STRESS- AND DRUG-DEPENDENT REGULATION OF METABOTROPIC GLUTAMATE RECEPTORS IN THE BED NUCLEUS OF THE STRIA TERMINALIS

By

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To my sister Stephanie, my oldest and greatest friend,

for her inspiring academic example;

To my parents for their unwavering love and encouragement;

To my husband Jon, my best friend;

and,

To the millions who suffer from addiction and anxiety disorders.

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ABBREVIATIONS

α1 Adrenergic Receptor-Mediated Long-Term Depression α1-LTD

Adenylate Cyclase ..... AC

Artificial Cerebrospinal Fluid ..... ACSF

Adrenocorticotropic Hormone ..... ACTH

(S)-(4-fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]oxadiazol-5-yl]-piperidin-1-yl}-methanone ADX47273

A Kinase Anchoring Protein 79/150 ..... AKAP79/150

N,*N*'-*Bis*(diphenylmethyl)-1,2-ethanediamine dihydrochloride AMN082

α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor ..... AMPAR

Adrenergic Receptor ..... AR

(3aS,6aS)-6a-naphthalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental[c]furan-1-on ..... Bay 36-7620

Basolateral Amygdala ..... BLA

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

Ca^{2+}/Calmodulin-Dependent Protein Kinase II ..... CaMKII

cyclic Adenosine Monophosphate ..... cAMP

Type 1 Endocannabinoid Receptor ..... CB_1R

3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide ..... CDDPB

Central Nucleus of the Amygdala ..... CeA

(R,S)-2-Amino-2-(2-chloro-5-hydroxyphenyl)acetic acid ..... CHPG

7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester ..... CPCCOEt

3,3′-difluorobenzaldazine (DFB), *N*-{4-Chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide ..... CPPHA

cAMP-Responsive Elements ..... CRE

cAMP-Responsive Element Binding Protein ..... CREB

Corticotropin-Releasing Factor ..... CRF

Dopamine ..... DA

3,3'-Diaminobenzidine ..... DAB

Diacylglycerol ..... DAG

Dopamine Transporter ..... DAT

Dopamine-β-Hydroxylase ..... DBH

Dorsal Subdivision of the Anterior BNST ..... dBNST

(*S*)-3,4-Dicarboxyphenylglycine ..... DCPG

(S)-3,5-Dihydroxyphenylglycine ..... DHPG

Endocannabinoid ..... eCB

Endocannabinoid-Mediated Long-Term Depression ..... eCB-LTD

Epinephrine ..... E

Elevated Plus Maze ..... EPM

Excitatory Postsynaptic Current ..... EPSC

Extracellular Signal-Regulated Kinase/
Mitogen-Activated Protein Kinase ..... ERK/MAPK

Field Excitatory Postsynaptic Potential ..... fEPSP

Fragile X Mental Retardation Protein ..... FMRP

γ-amino-butyric Acid ..... GABA

Glutamic Acid Decarboxylase ..... GAD

Glutamate Receptor Interacting Proteins 1 and 2 ..... GRIP1/2

Guanosine 5'-Diphosphate ..... GDP

G-Protein-Coupled Receptor ..... GPCR

Guanosine 5'-Triphosphate ..... GTP

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

High-Frequency Stimulation ..... HFS

Immediate-Early Gene ..... IEG

Ionotropic Glutamate Receptor ..... iGluR

Intraperitoneal ..... i.p.

1,4,5-Triphosphate ..... IP_3

3-4-Dihydro-2H-pyrano[2,3-b]quinolin-7-yl-(cis-4-methoxycyclohexyl)-methanone JNJ16259685

Kainate Receptor ..... KAR

Knockout ..... KO

L-(+)-2-Amino-4-phosphonobutyric acid L-AP4

Low-Frequency Stimulation ..... LFS

Long-Term Depression ..... LTD

Long-Term Potentiation ..... LTP

(2*S*)-2-Amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid LY341495

(S)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid LY367385

(1R,4R,5S,6R)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic Acid LY379268

Metabotropic Glutamate Receptor ..... mGluR

Metabotropic Glutamate Receptor 5-Mediated Long-Term Depression mGluR5-LTD

(5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate MK-801

6-(4-Methoxyphenyl)-5-methyl-3-(4-pyridinyl)-isoxazolo[4,5-*c*]pyridin-4(5*H*)-one hydrochloride ..... MMPIP

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

Medial Prefrontal Cortex ..... mPFC

3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride ..... MTEP

Noradrenaline ..... NA

Norepinephrine ..... NE

Norepinephrine Transporter ..... NET

N-methyl-D-aspartate Receptor ..... NMDAR

N-ethylmaleimide sensitive fusion protein ..... NSF

Nucleus Accumbens ..... NAc

Negative Allosteric Modulator ..... NAM

Periaqueductal Gray ..... PAG

Parkinson's Disease ..... PD

Prefrontal Cortex ..... PFC

N-Phenyl-7-(hydroxyimino)cyclopropa[*b*]chromen-1a-carboxamide ..... PHCCC

Phosphotoinositide ..... PI

Protein Interacting with C Kinase 1 ..... PICK1

Protein Kinase A ..... PKA

Protein Kinase C ..... PKC

Phospholipase C ..... PLC

Phospholipase D ..... PLD

Positive Allosteric Modulator ..... PAM

Protein Phosphatase 2B PP2B

Paired Pulse Ratios ..... PPR

Post-Synaptic Density ..... PSD

Post-Traumatic Stress Disorder ..... PTSD

Paraventricular Nucleus of the Hypothalamus ..... PVN

butyl (9H-xanthene-9-carbonyl)carbamate ..... Ro 67-4853

(*S*)-2-(4-fluorophenyl)-1-(toluene-4-sulfonyl)pyrrolidine ..... Ro 67-7476

Synapse-Associated Protein 97 ..... SAP97

Serotonin Transporter ..... SERT

Substantia Nigra ..... SNi

Spontaneous Excitatory Postsynaptic Current ..... sEPSC

Transient Receptor Potential Cation Channel M1 ..... TRPM1

Voltage-Gated Calcium Channel ..... VGCC

Ventral Noradrenergic Bundle ..... VNAB

Ventral Tegmental Area ..... VTA

(+/-)-cis-2-(3,5-dichlorphenylcarbamoyl)cyclohexanecarboxylic acid VU0155041

4-nitro-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide VU29

4-nitro-N-(1,4-diphenyl-1H-pyrazol-5-yl)benzamide VU71

Wild-Type ..... WT

6-amino-N-cyclohexyl-N,3-dimethylthiazolo[3,2-a]benzimidazole-2-carboxamide ..... YM 298198

CHAPTER I

INTRODUCTION

The work of the thesis presented here is composed of two different projects, and thus requires a broad background. In this introductory chapter, I will focus on glutamatergic signaling and the neural circuitry involved in drug addiction and stress/anxiety. First, I will explain glutamatergic signaling and the receptors that mediate it, focusing on metabotropic glutamate receptors and their role in modulating glutamatergic transmission. Then, I will shift topics to discuss the bed nucleus of the stria terminalis (BNST), the brain region at the center of this thesis. Finally, I will address the larger neural framework of drug addiction and stress/anxiety.

Glutamatergic Signaling

A synapse is a locus for communication between two neurons. During synaptic transmission, an electrical signal from one neuron, the presynaptic neuron, is transmitted chemically across the synaptic cleft to another neuron, the postsynaptic neuron (Kandel, *et al* 2000). Upon reaching the postsynaptic cell, the chemical signal is converted back to an electrical signal by ligand-gated ion channels on the postsynaptic cell surface. This electrical signal can then elicit a myriad of responses

from the postsynaptic cell, dependent on a multitude of factors such as which neurotransmitter was involved, which postsynaptic receptors were activated in response, the duration of the signal, etc. The modification of glutamatergic synapses over time is referred to as synaptic plasticity. The biochemical and structural changes underlying synaptic plasticity are thought to be responsible for long-term phenomena such as learning and memory.

Glutamate is the major excitatory neurotransmitter in the brain (Olney, 1989). It acts to excite the postsynaptic cell through two classes of receptors: ionotropic and metabotropic glutamate receptors. For the purposes of this thesis, this introduction will briefly outline synaptic plasticity at glutamatergic synapses before focusing on the roles of metabotropic glutamate receptors (mGluRs) in synaptic plasticity and their involvement in drug addiction and stress/anxiety disorders.

Fast Glutamatergic Transmission

Fast glutamatergic transmission is mediated by ionotropic glutamate receptors. These receptors are ligand-gated ion channels that, when activated, depolarize the membrane by allowing Na^+, K^+, and sometimes Ca^{2+} to flux through the channel. Ionotropic receptors are classified into 3 groups based on sequence homology and ligand sensitivity- AMPA receptors (AMPARs), NMDA receptors (NMDARs), and kainate receptors (KARs) (for review, see Dingledine et al, 1999).

GluAs

AMPA receptors (AMPARs) are so named because of their sensitivity to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). They are tetramers, made up

of 4 subunits: GluA1, GluA2, GluA3, and GluA4, in any combination (Rosenmund, *et al* 1998). Thus, an AMPAR can be a homomer or heteromer, depending on its subunit composition. Each subunit further confers specific properties to the AMPAR, the composition of which is regulated by factors such as animal age and RNA editing.

GluA1 is the most well-characterized AMPAR subunit. Phosphorylation sites on the C-terminal tail of GluA1 control multiple aspects of AMPAR function. Of the many sites of regulation, two serine residues, 831 and 845, serve as major regulators of AMPAR function via their phosphorylation state. Phosphorylation of Ser831 increases the single-channel conductance of the receptor, and can be mediated by either protein kinase C (PKC) or Ca^{2+}/Calmodulin-Dependent Protein Kinase II (CaMKII) (Derkach, *et al* 1999). Ser845 phosphorylation, which is predominantly mediated by protein kinase A (PKA), increases the open probability of the AMPAR, as well as the peak amplitude of the AMPAR-mediated current (Roche, *et al* 1996). Therefore, both Ser831 and Ser845 phosphorylation increase the net AMPAR current of the postsynaptic cell. (Ser845 phosphorylation also controls membrane targeting of the AMPAR.) Another residue, Ser818, can be phosphorylated by PKC to drive synaptic insertion of GluA1-containing AMPARs (Boehm, *et al* 2006). Finally, phosphorylation at a threonine residue, T840, has been recently identified as a potential age-dependent regulator of plasticity in the hippocampus (Lee, *et al* 2007).

