippocampus is normal (Selcher et al., 2001) or only subtly altered in the dorsal striatum (Mazzucchelli et al., 2002) in ERK1 knockout mice. Third, multiple labs have suggested that stimuli that promote these forms of synaptic plasticity may selectively activate ERK2 (Adams and Sweatt, 2002).

At present, very little is known about the distinctive roles of the ERK1 and ERK2 isoforms, as they are highly homologous (Chen et al., 2001). However, several studies, primarily in non-neuronal systems, have identified proteins and/or drugs that produce or facilitate selective activation of ERK1 versus ERK2. For example, MEK-binding partner

1 (MP1) selectively facilitates ERK1 activation and binds with considerable selectivity to ERK1 over ERK2 (Schaeffer et al., 1998). While little information exists for substrates differentially phosphorylated by ERK1 and ERK2, data from knockout mice point to differences in function. For example, while ERK1 knockout mice are viable and fertile (Pages et al., 1999; Selcher et al., 2001; Pages and Pouyssegur, 2004), targeted disruption of ERK2 produces a lethal mutation (Adams and Sweatt, 2002). Further, while much pharmacological evidence suggests that the MAP kinase signaling cascade plays an important role in NMDA-receptor dependent synaptic plasticity and learning and memory, hippocampal and amygdala based learning are unimpaired in ERK1 knockout mice (Selcher et al., 2001), and NMDA receptor-dependent plasticity is either normal (Selcher et al., 2001), or altered in complex ways in ERK1 null mice (Mazzucchelli et al., 2002).

Effectors through which these proteins kinase cascades act include proteins that regulate gene expression (cAMP-response element binding protein (CREB) and histone acetyltransferases (HAT)), as well as the voltage-gated potassium channel Kv4.2 (Adams and Sweatt, 2002). The last target represents a mechanism by which ERKs can regulate neuronal excitability. Other candidate molecules also exist (glutamate receptors, calcium channels, phosphatases, cytoskeletal proteins, and protein synthesis machinery).

Since English and Sweatt first provided evidence that ERK signaling is involved in plasticity by using the MEK inhibitor PD 98059 to block LTP induction in the hippocampus, much emphasis has been placed on the role of this signaling cascade in learning and memory, and in glutamatergic synaptic plasticity at a variety of synapses (Orban et al., 1999; Adams et al., 2000; Mazzucchelli and Brambilla, 2000; Adams and

Sweatt, 2002; Mazzucchelli et al., 2002; Gallagher et al., 2004; Sweatt, 2004). In particular, MEK inhibitors block the induction of several forms of NMDA-receptor dependent LTP and LTD throughout synapses of the hippocampus and the amygdala (for review see Sweatt, 2004). NMDAR-independent forms of LTD are also blocked by MEK inhibitors. Importantly, group I mGluR LTD in the hippocampus and cerebellum is dependent on ERK activation (Kawasaki et al., 1999; Gallagher et al., 2004).

ERK and Drug Addiction

A more recent, growing literature indicates that the ERK signaling cascade plays a critical role in behavioral alterations produced by psychostimulants. Inhibitors of the ERK signaling cascade administered *in vivo* reduce psychostimulant mediated behavioral sensitization (Valjent et al., 2005). It has also been suggested that ERK activation is involved in the incubation of cocaine craving in a region-specific manner (Lu et al., 2005). For instance, ERK signaling is necessary for VTA-BDNF stimulated long term enhancement of cocaine seeking during withdrawal (Lu et al., 2004). And importantly, *in vivo* psychostimulant administration produces activation of ERK in the reward circuitry including strong activation within the BNST (Valjent et al., 2000; Valjent et al., 2004). In addition to being involved in the rewarding properties of cocaine (Valjent et al., 2000), morphine, nicotine, cocaine and alcohol administration alters ERK activity (Narita et al., 2002; Sanna et al., 2002; Brunzell et al., 2003; Zhang et al., 2004). Also, consistent with a role of ERK in synaptic transmission in the BNST, addictive drugs activate ERK in the lateral BNST (Valjent et al., 2004).

Summary

There are many implications for mGluR function and the BNST in pathophysiological conditions such as stress/anxiety and drug addiction. Therefore, mGluRs and signal transduction mechanisms activated by these receptors within the BNST are potential targets for pharmacological treatment of drug related behaviors (stress induced reinstatement) and stress/anxiety conditions.

Hypothesis

Glutamate acts to modulate neuronal transmission in the BNST via mGluRs by recruiting signaling cascades previously shown to be involved in stress and addiction.

Specific Aims

1. Characterization of group II and/or III mGluR function as well as synaptic locus on excitatory transmission in the BNST.
2. Investigation of function, synaptic locus, downstream effectors and a behavioral correlate to drug addiction of group I mGluRs in the BNST.

CHAPTER II

MATERIALS AND METHODS

Animals

Male C57Bl/6j mice (5-10 weeks old, Jackson Laboratories), CB1 receptor (-/-) males 5-6 weeks of age, or ERK1 receptor (-/-) males 5-6 weeks of age, both of which were on a C57Bl/6j background, were used.

Brain Slice Preparation

For interface chamber recordings, the brains were quickly removed and placed in ice-cold artificial cerebro-spinal fluid (ACSF) (in mM: 124 NaCl, 4.4 KCl, 2 CaCl₂, 1.2 MgSO₄, 1 NaH₂PO₄, 10 glucose, and 26 NaHCO₃). Slices 300 μ m in thickness were prepared using a vibratome (Leica). Rostral slices containing anterior portions of BNST (Bregma 0.26 mm to 0.02 mm) (Franklin and Paxinos, 1997) were identified using the internal capsule, anterior commissure, fornix and stria terminalis as landmarks as previously described (Egli and Winder, 2003; Weitlauf et al., 2004; Egli et al., 2005). Slices were then transferred to either an interface recording chamber (field potential recordings, \sim 28°C), a submerged recording chamber (whole-cell patch clamp recordings, 24-25°C) or a submerged holding chamber (25°C) where they were perfused with oxygenated (95% O₂/5% CO₂) ACSF at a rate of 2 mL/minute. Slices were allowed to equilibrate in normal ACSF for one hour before experiments began.

Extracellular Field Potential Recordings

Low resistance (2-3 M Ω) extracellular electrodes were pulled with borosilicate glass on a Flaming-Brown Micropipette Puller (Sutter) and were filled with ACSF. Stimulating electrodes consisted of twisted, insulated nichrome bipolar wire. Stimulating electrodes were placed on the dorsal anterolateral BNST (dBNST) border of the internal capsule approximately 200-500 μ m dorsal to the anterior commissure. Field potential responses were evoked at a frequency of 0.05Hz using a stimulus range of 5-15 V at a duration of 100-150 μ s. Experiments were performed in a heated (\sim 28 $^{\circ}$ C) interface-style recording chamber (Fine Science Tools) as previously reported (Weitlauf et al., 2004; Grueter and Winder, 2005). All recordings were performed in the presence of 25 μ M picrotoxin. Data points are represented as averages of the peak amplitude at 1 minute intervals.

Local Stimulation in the dBNST Yields an Excitatory Response

Consistent with previous results (Weitlauf et al., 2004), as shown in Figure 6, brief, local, single pulse stimulation (50-100 μ s) in dBNST from 6-10 week old male C57Bl6/J mice yields a short latency extracellular waveform in an interface chamber which typically includes a biphasic negative potential in the presence of 25 μ M picrotoxin (Figure 6). The first downward deflection (referred to as N1) of the biphasic peak is abolished by the sodium channel blocker, tetrodotoxin (TTX, 1 μ M; Figure 6). Thus, the N1 is thought to be indicative of an axonal response and to reflect the number of axons/cells directly stimulated in the field. The second peak (referred to as N2) also is abolished by TTX, but in addition is sensitive to CNQX, an AMPA/kainate receptor

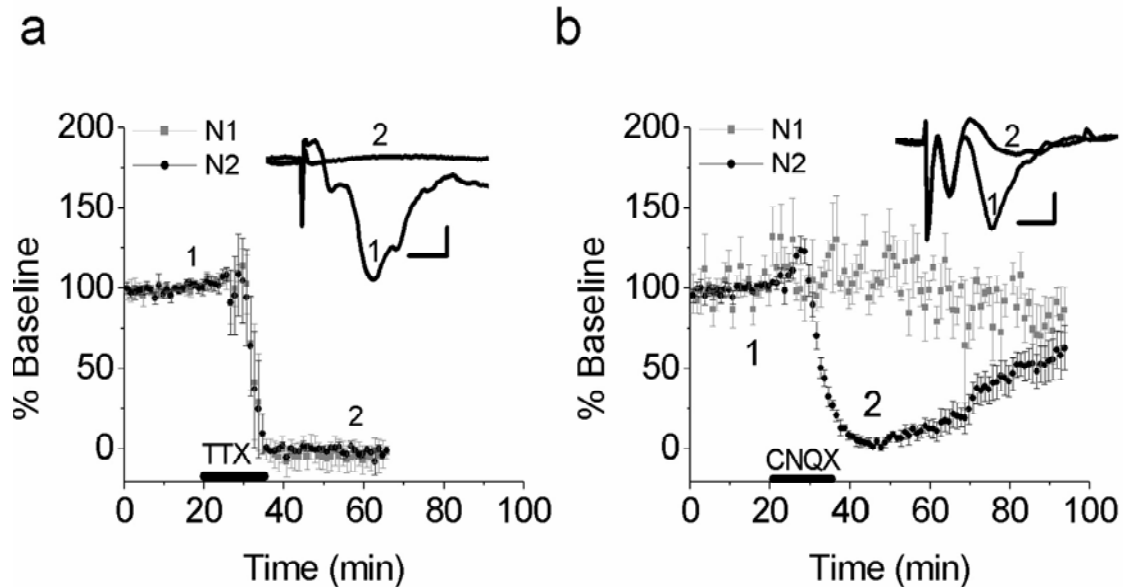


Figure 6. Characterization of field potential responses in BNST. a. The time course of the effects of TTX (1 μ M for 15 min, n=5) on the N1 and N2 of the BNST field potential. Inset, representative traces of a BNST field potential pre- and post TTX (1 μ M) application. b. The time course of the effects of CNQX (10 μ M for 15 min, n=6) on the N1 and N2 of the BNST field potential. Inset, representative trace of a BNST field response pre- and post-CNQX (10 μ M) application.

antagonist, suggesting that it is driven by excitatory glutamatergic transmission (Figure 6).

Whole-Cell Voltage-Clamp Recordings

Slices were prepared as above except the dissection solution was a low Na⁺/high sucrose solution. Recordings were performed in a submerged chamber continuously perfused at a rate of 2 ml/minute with oxygenated ACSF (24-25°C). Electrodes of 3.0-5.0 MΩ were pulled on a Flaming-Brown Micropipette Puller and were filled with (in mM): K⁺ gluconate or Cs gluconate (135), NaCl (5), HEPES (10), EGTA (0.6), ATP (4), GTP (0.4) and biocytin (0.1%). Effects of mGluR ligands on glutamatergic transmission obtained with these two internal solutions were comparable and therefore pooled. All cells recorded from were voltage-clamped at -70 mV. Excitatory post synaptic currents (EPSCs) of 100-400 pA were acquired by a Multiclamp amplifier (Axon Instruments), digitized and analyzed via pClamp 9.0 software (Axon Instruments). Input resistance (K⁺ 140-800 MΩ; Cs⁺ 400-5000 MΩ), holding current, and access resistance (12-45 MΩ) were all monitored continuously throughout the duration of the experiments (see Figure 8a). Experiments in which changes in access resistance were greater than 20% were not included in the data analysis. Stimulating electrodes and their placement were identical to field potential recordings. Stimulus intensity ranged from 6-30 V with a 100 μs duration. Events were recorded at a frequency of 0.17 Hz. Data is represented as a 1 minute average of the peak amplitudes.

Whole-cell patch clamp recordings were acquired in cells from the same region of the dBNST as the field potential recordings. The configuration recording differed in that whole-cell patch clamp recordings were performed in a submerged chamber. Current-

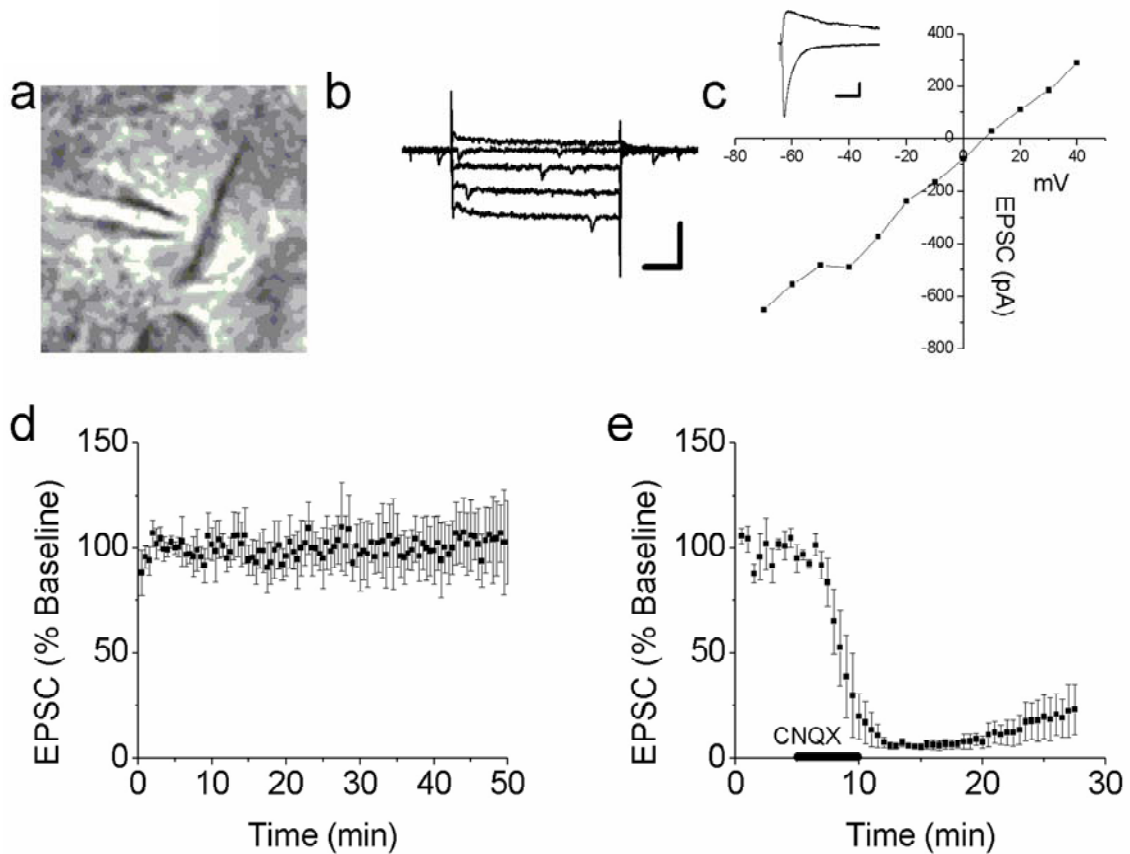


Figure 7. Characterization of synaptically evoked EPSCs in the BNST.

a. Representative trace of an I-V relationship of a BNST neuron. Cell was voltage clamped at -70 mV and stepped in 10 mV intervals from -100 to -20 mV. Scale indicates 100 pA and 2 msec. b. I-V plot of EPSC. Inset represents traces at -70 and +40 mV. c. Stability of evoked EPSCs in the BNST (n=4). e. Time course of effects of CNQX on EPSCs in the BNST (n=3).

voltage relationships obtained from these neurons (Figure 7) are consistent with those previously reported (Rainnie, 1999; Egli and Winder, 2003). Synaptic stimulation in the presence of 25 μ M picrotoxin, at a holding potential of -70mV elicited an EPSC (Figure 7). Unless otherwise reported, cells were allowed to equilibrate at least 10 minutes after whole-cell configuration before baseline was recorded. This EPSC had a reversal potential of near 0 mV when corrected for junction potential (Figure 7), was stable for long periods of time (Figure 6), and was abolished by CNQX at -70mV (Figure 7). The EPSC under basal stimulation is unaltered in the presence of the NMDA antagonist DL-AP5 (Data not shown). These data indicate that the postsynaptic currents elicited by a brief stimulus are primarily mediated by non-NMDA glutamate receptor subtypes.

A paired-pulse ratio (PPR) was acquired by applying a second stimulus of equal intensity 50 ms after the first stimulus. The magnitude of the facilitation that occurs in response to paired-pulse stimulation, a measure that has been shown to change in response to alteration in release probability, was compared before, during and after drug application. An alteration in the paired pulse ratio, that is the amplitude of the second pulse over the first pulse, suggests a presynaptic site of action.

Miniature EPSCs were collected at a holding potential of -90mV in the presence of 1 μ M TTX. 2 minute blocks of events were analyzed using Minianalysis software (Synaptosoft, Decatur, Georgia) with detection parameters set at greater than 5 pA amplitude and less than 3 ms rise time. All events were verified by eye.

Drug Application

All experiments were performed in the presence of picrotoxin (25 μ M). Where indicated in the text, the following drugs were bath applied: tetrodotoxin (TTX, 1 μ M) and 6-Cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX, 10 μ M) were purchased from Sigma-Aldrich (St. Louis, MO). (2*S*,2'*R*,3'*R*)-2-(2',3'-Dicarboxycyclopropyl)glycine (DCG-IV, 1 μ M), L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4), DL-2-Amino-5-phosphonovaleric acid (APV, 10 μ M), (2*S*)-2-Amino-2-Figure-3-(xanth-9-yl) propanoic acid, (LY 341495, 1 μ M), and (S)-3,4-Dicarboxyphenylglycine (DCPG, 30 μ M) were purchased from Tocris. Dimethyl Sulfoxide (DMSO) was the carrier for picrotoxin (0.02% v/v). (1*S*,2*S*,5*R*,6*S*)-2-aminobicyclo [3.1.0]hexane-2,6-dicarboxylic acid (LY 354740) was a gift from Dr. Darryle Schoepp (Eli Lilly).

CHAPTER III

GROUP II AND III METABOTROPIC GLUTAMATE RECEPTORS SUPPRESS EXCITATORY SYNAPTIC TRANSMISSION IN THE DORSOLATERAL BED NUCLEUS OF THE STRIA TERMINALIS

Introduction

Behavioral studies, both in rodent models of anxiety (Helton et al., 1998; Klodzinska et al., 1999; Linden et al., 2002; Tizzano et al., 2002; Linden et al., 2003) and in humans (Fitzjohn et al., 1998; Grillon et al., 2003) suggest that group II and III mGluR ligands may represent useful approaches to treat anxiety. These receptors regulate glutamatergic transmission in several different brain regions, in many cases inhibiting transmission (Heinbockel and Pape, 2000; Man et al., 2000; Kahn et al., 2001; Otani et al., 2002; Robbe et al., 2002), and in other cases enhancing transmission (Evans et al., 2000; Rush et al., 2001; Wu et al., 2004). Group II and III mGluRs have been shown to function in brain regions involved in stress responses. For instance, group II mGluRs provide tonic regulation of glutamatergic transmission in the hypothalamic-pituitary-adrenocortical axis (Scaccianoce et al., 2003) and inhibition of glutamatergic transmission in the PFC (Otani et al., 2002), Group III mGluRs induce a long-lasting potentiation of glutamatergic transmission in the BLA (Neugebauer et al., 1997) and inhibit glutamatergic transmission in the PVN (Schrader and Tasker, 1997). Both group II and group III mGluRs have been shown to inhibit glutamatergic transmission in the central nucleus of the amygdala (Neugebauer et al., 2000), and hippocampus (Capogna, 2004).

Immunohistochemical studies suggest expression of mGluR2, mGluR3 (Kinoshita et al., 1998; Tamaru et al., 2001), and mGluR7 in the BNST (Kinoshita et al., 1998) as well as mRNAs for mGluR7 but not mGluR4 (Kinzie et al., 1995; Ohishi et al., 1995). To date, the actions of mGluRs on glutamatergic transmission in the BNST have not been reported. Therefore, we utilized two distinct electrophysiological methods, extracellular field potential and whole-cell patch clamp recordings, to determine the effect of activation of group II and III mGluRs on excitatory transmission in BNST. We find that both group II and group III mGluRs elicit a long-lasting inhibition of excitatory synaptic transmission in the BNST.

Results

Group II mGluR Activation Inhibits Excitatory Field Potentials in the dBNST

To determine the role of activation of group II mGluRs on excitatory synaptic transmission in dBNST, we assessed the effects of application of specific group II mGluR agonists on both the extracellular N2 response and the EPSC. To evaluate the effect of group II mGluR activation on the CNQX-sensitive N2 potential in dBNST slices, the group II mGluR agonist LY354740 (1 μ M for 15 min) was bath applied in an interface recording chamber. LY354740 caused a transient, reversible depression of the field potential to $74.8 \pm 4.7\%$ of baseline that was not associated with any changes in the N1 response (Figure 8a). This action was blocked by pretreatment of the slice with the mGluR antagonist LY341495 (1 μ M, Figure 8b), which had no effect on basal transmission when applied alone (data not shown). Furthermore, the effect of LY354740

on field recordings was mimicked by another selective group II mGluR agonist DCG-IV. Perfusion of DCG-IV (1 μ M, 15 min, in presence of DL-APV to block potential direct actions of the drug at NMDA receptors) caused a reversible depression of the N2 amplitude to 70.0 ± 3.6 % of baseline (Figure 8c).

Group II MGLuR Activation Inhibits Synaptically-Evoked EPSCs in the dBNST in a Dose-Dependent Manner

The inhibition of the field potential response observed above could be through mGluRs acting at a number of different levels. To more specifically isolate glutamatergic synaptic transmission, we determined the effect of activation of group II mGluRs on voltage clamped EPSCs. Similar to field potential results described above, brief perfusion of LY354740 (1 μ M for 5 minutes) during whole-cell voltage clamp recordings of dBNST neurons caused a transient depression of EPSCs. Peak depression was 53.4 ± 3.9 % of baseline (Figure 9a,b). The LY354740 elicited inhibition of EPSC amplitude returned to baseline levels approximately 30 minutes post washout. As shown in representative fashion in Figure 4A, this depression of the EPSC was not associated with marked changes in the holding current or input resistance (R_m). LY354740-induced suppression of EPSCs was concentration-dependent, with an EC₅₀ of ~ 6.0 nM (Figure 9c).

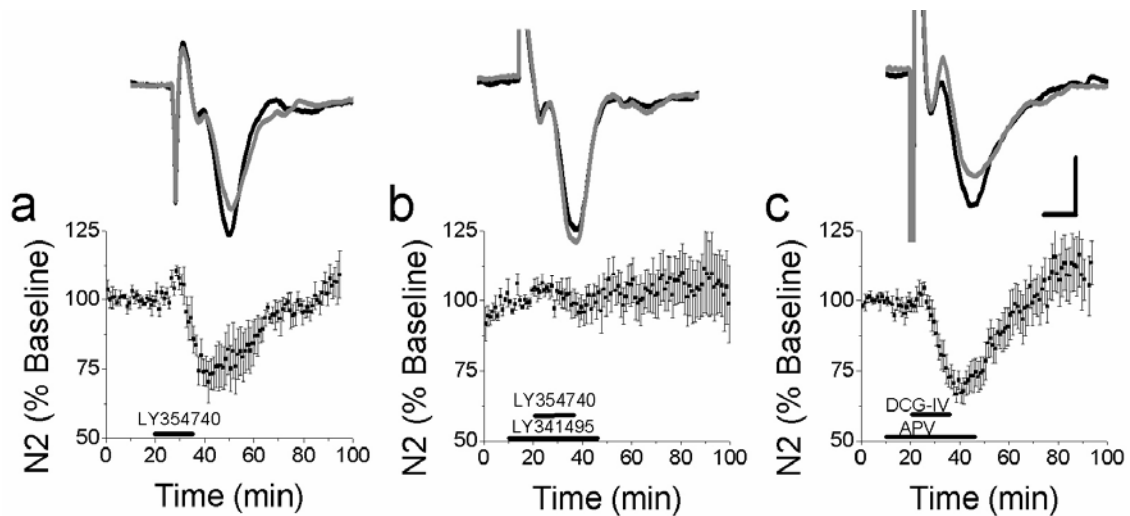


Figure 8. Group II activation depresses synaptic field potentials in the BNST. Time course of effects of group II mGluR compounds on excitatory transmission in the BNST. a. LY354740 (1 μM, 15 min, n=5) significantly reduced field potential amplitude in the BNST. The effects of LY354740 were completely reversible 30 minutes following washout. b. The effects of LY354740 (1 μM) were blocked by LY341495 (1 μM LY341495, n=5). c. DCG-IV (1 μM, 15 min, in presence of 100 μM AP-5, n=5) also inhibits N2 responses. Scale indicates 0.2 mV and 20 msec.

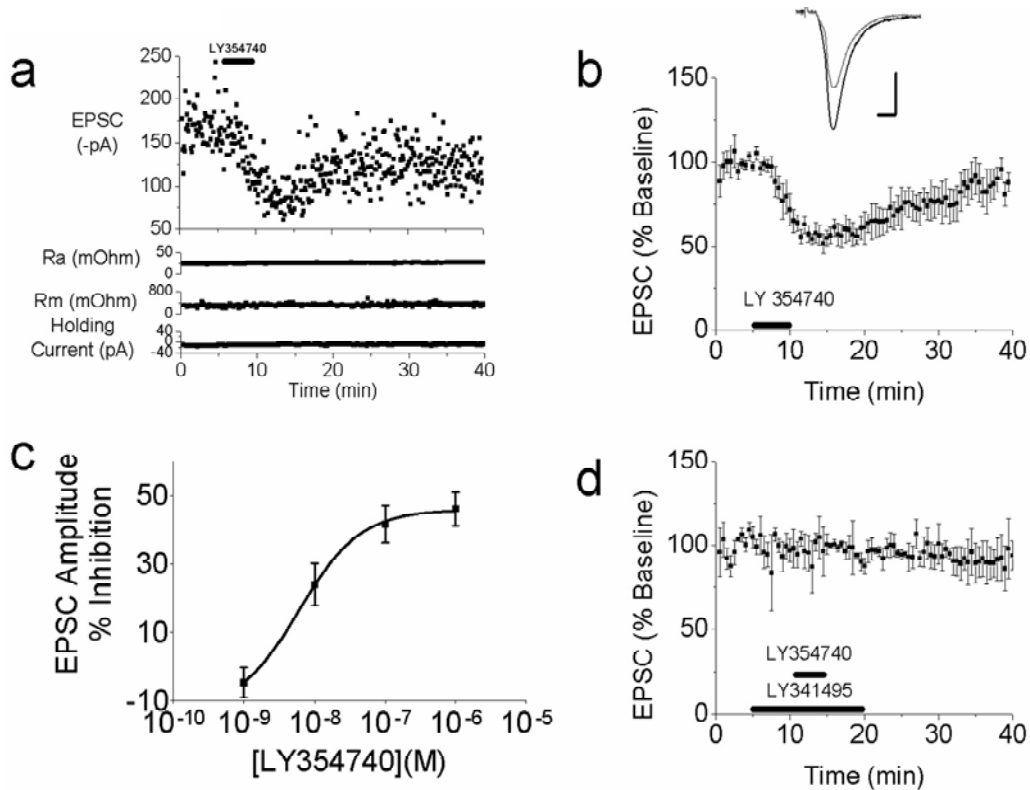


Figure 9. Inhibition of EPSCs in BNST neurons by activation of group II mGluRs is concentration dependent. a. Representative experiment illustrating the lack of postsynaptic effects and the depression of excitatory synaptic transmission by LY354740 (1 μ M, 5 min). b. Average results of 5 minute application of LY354740 (n=6). c. Depression of synaptic transmission by LY354740 is concentration dependent (n=3-6). d. The effects of LY354740 were blocked by LY341495 (n=3).

Similar to field potential effects, LY341495 (1 μ M) blocked the depression of EPSCs induced by LY35740. In the presence of LY341495, the depression induced by LY35740 was $101.3 \pm 10.1\%$ of baseline (Figure 9d). These results suggest a group II mGluR inhibits excitatory transmission in the dBNST.

Group II mGluR Activation Induces a Long Term Depression of Excitatory Transmission in the dBNST

At many glutamatergic synapses in the CNS, mGluR activation can recruit a lasting reduction in synaptic efficacy. In particular, group II mGluR activation has been shown to persistently reduce synaptic efficacy at several different CNS synapses (Otani et al., 1999; Man et al., 2000; Kahn et al., 2001; Kawasaki et al., 2004). While brief application of the group II mGluR agonist LY35740 produced only a transient depression of synaptic transmission, we find that more prolonged activation of the receptor produced a persistent depression of synaptic transmission in dBNST (Figure 10a). The effect is unlikely to be mediated simply by poor pharmacokinetics, as bath application of the antagonist LY341495 (1 μ M) 30 minutes following washout of the agonist failed to reverse the depression (Figure 10b). This concentration of LY341495 was sufficient to abolish the effects of LY35740 when applied immediately prior to agonist application (Figure 9d).

Group III mGluR Activation Depresses Glutamatergic Transmission in the dBNST

To test the potential role of group III mGluRs in regulating glutamatergic transmission in the dBNST, we bathed slices with increasing concentrations of the group

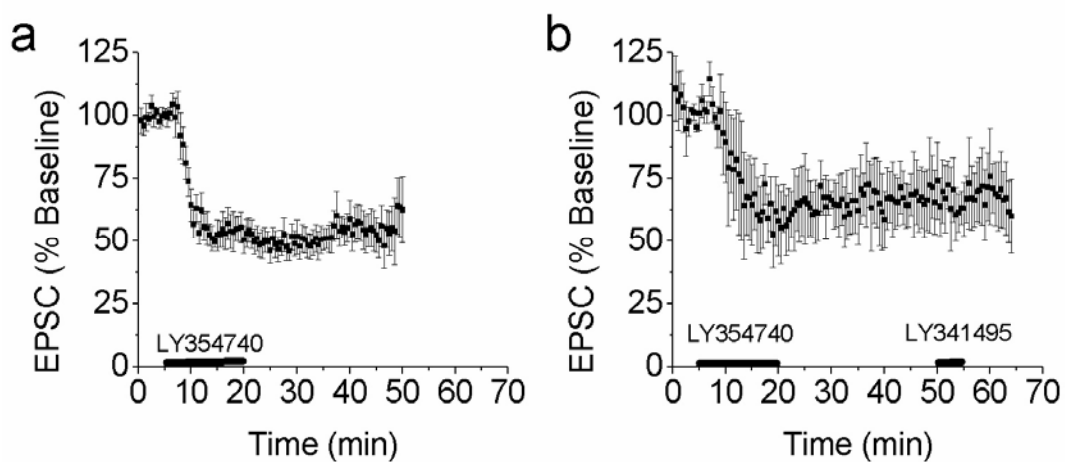


Figure 10. Group II mGluR activation induces long term depression in the BNST

- a. Application of LY354740 (1 μ M) for an extended time period (1 μ M, 15 min) resulted in a depression of synaptic transmission that persisted in the absence of the drug (n=9).
- b. Application of LY341495 (1 μ M) 30 min post LY354740 washout fails to reverse depression of EPSC (n=3).

III mGluR agonist, L-AP4. At the maximal concentration tested (1 mM), L-AP4 induced a depression of EPSC amplitude to 41.0 ± 6.9 percent of the baseline amplitude that only partially reversed to $69.6 \pm 9.9\%$ of baseline 40 minutes after washout (Figure 11a, c). No changes in postsynaptic properties were observed. The dose-response relationship suggests a low potency for L-AP4, since 1mM L-AP4 did not clearly saturate the effect (Figure 11c). To begin to determine which subtypes of group III mGluRs may participate in regulating glutamatergic transmission in dBNST, we also utilized the mGluR8 specific agonist DCPG. 30 μ M DCPG elicited a depression of EPSC amplitude to $70.4 \pm 7.9 \%$ of the baseline amplitude (Figure 11b).