The C-terminal domains of each AMPAR subunit also determine the interactions of the whole AMPAR with binding partner proteins. The C-terminus of GluA1 interacts with proteins containing PDZ domains, such as synapse-associated protein 97

(SAP97). SAP97, which forms a complex with A-kinase anchoring protein 79/150 (AKAP79/150), can recruit PKA to phosphorylate the Ser845 site (Colledge, *et al* 2000). Interestingly, this complex is also able to recruit protein phosphatase 2B (PP2B, also known as calcineurin), which can dephosphorylate the Ser845 site, leading to AMPAR internalization (Dell'Acqua, *et al* 2006). The bidirectional regulation of GluA1 by kinase/phosphatase activity suggests this subunit is responsible for the activity-dependence of AMPAR function (for review, see Kessels and Malinow, 2009).

The GluA2 subunit determines the Ca^{2+} sensitivity of the AMPAR. Greater than 95% of the time, the GluA2 mRNA is edited (Q/R edit), making GluA2-containing AMPARs impermeable to Ca^{2+} (Sommer, *et al* 1991). However, in AMPARs containing unedited GluA2, or lacking GluA2, Ca^{2+} can be fluxed through the receptor, in addition to Na^{+} and K^{+}. Insertion of GluA2-containing AMPARs at the synapse is regulated via phosphorylation of Ser880 (Chung, *et al* 2000). Specifically, phosphorylation of Ser880 by PKC is required for endocytosis of GluA2-containing AMPARs (Chung, *et al* 2003).

C-terminal tyrosine (Tyr) residues have also been shown to regulate AMPAR subunit function and binding. Tyr876 on GluA2 can be phosphorylated by a number of tyrosine kinases. When this residue is phosphorylated, GluA2 interactions with glutamate receptor interacting proteins 1 and 2 (GRIP1/2) are prevented, leading to internalization of the receptor (Wang, *et al* 2005). GluA2 can also bind to protein interacting with C kinase 1 (PICK1) and N-ethylmaleimide sensitive fusion protein (NSF), allowing for more stable localization at the synapse (Lin and Huganir, 2007).

GluA3 and GluA4 are somewhat less well-studied subunits. GluA4 is the most rapidly desensitizing of the four subunits (Wang, *et al* 2005). Also, PKA/PKC phosphorylation of this subunit at residue Ser842 leads to AMPAR localization to the synapse (Nuriya, *et al* 2005).

GluNs

N-methyl-D-aspartate or NMDA receptors (NMDARs) are a second major class of iGluRs. At first glance, they share many similarities with AMPARs. NMDARs, like AMPARs, are tetrameric ligand-gated cation channels. NMDARs almost always exist as heteromers, comprised of two of the requisite GluN1 subunits, and two subunits of either the GluN2 (2A, 2B, 2C, or 2D) or GluN3 varieties. Both are mediators of fast glutamatergic transmission. As with AMPARs, the subunit composition of an individual NMDAR determines its physiological and pharmacological profile (for review, see Lau and Zukin, 2007).

However, NMDARs differ from AMPARs in several key ways. Unlike AMPARs, NMDARs readily flux Ca^{2+} ions, in addition to Na^{+} and K^{+}. NMDARs also require glycine binding as a co-agonist with glutamate (Thomson, 1989). But perhaps most importantly, NMDARs are not active under basal synaptic conditions (Paoletti and Neyton, 2007). This is due to the presence of a Mg^{2+} ion in the pore of the ion channel, blocking the flow of ions. However, when the membrane becomes depolarized, the NMDAR undergoes a voltage-dependent conformational change, dislodging the Mg^{2+} ion. If glutamate and glycine are present when the Mg^{2+} block is relieved, the NMDAR can then be activated. This dual voltage-dependent and ligand-gated activation makes the NMDAR a unique “coincidence detector”, because

its activation only occurs under a special set of circumstances. NMDAR activation is usually recruited after AMPARs, KARs, and other ion channels are activated and depolarize the membrane. While primarily postsynaptically expressed, some reports have shown evidence for the existence of presynaptic NMDARss in some brain regions, including the BNST (Brasier and Feldman, 2008, Gracy and Pickel, 1995).

As mentioned above, NMDAR subunit composition determines many of the receptor's qualities (for review, see Paoletti and Neyton, 2007). GluN2A-containing receptors have lower affinity for glutamate, greater open probability of the channel, faster decay kinetics (tau), and a larger degree of Ca^{2+}-dependent desensitization than other subtypes. GluN2B, in contrast, confers slower decay kinetics and decreased open probability. Both GluN2C and 2D provide weakened sensitivity to the Mg^{2+} block and low channel conductance. GluN3 is unique in that it reduces Ca^{2+} permeability and overall surface expression of NMDARs. The expression of GluN3, however, is somewhat rare. The vast majority of central NMDARs are of the GluN1/GluN2 variety.

The coincidence detector function of the NMDAR allows the receptor to respond only when both presynaptic and postsynaptic activity are occurring. Upon activation, NMDARs flux Ca^{2+} into the postsynaptic cell (Dingledine, *et al* 1999). This increase in postsynaptic Ca2+ concentration is a critical induction step in many forms of synaptic plasticity, and triggers many Ca^{2+}-activated signaling cascades, activating a myriad of kinases such as CaMKII, Extracellular Signal-Regulated Kinase/Mitogen-Activated Protein Kinase (ERK/MAPK), PKA, and PKC. These

kinases can then regulate a wide array of cellular processes, including AMPAR insertion/removal from the synapse, conductance, and open probability, which alter the strength of the synapse, conferring plasticity. These signaling cascades will be discussed in greater detail below.

Long-Term Potentiation and Long-Term Depression

While some alterations in synaptic strength may soon be reversed, frequently these changes persist over time. Such storage of a synapse's history, over greater periods of time, was long ago postulated to be the mechanism by which the brain as a whole stores information (Hebb, 1949). In 1973, this was first demonstrated by Bliss and Lomo in the hippocampus, a structure first identified as being important for memory storage years earlier by study of the brain damaged patient, H.M. (Scoville and Milner, 1957). Bliss and Lomo were working in the hippocampus using electrophysiology to stimulate axonal firing and record the subsequent neuronal response electrically. They observed that after high frequency stimulation (HFS), the subsequent responses they recorded were enhanced compared to the pre-HFS responses (Bliss and Lomo, 1973). It was as if the burst of HFS had sensitized the postsynaptic neurons, so that subsequent glutamate release caused greater depolarization than it would under basal conditions. Even more surprising, this enhanced synaptic transmission persisted for hours.

The name given to the form of plasticity Bliss and Lomo discovered was long-term potentiation (LTP). LTP can be induced in a variety of ways and can require any number of factors, depending on which cell types and neurotransmitters are

involved (Bear and Malenka, 1994; Malenka and Bear, 2004). A typical LTP induction pathway involves recruitment of NMDARs, increased intracellular Ca^{2+}, activation of CaMKII and PKA, increased AMPAR current (through phosphorylation or increased insertion), and frequently, ERK/MAPK activation. In order to be maintained over large periods of time, many forms of LTP also require gene transcription and/or the translation of new proteins.

LTP has long been postulated to be the cellular mechanism of learning and memory, a hypothesis still being supported by a recent study. First, rats that underwent single-trial avoidance learning, a hippocampal-dependent task, were found to exhibit many of the cellular markers of LTP, compared to yoked or trained-only control rats (Whitlock, *et al* 2006). Remarkably, the experimental animals showed enhanced postsynaptic responses, recorded *in vivo*, suggesting that the learning task had induced LTP. This enhancement occurred in a spatially restricted area. Subsequent attempts to elicit HFS-induced LTP, a very well characterized form of plasticity in the hippocampus, were occluded at the locations were increased responses were seen *in vivo*, but LTP was able to be induced at more distal sites.

Synaptic plasticity can also occur in the opposite direction. A sustained decrease in synaptic strength over time is known as long-term depression (LTD). Discovered long after LTP, a reproducible protocol for LTD induction (low-frequency stimulation, or LFS) was not firmly established until 1992 (Dudek and Bear, 1992). While at first believed to merely be "depotentiation", or the undoing of LTP, LTD is now considered to be a critical form of synaptic plasticity in its own right. Mechanisms of LTD are thought to be involved in experience-dependent

development, drug addiction, learning and memory, mental retardation, and stress (Heynen, *et al* 2003, Thomas, *et al* 2001, Brigman, *et al* 2010, Dolen, *et al* 2007, McElligott and Winder, 2008; for review, see Malenka and Bear, 2004).

LTP and LTD, besides their potential as mechanisms of learning and memory, have proven to be critical experimental phenomena for the advancement of neurophysiology (Malenka and Bear, 2004). By using protocols or drugs to induce LTP and LTD, the dynamic range of synaptic transmission at a set of synapses can be explored, alongside other parameters like cell type or circuit connectivity. Furthermore, both forms of plasticity have been invaluable serving as assays for experience-induced changes in synaptic plasticity. Numerous human diseases and disorders have now been classified by their ability to enhance, disrupt, alter, or leave intact some form of plasticity, which has been critical for identifying circuitry or molecular targets that may be underlie a certain pathology (for review, see Malenka and Bear, 2004).

Signal Transduction Mechanisms of Plasticity

The full list of molecules involved in the signal transduction of plasticity is immense, and new components are continually being identified. For the purpose of introducing this thesis, I provide here only a few examples of key players in the process. Both LTP and LTD involve large signal transduction networks within the postsynaptic cell (and frequently, the presynaptic axon terminal as well). Induction of LTP, as stated previously, frequently involves increased intracellular Ca^{2+}. As Ca^{2+} enters the cell, Ca^{2+}-sensitive kinases such as Ca^{2+}/Calmodulin-Dependent Protein

Kinase II (CaMKII) become activated (Malinow, *et al* 1988). Once activated in the presence of Ca^{2+}, CaMKII can autophosphorylate at Thr286 to achieve Ca^{2+}-independent function. CaMKII is a serine/threonine kinase with many targets, including AMPARs (GluA1), NMDARs (GluN2B), structural proteins (α-actinin 2), and scaffolding proteins (SAP97). Thus, CaMKII can mediate a wide variety of responses to changes in synaptic strength (for review, see Wayman, *et al* 2008). Protein kinase A (PKA) and protein kinase C (PKC) are two more protein kinases activated by synaptic activity and involved in synaptic plasticity. PKA is stimulated by increased cyclic adenosine monophosphate (cAMP) accumulation, and phosphorylates a wide array of substrates including GluA1 (for review, see Meinkoth, *et al* 1993). PKC, activated by many forms of LTD, can phosphorylate AMPARs on subunits GluA1 and GluA2 (Derkach, *et al* 1999; Chung, *et al* 2000). (Their respective effects on AMPAR function and localization have been described above.) CaMKII, PKA, and PKC can each activate the ERK/MAPK pathway, which is important for a myriad of cellular processes including transcription and translation. Besides activation of PKA, cAMP can regulate transcription at sites called cAMP Responsive Elements (CRE) with the help of CREB (cAMP responsive element binding protein). CREB binding at CRE sites will drive the transcription of the genes with those elements, which includes immediate early genes (IEGs).