Activation of Group II MGLuRs does not Enhance Group III MGLuR-Mediated Inhibition

Multiple brain regions provide glutamatergic input into the dBNST, including the BLA, subiculum and prelimbic cortical areas. While both group II and group III mGluR activation robustly inhibits EPSCs in the dBNST, neither completely abolishes the response. To begin to determine whether group II and group III mGluRs act at the same synapses through the same mechanisms, we co-applied these agonists at the maximal concentrations we utilized. Co-application of 1 μ M LY354740 (a saturating concentration based on the concentration-response curve) and 1mM L-AP4 for 5 minutes resulted in a depression of EPSC amplitude to $34.5 \pm 7.2\%$ (Figure 12). The effect partially reversed to a magnitude of $64.9 \pm 11.2\%$ baseline. The coapplication of LY354740 and L-AP4 did not significantly alter EPSCs compared with the effects of L-AP4 at the concentrations used ($34.5 \pm 7.2\%$ vs. $41.0 \pm 6.9\%$ of baseline for LY354740 + L-AP4 and L-AP4 alone,

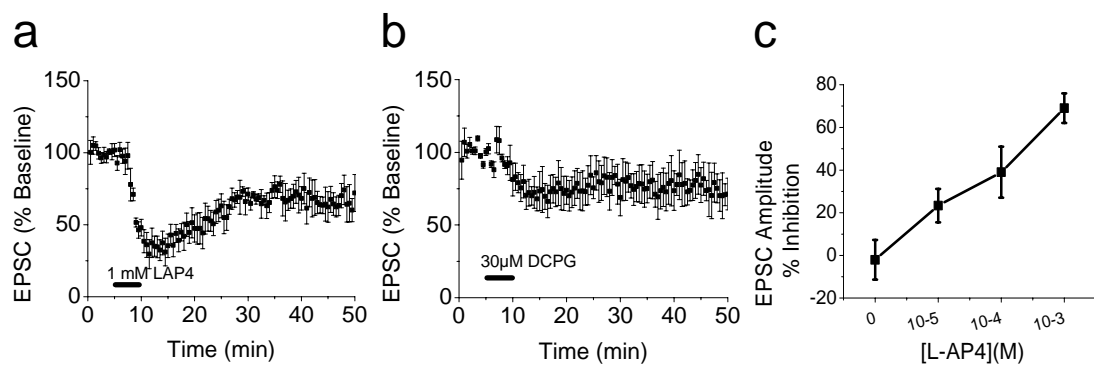


Figure 11. Group III mGluR activation inhibits EPSCs in the BNST. a. 1 mM L-AP4 caused a lasting depression in synaptic transmission (n=6). b. Effects of 30 μM DCPG on synaptic transmission in the BNST. c. Effects of 10 μM, 100 μM and 1 mM L-AP4 on EPSC amplitude (n=5-6).

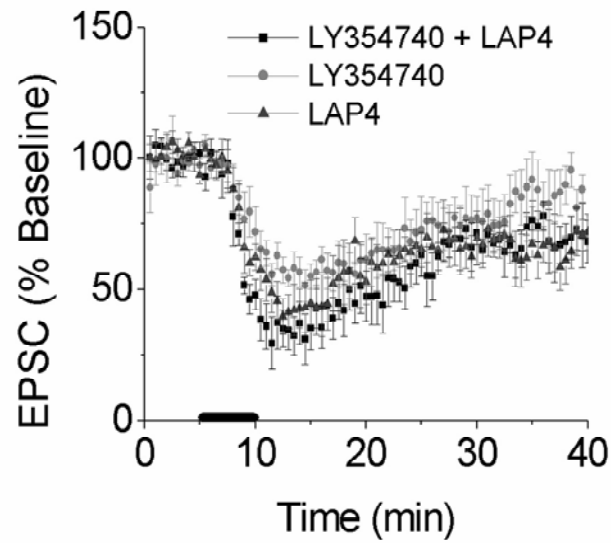


Figure 12. Group II receptor activation fails to enhance group III mediated inhibition of EPSCs in the BNST. Time course of effects of LY354740 (1 μ M), L-AP4 (1 mM) and coapplication of the agonists for 5 min on EPSCs in the BNST (n=4).

respectively; $p > 0.05$). Thus these data suggest that the mGluRs mediating the actions of these drugs may be expressed on a common set of synapses.

Group II and III mGluR Depression of EPSC Amplitude is Associated with Enhancement of Paired-Pulse Ratios in the dBNST

To begin to determine the locus of the actions of group II and III mGluRs in regulating EPSC amplitude, we incorporated paired pulse ratio (PPR) measurements in our experimental design. Alterations in PPR are classically interpreted as suggestive of a change in presynaptic function. As seen in figure 13a, 5 min application of LY354740 caused a marginal increase in PPR. A more robust and persistent increase in PPR was seen with the longer application of LY354740 (Figure 13b). Interestingly, the enhancement of group II mGluR mediated effects on PPR mirrored that of the inhibition of the EPSCs. Activation of group III mGluRs by L-AP4 also led to a long lasting increase in PPR that mirrored the inhibition of the EPSCs (Figure 13c). As shown in Figure 13, both LY354740 and L-AP4 significantly increase PPR.

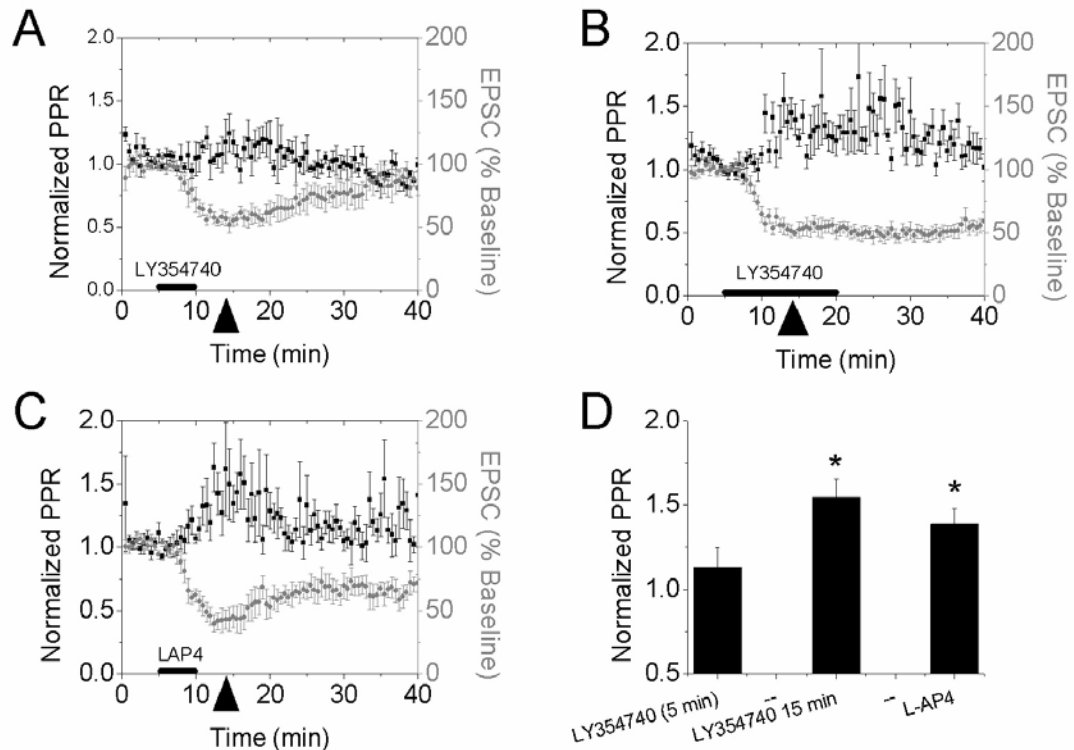


Figure 13. Group II or III mGluRs function has a presynaptic locus
a. Time course of PPR from cells in which LY354740 was applied over 5 minutes (n=6). Insets are representative traces from time points indicated for a-c. b. Time course of PPR from cells in which LY354740 was applied over 15 minutes (n=9). c. Time course of PPR from cells in which 1 mM L-AP4 was applied (n=6). d. Graph of PPR at the initial peak depression of the EPSC. Arrow indicates time at which analysis was performed. Scales indicate 100 pA and 20 ms.

Discussion

The BNST, and in particular glutamatergic transmission within the BNST is believed to be a critical substrate for stress/anxiety pathways. Metabotropic glutamate receptor ligands, in particular group II and group III mGluR agonists, produce anxiolytic effects on animal behavior in a variety of tasks (Fitzjohn et al., 1998; Klodzinska et al., 1999; Pilc et al., 2002; Wieronska et al., 2004), however to date, the sites of actions responsible for these effects are unknown. In this study, we report that fast glutamatergic transmission in the BNST of adult mice is depressed by these ligands.

Consistent with the function of group II mGluRs in other brain regions (Bushell et al., 1996; Macek et al., 1996; Kilbride et al., 1998), we find selective activation of group II mGluRs in the BNST can produce a reversible depression of synaptic transmission. We find that two group II mGluR selective agonists, DCG-IV and LY354740 (Fitzjohn et al., 1998), both inhibited glutamate-dependent field potentials and EPSCs, and that this effect was blocked by LY341495 at concentrations selective for group II mGluRs. The effect of LY354740 on EPSCs is potent and concentration dependent, with maximal depression elicited by 100 nM LY354740. Because DCG-IV has agonist activity at the NMDA receptor at higher concentrations (Uyama et al., 1997), we performed experiments with this compound in the presence of the NMDA receptor antagonist DL-AP5.

For the study of group III mGluRs we also used two agonists, L-AP4 and DCPG. L-AP4, the most commonly used selective group III agonist has a high affinity for mGluR4/6/8 and a low affinity for mGluR7 (Cartmell and Schoepp, 2000). DCPG, a more recently described group III mGluR agonist, is a relatively specific mGluR8 agonist

(Linden et al., 2003). The dose-response relationship for L-AP4 suggests the involvement of mGluR7, since high concentrations of L-AP4 did not produce saturating actions. Consistent with this idea, anatomical evidence points to the expression of mGluR7, with weaker, if any, expression of mGluR4 in dBNST (Kinzie et al., 1995; Ohishi et al., 1995; Kinoshita et al., 1998). However, significant effects were observed with low concentrations of L-AP4, suggesting the possible involvement of other group III mGluRs. Consistent with this, we find that the mGluR8 agonist DCPG also decreases excitatory transmission. At present it is unclear where mGluR8 is localized within BNST.

Group II mGluRs Modulate Excitatory Transmission in the dBNST

Group II mGluRs are located primarily presynaptically and function to modulate transmitter release (Gereau and Conn, 1994; Shigemoto et al., 1996; Lujan et al., 1997; Anwyl, 1999), although there is also evidence for the function of group II mGluRs postsynaptically (Otani et al., 2002). Group II mGluRs can couple to a variety of effector systems (Gereau and Conn, 1994), including regulation of cAMP production, direct modulation of ion channels, and in some cases activation of PLC and PLD (Otani et al., 2002). Consistent with immunohistochemical evidence of the presence of mGluR2 and mGluR3 in the BNST (Kinoshita et al., 1998; Tamaru et al., 2001), our data suggests group II mGluRs function to decrease excitatory transmission in this region. The depression of glutamatergic transmission by group II mGluR agonists that we observed was accompanied by marked alterations in PPR, suggesting that the actions of the group II mGluRs in this case is likely a presynaptic one.

Interestingly, we found that while brief application of the group II agonists produced a reversible depression of glutamatergic transmission, more prolonged activation of the receptors produced a persistent, likely presynaptically mediated, depression of synaptic transmission in the BNST. This persistent depression does not appear to be due to poor pharmacokinetics or a constitutively activated group II mGluR since late application of the antagonist LY341495 did not reverse the depressed EPSC.

Long-term depression of excitatory transmission (LTD) induced by activation of group II mGluRs has been reported in a number of brain regions. For example, group II mGluR-dependent LTD has been demonstrated in the BLA (Man et al., 2000), the nucleus accumbens (Robbe et al., 2002), the striatum (Kahn et al., 2001) and the mossy fiber-CA3 synapse (Kobayashi et al., 1996). In these regions, evidence suggests that the persistent depression is mediated presynaptically via a reduction in glutamate release. In contrast, thalamic inputs to the lateral nucleus of the amygdala (Heinbockel and Pape, 2000), as well as glutamatergic synapses in the dentate gyrus and medial prefrontal cortex (Otani et al., 2002; Kawasaki et al., 2004) undergo a group II mGluR-mediated LTD that appears to be postsynaptically elicited. The LTD that we observe in the BNST is associated with a persistent alteration in paired-pulse ratios, suggesting that it may be mediated by presynaptic alterations in glutamate release. In contrast to group I mGluR LTD in the hippocampus (Doherty et al., 1997), yet similar to group II LTD in the striatum (Kahn et al., 2001), LTD in the BNST was not caused by slow washout of LY354740 or a constitutively activated receptor because application of the group II antagonist LY341495 during the washing period did not affect the magnitude of LTD.

Group III mGluRs Modulate Excitatory Transmission in the dBNST

In addition to the effects of group II mGluR activation, the group III agonist L-AP4 clearly inhibited the synaptically-evoked responses of BNST neurons. Like group II mGluRs, group III mGluRs are coupled to a variety of effectors, including inhibition of cAMP production, and direct modulation of ion channels (Gereau and Conn, 1994).

As with the group II mGluR activation, we find that activation of group III mGluRs can elicit a lasting, presynaptically mediated depression of EPSCs in the BNST, suggesting that activation of group III mGluRs may also produce LTD in this region. Indeed, LTD requiring group III mGluRs has been observed at glutamatergic inputs on interneurons in CA3 (Laezza et al., 1999). Unfortunately however, at present we cannot rule out the possibility that the persistent depression is a consequence of poor drug washout since antagonist development for group III mGluRs has lagged behind the other groups.

Group II mGluR Activation Fails to Enhance Inhibitory Effects of Group III mGluR Activation

Multiple mGluRs with different signaling cascades have been shown to function at the same synapses (Kawasaki et al., 1998). We find that activation of group II or group III mGluRs does not completely block excitatory transmission in the BNST. We therefore hypothesized that either 1) the receptors function at differing afferent pathways, or 2) there was incomplete modulation of glutamatergic transmission by activation of mGluRs at each synapse. In order to begin to test if group II and group III mGluRs act on different inputs or different signaling pathways we co-applied specific agonists. Activation of group II mGluRs did not enhance the inhibition induced by the group III

agonist. This suggests the interesting possibilities that either these mGluRs function at a common pool of afferent inputs, and/or that they converge to some degree on common effector pathways. Future higher resolution anatomical studies combined with more mechanistic studies will be necessary to address these possibilities.

Behavioral Relevance

The present findings are consistent with the anxiolytic properties of agonists of group II and group III receptors. AMPA receptor antagonists directly injected into the BNST reduce potentiated startle responses (Walker and Davis, 1997), suggesting that dampening fast glutamatergic signaling in the BNST can produce anxiolytic responses. Thus the depression of this transmission produced by group II and group III mGluR agonists that we observe in the present study would be predicted to have similar outcomes on behavior. Reduced glutamatergic drive within the BNST would likely decrease output to the stress and reward circuitry, potentially reducing recruitment of the HPA axis.

Conclusions

The BNST plays a critical role in the response to stress and anxiety through a mechanism that involves excitatory glutamatergic transmission. The present study has demonstrated that excitatory transmission in the BNST is modulated by activation of group II and III mGluRs. Thus mGluRs within the BNST represent a candidate therapeutic target for the treatment of anxiety disorders. Behavioral studies will be necessary to test this possibility.

CHAPTER IV

ERK1-DEPENDENT MGLUR5-INDUCED LTD IN THE BED NUCLEUS OF THE STRIA TERMINALIS IS DISRUPTED BY COCAINE SELF-ADMINISTRATION.

Introduction

A major function of group I mGluR activation is to produce a persistent weakening of glutamatergic transmission at synapses in the CNS. There appear to be at least two major mechanisms through which this is accomplished. One involves the recruitment of endocannabinoid signaling and presynaptic alterations, and has been observed in the dorsal and ventral striatum (Gerdeman and Lovinger, 2001; Sung et al., 2001; Robbe et al., 2002). A second major mechanism is through endocannabinoid-independent signaling mechanisms (Rouach and Nicoll, 2003), and has been extensively characterized in the hippocampus. Both presynaptic (Gereau and Conn, 1995; Watabe et al., 2002; Zakharenko et al., 2002) and postsynaptic mechanisms (Huber et al., 2000; Huber et al., 2001; Snyder et al., 2001; Nosyreva and Huber, 2005) have been described for endocannabinoid-independent forms of group I mGluR LTD.

Endocannabinoid-dependent group I mGluR-induced LTD is disrupted in the ventral striatum in animals previously exposed to cocaine, suggesting that alterations in this form of plasticity may play a role in cocaine-induced alterations in animal behavior (Swanson et al., 2001). To date, however, the role of endocannabinoid-independent group I mGluR LTD has not been explored. We have addressed this question by examining effects of activation of group I mGluRs at excitatory synapses within the bed nucleus of the stria terminalis (BNST), a region critical in mediating stress-reward

interactions (Delfs et al., 2000; Stewart, 2000). We find that activation of group I mGluRs produces two distinct effects. First, a combined mGluR1 and mGluR5 activation induced a transient depression that is cannabinoid 1 receptor (CB1R) dependent. Second, as with endocannabinoid-independent group I mGluR LTD in the adult hippocampus, we find that activation of mGluR5 induces an ERK-dependent LTD. Surprisingly, our data demonstrate that this LTD requires the ERK1 rather than ERK2 isoform, establishing a key role for this isoform in the CNS. Finally, we find that this LTD is dramatically reduced following cocaine self-administration, suggesting a role for this form of plasticity in the actions of psychostimulants on anxiety and reward circuitries and their emergent control of animal behavior.