Metabotropic Glutamate Receptors

Metabotropic glutamate receptors (mGluRs) are a separate class of glutamate receptors in the central nervous system. Whereas ionotropic glutamate receptors such as AMPARs, NMDARs, and KARs act as ligand-gated ion channels, mGluRs signal through interactions with G proteins. mGluRs provide a way for glutamate to signal as a neuromodulator, via second messenger signaling cascades (Conn and Pin, 1997).

Structure and Classification

Metabotropic glutamate receptors are seven transmembrane, G-protein-coupled receptors (GPCRs). GPCRs are the most abundant family of receptor genes in the human genome (Niswender and Conn, 2010). mGluRs are class C GPCRs, a group characterized by their large, extracellular N-terminal domains. Often referred to as a "clam shell" or "venus fly trap" domain due to its shape, this large extracellular domain is the sight of the orthosteric pocket where glutamate binds to mGluRs, inducing a structural change (Jingami, *et al* 2003). mGluRs have been theorized to exist as constitutive dimers, with the extracellular domain postulated to be the locus of dimerization (reviewed in Pin, *et al* 2003). It is currently unclear whether glutamate binding to one extracellular domain of the dimer is sufficient for receptor activation, or whether binding to both is necessary and/or favorable (Kammermeier and Yun, 2005; Kniazeff, *et al* 2004; Suzuki, *et al* 2004).

Upon binding of ligand to the extracellular domain, the membrane-bound receptor undergoes a confirmational change, activating the G protein on the intracellular surface of the membrane. The G protein is a hydrolase consisting of 3 subunits: α, β, and γ. In basal conditions, the α subunit is bound to guanosine 5'-diphosphate (GDP), which is converted to guanosine 5'-triphosphate (GTP) upon activation of the G protein. Once activated, the G protein subunits can then modulate a myriad of intracellular signaling molecules, such as protein kinases, ion channels, or transcription factors. G proteins can directly bind some substrates, such as ion channels, or activate/inactive second messenger cascades, which can exert effects on virtually every process within the cell. Eight known mGluR subtypes have been identified, and they have been classified into three groups (Groups I-III) based on sequence homology, pharmacology, and G-protein coupling.

Group I mGluRs

Signaling

Group I mGluRs, comprised of mGluR1 and mGluR5, couple to $G\alpha_q/G\alpha_{11}$ proteins. This pathway activates phospholipase C (PLC), which leads to phosphotoinositide (PI) hydrolysis, generating inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), eventually leading to activation of PKC (Conn and Pin, 1997). IP_3, a potent second messenger, acts as a ligand at IP_3 receptors expressed on the endoplasmic reticulum (as well as the sarcoplasmic reticulum), triggering the release of intracellular Ca^{2+}, an important second messenger. Increased intracellular Ca^{2+} can regulate the activity of channels (through voltage gating) and Ca^{2+}-sensitive proteins

such as CaMKII, making Ca^{2+} an important mediator of glutamate's effects on neurons (Song, *et al* 2009).

The production of DAG induced by Group I mGluR activation may also trigger the production of the endocannabinoid (eCB) molecules anandamide and 2-AG (Swanson, *et al* 2001, Robbe, *et al* 2002, Gerdeman and Lovinger, 2001, Grueter, *et al* 2006). eCBs are lipids, enabling them to leave the postsynaptic cell and activate presynaptic eCB receptors. Once activated, presynaptic eCB receptors signal to alter the release of a neurotransmitter presynaptically. This retrograde signaling pathway provides a presynaptic mechanism by which mGluR1/5 activation can alter neurotransmitter release, and has been implicated in several forms of plasticity. Group I mGluRs have also been shown to activate/inactivate adenylate cyclase (AC), through $G\alpha_s$ and $G\alpha_{i/o}$ proteins, respectively, as well as phospholipase D (PLD) and a variety of protein kinases. In nearly all cell types/neuronal populations, Group I mGluRs have been shown to signal to the extracellular-signal regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway, a critical mediator of several crucial cellular processes (for review, see Roux and Blenis, 2004). It is through the ERK pathway that Group I mGluRs exert the majority of their effects on synaptic plasticity (Malenka and Bear, 2004). Though rare, presynaptic Group I receptors do occur, and are thought to promote release of glutamate when activated (Cochilla and Alford, 1998).

Pharmacology

The first pharmacological compounds created to mimic the effects of glutamate at Group I mGluRs were phenylglycine derivatives. These first compounds were

designed to bind in the glutamate binding pocket, making them orthosteric compounds. The most selective and widely used orthosteric agonist for Group I mGluRs is (S)-3,5-Dihydroxyphenylglycine (DHPG), which activates mGluR1/5 with similar potencies. mGluR5 can be selectively (but more weakly) activated by (R,S)-2-Amino-2-(2-chloro-5-hydroxyphenyl)acetic acid (CHPG). Currently, no high potency, selective mGluR1 orthosteric agonists exist. For selective blockade of mGluR1 over mGluR5, the most potent orthosteric antagonist is (S)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid (LY367385) (Schoepp, *et al* 1999). There are no widely-used mGluR5-selective orthosteric antagonists.

A new class of compounds for mGluRs was discovered in 1996 with the advent of 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt), a highly selective compound targeting mGluR1 (Ott, *et al* 2000). CPCCOEt is an allosteric modulator, meaning it binds outside of the glutamate binding pocket, at an allosteric site. Allosteric modulators can be positive or negative, meaning they increase or decrease the potency of an orthosteric ligand's effect, respectively. Importantly, allosteric modulators should not affect receptor activity on their own; they merely shift the receptor's response to an orthosteric ligand. This type of compound has opened the doors for increasingly selective activation of mGluR subtypes.

A wide variety of positive and negative allosteric compounds have been characterized for Group I mGluRs (for review, see Niswender and Conn, 2010). Selective mGluR1 positive allosteric modulators (PAMs) include (*S*)-2-(4-fluorophenyl)-1-(toluene-4-sulfonyl)pyrrolidine (Ro 67-7476), butyl (9H-xanthene-

9-carbonyl)carbamate (Ro 67-4853), and 4-nitro-N-(1,4-diphenyl-1H-pyrazol-5-yl)benzamide (VU71), while 3,3′-difluorobenzaldazine (DFB), *N*-{4-Chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide (CPPHA), 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide (CDDPB), 4-nitro-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide (VU29), and (S)-(4-fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]oxadiazol-5-yl]-piperidin-1-yl}-methanone (ADX47273) are selective mGluR5 PAMs. Highly selective and reliable negative allosteric modulators (NAMs) exist for mGluR1 and mGluR5 as well. Besides CPCCOEt, mGluR1 NAMs include (3aS,6aS)-6a-naphthalen-2-ylmethyl-5-methyliden-hexahydro-cyclopental[c]furan-1-on (Bay 36-7620), 3-4-Dihydro-2H-pyrano[2,3-b]quinolin-7-yl-(cis-4-methoxycyclohexyl)-methanone (JNJ16259685), and 6-amino-N-cyclohexyl-N,3-dimethylthiazolo[3,2-a]benzimidazole-2-carboxamide (YM 298198). Two compounds, 2-methyl-6-(phenylethynyl)pyridine (MPEP) and its derivative 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride (MTEP), are the gold standard NAMs of mGluR5 in terms of potency, selectivity (MTEP is slightly more selective), and bioavailability (see Table 1).

Localization

Both mGluR1 and mGluR5 are fairly widely expressed across brain regions. Because expression of mGluR1/5 is largely postsynaptic, mRNA is a good marker for the cell types that express these proteins. mGluR1 mRNA is found in cortex, striatum, hippocampus, cerebellum, hypothalamus, thalamus, olfactory bulb, septum, globus pallidus, and several other brain regions. mGluR5 mRNA is present in BNST, olfactory bulb, striatum, nucleus accumbens (NAc), septum, hippocampus, cortex,

Table 1. Group I mGluR Pharmacology.

	Group I	mGluR1	mGluR5
agonist	DHPG	-	CHPG
antagonist	-	LY367385	-
positive allosteric modulator	-	Ro 67-7476, Ro 67-4853, VU71, and others	DFB, CPPHA, CDDPB, VU29, ADX47273, and others
negative allosteric modulator	-	CPCCOEt, Bay 36-7620, JNJ16259685, YM 298198, and others	MPEP, MTEP

thalamus, hypothalamus, amygdala, spinal cord, as well as in other areas (Allen Brain Atlas). Besides being expressed in neurons, mGluR5 is also expressed on glial cells (Haydon, *et al* 2009)

Group II mGluRs

Because they were not the focus of this thesis, this introduction will only briefly touch on Group II mGluRs. Comprised of mGluRs 2 and 3, Group II receptors are primarily expressed presynaptically (Tizzano, *et al* 2002, Bellisi and Conti, 2010, Niswender, *et al* 2008). They couple to $G\alpha_{i/o}$ proteins, as do the Group III mGluRs (this signaling is discussed in greater detail below). Group II mGluRs generally serve as presynaptic autoreceptors throughout the brain, working to inhibit the release of glutamate when activated (Doi, *et al* 2002, Marti, *et al* 2001). They have been implicated in several disorders, including anxiety, schizophrenia, and Parkinson's disease.

Group III mGluRs

Signaling

Group III mGluRs (comprised of mGluR4, mGluR6, mGluR7, and mGluR8), similar to Group II mGluRs, also couple to $G\alpha_{i/o}$ proteins. This Gα subunit typically signals to inhibit the activity of adenylate cyclase (AC), a membrane-bound protein that catalyzes the conversion of ATP to cyclic adenosine monophosphate (cAMP). Activation of AC leads to intracellular cAMP accumulation, which stimulates

transcription and activates the protein kinase A (PKA) signaling pathway. Group III receptor activation inactivates AC, thus opposing the activation of the PKA pathway and the transcription of cAMP-responsive gene transcription. Once activated, the βγ subunits can also signal to membrane-bound target proteins such as ion channels, regulating their activity. However, as with most GPCRs, some overlap in G protein activation and signaling may occur with Group III mGluRs, allowing for the potential activation of other intracellular signaling cascades.

Pharmacology

The availability of selective pharmacological tools for Group III receptor subtypes is scarce, hindering the progress of studying these receptors. The most widely used and selective (with respect to other mGluRs, and iGluRs) Group III agonist is L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4). While the potency of L-AP4 at mGluR4, mGluR6, and mGluR8 ranges from sub- to low micromolar, its potency at mGluR7 is in the millimolar range (Schoepp, *et al* 1999). Interestingly, this extreme contrast in potencies mirrors the difference in the affinity of glutamate for these receptors, with mGluR7 showing a much lower affinity for its endogenous ligand than other Group III subtypes. The only subtype-selective Group III agonist is (*S*)-3,4-Dicarboxyphenylglycine (DCPG), which has approximately 100-fold selectivity for mGluR8 over mGluR4 (Linden, *et al* 2003). The most potent Group III antagonist is (2*S*)-2-Amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495), although it is "Group II-preferring" (Niswender, *et al* 2008). No subtype-selective antagonists exist for Group III receptors in the current literature.