Results

Group I mGluRs Depress Excitatory Transmission in the BNST

To study the effects of group I mGluR activation on excitatory synaptic transmission in the BNST, we used local afferent stimulation and both extracellular and whole-cell patch clamp recording of postsynaptic responses as previously described; (Weitlauf et al., 2004; Egli et al., 2005; Grueter and Winder, 2005). To activate group I mGluRs, the specific agonist (RS)-3,5-Dihydroxyphenylglycine (DHPG) was briefly bath applied. Under extracellular recording conditions, following 15 min superfusion of DHPG (100 μ M), the amplitude of the N2 (synaptic, glutamatergic component) decreased to 79 \pm 4 % of baseline. The N2 amplitude 40 minutes following DHPG remained depressed at 86 \pm 2 % of baseline (Figure 14; $p < 0.05$). These effects were independent of

changes in the N1, the nonsynaptic, predominantly axonally-mediated response, indicating that depression of the synaptically-evoked response occurs independent of gross alteration of the number of axons recruited by the local afferent stimulation.

DHPG also reduced excitatory postsynaptic currents (EPSCs) in a concentration-dependent manner (Figure 14). 100 μ M DHPG reduced EPSCs to 52 \pm 5% of baseline at the peak effect that remained depressed (62 \pm 6% of baseline) 30 minutes post-DHPG application (Figure 14). The DHPG-induced LTD of EPSCs was independent of NMDA receptor function as robust LTD was elicited in slices pretreated with the NMDA receptor antagonist, DL -AP5 (100 μ M, peak effect: 46 \pm 11% of baseline, 30 min. post-DHPG: 65 \pm 8% of baseline; Figure 14).

To begin to identify the group I mGluR subtype, mGluR1 and/or mGluR5, involved in the DHPG-induced depression of EPSCs we applied DHPG in the presence of selective mGluR1 and mGluR5-specific antagonists. In the presence of the mGluR5-specific antagonist, MPEP (10 μ M), the initial component of the depression was not significantly altered (69 \pm 8% vs. 52 \pm 5% of baseline), however, 30 minutes post-DHPG, the depression was significantly attenuated (94 \pm 3% vs. 62 \pm 6% of baseline, p <0.05; Figure 15). While application of MPEP alone produced no effect on basal transmission, application of MPEP in combination with the mGluR1 selective antagonist LY367385 (100 μ M), produced a slight ~10% depression of the EPSC (data not shown). When DHPG was then applied in the presence of both of these antagonists, both the initial DHPG-induced depression (74 \pm 8% vs. 52 \pm 6% of baseline, Figure 15, p <0.05) and the

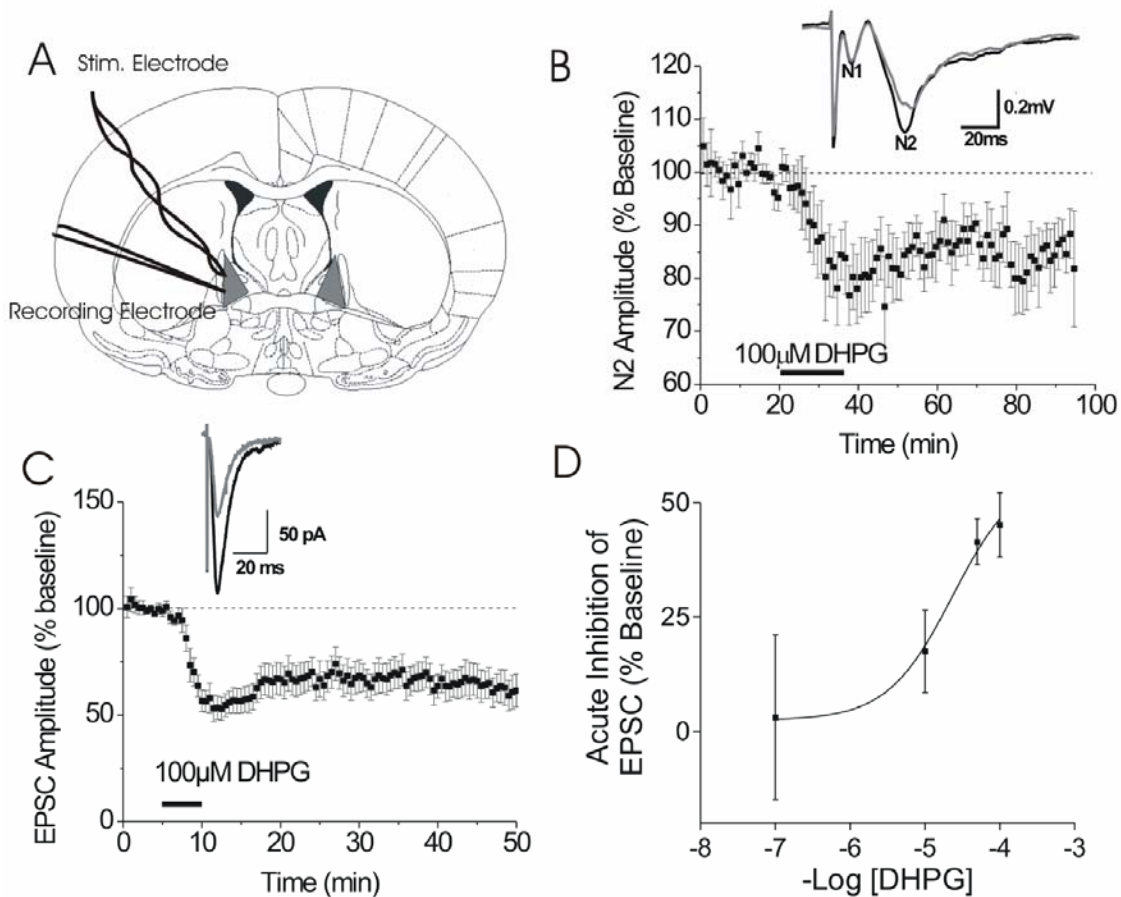


Figure 14. Group 1 mGluR activation in the BNST results in LTD of excitatory transmission.

(A) Diagram of coronal mouse brain containing the BNST. (B) Application of DHPG-induced persistent reduction in the synaptic response (N2), as measured by the peak amplitude of field potential responses, normalized to the average value during the basal period prior to drug application. Inset: Sample traces from one experiment before (heavy line) and after (light line) DHPG application, average of 6 consecutive traces at 18-20 and 38-40 min. Error bars, SEM. (C) Timecourse of DHPG-induced reduction in EPSC amplitude, normalized to the average value during the basal period prior to drug application. Inset: Representative EPSC traces from BNST neurons pre and post DHPG application, average of 10 consecutive traces from min 4-5 and min 12-13. (D) Concentration-response curve of peak DHPG-induced depression of EPSCs (n=3-12).

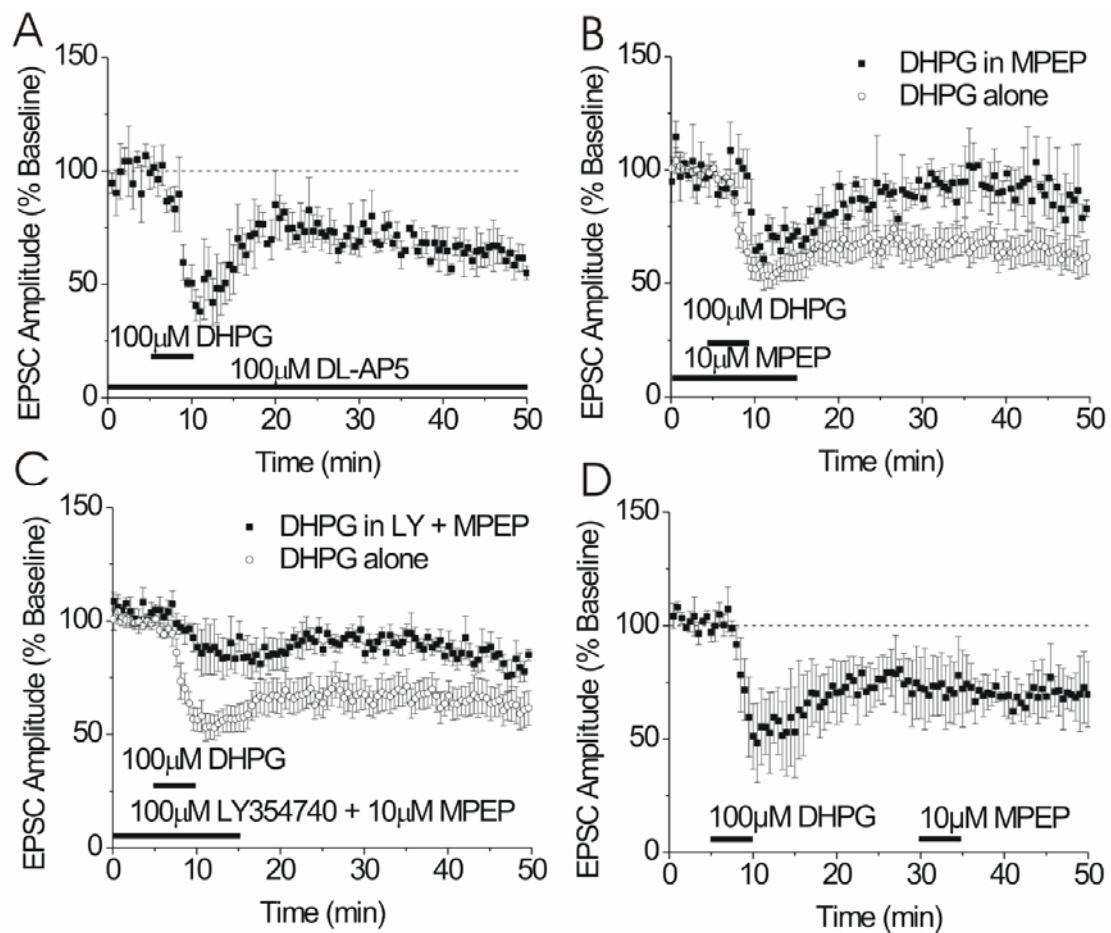


Figure 15. Pharmacological antagonists show DHPG induces a dual component in the BNST, the late component being mGluR5 dependent.

(A) The NMDA receptor antagonist, DL-APV (100 μ M) does not alter DHPG induced depression of EPSCs (n=3). (B) MPEP blocks the late but not the peak effect fo DHPG on EPSCs (n=3). (C) Both the mGluR1 antagonist LY 367385 (100 μ M) and MPEP (10 μ M) are required to attenuated the peak DHPG effect on EPSCs (n=3). D. MPEP does not reverse DHPG-induced LTD (n=3).

persistent depression ($89\pm 7\%$ vs. $62\pm 6\%$ of baseline, $p < 0.05$) were significantly attenuated compared to DHPG alone. Thus, these data suggest that DHPG-induced persistent depression of excitatory transmission in the BNST requires mGluR5 activation, and that mGluR1 activity is additionally required for the early component.

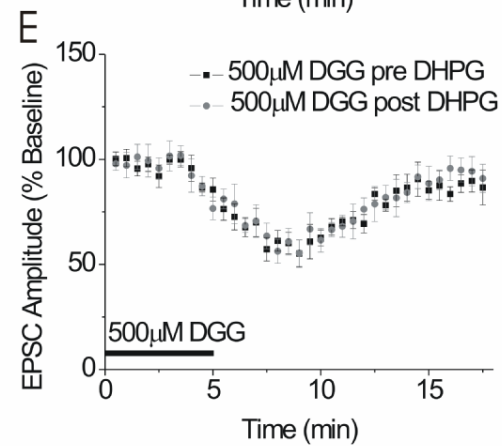
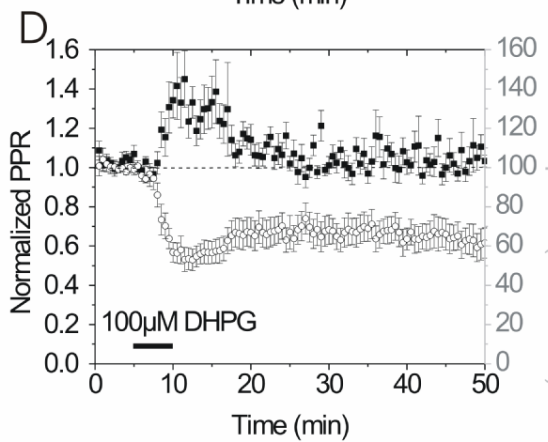
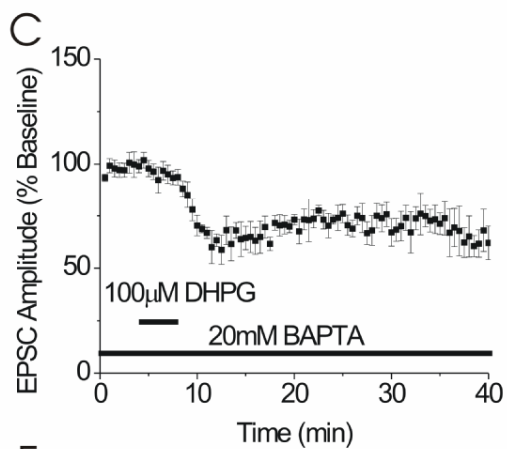
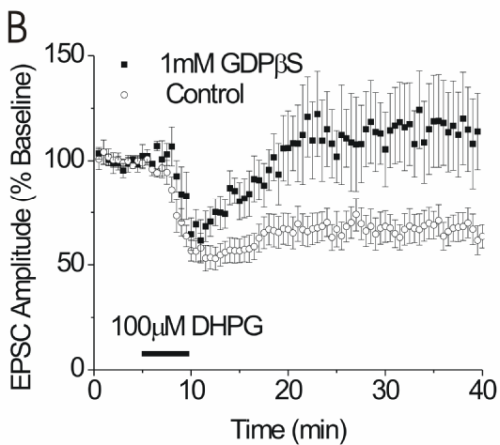
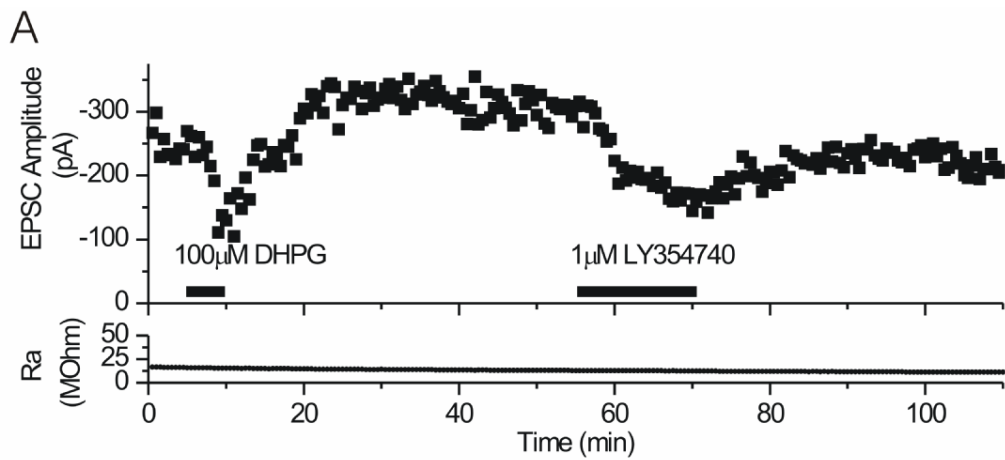
To verify that DHPG-induced persistent depression was not simply the result of poor washout of DHPG, or perhaps of induction of constitutive activity of group I mGluRs, we again applied MPEP, this time 20 minutes after washout of DHPG (Figure 15). At this time point, MPEP failed to alter EPSC amplitude, suggesting that transient activation of mGluR5 induces LTD.

Postsynaptic Induction of mGluR5-Dependent LTD in BNST

In the hippocampus, mGluR5-dependent LTD is thought to be induced postsynaptically (Watabe et al., 2002). To begin to determine the site of induction of mGluR5-dependent LTD in the BNST, we replaced GTP with 1 mM guanosine-5'-O-(2-thiodiphosphate) (GDP β S) in our standard potassium gluconate-based electrode-filling solution to inhibit activation of postsynaptic G proteins. Dialyzing GDP β S into the postsynaptic cell blocked DHPG-induced LTD ($114\pm 20\%$ of baseline) in the BNST while having no effect on the early component ($66\pm 8\%$ of baseline; Figure 16). In contrast, we tested the effects of GDP β S in the postsynaptic cell on depression of synaptic transmission induced by activation of group 2 mGluRs. In the BNST, group II mGluR activation produces LTD at excitatory synapses that likely results from a presynaptic action (Grueter and Winder, 2005). Inclusion of GDP β S in the pipette did not alter the ability

Figure 16. Postsynaptic G-proteins mediated the DHPG-induced LTD while no changes in probability of release or synaptic cleft glutamate concentrations are detected.