Some progress has been made identifying subtype-selective allosteric compounds for Group III mGluRs. *N*-Phenyl-7-(hydroxyimino)cyclopropa[*b*]chromen-1a-carboxamide (PHCCC), a PAM of mGluR4, shows good selectivity and micromolar potency at mGluR4, when glutamate is present (Maj, *et al* 2003). More recently, two potential mGluR4-selective compounds have been identified at Vanderbilt, (+/-)-cis-2-(3,5-dichlorphenylcarbamoyl)cyclohexanecarboxylic acid (VU0155041) and VU0080421 (Niswender, *et al* 2008). One of these, VU0155041, shows allosteric agonist activity, meaning it can activate mGluR4 in the absence of an orthosteric ligand such as glutamate. This difference in action from PHCCC suggests VU0155041 may be working through a separate allosteric site on the receptor.

A few mGluR7-selective compounds are also currently available. The most frequently used of these is *N*,*N*'-*Bis*(diphenylmethyl)-1,2-ethanediamine dihydrochloride (AMN082), which is reported to have allosteric agonist activity at mGluR7 (Mitsukawa, *et al* 2005). However, this activity appears to be confined to certain cell types, cell lines, and brain regions, as not all studies replicate agonist activity (Ayala, *et al* 2008, Niswender, *et al* 2010). Similar inconsistent effects are seen with the mGluR7 NAM 6-(4-Methoxyphenyl)-5-methyl-3-(4-p yridinyl)-isoxazolo[4,5-*c*]pyridin-4(5*H*)-one hydrochloride (MMPIP). It is hypothesized that MMPIP may only block some certain mGluR7 signaling pathways, while leaving others intact (reviewed in Kenakin, 2005).

Recently, a novel PAM of mGluR8 has been reported, AZ12216052 (Duvoisin, *et al* 2010b). This compound induces a modest 1.8 fold leftward shift in the concentration-response curve of glutamate, and exhibits similar selectivity for

mGluR4/8. It was also reported that AZ1216052 had effects even at extremely low glutamate concentrations, which could suggest this compound is an agonist, rather than a modulator compound. However, this apparent intrinsic agonist activity of AZ12216052 could be explained by the possible presence of endogenous glutamate in the preparation, which would be provide a permissive environment for a PAM activity (see Table 2).

Localization

Group III receptors exhibit very diverse expression patterns. In general, these receptors are thought to be primarily presynaptically localized. In many brain regions, they have been shown to have a classic autoreceptor function, inhibiting the release of glutamate when activated (Ayala, *et al* 2008, Valenti, *et al* 2005, Abitbol, *et al* 2008, Schmid and Fendt, 2006). However, mGluR4/8 can exist postsynaptically, such as in the hippocampus and retina, and are both expressed in the periphery (Bradley, *et al* 1996, Koulen and Brandstatter, 2002).

The expression of individual Group III subtypes is largely inferred by pharmacology, as antibodies selective for these subtypes are of limited availability/selectivity. While mRNA expression patterns exist, they are difficult to infer protein expression from when looking at a presynaptic protein, due to the possibility of axon terminals expressing the protein projecting to other regions. mGluR4 protein expression is highest in the cerebellum, but mRNA is expressed in other brain regions, such as olfactory blub, cortex, striatum, NAc, septum, thalamus, amygdala, and hippocampus (Knopfel and Grandes, 2002; Allen Brain Atlas). mGluR6 is only expressed in retinal bipolar cells, and is not thought to be expressed in other regions

Table 2. Group III mGluR Pharmacology. (* denotes allosteric agonist activity)

	Group III	mGluR4	mGluR7	mGluR8
agonist	L-AP4	Z-cyclopentyl L-AP4	-	DCPG
antagonist	LY 341495	-	-	-
positive allosteric modulator	-	PHCCC, AZ12216052, VU0155041*, VU0080421	-	AZ12216052
negative allosteric modulator	-	-	AMN082, MMPIP	-

(Nakajima, *et al* 1993). mGluR7 is expressed in the active zone of glutamatergic synapses, and is expressed at low levels throughout the brain (Shigemoto, *et al* 1996; Allen Brain Atlas).

The expression of mGluR8 is somewhat unusual compared to other Group III subtypes. While widely expressed during development, its expression tapers down to discrete pockets in the adult brain (Duvoisin, *et al* 1995). mRNA is expressed in the adult amygdala and extended amygdala, olfactory bulbs, retina, and prefrontal cortex. Recent work also suggests mGluR8 expression may also be sexually dimorphic, as sex differences in response to mGluR8 ligands have been reported (Duvoisin, *et al* 2010a).

Involvement in Plasticity

Group I mGluRs

Group I receptors have been implicated in several forms of plasticity. Activation of these receptors, in general, leads to depolarization and increased excitability of a neuron. This "activating" function makes Group I receptors somewhat unique among mGluRs, as the activation of Group II-III receptors generally opposes, rather than facilitates, transmission. In the earliest studies of mGluR-dependent plasticity, low-frequency stimulation (LFS) or pairing protocols elicit Group I mGluR-dependent forms of LTD in such diverse brain regions as the hippocampus and cerebellum (Dudek and Bear, 1992; Oliet, *et al* 1997; Inoue, *et al* 1992). Alternately, application of the Group I agonist DHPG also leads to LTD of excitatory transmission in many brain regions, such as the hippocampus.

mGluR1 mediates a form of LTD in the VTA. However, in the NAc, mGluR5 activation is required for a form of LTD that is also mediated presynaptically by endocannabinoids (eCB-LTD) (Fourgeaud, *et al* 2004). mGluR5 also mediates LTD in the dorsal striatum, and regulates plasticity in the hippocampus and visual cortex (Tsanov and Manahan-Vaughan, 2009).

Group II mGluRs

As with Group I receptors, many form of plasticity involving Group II receptors have been elucidated. Group II receptors can be heteroreceptors, regulating release at inhibitory and neuromodulatory terminals, as well as at excitatory synapses as homosynaptic autoreceptors (Doi, *et al* 2002, Marti, *et al* 2001). While largely thought to be expressed presynaptically, postsynaptic mGluR2/3 has been reported in certain brain regions, such as the amygdala (Muly, *et al* 2007). Group II receptors are important inducers of LTD in several brain regions (for review, see Pinheiro and Mulle, 2008). In the hippocampus, presynaptic mGluR2 mediates LFS-induced LTD at the mossy fiber/Ca3 pyramidal cell synapse (Kobayashi, *et al* 1996). Activation of presynaptic Group II receptors in the NAc and the BNST leads to LTD (Robbe, *et al* 2002; Grueter and Winder, 2005; Muly, *et al* 2007). mGluR2/3 have also been implicated, along with Group I receptors, in LTD in the prefrontal cortex (PFC) (Otani, *et al* 1999).

Group III mGluRs

The Group III mGluRs expressed in brain (mGluR4, mGluR7, and mGluR8) all serve important roles in neuroplasticity, though they have not been widely studied electrophysiologically due to the lack of specific pharmacological compounds. As

with Group II receptors, Group III receptors can be heteroreceptors, regulating the release of GABA and neuromodulators, in addition to glutamate. In the paraventricular nucleus of the hypothalamus (PVN), presynaptic Group III mGluRs are tonically inhibited (Gordon and Bains, 2003, 2005). However, activation of adrenergic signaling disinhibits these receptors, and their subsequent activation induces a metaplastic depression in transmission (Kuzmiski, *et al* 2009). Unfortunately, the receptor subtypes mediating this depression are currently unknown.

mGluR4 is required for several forms of cerebellar plasticity implicated in motor learning. Mice lacking mGluR4 fail to learn difficult motor tasks, have poor spatial memory, and show abnormal cerebellar plasticity (Pekhletski, *et al* 1996). mGluR7, highly localized to the active zone of glutamatergic synapses throughout the brain and with a low affinity for glutamate, has been postulated to serve as a failsafe autoreceptor to prevent glutamate overstimulation (Shigemoto, *et al* 1996). This receptor has also been implicated in certain forms of amygdala-driven learning, based on the phenotype of mGluR7-deficient mice (Masugi, *et al* 1999). mGluR8 activation depresses transmission at the SC-CA1 synapse in the hippocampus in neonatal rats, but this effect is lost in adult animals, when mGluR7 is the dominant Group III receptor present (Ayala, *et al* 2008). However, the mGluR8 agonist DCPG induces a persistent depression in excitatory transmission in BNST (in 5-10 week old mice) that is consistent with LTD, suggesting this receptor may serve to regulate transmission in adult animals in some regions (Grueter and Winder, 2005).

Implications in Behaviors and Disease States

As an increasing number of disorders are theorized to involve dysregulation of glutamatergic signaling, new roles for mGluRs in the pathology and treatment of these disorders are emerging. Their widespread localization and specialized functions show the great potential of mGluRs as therapeutic targets. In many instances, the first implication of mGluR involvement came from the phenotype of one of the eight mGluR knockout mice (for review, see Swanson, *et al* 2005).

Group I mGluRs

Group I receptors are easily the most well-studied group of mGluRs, with a huge literature dedicated to them. Both mGluR1 and mGluR5 have been implicated in several specific forms of learning and memory, and both knockout animals show deficits in prepulse inhibition, a sensorimotor gating behavior that is altered in schizophrenics and reversed by antipsychotic compounds (Brody, *et al* 2004). mGluR1 also plays a critical role in cerebellar development, and has been implicated in cerebellar ataxia (Levenes, *et al* 1997).

mGluR5 has been implicated in a wide variety of disorders. Supporting a role in drug addiction, studies have shown mice lacking mGluR5 will not self-administer cocaine and do not exhibit locomotor sensitization to cocaine, and mGluR5-mediated forms of plasticity are disrupted by drug exposure (Chiamulera, *et al* 2001, Robbe, *et al* 2002, Fourgeaud, *et al* 2004, Grueter, *et al* 2006). mGluR5 may also be involved in anxiety, as compounds blocking mGluR5 activation, such as MPEP, are anxiolytic. Most recently, a role for mGluR5 is emerging in fragile X syndrome, the most common heritable form of mental retardation, and part of the

autism spectrum of disorders (Richards, *et al* 1981). Fragile X has been modeled in a knockout mouse model where the fragile x mental retardation protein (FMRP) has been deleted (Huber, *et al* 2002). The phenotype of these mice can be significantly rescued by a reduction of mGluR5 protein, suggesting mGluR5 is a critical mediator of Fragile X Syndrome (Dolen, *et al* 2007). Antagonism of mGluR5 has also been suggested as a strategy for reducing dyskinesia in Parkinson's disease (Rylander, *et al* 2009).

Group II mGluRs

Group II receptors are also involved in several pathologies. Evidence suggests a role for mGluR2 in cognitive impairment, and that compounds preventing mGluR2 activation could be used to treat cognitive disorders (Higgins, *et al* 2004). This receptor is also thought to be the primary mediator of the effectiveness of Group II ligands in the treatment of psychotic symptoms (Woolley, *et al* 2008). Group II receptors have also been implicated in anxiety disorders, as mGluR2/3 agonists have anxiolytic properties, such as a reduction in fear potentiated startle or increased open arm entries in an elevated plus maze (for review, see Swanson, *et al* 2005). Furthermore, a role for Group II receptors is emerging in Parkinson's disease (PD). Group II receptors in the basal ganglia may play a key role in the control of movement, and mGluR2/3 antagonist binding is altered in PD brains post-mortem (Samadi, *et al* 2009). And, Group II receptor activation via 3,3'-Diaminobenzidine (LY379268) actually reverses dopamine depletion-induced spine loss on striatal medium spiny neurons *in vitro* (Garcia, *et al* 2010).

Group III mGluRs

Group III receptors have also been implicated in a variety of behaviors and disorders. As is the case with Group II receptors, it is difficult to know which receptor subtype or subtypes is/are mediating the effects of a general group agonist or antagonist. Compounds with subtype selectivity are thus critical in situations were multiple receptor subtypes are expressed. Without such compounds, much of the role of a receptor subtype must be inferred from the phenotype of that subtype's knockout mouse.

Of all Group III mGluRs, mGluR4 is currently receiving the most attention as a therapeutic target. Recently, novel mGluR4 PAMs have been shown to reduce catalepsy and akinesia, two behavioral models of Parkinsonian symptoms (Niswender, *et al* 2008). mGluR4 KO mice have motor learning deficits, consistent with the important role of this receptors in cerebellar motor learning (Pekhletski, *et al* 1996). Much effort is now underway towards the development of more selective, potent, and biologically active compounds targeting mGluR4.

Expression of mGluR6 is restricted to the retina. Within the retina, mGluR6 is expressed primarily in retinal ON bipolar cells, and is a critical mediator of the ON response to light. Mice lacking mGluR6 exhibit acute visual defects, including a lack of response to light onset (Masu, *et al* 1995). Recent work has identified TRPM1 as the ion channel gated by mGluR6 that is required for the depolarizing response to light of retinal ON bipolar cells (Morgans, *et al* 2009). This evidence of an mGluR-gated ion channel adds to the diverse function of mGluRs throughout the brain and body.

Both mGluR7 and mGluR8 have been implicated in anxiety (Swanson, *et al* 2005). This was, at first, largely due to the initial success of general Group III compounds as anxiolytics, but was later reinforced by the mGluR7 and mGluR8 knockout mouse phenotypes. mGluR7-deficient mice exhibit an anxiolytic phenotype in behavioral tests of anxiety, including elevated plus maze (EPM), light-dark box, and stress-induced hypothermia test (Cryan, *et al* 2003). Interestingly, mGluR8 knockout mice have the opposite phenotype. These mice exhibit a basal anxiety phenotype in EPM that is similar to the behavior of mice tested under stressful conditions (Linden, *et al* 2002, 2003; Duvoisin, *et al* 2005). When stressed, mGluR8 KO mice did not show further increases in anxiety-related behaviors, suggesting anxiety circuitry is already engaged in these mice (Linden, *et al* 2002). Currently, more selective and biologically active compounds are needed to parse out the mechanisms for such paradoxically different knockout phenotypes.

The Bed Nucleus of the Stria Terminalis (BNST)

The Extended Amygdala

Now that I have introduced the basic mechanisms of glutamatergic signaling, synaptic plasticity, and the involvement of mGluRs, the rest of the introduction will focus on the brain regions and neural networks studied in my thesis work. The bed nucleus of the stria terminalis (BNST) is a limbic forebrain region considered to be part of the extended amygdala. The extended amygdala is a loose grouping of interconnected nuclei sharing similar cytoarchitecture and circuitry (for review, see

Heimer and Alheid, 1991). Besides the BNST, the extended amygdala is comprised of the central nucleus of the amygdala (CeA) and the "shell" subregion of the nucleus accumbens (NAc). Each of these regions receives multiple projections from limbic brain regions such as the hippocampus and basolateral amygdala (BLA), and projects to regions involving reward, ingestive behaviors, and stress (Alheid, *et al* 1998).

Anatomy

The BNST is centered in the rostral portion of the basal forebrain, and extends from the caudal end of the nucleus accumbens (NAc) to the rostral end of the 3rd ventricle, ventral to the lateral septum and ventricles, and surrounding the anterior commissure (Herman and Cullinan, 1997). The BNST has been divided anatomically into two main divisions, posterior and anterior, and the latter has further been divided into dorsal, lateral, and ventral subdivisions (Forray and Gysling, 2004). Currently, 12 different subnuclei have been identified based on cellular and anatomical properties (Dong, *et al* 2001). This work will focus on the dorsal subdivision of the anterior BNST (dBNST). The dBNST is easy to identify in a coronal section from a mouse brain. Directly below the base of the lateral ventricle, sitting above the decussation of the anterior commissure, and demarcated laterally from the striatum by the internal capsule, the dBNST is shaped like a small triangle. The fibers of the stria terminalis cross through the medial half of the dBNST, entering at the dorsal apex of the triangle.

The majority of the cells in the BNST stain positive for GAD (glutamic acid decarboxylase), a marker for GABA (γ-Aminobutyric acid) synthesis (Bowers, *et al* 1998). Besides GABA, BNST neurons express a variety of neuropeptides and catecholamines (Georges and Aston-Jones, 2002; Walter, *et al* 1991). The properties of BNST neurons were first reported by the Rainnie and Winder labs (Rainnie, 1999; Egli and Winder, 2003).

Circuitry

Being an extremely heterogeneous brain region with many subnuclei and neurotransmitter systems, it should come as no surprise how complex the circuitry of the BNST seems to be. The BNST receives excitatory inputs from limbic regions such as the hippocampus (ventral subiculum), the infralimbic region of the prefrontal cortex (PFC), and BLA. (Dong, *et al* 2001; Cullinan, *et al* 1993) (Figure 1). These regions are critical centers for cognitive and emotional processing. Besides glutamate, the BNST also receives noradrenergic, dopaminergic, seratonergic inputs. The afferents from the ventral noradrenergic bundle (VNAB) to the BNST are among the densest noradrenergic projections in the brain (Forray and Gysling, 2004). Dopaminergic afferents enter the BNST from most of the brain's dopaminergic centers, including the ventral tegmental area (VTA), periaqueductal gray (PAG), substantia nigra (SNi), dorsal raphe, and brain stem nuclei (Hasue and Shammah-Lagnado, 2002, Meloni, *et al* 2006). In addition, the BNST shares reciprocal connections with many of these nuclei, including the VTA and CeA.

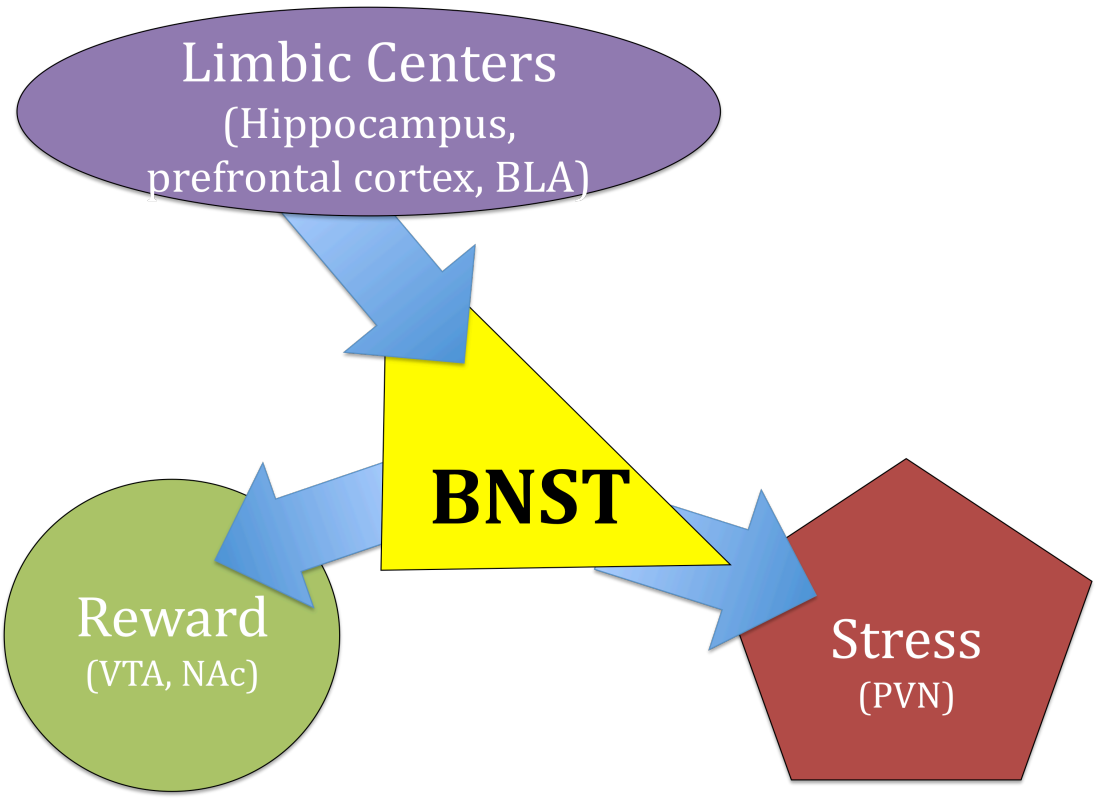


Figure 1. Circuit Diagram of the BNST. The BNST receives limbic inputs from the Hippocampus, Prefrontal Cortex, and Basolateral Amygdala, and sends projections to the brain's main stress (Paraventricular Nucleus of the Hypothalamus) and reward centers (Ventral Tegmental Area and Nucleus Accumbens).

With excitatory information entering from cortical and limbic regions (Dong, *et al* 2001; McDonald, 1998; Cullinan, *et al* 1993), and modulatory inputs from other brain nuclei, including the other members of the extended amygdala, the BNST serves as a critical intermediary in the brain's stress response, sending important regulatory projections to the paraventricular nucleus (PVN) of the hypothalamus, the master regulator of the body's hypothalamic-pituitary-adrenal (HPA) stress response (Dong and Swanson, 2004, Radley, *et al* 2009) (Figure 1). Interestingly, some of these projections stimulate HPA axis activity, while others are inhibitory (Herman, *et al* 1994; Dunn, 1987). Under basal conditions, it is thought that the inhibitory tone of BNST projections to the PVN prevails (Ulrich-Lai and Herman, 2009). This inhibition must then be disinhibited for HPA activation.