(A) Upper panel: Representative experiment showing blockade of DHPG-induced LTD but not LY354740-induced LTD by intracellular GDP β S (1mM). Lower Panel: Access resistance is unaltered in the presence of GDP β S during the duration of the experiment. (B) Blockade of postsynaptic GPCR-signaling with GDP β S prevents DHPG-induced depression of EPSCs (closed squares, n=5) but not control cells containing GTP (open circles). (C) Chelation of postsynaptic Ca²⁺ with 20mM BAPTA in the intracellular solution does not alter DHPG-induced depression of EPSCs (n=6). (D) Timecourse of normalized PPR illustrating transient increase in release probability in response to DHPG (left axis). As illustrated, DHPG causes a transient increase in PPR during the peak effect on EPSC amplitude but returns to basal levels during the LTD (closed squares, PPR; open circles EPSC amplitude). (E) Percent inhibition of EPSCs by γ DGG is unchanged before and after DHPG application (n=5).



of the group II mGluR agonist LY 354740 (1 μ M) to reduce synaptic transmission (Figure 16). Further, GDP β S did not alter basal transmission in time-matched recordings (data not shown). These results indicate a postsynaptic G-protein is necessary for induction of LTD though it does not rule out the possibility of presynaptic maintenance of this LTD.

Results in the literature are mixed as to whether postsynaptic calcium rises are required for mGluR5-dependent LTD (Bolshakov and Siegelbaum, 1994; Oliet et al., 1997; Maejima et al., 2001; Rae and Irving, 2004). To begin to test for postsynaptic calcium dependency, we added 20 mM BAPTA to our standard potassium gluconate-based electrode-filling solution to chelate intracellular calcium. Introduction of 20 mM BAPTA to the postsynaptic cell had no significant effect on DHPG LTD (66 \pm 8% of baseline, Figure 16).

Synaptic Locus of Group I mGluR-Mediated Effects

To begin to assess the synaptic mechanisms involved in DHPG-induced depression of transmission, we examined the effects of DHPG application on the ratio of EPSC amplitudes in response to closely spaced (50 ms) paired stimuli (paired-pulse ratio, PPR) to query whether DHPG produces alterations in presynaptic release probability. During the initial, peak depression of the EPSC produced by DHPG, we found a significant increase in the PPR (120 \pm 6%, p <0.05, Figure 16). However, there was no significant change in PPR during DHPG LTD (104 \pm 5%, p =0.37, Figure 16). In comparison, activation of either group 2 or group 3 mGluRs, which commonly function presynaptically as autoreceptors, caused a persistent increase in PPR in the BNST

(Grueter and Winder, 2005). These data suggest that while the transient peak effect of DHPG involves an alteration in release probability, the LTD does not.

While the PPR data suggest that alterations in release probability cannot account for the LTD induced by DHPG, presynaptic mechanisms that altered either vesicular glutamate concentration, fusion pore release dynamics, or synaptic glutamate buffering could still account for DHPG-induced LTD. To begin to address these possibilities, we performed experiments comparing the effects of bath application of a low affinity AMPA receptor antagonist, γ DGG (Chen and Diamond, 2002), before and after DHPG application (Figure 15E). Alterations in synaptic cleft glutamate concentration by DHPG would be predicted to alter the antagonism of the EPSC by γ DGG. We found that brief application of 500 μ M γ DGG produced an ~50% inhibition of the EPSC, which nearly completely reversed upon washout. The percent inhibition by this application was unchanged when γ DGG was again applied 30 minutes after DHPG application (Figure 16E). These data suggest that DHPG-induced LTD is not likely due to alterations in glutamate concentrations at the synaptic cleft.

Spontaneous, CNQX-sensitive (data not shown) miniature excitatory postsynaptic currents (mEPSCs) were also analyzed in the BNST following DHPG application to further assess mechanisms by which transmission is modulated. Representative traces of mEPSCs measured at -90 mV before and after DHPG application are shown in Figure 17. Application of 100 μ M DHPG significantly decreased frequency of mEPSCs both at the transient time point (68.6 \pm 6% of baseline, p <0.05, Figure 17) and at the late time point (64.4 \pm 9% of baseline, p <0.05, Figure 17). However, there was no significant effect on mEPSC amplitude at either the transient time point (96.8 \pm 5% of baseline, p >0.05,

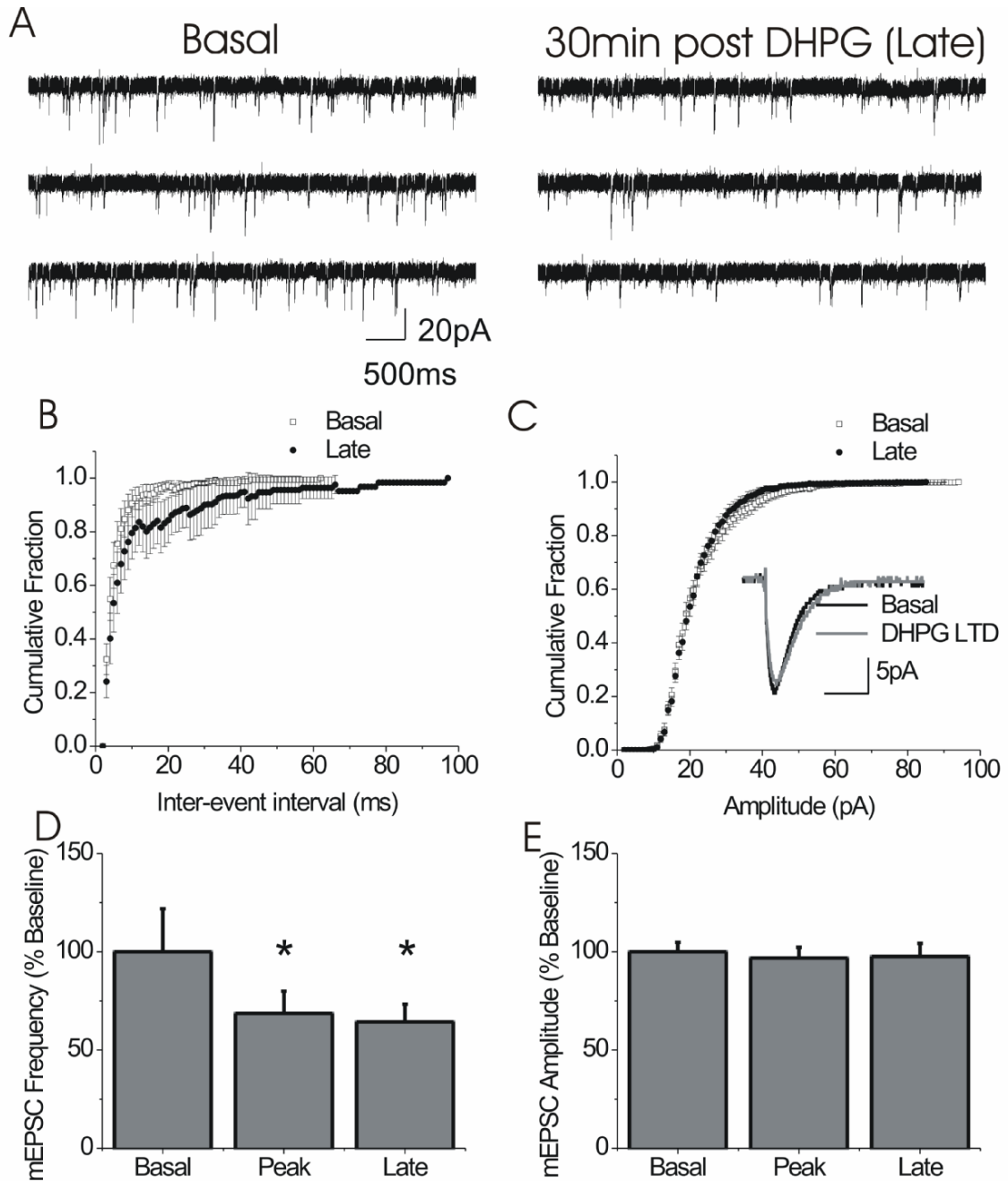


Figure 17. DHPG decreased the frequency but not amplitude of BNST mEPSCs.

(A) Sample current traces of mEPSCs in a BNST neuron before DHPG application (basal) and at 30 min post (late) DHPG. (B) Cumulative probability plot of DHPG induced effects on mEPSC inter-event interval within the basal (3-5 min, open squares) and late (min 35-37 closed circles) time periods. ($n=7$; $p<0.05$ K-S test). (C) Cumulative probability of effects of DHPG on mEPSC amplitude within the same current recordings as shown in B. Inset, representative traces before DHPG application and during DHPG mediated LTD. (D, E, F) Percent of mean effect of DHPG on mEPSCs frequency (D), and amplitude (E, F) during the peak and 30 minutes post (late) DHPG ($*p<0.05$).

Figure 17) or the late time point ($97.6 \pm 7\%$ of baseline, $p > 0.05$, Figure 17). Additionally, no gross changes in the kinetics of mEPSCs were observed after DHPG application (inset, Figure 17), however, a small but significant change in decay time (τ) was observed (2.38 ± 0.39 ms basal vs. 2.68 ± 0.51 ms 30 min post DHPG; $n = 7$, $p < 0.05$).

DHPG-Induced Transient Effects, but not LTD, are Reduced in CB1R Knockout Mice

Group I mGluR-induced LTD has been demonstrated to be presynaptically maintained and dependent on endocannabinoid receptor CB1 (CB1R) signaling in the nucleus accumbens and striatum (Maejima et al., 2001; Robbe et al., 2003) while being independent of cannabinoid signaling in the hippocampus (Rouach and Nicoll, 2003). To test whether the CB1R is involved in effects of DHPG on EPSCs in BNST, we examined the effects of DHPG ($100 \mu\text{M}$) on EPSCs recorded from BNST neurons in slices obtained from CB1R knockout mice (Zimmer et al., 1999). Interestingly, the initial depression of the EPSC by DHPG was significantly blunted in neurons from CB1R knockout mice ($84 \pm 5\%$ of baseline, $p < 0.05$) compared to control animals at the same time point (Figure 18). Consistent with these data, we found that in contrast to results obtained in wildtype mice, PPR following DHPG application was unaltered in these experiments ($108 \pm 6\%$ of baseline, $p > 0.05$, Figure 18). The CB1R knockout mice did not have a generalized deficit in receptor regulation of presynaptic function, however, as activation of group II mGluRs in the BNST of CB1R knockout mice resulted in an increase in PPR ($154 \pm 4\%$ of baseline, data not shown) as previously described in wildtype mice (Grueter and Winder, 2005).

While the acute effect of DHPG on EPSCs was blunted in the CB1R knockout mice, DHPG-induced LTD was still robustly elicited in these animals ($73\pm 7\%$ vs. $62\pm 6\%$ of baseline 30 minutes after DHPG administration, Figure 18). These data suggest that the initial component of the DHPG-induced depression of the EPSC, as in the hippocampus, is mediated through the CB1R, while the mGluR5-mediated LTD in the BNST is independent of CB1R signaling.

DHPG-Induced Depression of Synaptic Transmission in the BNST is ERK1 Dependent

CB1R-independent group I mGluR-dependent LTD in the hippocampus has been reported to require ERK activation (Gallagher et al., 2004). Further, group I mGluRs recruit ERK activity in a variety of contexts (Mao and Wang, 2003; Mao et al., 2005). In order to examine the role of ERK in DHPG-induced depression of EPSCs in the BNST we utilized two converging approaches. First, we applied DHPG ($100\ \mu\text{M}$) in the presence of the MEK inhibitor (U0126, $20\ \mu\text{M}$) to BNST slices while recording EPSCs. The presence of the MEK inhibitor significantly attenuated, but did not ablate, the early component of the DHPG-mediated depression of transmission to $69\pm 5\%$ vs. $52\pm 5\%$ of baseline (Figure 19). However, the DHPG-induced LTD was abolished in the presence of the MEK inhibitor ($92\pm 10\%$ of baseline, 30 min post DHPG, Figure 19).

We next examined the effects of DHPG on EPSCs in the BNST of ERK1 knockout mice. Similar to the U0126 experiments, application of DHPG in the BNST of ERK1 knockout mice produced an initial depression of the EPSC that was significantly attenuated, though still present ($74\pm 4\%$ of baseline). Surprisingly, however, LTD was

Figure 18. The peak component of the EPSC is CB1 receptor dependent but the LTD is CB1 R independent.

(A) Summary of the effects of DHPG (100 μ M) on EPSCs in BNST from CB1 receptor null mice (black squares; n=9) and control animals (gray circles; n=12). (B) The PPR is unchanged in the CB1R null BNST following DHPG application. (C) Bar graph representing the basal, peak and late effects of DHPG on PPR in control and CB1R null mice (n=9 and 12, respectively; * p<0.05 compared to basal).

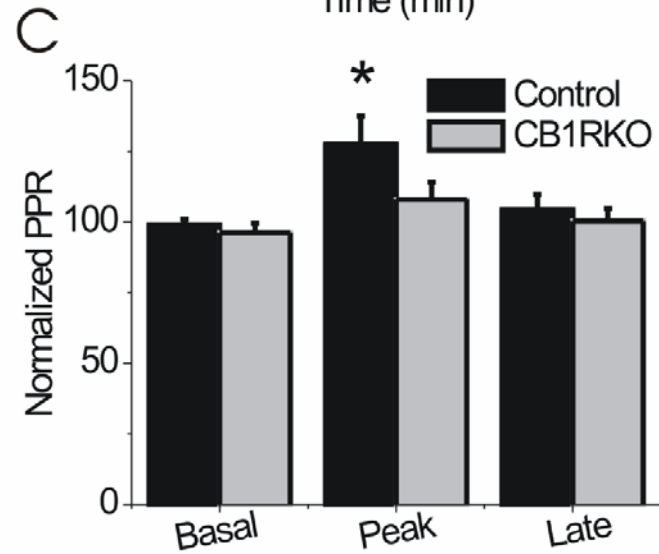
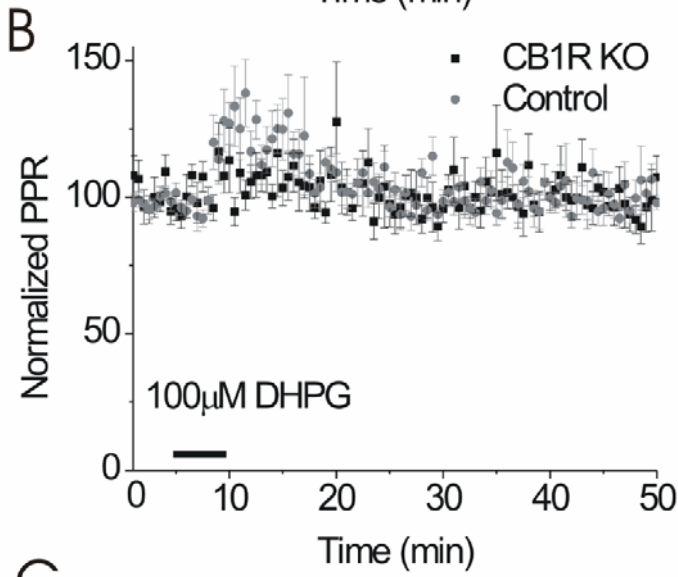
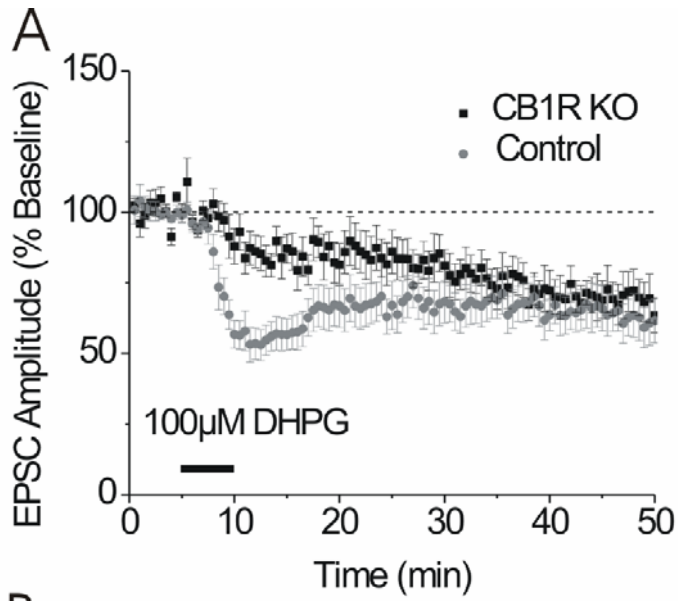
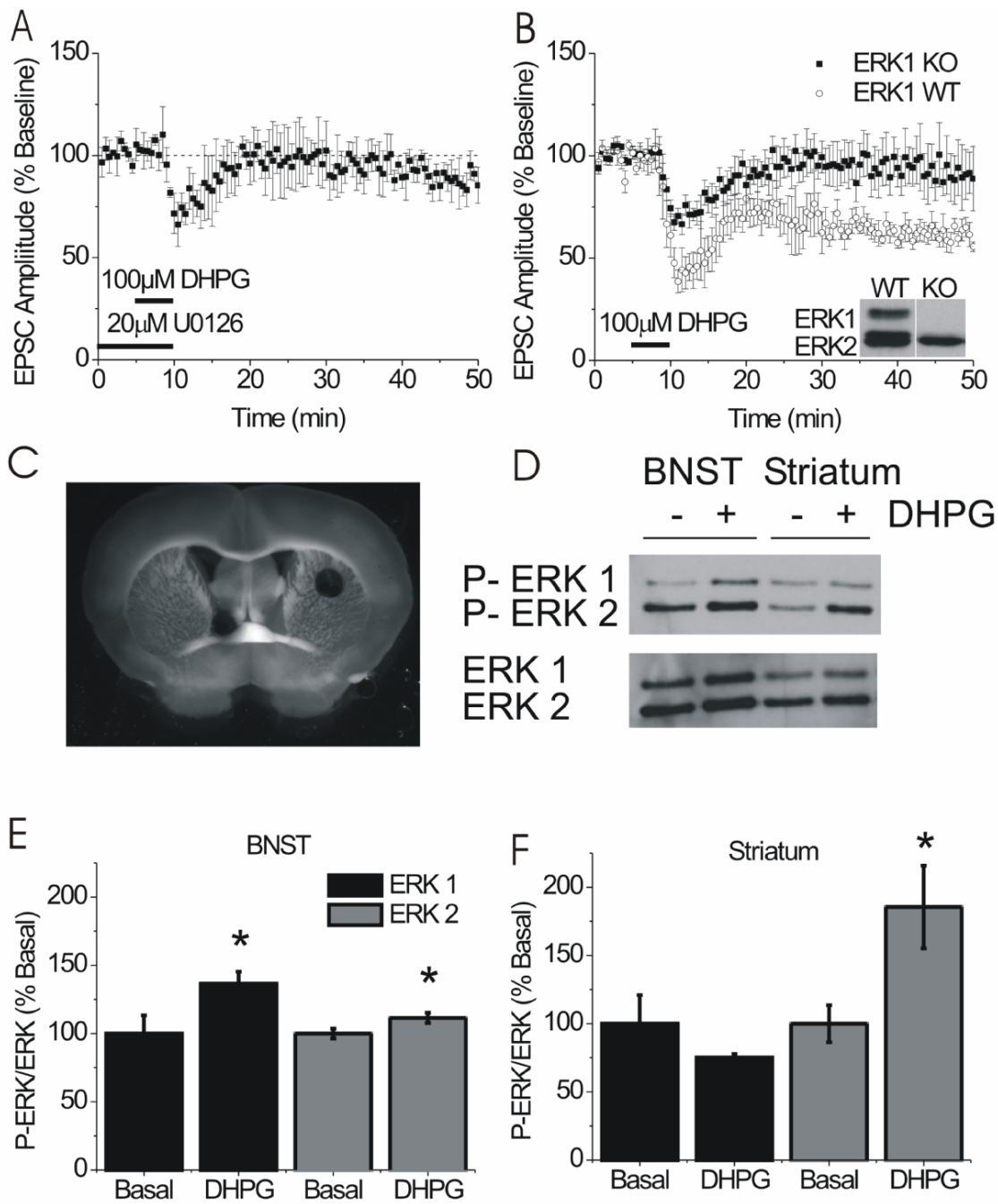


Figure 19. mGluR5-induced LTD in the BNST is ERK dependent.

(A) DHPG induced LTD of EPSCs is abolished by the presence of the MEK inhibitor, U0126 (20 μ M, n=6). (B) EPSCs from BNST neurons from ERK1 null mice do not elicit LTD following DHPG application (n=6). Inset represents presence/absence of ERK1 in control and ERK1 (-/-) mice. (C) Representative slice illustrating size and location of BNST and striatal punches taken for biochemical analysis. (D) Representative immunoblot for phosphorylated ERK1/ERK 2 and pan ERK1/ERK2 from either the BNST or the striatum in the absence or presence of DHPG. (E and F) Quantitative analysis of ERK1 and ERK2 phosphorylation in the BNST (E; n=8; *p<0.05) and the striatum (F, n=8; *p<0.05).



completely ablated ($102\pm 7\%$ of baseline, Figure 19). The effects of DHPG on EPSCs in the BNST from wild-type littermates were not different from C57Bl6j mice (Figure 19).

DHPG Activates ERK in BNST

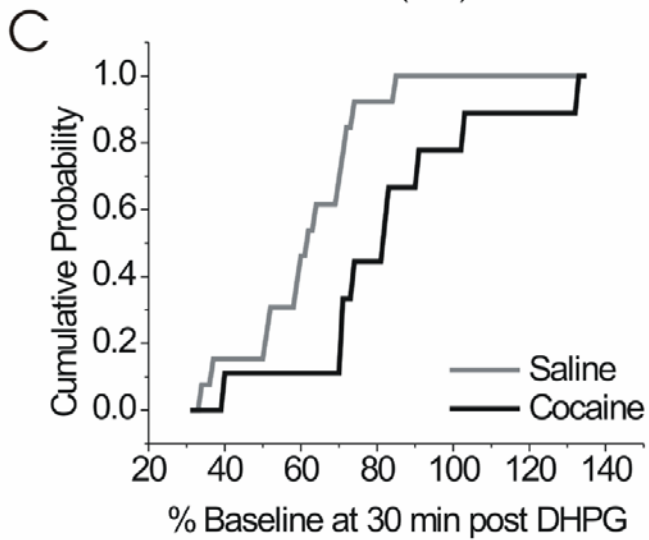
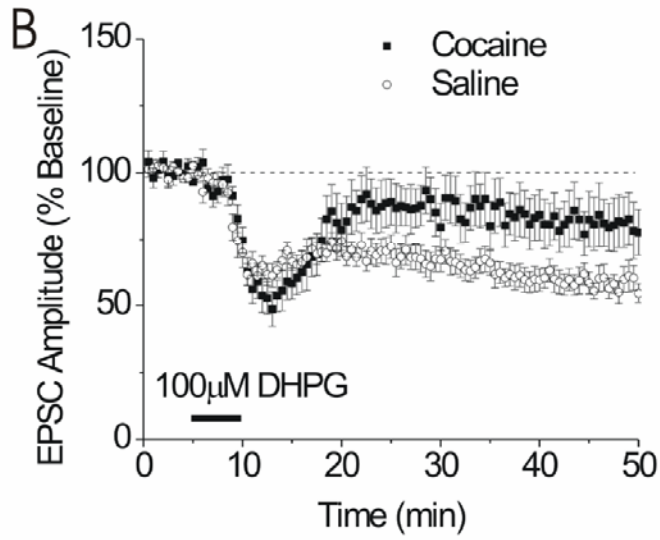
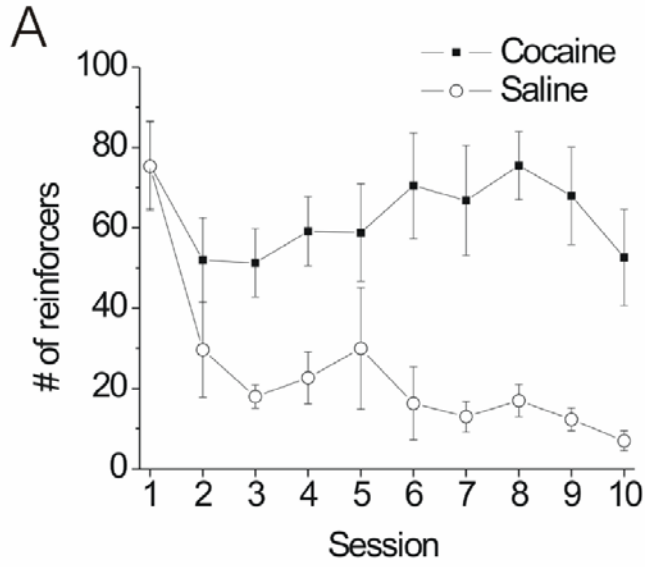
The above results suggest that ERK1 activity is required for DHPG-induced LTD. Since group I mGluR activation has been shown to activate ERK1/2 in other brain regions (Choe and Wang, 2001; Adwanikar et al., 2004; Gallagher et al., 2004), we tested whether DHPG application activated ERK1 and ERK2 in the dorsal BNST. Frozen tissue punches from the BNST and striatum were obtained following a 5 min exposure to DHPG ($100\mu\text{M}$) (Figure 19).

First, as has been previously reported, we found that DHPG produced robust activation of ERK in striatum (Choe and Wang, 2001); Figure 19). Striatal ERK2 phosphorylation was increased almost two fold in response to DHPG ($185\pm 4\%$ of basal, $p < 0.05$, Figure 19) while ERK1 phosphorylation was not significantly altered ($75\pm 3\%$ of basal, $p > 0.05$, Figure 19). Thus, as is the case in many brain regions, ERK2 appears to be the predominant isoform activated by group I mGluRs in the striatum. Interestingly, in the BNST, the relative ratio of ERK1 to ERK2 expression was higher than in striatum (Figure 19). Moreover, we observed a relatively robust increase in ERK1 phosphorylation in the BNST in the presence of $100\mu\text{M}$ DHPG ($137\pm 9\%$ of basal, $p < 0.05$, Figure 19). DHPG treatment caused only a very small increase in ERK2 activation ($111\pm 4\%$ of basal, $p < 0.05$, Figure 19). These data suggest activation of group I mGluRs results in ERK1 and to a lesser extent ERK2 activation in the BNST.

MGluR5-Dependent LTD in the BNST is Greatly Attenuated by Contingent *in vivo* Cocaine Administration

Intraperitoneal cocaine administration disrupts endocannabinoid dependent, group I mGluR-induced LTD in the nucleus accumbens (Swanson et al., 2001; Fourgeaud et al., 2004), and activates ERK in the BNST (Valjent et al., 2004). Thus we examined the effects of self-administration of cocaine on mGluR5-dependent LTD in the BNST. After food training, mice were allowed 3 hour daily sessions of access to either cocaine or saline on a fixed ratio 1 (FR1) schedule. Animals in the cocaine group lever pressed significantly more than saline controls (Figure 20). Twenty four hours after the last day of self-administration (10 days total), the mice were sacrificed, and brain slices prepared for electrophysiological analysis. In studies in which the experimenter was blinded to the conditions, the initial component did not differ in slices prepared from the DHPG-induced depression for cocaine self-administering ($51\pm 6\%$ of baseline, Figure 20) and saline administering ($61\pm 4\%$ of baseline, Figure 20) mice. However, the persistent depression of EPSCs by DHPG was significantly attenuated in slices prepared from cocaine self-administering mice ($83\pm 9\%$ of baseline, when compared to saline control animals, $63\pm 5\%$ of baseline, $p < 0.05$). The cumulative probability of DHPG-induced LTD is also significantly altered in cocaine vs. saline treated mice (Figure 20; KS-Test, $D = 0.57$ with a $p < 0.05$). Operant training did not alter DHPG-induced LTD as there is no difference between animals self-administering saline and naïve animals, $61\pm 4\%$ vs. $62\pm 6\%$, respectively. PPR from cocaine treated and saline treated groups were not different than naïve animals (data not shown).

Figure 20. mGluR5-dependent LTD is attenuated by contingent *in vivo* cocaine. (A) Summary of active lever presses for cocaine/saline (* $p < 0.05$). B. Summary of the effects of cocaine self-administration on mGluR5-induced LTD in the BNST (cocaine, $n=9$; saline, $n=13$). C. Cumulative probability of DHPG induced depression of excitatory transmission from saline administered (dotted line) and cocaine administered (solid line) mice.



Discussion

Group I mGluRs are necessary for many of the behavioral alterations produced by cocaine administration (Chiamulera et al., 2001; McGeehan and Olive, 2003; Herzig and Schmidt, 2004; Kenny et al., 2005; Lee et al., 2005). Several studies indicate that signaling by mGluR5 in the nucleus accumbens is altered by cocaine administration (Swanson and Kalivas, 2000; Swanson et al., 2001). The BNST is a brain region uniquely positioned to modulate the integration of motivational and higher order processive innervations, receiving input from limbic regions such as the amygdala and hippocampus, and projecting to areas involved in the reward/motivation and stress circuitry, the VTA, NAc and PVN (Herman and Cullinan, 1997; McDonald et al., 1999; Georges and Aston-Jones, 2002). Thus in the present studies, we have examined group I mGluR regulation of excitatory transmission in the BNST.

The data presented in the present work demonstrates that activation of group I mGluRs in the BNST leads to a postsynaptically-induced persistent weakening of excitatory transmission. This depression occurs through two temporally distinct mechanisms as detailed below. Moreover, we define a novel role for the ERK1 MAP kinase isoform, which has to this point received little attention in neuronal function. Finally, both group I mGluRs and ERK have been shown to play important roles in the behavioral actions of psychostimulants. Consistent with this, we find that the DHPG-induced LTD in the BNST is disrupted following cocaine self administration.

Group I mGluR Activation Depresses Excitatory Transmission in the BNST via Temporally Distinct Phenomena

We find that activation of group I mGluRs in the BNST results in two temporally and mechanistically distinct effects on excitatory synaptic transmission (Figure 21). DHPG-induced acute depression differs from the persistent depression in a number of respects. Using mGluR-subtype specific antagonists, we present data suggesting that the persistent depression is induced primarily by mGluR5 activation, while mGluR1 activation is additionally required for the acute depression induced by DHPG application. This differentiation between the role of mGluR1 in the acute depression and mGluR5 in the LTD is consistent with the distinct action of these group I mGluR subtypes in the hippocampus on LTD and excitability of hippocampal neurons (Mannaioni et al., 2001; Faas et al., 2002). We used multiple pharmacological, genetic and biophysical tools to further differentiate these processes, as discussed below.

CB1R is Required for the Acute Presynaptic Phase of DHPG-Induced LTD

In contrast to the persistent depression described in detail below, the acute depression is associated with a robust increase in PPR of evoked EPSCs and a decrease in mEPSC frequency, suggesting that it involves a decrease in release probability. At many CNS synapses, presynaptic actions of group I mGluRs are mediated by the endocannabinoid system (Maejima et al., 2001). Similar to findings in the hippocampus (Rouach and Nicoll, 2003) and NAc (Robbe et al., 2002), we found that the initial phase of the DHPG-mediated depression is ablated in CB1R knockout mice. These data suggest that the acute depression is mediated by mGluR1/5-dependent production of endocannabinoids which then act on presynaptic CB1 receptors to depress transmission.

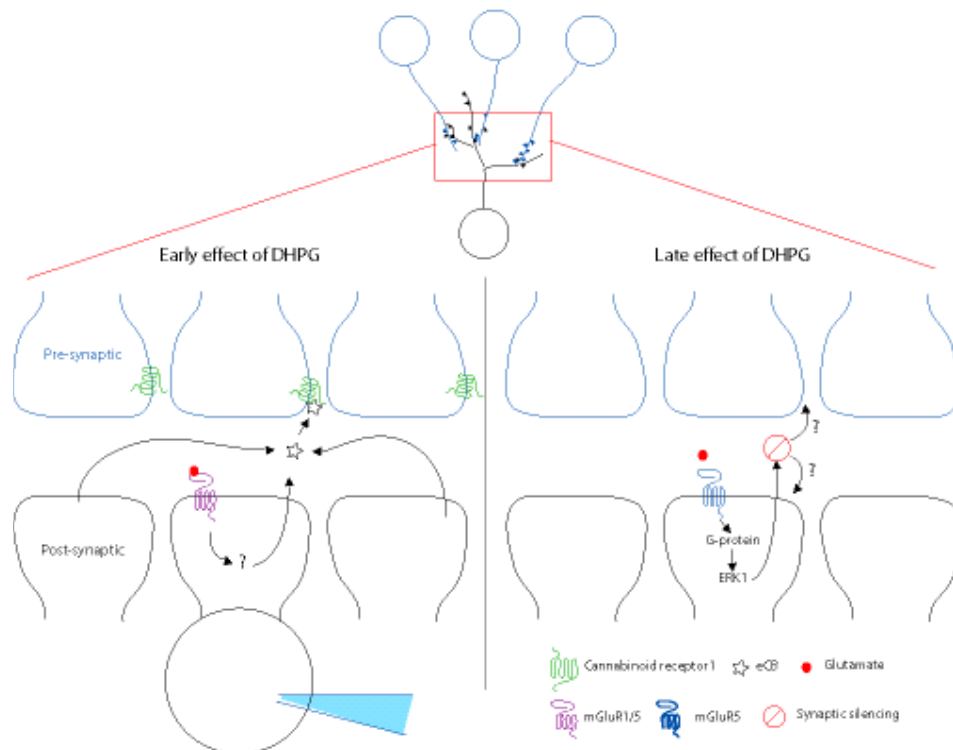


Figure 21. Model illustrating group I mGluR activation at excitatory BNST synapses. Left: Endocannabinoids act presynaptically to inhibit probability of release following mGluR1/5 activation. Right: A postsynaptic G-protein regulates ERK1/mGluR5-dependent LTD in the BNST resulting in a silencing of synapses.