Finally, the BNST is well positioned to regulate the brains reward centers (Figure 1). The VTA, NAc, and PFC make up the mesolimbic dopamine system activated by drugs of abuse, which will be covered in greater detail below. As mentioned above, the BNST is interconnected with the VTA. This VTA projection regulates the firing of VTA dopaminergic neurons, which are the heart of the mesolimbic dopamine system (Georges and Aston-Jones, 2002). The BNST also projects to the NAc, providing another level of reward circuitry regulation (Dong, *et al* 2001). Thus, the BNST is uniquely prepared first to process stressful or emotional cues, and then to respond via the reward or stress circuits.

Drug addiction

Reward Circuitry

The existence of brain regions mediating reward-seeking behaviors was identified in a rudimentary basis by self-stimulation experiments over 50 years ago (Olds and Milner, 1954). These studies pointed to the VTA as sensitive area, and subsequent decades of work outlined the mesolimbic dopamine system as being a critical reward circuit. The mesolimbic dopamine system is comprised of dopaminergic cell bodies in the VTA that project to the NAc and PFC. The NAc is involved in motivation, and dopamine in the NAc is critical for the sensation of reward. The PFC determines the motivational significance of a stimulus and determines the organism's behavioral response based on that significance.

The mesolimbic dopamine system has been implicated most heavily in psychostimulant abuse, and is responsible for the acute rewarding effects of drugs such as cocaine and methamphetamine. While it was thought for some time to be the final common pathway for reinforcement of all drugs of abuse, this was ultimately proven incorrect. Currently, it is hypothesized that while all drugs of abuse will activate the mesolimbic dopamine system, regulation of motivation and reinforcement occurs in the NAc, suggesting overlapping circuitry is also crucial for the reinforcing effects of an addictive substance (Koob, 1999; Nestler, 2004).

Dopamine and Other Biogenic Amines

The research presented and discussed herein focuses on cocaine and its molecular targets. Cocaine, along with amphetamine, falls into the psychostimulant class of drugs. Psychostimulants are compounds that act to increase motor activity, as the name suggests. Both cocaine and amphetamine acutely target monoamine transporter proteins (for review, see Kahlig and Galli, 2003). Transporters function to remove excess ligand from the extracellular space around synapses, thereby curtailing the signal of that particular ligand. Cocaine is a nonselective monoamine transport blocker, binding to and subsequently blocking the dopamine transporter (DAT), serotonin transporter (SERT), and norepinephrine transporter (NET). Blockade of these transporters results in an increase in the intensity and duration of monoamine signaling events. Amphetamine, in contrast, can be taken up by monoamine transporters as if it were a monoamine. Once inside the presynaptic terminal, amphetamine will target vesicular monoamine transporters, leading to increasing concentrations of intracellular monoamines. Eventually, the monoamine transporter, whose utility is linked to the monoamine concentration gradient, will start to efflux the built up monoamines, functioning exactly opposite of its physiological role (Khoshbouei, *et al* 2003). Both cocaine and amphetamine have positive reinforcing effects, and are considered to be highly rewarding. These qualities lend themselves well to the study of drug-seeking behaviors. Norepinephrine was the first monoamine postulated to play a role in reward (Stein, 1962), followed closely by dopamine (Wise, 1978). Since that time, addiction research has traditionally focused on dopamine as the central mediator of reward

signaling. An increase in dopamine in the NAc shell has become the hallmark of an addictive substance. [Interestingly, it has been demonstrated that drug-induced dopamine levels rise more and with greater drug sensitivity in BNST than in NAc shell (Di Chiara, *et al* 1999)]. While other neuromodulators and neurotransmitters are now known to be involved in the brain's response to drugs of abuse, glutamatergic transmission is emerging as an important mediator of reward processes, and a substrate targeted by addictive substances.

Self-Administration as an Animal Model of Addiction

Addiction involves a complex pattern of behaviors, broken down into stages over the lifetime of the individual. First is *Binge/Intoxication*, followed by *Withdrawal/Negative Affect*, and finally, *Preoccupation/Anticipation* (Koob and Volkow, 2009). The development of animal models has proven critical for the understanding of many human disorders, though they are frequently difficult to institute. The oldest and most widely used animal models of addiction are *Binge/Intoxication* models.

The first, and most obvious, way to model substance abuse in animals is to give the animals access to addictive substances and monitor their use. Unfortunately, rodents (the primary animals for addiction models) will not readily consume drugs; they must first be trained to receive a palatable substance (such as sweetened condensed milk) via operant conditioning. In these paradigms, mice or rats learn to associate pressing a lever with receiving a reward (Olsen and Winder, 2006), after which they will learn to lever press for cocaine. This model of drug seeking

behavior is called "self-administration", because the animal is contingently receiving a drug that he is motivated to seek. Biochemical and electrophysiological studies of animals trained to self-administer drugs have elucidated numerous pathways and targets of addiction-related behaviors. These studies are especially valuable when compared to findings from mice or rats given drugs non-contingently (such as via i.p. injection). Non-contingent drugs can cause addiction in animal models, but fail to engage the motivational, drug-seeking aspects of an addict's behavior, and could thus have slightly different targets (for review, see Koob, 2009).

Glutamatergic Transmission as a Substrate of Drug Exposure

While monoamines are undoubtedly important in the pathophysiology of cocaine addiction, recent work has focused on how those affected monoamine systems alter glutamatergic signaling and plasticity. Synaptic plasticity such as LTP and LTD, as mentioned previously, is thought to underlie important behavioral processes such as learning and memory. Addiction, therefore, could be hijacking the brain's learning and memory mechanisms, leading to drug dependence.

AMPAR/NMDAR Ratios

In 2001, the Bonci laboratory demonstrated that a single, *in vivo* exposure to cocaine induces LTP at excitatory synapses on VTA dopamine neurons (Ungless, *et al* 2001). The mechanism for this was increased AMPAR currents, leading to an increase in the ratio of AMPARs to NMDARs at these synapses, a change that could occur in LTP. AMPAR/NMDAR ratios are determined by several methods, but in general, the total postsynaptic current is measured while NMDARs are blocked, allowing for isolation

of the AMPAR component of the current. This AMPAR component is then subtracted from the total current, and the difference equals the contribution of NMDARs. The ratio of AMPAR current: NMDAR current is calculated in basal conditions, and then compared to the ratio obtained after an experimental manipulation (in this case, *in vivo* drug exposure). An increase in this ratio roughly correlates to LTP at excitatory synapses, and a decrease indicates a weakened synaptic strength, similar to LTD. Generally, the change in AMPAR/NMDAR ratio involves changes in the AMPAR-mediated current, although this is not always the case. It is important in these studies to control for any potential change in glutamate release, as this would also alter the amplitude of postsynaptic currents.

The Bonci study was the first study to show glutamatergic transmission to be a substrate of a single, *in vivo* drug exposure. Further experiments demonstrated an increase in the AMPAR-mediated current after cocaine (Ungless, *et al* 2001). As the total protein levels of AMPAR subunits GluA1 and GluA2 was unchanged by cocaine exposure, an increase in synaptic localization and/or phosphorylation events increasing AMPAR currents is the most likely mechanism by which cocaine increased AMPAR current.

Importantly, the observed increase in AMPAR/NMDAR ratio mapped onto 2 important aspects of drug exposure. First, the duration of the change in the AMPAR/NMDAR ratio of VTA DA neurons was transient (Ungless, *et al* 2001). While present 1 and 5 days after a single exposure to cocaine, the ratio had returned to normal by 10 days after cocaine exposure. This timecourse fits well with the known transient role of the VTA in the development of addiction neurophysiologically.

Secondly, the cocaine-induced change in AMPAR/NMDAR ratio was blocked by infusion of the NMDAR antagonist (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801), which also blocks behavioral sensitization to cocaine (Kalivas and Alesdatter, 1993). Intra-VTA MK-801 also blocks NMDAR-dependent LTP in the VTA, a form of LTP which is mediated by increased AMPAR number and function (Barria, *et al* 1997). Taken together, the Bonci study was the first to provide a mechanistic correlation between NMDAR-dependent LTP and increased AMPAR/NMDAR ratios in the VTA, and suggests these changes could underlie drug-induced behavioral changes like sensitization.

A later study by Bellone and Luscher more closely examined cocaine-induced changes in AMPAR/NMDAR ratios in the VTA (Bellone and Luscher, 2006). After a single exposure to cocaine, AMPARs in the VTA were found to be susceptible to the effects of Joro spider toxin, a polyamine that causes inward rectification of calcium-permeable AMPARs. This new polyamine sensitivity suggested a switch in AMPAR subunit composition at the synapse, induced by cocaine. AMPAR redistribution is dependent on protein-protein interactions with the C-terminal tails of AMPAR subunits, and can be mediated by changes in the phosphorylation of sites within the C-terminal domains (as discussed above). The polyamine sensitivity after cocaine could be mediated either by insertion of calcium-permeable AMPARs into the synapse, and/or the removal of GluA2-containg (calcium-impermeable) AMPARs from the synapse. The experimenters decided to manipulate PICK1, a protein responsible for regulating the localization of GluA2-containg AMPARs at the synapse. When PICK1 was knocked down via peptide inhibition, cocaine-induced

polyamine sensitivity was lost. This suggested there was a loss of GluA2-containing receptors was induced by cocaine exposure (Bellone and Luscher, 2006). Bellone and Luscher went on to try and rescue the drug-induced loss of GluA2-containing AMPARs. GluA2-containing AMPARs are known to be critical for expression of mGluR-LTD in the VTA. Therefore, mGluR-LTD in the VTA could reverse the changes in AMPAR/NMDAR ratios induced by cocaine exposure (Bellone and Luscher, 2006). Indeed, LTD induced via mGluR1 activation or low frequency stimulation reversed the changes in polyamine sensitivity in slices from cocaine-exposed mice. *In vivo* activation of mGluR1 after cocaine exposure also reversed the cocaine-induced changes in AMPAR subunit composition and the increase in AMPAR/NMDAR ratio. This ability to oppose cocaine-induced plasticity through pharmacological means holds great promise for future treatment of addiction.

Drug-Induced Disruption of ex vivo Plasticity

Along with the ability to confer plasticity after exposure, cocaine has also been shown to disrupt many characterized *ex vivo* forms of plasticity. The Manzoni group has characterized a form of LTD in nucleus accumbens (NAc) slices, evoked by mimicking natural frequencies of cortical afferents (Robbe, *et al* 2002). This LTD is mediated postsynaptically by mGluR5-induced internal Ca^{2+} release, and presynaptically by type 1 endocannabinoid receptors (CB_1Rs). mGluR1/5 can activate presynaptic CB_1Rs through the PLC pathway, as previously mentioned. This pathway generates endocannabinoid molecules within the postsynaptic cell and releases them to as a retrograde signal, at presynaptic receptors. Activation of CB_1Rs decreases the release of glutamate, causing a depression in transmission.