As seen with DHPG-induced acute depression in cerebellar Purkinje cells (Maejima et al., 2001), the mGluR-mediated early CB1 receptor-dependent signaling may be through a Ca^{2+} independent pathway as the presence of 20mM BAPTA in the postsynaptic cell did not alter DHPG induced depression of excitatory transmission or PPR. An endocannabinoid candidate, 2-arachidonoylglycerol (2-AG), is thought to be produced downstream of PLC activity in a largely calcium-independent manner. Another, endocannabinoid candidate, anandamide, appears to be more calcium dependent (Maejima et al., 2001; Varma et al., 2001; Freund et al., 2003; Hashimoto et al., 2005). Thus, one possibility is that the CB1R-dependent component of DHPG-induced depression that we observe is mediated through the generation of 2-AG. However, it is important to note that we cannot exclude the possibility that endocannabinoids generated by neighboring cells in a Ca^{2+} -dependent manner contribute to these effects.

The Late, Persistent Phase of the mGluR5-Dependent LTD is Postsynaptically Induced

In addition to the acute effects of group I mGluR activation, we find that a persistent depression is elicited through independent mechanisms. As mentioned above, pharmacological evidence suggests the late phase of the DHPG-induced LTD is mGluR5 dependent as well as CB1R independent. The site of action of DHPG to produce the persistent depression of transmission is a bit more elusive. The induction of LTD appears to have a postsynaptic component, as dialysis of the postsynaptic cell with GDP β S blocked DHPG-induced LTD. The LTD was not associated with alterations in PPR, consistent with the lack of CB1R dependence, suggesting the maintenance of this LTD is not through an alteration of release probability. It is possible that the DHPG-induced

LTD is due to alterations in glutamate concentrations at the synapse (i.e., changes in vesicular loading or reuptake of glutamate). To test this possibility assessed the effectiveness of the low-affinity AMPAR antagonist γ DGG before and 30 min after DHPG application. If synaptic cleft concentrations of glutamate were reduced following DHPG-induced LTD we would expect an increase in the γ DGG mediated depression of excitatory transmission (Chen and Diamond, 2002). As this was not the case we conclude that vesicular content and/or reuptake of glutamate are not altered during DHPG-induced LTD.

Similar to what is seen in the hippocampus (Snyder et al., 2001), analysis of mEPSCs after DHPG treatment revealed a persistent decrease in the mEPSC frequency but not amplitude. As this mEPSC frequency decrease was not associated with an alteration in PPR in the evoked EPSCs, we interpret these data to suggest that DHPG-induced LTD is due to a reduction in n (number of available synaptic sites) rather than p (release probability). Such a “silencing” of synapses could occur through either pre- or postsynaptic actions, or a combination of the two. This idea is consistent with trafficking of AMPA receptors from the synapse following DHPG application as was shown in the hippocampus (Snyder et al., 2001; Xiao et al., 2001; Mangiavacchi and Wolf, 2004; Nosyreva and Huber, 2005).

A Novel Role for ERK1 in MGluR5-LTD

The MAPK/ERK signaling cascade plays a crucial role in a variety of cell regulatory events. Much emphasis has been placed on the role of this signaling cascade in learning and memory, and in glutamatergic synaptic plasticity at a variety of synapses

(Adams and Sweatt, 2002; Sweatt, 2004). In particular, MEK inhibitors block the induction of several forms of NMDA-receptor dependent LTP and LTD throughout synapses of the hippocampus and the amygdala (for review see Sweatt, 2004). A more recent, growing literature indicates that the ERK signaling cascade plays a critical role in behavioral alterations produced by psychostimulants. Inhibitors of the ERK signaling cascade administered *in vivo* reduce psychostimulant mediated behavioral sensitization (Valjent et al., 2005). It has also been suggested that ERK activation is involved in the incubation of cocaine craving in a region-specific manner (Lu et al., 2005). Further, *in vivo* psychostimulant administration produces activation of ERK in the reward circuitry including, strong activation within the BNST (Valjent et al., 2000; Valjent et al., 2004).

To date, evidence has suggested that the primary ERK isoform involved in these processes is ERK2 (Sweatt, 2004). First, ERK2 is the predominant isoform expressed in the hippocampus and amygdala. Second, these forms of synaptic plasticity are either normal (Selcher et al., 2001) or only subtly altered (Mazzucchelli et al., 2002) in ERK1 knockout mice. Third, multiple labs have suggested that stimuli that promote these forms of synaptic plasticity may selectively activate ERK2 (Adams and Sweatt, 2002).

Here, we provide evidence that ERK1 rather than ERK2 plays a critical role in mGluR5-induced LTD in the BNST. By using an inhibitor (U0126) targeting the MEKs that activate ERK1/ERK2 we were able to show that the ERK signaling cascade is necessary for mGluR5-induced LTD in the BNST. MEK inhibitors like U0126 do not allow for distinction of the relative roles of ERK1 and ERK2. Because ERK1 expression relative to ERK2 is stronger in the BNST than many other regions (Figure 18), we took

advantage of the availability of mice with a targeted deletion of ERK1. As seen in Figure 18, in ERK1 knockout mice, DHPG application in the BNST does not induce LTD. Further, consistent with a role specifically for ERK1 in DHPG-induced LTD in the BNST, we find that DHPG application preferentially activates ERK1 in the BNST, in contrast to the striatum, where ERK2 is the predominant isoform activated.

At present, very little is known about the distinctive roles of the ERK1 and ERK2 isoforms, as they are highly homologous (Chen et al., 2001). However, several studies, primarily in non-neuronal systems, have identified proteins and/or drugs that produce or facilitate selective activation of ERK1 versus ERK2. For example, MEK-binding partner 1 (MP1) selectively facilitates ERK1 activation and binds with considerable selectivity to ERK1 over ERK2 (Schaeffer et al., 1998). While little information exists for substrates differentially phosphorylated by ERK1 and ERK2, data from knockout mice point to differences in function. For example, while ERK1 knockout mice are viable and fertile (Selcher et al., 2001; Pages and Pouyssegur, 2004), targeted disruption of ERK2 produces a lethal mutation (Adams and Sweatt, 2002). Further, while much pharmacological evidence suggests that the MAP kinase signaling cascade plays an important role in NMDA receptor-dependent synaptic plasticity and learning and memory, hippocampal and amygdala based learning are unimpaired in ERK1 knockout mice (Selcher et al., 2001), and NMDA receptor-dependent plasticity is either normal (Selcher et al., 2001), or altered in complex ways in ERK1 null mice (Mazzucchelli et al., 2002).

Group I mGluRs and Drug Addiction

Our results indicate that mGluR5 is responsible for the DHPG-induced LTD in the BNST. As mentioned above, behavioral studies demonstrated that genetic deletion of mGluR5 as well as pharmacological blockade of mGluR5 resulted in reduced cocaine induced hyperactivity and reinforcing properties as well as reduced rewarding properties of cocaine (Chiamulera et al., 2001; Herzig and Schmidt, 2004; McGeehan et al., 2004; Lee et al., 2005). While the specific site of action of mGluR5 function in cocaine mediated behavioral and rewarding properties are still unknown, we show that, like in the NAc (Swanson et al., 2001; Fourgeaud et al., 2004), *in vivo* cocaine administration attenuates DHPG-induced LTD. Therefore, alterations in mGluR5 function in the BNST may play a key role in behavioral properties of addiction.

Excitatory Transmission and Drug Addiction

Lasting changes in the efficacy of glutamatergic synaptic transmission in specific brain regions has been suggested to play an important role in the neuroadaptations that occur after substance abuse (Hyman and Malenka, 2001; Winder et al., 2002; Kalivas, 2004; Wolf et al., 2004). Recent work suggests both psychostimulants and stress induce LTP in the VTA and psychostimulants alter NAc synaptic plasticity (Swanson et al., 2001; Thomas et al., 2001; Saal et al., 2003; Dong et al., 2004). As the BNST projects to the VTA and NAc it is possible that the BNST plays a role in the integration of information leading to adaptive changes in synaptic responses following administration of drugs of abuse. Indeed, this brain region plays a critical role in stress-induced reinstatement of drug seeking (Erb et al., 1996; Shaham et al., 2000), as well as morphine

withdrawal-induced conditioned place aversion (Aston-Jones et al., 1999). Moreover, evidence suggests that this region, and in particular glutamatergic transmission within this region, plays a critical role in anxiety-like behavior in rodents (Walker et al., 2003). Thus persistent regulation of glutamatergic transmission in this region by group I mGluRs may play an important role in regulating generalized anxiety states.

It is interesting to note that cocaine self administration has recently been reported to enhance AMPA/NMDA response ratios in the ventral BNST (Dumont et al., 2005), and intraperitoneal administration of cocaine enhances this ratio on dopaminergic neurons within the VTA (Borgland et al., 2004), a major efferent site from the BNST. Our data provide a potential mechanism contributing to these changes, as a reduction in the inhibitory constraint provided by group I mGluRs, as we have demonstrated, would likely facilitate the induction of NMDA-receptor dependent synaptic plasticity in these regions.

Conclusions

Our data, showing a lack of LTD in the BNST in *ERK1* mutants and after pharmacological blockade of MEK, establish a critical role for this isoform in regulating synaptic function. Moreover, the recent idea that neural adaptations to drugs of abuse may involve the same molecular elements as those essential to behavioral plasticity is strongly confirmed by our results, as mGluR5-dependent LTD is greatly attenuated in animals self-administering cocaine. These data suggest the possibility that hyperphosphorylation of ERK1 in the BNST (Valjent et al., 2004) in response to cocaine may be downstream of mGluR5 activation. Interestingly, the mGluR LTD phenotype

seen in the BNST following cocaine administration resembles that found in a NAc upon repeated cocaine administration and is believed to be a crucial molecular substrate for long-term adaptations to drug abuse (Swanson et al., 2001; Fourgeaud et al., 2004). Future work with *in vivo* pharmacological blockade of ERK activation during cocaine administration will certainly help to elucidate the still largely obscure signaling mechanisms underlying the synaptic plasticity and pathophysiology of drug addiction involving the BNST.

CHAPTER V

GENERAL DISCUSSION

The integration of information from neurocircuits innervating the reward circuitry is becoming increasingly recognized. For instance, the roles of stress/anxiety pathways in modulating specific effects of the reward system are becoming more apparent. Behavioral data suggests the BNST is involved in integrating stress information and relaying this information to the stress and reward pathways. Moreover, behavioral data suggests glutamatergic transmission in the BNST as well as other regions in the stress/reward pathways plays an integral role in regulating stress and drug addiction behaviors. Synaptic plasticity in this region could in part underlie the persistent behavioral alterations in generalized anxiety and addiction. However, little physiological (functional) data has been generated discerning the signaling pathways involved in BNST synaptic plasticity. Utilizing electrophysiological techniques and incorporating pharmacological, biophysical, genetic and behavioral manipulations this thesis has been aimed at elucidating synaptic signaling pathways potentially involved in complex behaviors such as drug addiction in the BNST.

Function of Group II mGluRs in Excitatory Transmission in the BNST

The data I describe in this document suggest excitatory drive to the BNST, whether originating from the Ventral Subiculum, limbic cortex or the BLA, is modulated presynaptically by group II mGluRs. Consistent with the function of group II mGluRs in

other brain regions (Bushell et al., 1996; Macek et al., 1996; Kilbride et al., 1998), we find selective activation of group II mGluRs in the BNST can produce a reversible depression of synaptic transmission. In other brain regions, group II mGluRs act as autoreceptors at glutamatergic synapses which, upon activation, results in a reduction in the probability of synaptic vesicle release. Likewise, experiments designed to test for changes in release probability, PPR analysis, indicate a decrease in the probability of vesicular release following prolonged group II receptor activation in the BNST.

There are many potential targets downstream of mGluR2/3 receptor activation. As would be expected of PTX sensitive $G_{\alpha i}$ -mediated effects, group II mGluRs have been shown to regulate adenylyl cyclase activity (Gereau and Conn, 1994), modulate ion channels, and in some cases activate PLC and PLD (Otani et al., 2002). Other potential downstream targets implicated in vesicular release are the VGCCs. Modulation of these channels located at the presynaptic terminals can reduce influx of calcium ultimately resulting in a decrease in vesicular release probability. Future studies investigating the downstream effectors of group II mGluRs in the BNST such as PKA, PLC, PLD and VGCCs could prove beneficial in determining how these signaling pathways are involved in modulating vesicular release probability in the BNST.

Interestingly, prolonged activation of group II mGluRs resulted in LTD while a brief activation of these receptors resulted in a short term, reversible depression. It is conceivable that these receptors in the BNST undergo prolonged activation during extended periods of stress/anxiety and therefore could be targets for anxiolytics. Consistent with this, behavioral studies, both in rodent models of anxiety (Helton et al., 1998; Klodzinska et al., 1999; Linden et al., 2002; Tizzano et al., 2002; Linden et al.,

2003) and in humans (Fitzjohn et al., 1998; Grillon et al., 2003) suggest that group II and III mGluR ligands when given systemically may represent useful approaches to treat anxiety.

Function of Group III MGluRs in Excitatory Transmission in the BNST

We found a persistent depression of synaptic transmission in studies in which the group III mGluR agonist L -AP4 was added. This effect is likely mediated by the mGluR7 and/or 8 subtypes, as L -AP4 has a much higher affinity for mGluR4 than 7 and in the BNST a relatively high concentration of L -AP4 is required to produce significant weakening of excitatory transmission. Also, the specific mGluR8 agonist DCPG produces a depression of EPSCs implicating a role for this receptor in the BNST. Consistent with this, anatomical evidence points to the expression of mGluR7, with weaker, if any, expression of mGluR4 in dBNST (Kinzie et al., 1995; Ohishi et al., 1995; Kinoshita et al., 1998). However, significant effects were observed with low concentrations of L -AP4, suggesting the possible involvement of mGluR4 as well. Also consistent with anatomical data showing mGluR7 at the axon terminal, we find the functional output, as assessed by PPR, is a result of a reduced vesicular release probability.

Implications for Group II and III MGluR Activation in the BNST

While direct activation of group II and/or III mGluRs in the BNST has not been studied *in vivo*, other behavioral studies in which the AMPA receptor antagonist (NBQX) was injected into the BNST and global administration of mGluR agonists/antagonists can

give us insight into the behavioral consequences of group II and III mGluR function in the BNST. One can speculate that as activation of these receptors function to decrease synaptic efficacy in this region, a decrease in the functional output of the BNST would be observed. In other words, as antagonism of AMPARs results in the complete blockade of output from the BNST, the modulatory effect of reducing this output could have a similar result although perhaps less severe. Future experiments designed to specifically target group II or III mGluRs in the BNST could provide insight into the legitimacy of this prediction. For instance, one could knock down these receptors in the BNST using virally mediated methods followed by behavioral paradigms designed to test for anxiolytic states.

It is difficult to make clear predictions regarding the net effect of group II and III activation in the BNST on propagation of a signal through the stress and reward circuitries. However, one could speculate that reduced glutamatergic drive within the BNST would likely decrease output to the stress and reward circuitry, potentially reducing recruitment of the HPA axis (Figure 22). Therefore, as many pathophysiological disorders/syndromes have been linked to altered mGluR function, including stress/anxiety and drug addiction, mGluRs in the BNST, could be the targets of the anxiolytic effects of the group II and III compounds. (Kim and Vezina, 1999; Kim et al., 2005).