Simultaneous to that, activated mGluR5 triggers intracellular Ca^{2+} signaling in the postsynaptic cell, which could act to depress transmission. Therefore, mGluR5-LTD in the NAc has both pre- and postsynaptic mechanisms.

mGluR5-LTD in the NAc is disrupted by a single *in vivo* exposure to cocaine (Fourgeaud, *et al* 2004). This disruption is accompanied by an increase in the expression of long Homer isoforms in the NAc, and a decrease in surface mGluR5 protein. Homer proteins are important scaffolding proteins that link Group I mGluRs to intracellular Ca^{2+} stores (Fagni, *et al* 2002). The long homer proteins consist of Homer1b-g, Homer2a/b, and Homer3, the products of three separate genes in mammals (for review, see Szumlinski, *et al* 2006). Long homer isoforms are expressed steadily under basal conditions. In addition to EVH1 domains through which they associate with mGluR1/5, IP_3 receptors, and other target proteins, long homers also contain coil-coil domains necessary for dimerization; these dimers are necessary for the scaffolding function of homers. However, after certain *in vivo* events (such as ischemia, stress, or seizure), Homer1a expression is induced (for review, see de Bartolomeis and Iasevoli, 2003). Homer1a is a short isoform homer protein, containing the EVH1 interaction domains but lacking the coil-coil domains of long isoforms, and is expressed as an immediate early gene product. Studies have shown homer1a to have a dominant-negative function on basal homer protein function, disrupting the scaffolding/coupling of mGluR1/5 to internal Ca^{2+} stores (Ango, *et al* 2001). This is thought to occur via homer1a binding to target sequences, preventing long homer isoform binding to those sites, thus removing the long homer scaffolds.

Both long and short homer isoforms are regulated by exposure to drugs of abuse. Repeated exposure to ethanol increases Homer2 mRNA levels in the NAc and amygdala, and overexpression of Homer2b in the NAC of WT mice increases alcohol drinking in a free-access model (Szumlinski, *et al* 2005). In the neocortex and NAc, homer1a mRNA is upregulated after acute and repeated exposure to psychostimulants (Fujiyama, *et al* 2003; Szumlinski, *et al* 2006). Repeated nicotine exposure increases homer1b/c mRNA and protein in the amygdala and VTA, and homer2 isoforms in NAc, amygdala, and VTA (Kane, *et al* 2005). In summary, drug-induced changes in homer protein expression (and conversely, homer-induced changes in drug-sensitive behaviors) point to mesolimbic and mesocortical glutamate as an important substrate of addictive substances and an important mediator of addictive behaviors.

<u>mGluR5-LTD in the BNST is Disrupted by Cocaine Exposure</u>

Our laboratory has previously characterized mGluR5-mediated LTD in the BNST (Grueter, *et al* 2006). Similar to endocannabinoid-mediated mGluR-LTD in the NAc, the early component of DHPG-induced depression of transmission requires CB1Rs. However, the LTD generated by DHPG is independent of CB1Rs and is mediated by G-protein signaling and extracellular-signal regulated kinase 1 (ERK1) activation. mGluR5-LTD is independent of NMDARs, but dependent on protein synthesis. The maintenance of mGluR5-LTD further requires clathrin-dependent endocytosis and rearrangement of the actin cytoskeleton within the postsynaptic cell.

Importantly, mGluR5-LTD is disrupted by exposure to cocaine (Grueter, *et al* 2006). In animals trained to self-administer cocaine, mGluR5-LTD is ablated 24 hours after the last session. Further, mice given an injection of 20 mg/kg cocaine for 10 days showed disrupted mGluR5-LTD, while saline controls looked normal. However, a single injection of cocaine was not sufficient to disrupt mGluR5-LTD. Because cocaine disrupted mGluR5-LTD in both contingent and non-contingent drug exposure paradigms, this suggests direct targeting of mGluR5 function by cocaine *in vivo*. To test this hypothesis directly, MPEP (the mGluR5 antagonist) was given to mice 30 minutes prior to cocaine for 10 days (Grueter, *et al* 2008). Surprisingly, mGluR5-LTD was intact in animals given MPEP prior to cocaine, as compared to controls. Therefore, similar to what was reported with mGluR5-LTD in the NAc, mGluR5-LTD in the BNST is disrupted by cocaine by a mechanism targeting mGluR5 function.

Stress and Anxiety

As mentioned above, the BNST is also heavily interconnected with the brain's stress circuitry. Stress-related disorders, such as anxiety and depression, are a growing health concern in the modern world (Wong and Licinio, 2004). *Stress* has been defined as the imposition or perception of an environmental or physical change (be it negative or positive) that elicits a spectrum of physiological changes construed as adaptive to the organism (Herman and Cullinan, 1997). *Anxiety* is a state of uncertainty, apprehension, vigilance, or even fear due to the anticipation of a real or

imagined threat. The umbrella of anxiety disorders includes generalized anxiety disorder, panic attacks, post-traumatic stress disorder (PTSD), and social phobias. Stress and anxiety are thought to share the common mechanism of uncontrolled neural excitability in select brain circuitry including limbic brain regions such as the BSTN and extended amygdala. It is this circuitry that is the target not only of therapeutic agents, but also animal models of anxiety behaviors.

Stress Circuitry

The body's primary stress response system, the Hypothalamic-Pituitary-Adrenal axis (HPA axis), begins in the hypothalamus. Hypothalamic neurons containing corticotropin-releasing factor (CRF) project to the pituitary gland, stimulating the release of adrenocorticotropic hormone (ACTH). ACTH then travels through the bloodstream to the adrenal glands, where it induces the release of corticosterone, the principal stress hormone. Coricosterone then acts throughout the brain and body on glucocorticoid and mineralocorticoid receptors, which regulate a variety of cellular processes such as gene transcription (Makino, *et al* 2002). At every step of this pathway, feedback mechanisms exist to regulate the release of CRF from the hypothalamus, ACTH from the pituitary, and corticosterone from the adrenal glands. In the brain, CRF signaling outside the hypothalamus acts as a neuromodulatory neuropeptide. Extra-hypothalamic CRF has been implicated in a variety of anxiety- and fear-related behaviors, and more recently, has been postulated as a link between stress and drug addiction (Koob, 1999). The BNST is one of the CRF-richest areas in the brain, and receives a CRF-ergic connection from the CeA (Erb, *et*

al 2001). Within the BNST, dopamine enhances glutamatergic transmission in a CRF receptor-dependent fashion. Further, NMDAR-dependent LTP in the BNST is enhanced after cocaine exposure in a CRF receptor-dependent manner. Taken together, these data suggest drugs of abuse may induce CRF release in the BNST via dopamine (Kash, Nobis, *et al* 2008).

Compounds Targeting Glutamatergic Signaling are Anxiolytic

For many years, the most common treatments of anxiety disorders focused on the brain's inhibitory GABAergic (targeted by benzodiazepines) or neuromodulatory monoaminergic (targeted by most antidepressants) systems. However, these medications come with complex side effects, and are not effective in treating anxiety in many cases (Amiel and Matthew, 2007). The search for novel therapeutic targets quickly ended with the glutamatergic system. While some compounds targeting iGluRs are effective anxiolytics, it is metabotropic glutamate receptors that hold the greatest therapeutic potential. With more specialized patterns of expression than iGluRs and greater potential to modulate glutamatergic transmission (rather than stimulate or block it completely), mGluRs are well-suited to be therapeutic targets. As stated previously, agonists of Group II and Group III receptors as well as antagonists of Group I receptors all have anxiolytic behavioral effects. Hence, glutamatergic transmission and signaling must be a neural substrate for stress/anxiety.

Animal Models of Anxiety

Types of Behavioral Stressors

A wide variety of behavioral stressors exist for rodents. These generally fall into 2 classes- processive stressors and systemic stressors. Processive stressors engage brain regions involved in decision making to determine whether the perceived stimulus is a threat worthy of engaging the stress response. Some examples of processive stressors include social defeat stress, restraint stress, and maternal separation. In contrast, hypothermia, hypoxia, and hemorrhage are all types of systemic stressors, which do not require any higher order processing to elicit a neural response (Herman and Cullinan, 1997). As a limbic regulator of the brain's HPA axis, both systemic and processive stressors would likely recruit BNST activation.

Assaying for Anxiety-Related Behaviors in Rodents

Several behavioral tests are used to measure anxiety-related behaviors in mice or rats (for review, see Cryan and Holmes, 2005). Many of these tests take advantage of some basic stress-induced avoidance behaviors exhibited by rodents, including freezing (decreased locomotion) and the avoidance of bright or open spaces. The most commonly used test for rodent anxiety-related behavior is the elevated plus maze (EPM). The EPM is shaped like plus sign, with two of the arms having walls and an end, and the other two arms being open. The entire maze is elevated several feet off of the floor. Mice and rats prefer to spend their time in the closed arms of the EPM, and will explore the open arms very little. This effect is magnified under bright lighting conditions, which are aversive to rodents. Animals given anxiolytics,

however, will markedly increase their time spent in the open arms without causing a change in total locomotor activity. The reproducibility of this behavior has made the EPM a critical test for potential anxiolytic compounds. Another common behavioral test is the open field. Because mice tend to avoid open spaces, they prefer to run around the perimeter of the open field chamber. Thus, measuring the time spent in the center of the open field is a measure of anxiety, with increased center time correlating with decreased anxiety.

Genetic Mouse Models

Finally, the EPM and open field have also been used to identify inherent differences between mice of different strains or genotypes. Several genetic mouse models have been identified as having anxiolytic or anxiogenic phenotypes. As mentioned previously, mGluR7-deficient mice have an anxiolytic behavioral profile, whereas mGluR8-deficient mice show a basal anxiety phenotype (Cryan, *et al* 2003, Linden, *et al* 2002). Loss of critical adrenergic proteins has also been shown to impart anxiety phenotypes as well, with α2A adrenergic receptor (AR) KO mice showing decreased open arm time in the EPM (Lahdesmaki, *et al* 2002).

Anxiety Versus Fear

It is important for me to pause here and clarify the difference between anxiety and fear. Though similar, these two emotional responses differ in behavioral expression and brain circuitry. Fear is elicited by the eminent, sensory-engaging threat of a predator's presence, while anxiety is the response evoked by the potential presence of the predator. Interestingly, anxiolytics will block anxiety but not fear (Blanchard,

et al 1993). Generally, the BNST is the extended amygdala nucleus that mediates anxiety-related behaviors, with the CeA tending to be more involved in fear (Walker and Davis, 1997). However, in animal models, anxiety-like behaviors are sometimes referred to as types of fear-mediated behaviors, greatly confusing the matter. "Sustained fear", for example, is a term applied to the steady, sustained level of anxiety maintained by a vigilant animal, and is probably BNST-mediated rather than CeA-mediated (de Jongh, *et al* 2003). This vigilance behavior appears to model several clinical anxiety phenotypes and can be modified by anxiolytics, suggesting it is indeed "anxious" in nature.