The Hypothalamo-Pituitary-Adrenocortical Axis Regulates the Stress Response

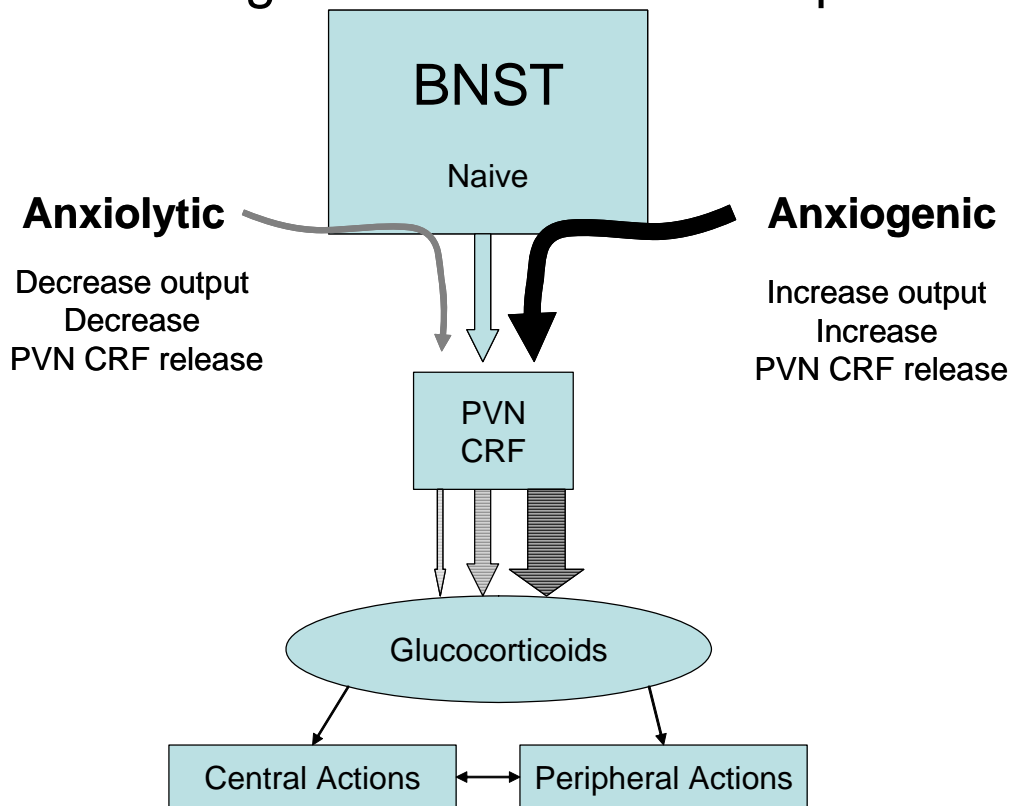


Figure 22. Schematic showing potential effects of anxiolytic and anxiogenic stimuli on stress/anxiety pathway.

Group I mGluR Activation Weakens Excitatory Transmission in the BNST

Group I mGluRs have been shown to function at many synapses in the CNS. As was the case in the BNST, a major function of group I mGluR activation is to produce a persistent weakening of glutamatergic transmission. We investigate the synaptic locus as well as downstream effectors of group I mGluRs in the BNST. Specifically, the role of CB1 receptor function and ERK in group I mGluR LTD were investigated. In many ways the results we observed mapped onto the results of multiple labs studying group I mGluR function in the hippocampus (Huber et al., 2000; Huber et al., 2001; Snyder et al., 2001; Nosyreva and Huber, 2005).

Cannabinoids and Group I mGluRs

As suggested previously, there appear to be at least two major mechanisms through which group I mGluR LTD is accomplished. One involves the recruitment of endocannabinoid signaling and presynaptic alterations, and has been observed in the dorsal and ventral striatum (Gerdeman and Lovinger, 2001; Sung et al., 2001; Robbe et al., 2002). A second major mechanism is through endocannabinoid-independent signaling mechanisms (Rouach and Nicoll, 2003), and has been extensively characterized in the hippocampus. Consistent with PPR and mEPSC data, the early component of the DHPG mediated LTD was CB1R dependent. However, the lasting depression was still present in the CB1R KO. It is important to note that group I mGluR function in the BNST could be dependent on the retrograde signaling of CB's. For instance, in the hippocampus, group I mGluR mediated LTD of inhibitory transmission is dependent on CB1R signaling

(Chevaleyre and Castillo, 2003, 2004). Future studies of CB signaling in the BNST could lead to new discoveries of the role of CB's in stress/anxiety and drug addiction.

The Role of ERK in Group I mGluR LTD

As mentioned previously, much emphasis has been placed on the role of the MAPK/ERK signaling cascade in learning and memory, and in glutamatergic synaptic plasticity at a variety of synapses (Orban et al., 1999; Adams et al., 2000; Mazzucchelli and Brambilla, 2000; Adams and Sweatt, 2002; Mazzucchelli et al., 2002; Gallagher et al., 2004; Sweatt, 2004). It is not surprising then that this pathway has been implicated in drug addiction as well as indicated by recent work from the Caboche laboratory (Valjent et al., 2000; Valjent et al., 2004; Lu et al., 2005; Valjent et al., 2005).

In this body of work we present the first evidence that ERK1 rather than ERK2 plays a critical role in neuronal function. More specifically, that mGluR5-induced LTD in the BNST is dependent on ERK1. At present, very little is known about the distinctive roles of the ERK1 and ERK2 isoforms, as they are highly homologous (Chen et al., 2001). However, several studies, primarily in non-neuronal systems, have identified proteins and/or drugs that produce or facilitate selective activation of ERK1 versus ERK2. For example, MEK-binding partner 1 (MP1) selectively facilitates ERK1 activation and binds with considerable selectivity to ERK1 over ERK2 (Schaeffer et al., 1998). While little information exists for substrates differentially phosphorylated by ERK1 and ERK2, data from knockout mice point to differences in function. For example, while ERK1 knockout mice are viable and fertile (Pages et al., 1999; Selcher et al., 2001; Pages and Pouyssegur, 2004), targeted disruption of ERK2 produces a lethal

mutation (Adams and Sweatt, 2002). Further, while much pharmacological evidence suggests that the MAP kinase signaling cascade plays an important role in NMDA-receptor dependent synaptic plasticity and learning and memory, hippocampal and amygdala based learning are unimpaired in ERK1 knockout mice (Selcher et al., 2001), and NMDA receptor dependent plasticity is either normal (Selcher et al., 2001), or altered in complex ways in ERK1 null mice (Mazzucchelli et al., 2002). Thus in future studies it will be important to determine why ERK1 is specifically required for DHPG-induced LTD in the BNST, and to determine whether this is a broader feature of mGluR5-dependent regulation of transmission, or whether it is specific to restricted regions like the BNST.

Downstream of ERK in Group I mGluR LTD

In neurons, ERK activation has been shown to be involved in learning based remodeling of dendritic synapses, interactions with dendritic spine stabilizing proteins, interaction with scaffolding proteins and regulation of protein synthesis (Sweatt, 2004). In the hippocampus, group I mGluR-induced LTD is dependent on dendritic protein synthesis (Huber et al., 2000; Huber et al., 2001; Gallagher et al., 2004) and results in alterations in protein synthesis dependent AMPA receptor trafficking (Mangiavacchi and Wolf, 2004; Nosyreva and Huber, 2005; Xiao et al., 2001; (Snyder et al., 2001; Collingridge et al., 2004). As group I mGluR LTD in the BNST is dependent on ERK activation, we investigated the possibility that this LTD is protein synthesis dependent and dependent on AMPA receptor trafficking. However, neither of two chemically

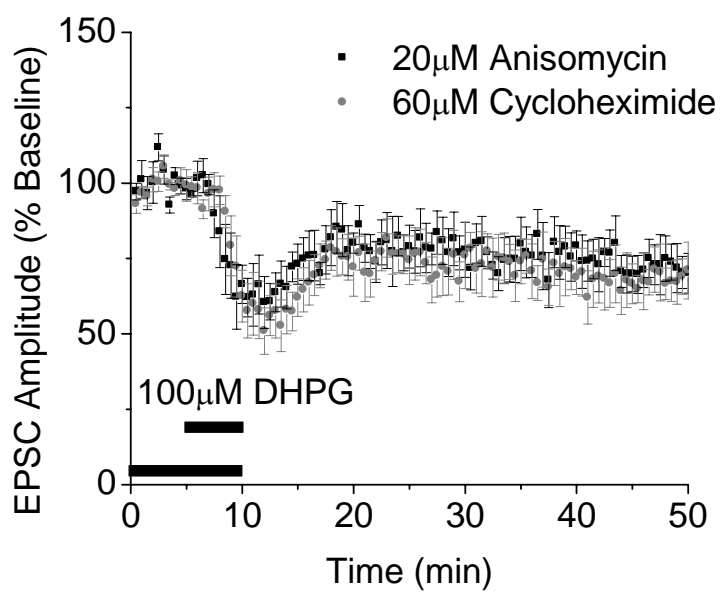


Figure 23. DHPG-induced LTD is unchanged by preincubation with the mechanistically distinct protein synthesis inhibitors Anisomycin or Cycloheximide.

distinct protein synthesis inhibitors altered group I mGluR LTD in the BNST (Figure 23). This suggests, unlike the group I mGluR-mediated LTD reported by Huber and colleagues and under the conditions of these recordings, protein synthesis is not involved in group I mGluR LTD in the BNST. However, we cannot rule out the possibility of a role for protein synthesis in group I mGluR-mediated LTD in the BNST as there is not a good positive control for this data.

AMPA internalization is emerging as a major mechanism for synaptic depression as was emphasized in the introduction. AMPAR internalization is dependent on the clathrin-mediated pathway involving the GTPase dynamin (Cremona and De Camilli, 1997; Collingridge et al., 2004), and is enhanced by factors that can induce synaptic depression, such as NMDAR stimulation (Beattie et al., 2000; Ehlers, 2000; Lin et al., 2000; Man et al., 2000; Man et al., 2000). Group I mGluR activation has been shown to enhance endocytosis as well (Mundell et al., 2001; Snyder et al., 2001; Xiao et al., 2001; Kawasaki et al., 2004).

To begin to investigate a role for endocytosis we studied the effects of intracellular Jasplakinolide on the group I mGluR-mediated LTD in the BNST. Jasplakinolide is a toxin that acts to stabilize actin and therefore disrupts endocytosis. Results in hippocampal slices indicate this drug is sufficient to prevent DHPG induced LTD (Xiao et al., 2001). In 4 of 5 experiments in which Jasplakinolide was introduced into postsynaptic BNST neurons group I mGluR-mediated LTD was greatly attenuated (Figure 24). Future studies introducing the dynamin-amphiphysin inhibitory peptide, which has been shown to block endocytosis, into the postsynaptic cell will help determine if endocytosis is involved in maintaining group I mGluR LTD in the BNST. A positive

result from these experiments will then lead us to test if GluR2 or perhaps mGluR5 are being endocytosed following activation of group I mGluRs. More specifically, we will begin to test for a role of the specific AMPAR subunits in group I mGluR-mediated LTD in the BNST.

As trafficking of the GluR1 subunit of the AMPAR may be a target for maintenance of group I mGluR LTD we tested the effects of DHPG on EPSC amplitude in BNST neurons from GluR1 knockout mice. In the GluR1 knockout mouse, the number of synaptic AMPARs is normal but the extrasynaptic receptors are greatly diminished (Zamanillo et al., 1999). Utilizing the GluR1 KO mice we investigated the role of GluR1 in group I mGluR LTD in the BNST. There is no change in DHPG-induced LTD in the BNST neurons from GluR1 KO mice suggesting this AMPAR subunit is not critical for this form of LTD (Figure 24). However, it is still possible that GluR2 containing AMPA receptors are involved in DHPG-induced LTD and therefore experiments designed to inhibit GluR2 trafficking or utilizing GluR2 knockout mice would be beneficial.

Group I mGluR LTD of Excitatory Synaptic Transmission is Attenuated in the BNST from Animals that Self-Administered Cocaine

A number of labs have shown that group I mGluRs play key roles in behavioral responses to psychostimulants. Genetic disruption of either mGluR5 (Chiamulera et al., 2001) or the homer family of group I mGluR interacting proteins (Swanson et al., 2001) produces profound alterations in responses to psychostimulants, as does pharmacological inhibition of group I mGluRs (McGeehan and Olive, 2003; Herzig and Schmidt, 2004).

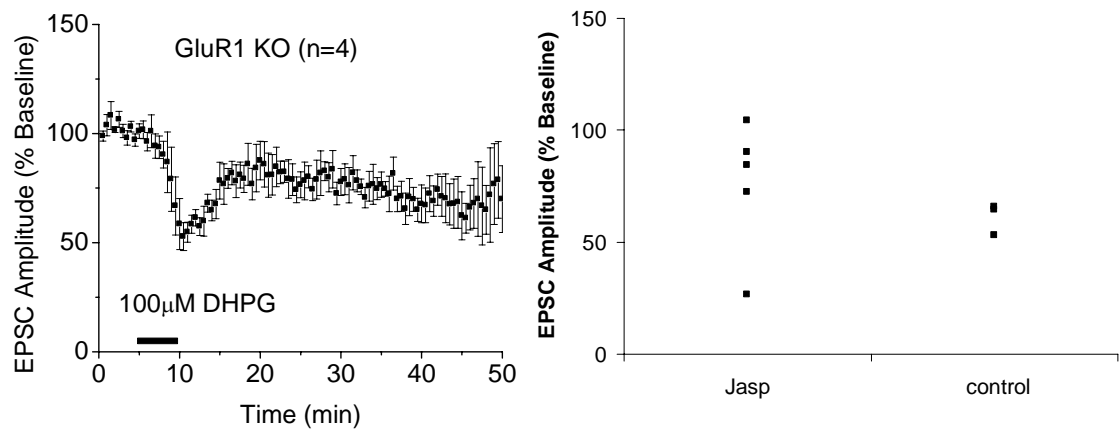


Figure 24. a. DHPG-induced LTD in the BNST is independent of GluR1. b. The actin stabilizing compound Jasplakinolide alters postsynaptic induced LTD (30 min post DHPG) in 4 of 5 BNST neurons.

A potential explanation for the attenuation of mGluR5-mediated LTD observed in the BNST following cocaine administration could potentially be downregulation of mGluR5 or perhaps disruption of mGluR5 mediated effects due to changes in scaffolding complexes involving Homer proteins.

Interestingly, it has been shown that puffing of glutamate into the BNST results in an increase in DA neuron firing in the VTA (Georges and Aston-Jones, 2002). However, upon greater stimulation of the BNST by glutamate, there is a blockade of VTA DA neuron firing (Georges and Aston-Jones, 2002). As activation of group I mGluRs in the BNST results in a decrease in BNST output, it is possible that this acts to prevent the switch from increased firing to no firing of VTA neurons. This could therefore explain the lack of a cocaine self-administration phenotype in mGluR5 knockouts and in experiments where mGluR5 signaling is blocked.

As mentioned in the introduction, remodeling of dendrites, axons, and synapses are proposed mechanisms for drug-induced changes in neuronal signaling. These changes could ultimately lead to changes in synaptic strength, generation of new synapses, or silencing of existing synapses and, thus, to the reorganization of circuits (Sala et al., 2001). Such long lasting changes are potentially due to drug-induced alteration in gene expression or in protein translation. Although under the experimental parameters used in the study of protein synthesis dependence of mGluR LTD we did not find any effect, it is still possible that *in vivo*, mGluR5 activity may lead to changes in gene expression and ultimately protein synthesis.

This thesis has revolved around laying the groundwork to study the phenomena of drug-induced changes in neuronal signaling in the BNST. Future directions looking at

changes in mGluR5 function, changes in AMPAR subunit expression and ERK pathway desensitization in the BNST could elucidate the correlation between the mechanism and behavioral changes induced by cocaine and other drugs of abuse. For instance, as is speculated with the homer2 KOs, it is possible that attenuated mGluR5 LTD in the BNST may be due to desensitization of mGluR5 (Sala et al., 2001). Another possibility is downregulation of AMPAR expression as well as a disconnection of the proposed mGluR5/AMPA interaction. Downregulation of AMPARs following cocaine treatment would suggest a cocaine-induced LTD of EPSCs that would, in turn, occlude the group I mGluR-mediated LTD.

Implications for Group I mGluR Function in the BNST on Stress/Anxiety

Lesioning the lateral BNST results in decreased CRH mRNA in the PVN and also decreased plasma corticosterone levels, which directly regulated by the PVN (Dunn, 1987; Herman et al., 1994). Decreased HPA output suggests that under basal conditions, activation of the PVN is consequent to activation of the lateral BNST. Therefore, a reduction in excitatory drive via mGluR activation could result in a reduction in PVN activation which ultimately results in an anxiolytic phenotype. This is consistent with the behavioral data suggesting anxiolytic properties of group II and III agonists (Fitzjohn et al., 1998) however contradicts the anxiolytic properties reported from group I mGluR antagonists (Pilc et al., 2002). However, to illustrate the complexity of the BNST circuitry, Dunn et al found that electrical stimulation of the lateral BNST resulted in a decrease while stimulation of the medial BNST caused an increase in plasma corticosterone levels (Dunn, 1987). This would be consistent with the group I mGluR

antagonists could acting as an anxiolytic like compound at the level of synapses onto the BNST yet contradicts the group II and III agonist effects.

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