Anatomical Circuit Distinction of Anxiety Versus Fear

Fear and anxiety are also mediated by discrete neural circuitry. For example, light-enhanced startle is a model of unconditioned fear that may be similar to CRF-enhanced startle, an anxiety-like paradigm (Walker and Davis, 1997, 2008). Fear-potentiated startle, on the other hand, models conditioned fear responses. Both are mediated by BLA projections to extended amygdala nuclei. The visual stimuli for each paradigm is identical, differing only in duration and the conditioning history of the animal. But while the behavioral expression of fear-potentiated startle is blocked by pharmacological inactivation of the CeA, light-enhanced startle is blocked by inactivation of the BNST (Lee and Davis, 1997). The BNST is therefore involved in fear-mediated behaviors involving vigilance and reaction to novel, unconditioned stimuli, whereas the CeA mediates faster, conditioned fear-related behaviors. These studies further support the idea that while sharing many similar inputs and related functions, the roles of extended amygdala nuclei can be parsed

apart behaviorally, as well as anatomically, and support a role for the BNST in shaping more anxiety-related behaviors.

Adrenergic Signaling

Besides being heavily connected to the PVN and the body's stress response pathway, the BNST is also richly innervated by noradrenergic nuclei in the brainstem (Forray and Gysling, 2004). As the chemical mediator of the body's fight-or-flight response, the adrenergic system is a critical mediator of stress/anxiety. Interestingly, adrenergic signaling may also mediate affective disorders and stress-induced relapse to drug seeking (Shaham, *et al* 2000). Norepinephrine (NE), also known as noradrenaline (NA), is a neuromodulatory biogenic amine that functions as a neurotransmitter within the CNS. It is synthesized from dopamine by dopamine-β-hydroxylase (DBH), which is useful as a marker for noradrenergic terminals. NE exerts its effects on cells via adrenergic receptors (ARs), which are comprised of 2 types- α ARs and β ARs (Hein and Kobilka, 1997). α ARs can be further divided into α1 and α2 types, with each having 3 subtypes (α1A, 1B, and 1D; α2A, 2B, and 2C). ARs are GPCRs, with α1 ARs being G_q-coupled, α2 ARs being G_i-coupled, and β ARs being generally G_s-coupled. Adrenergic receptors are expressed throughout the CNS and the periphery, and can also be activated by adrenaline and epinephrine (which are produced in the adrenal glands). As mentioned previously, the BNST receives one of the densest NE projections in the brain, from the VNAB. Both α and β ARs are expressed in the BNST, poised to respond to a change in adrenergic tone in the region in response to activation of the brain NE system.

AR-Mediated Behaviors

NE/E is the chemical messenger of the autonomic, fight-or-flight response to a stressor throughout the body. Therefore, it is not surprising that the brain NE system is also activated by acute stress (Morilak, *et al* 2005). Neural NE is largely thought to organize or facilitate an organism's stress response. NE has also been implicated in behaviors such as arousal and vigilance (Berridge and Dunn, 1989). Groundbreaking work from the Morilak group has highlighted a role for BNST ARs in the acute response to stress (Cecchi, *et al* 2002). Using microdialysis, they demonstrated a large spike in BNST extracellular NE levels in response to a single restraint stress. Direct microinjection of either α1 or β antagonists into the BNST was shown to block the anxiety phenotype of the stressed mice in the EPM. Finally, intra-BNST infusion of α1 AR antagonist reduced plasma levels of ACTH following a stressor, showing direct regulation of the HPA axis by α1 ARs in the BNST. This single study pinpointed BNST ARs as being critical regulators of both the behavioral and physiological stress response.

α1 Adrenergic Receptors (α1 ARs)

In the brain, α1 ARs activation has been shown to alter synaptic plasticity. In both the hippocampus and visual cortex, α1 ARs can induce LTD of excitatory transmission (Kirkwood, *et al* 1999; Scheiderer, *et al* 2004). In the hypothalamus, however, α1 AR activation enhances glutamatergic transmission (Gordon and Bains, 2003, 2005). Interestingly, this enhancement is mediated partially by presynaptic inhibition of Group III mGluRs and is mimicked by an *in vivo* stressor (Kuzmiski, *et*

al 2009), suggesting the α1 AR may be an important regulator of glutamatergic transmission during and after exposure to stress.

α1-LTD in the BNST

Activation of α1 ARs in the BNST via the α1 agonist methoxamine induces LTD *ex vivo* (McElligott and Winder, 2008). This LTD differs from other forms of α1-dependent plasticity in that it does not require NMDAR activation. L-type voltage-gated Ca^{2+} channel (VGCC) activation is required, however. This α1-LTD is mechanistically distinct from other G_q-coupled forms of LTD in the BNST (specifically mGluR-LTD), most notably in their different AMPAR subunit requirements for expression (McElligott, *et al* 2010). Further, while mGluR5-LTD is disrupted after *in vivo* cocaine exposure but intact after *in vivo* stress, α1-LTD showed exactly the opposite phenotype.

Importantly, α1-LTD can be induced by bath application of the endogenous ligand, NE. However, NE-induced α1-LTD exhibits the unique property of time dependence. While a 10 minute application of NE causes a transient decrease in excitatory responses, a 20 minute application is required to induce α1-LTD (McElligott and Winder, 2008). This time dependence may be partially due to the high rate of NE clearance in the BNST, mediated by high levels of NET. However, it could suggest that prolonged activation of the adrenergic system may be necessary to trigger the stress response mediated by α1 ARs in the BNST. Regardless, these studies show a clear role for NE in altering glutamatergic transmission in the BNST, an alteration critical for recruiting the body's stress response.

Summary

In summary, both drug addiction and stress/anxiety are thought to be mediated by changes in glutamatergic transmission and signaling. In both instances, the data suggest a role for mGluRs as important substrates of each disorder. In the first half of this thesis, I will present work examining the relationship between mGluR5 signaling, ERK activation, and cocaine exposure. In the latter half, my data will focus on the emerging role of mGluR8 as a target of stress regulation. My overarching hypothesis is that a specific type of *in vivo* experience (in this thesis, this means exposure to stress or cocaine) will increase glutamatergic signaling in the BNST, causing activation of specific mGluR subtypes (mGluR5 by cocaine, mGluR8 by stress) and subsequent selective disruption of their function.

Global Hypothesis and Specific Aims, Part I

Hypothesis

I hypothesize that cocaine disrupts *ex vivo* mGluR5-LTD in the BNST via alteration of mGluR5 activation and/or surface expression, thus altering the ability of mGluR5 to activate ERK, and blocking the LTD.

Specific Aims

1. Test the hypothesis that *in vivo* cocaine recruits the activation of mGluR5, and that this activation is necessary for cocaine-induced ERK activation in the BNST.

2. Test the hypothesis that cocaine disrupts the activation and/or surface expression of mGluR5 in the BNST by disrupting mGluR5 signaling, thereby blocking (or occluding) induction of mGluR5-LTD and subsequent activation of ERK.

Global Hypothesis and Specific Aims, Part II

Hypothesis

I hypothesize that mGluR8 is the primary Group III mGluR subtype regulating excitatory transmission in the BNST, and is itself regulated by exposure to stress and/or drugs of abuse.

Specific Aims

1. Determine the specific Group III mGluR subtypes mediating the effects of Group III agonists on excitatory transmission in the BNST.
2. Test the hypothesis that mGluR8 regulation of glutamatergic transmission in the BNST is effected by *in vivo* exposure to stress and/or drugs of abuse.

CHAPTER 2

MATERIALS AND METHODS

Animals

Unless otherwise noted, all mice used in these experiments were male C57Bl/6J mice from Jackson Laboratories. In some cases, male mGluR8$^{-/-}$ mice or α_{2A} adrenergic receptor$^{-/-}$ mice, both backcrossed into a C57Bl/6J background, were used. The mGluR8 KO mice were obtained from our collaborator Robert Duvoisin and bred in-house in a het x het breeding scheme (see Duvoisin, *et al* 2005 for characterization of this strain). The α_{2A} AR KO mice were bred in-house from KO x KO breedings. For a few immunohistochemical control samples, tissue from ERK1$^{-/-}$ and mGluR5$^{-/-}$ mice was used to ensure antibody specificity. All mice were aged 5-10 weeks, unless otherwise noted.

Brain Slice Preparation

Animals were anesthetized with isoflurane and euthanized according to IACUC-approved procedure. The brain was rapidly removed from the skull and immersed in ice-cold, oxygenated slicing solution (ACSF in which the sodium has been replaced with sucrose). The brain was placed in a metal brain matrix for crude sectioning, with the ventral surface facing up, and a coronal block of tissue

containing the BNST was extracted. The tissue block was hemisected and mounted to a metal specimen disk with cold-setting superglue. The tissue was then submerged in a bath of ice-cold, oxygenated, slicing solution and sliced into 300 μm thick slices on a Leica VT1000S vibratome (Wetzlar, Germany). Immediately after slicing, the slices were transferred to warm (28°C), oxygenated ACSF (in mM: 124 NaCl, 4.4 KCl, 1.2 $MgSO_4$• $7H_2O$, 1 NaH_2PO_4, 2.5 $CaCl_2$ • $2H_2O$, 26 $NaHCO_3$, 10 glucose) in either tissue incubation chambers (for chamber slice experiments) or humidified interface chambers (for field potential experiments).

Field Potential Recordings

Immediately after slicing, BNST slices were transferred to humidified interface chambers containing warm (28 °C), oxygenated ACSF perfused continuously at a rate of 2-3 ml/min. After recovering for 30 minutes, picrotoxin (25μM) was added to the slices to block transmission through $GABA_A$ receptors. In experiments with AZ12216052 and vehicle control, 0.1% DMSO was also added to the ACSF at this time. Slices then recovered for another 30 minutes prior to the onset of recording.

A stimulating electrode was placed at the dorsal apex of the dorsal BNST (dBNST). Local afferents were stimulated from 3-30 V to determine the range of a response's amplitudes, and a stimulation intensity corresponding to ~50% of the maximum response amplitude was used. For synaptic depression/LTD experiments, a 50-60%

maximum response amplitude was used. Responses were evoked at a frequency of 0.05 Hz, with a stimulus duration of 0.065 msec.

Field potential responses were recorded using borosilicate glass pipettes (resistance 1-3 MΩ) pulled on a Flaming-Brown micropipette puller (Sutter Instrument Co., Novato, CA). Pipettes were filled with ACSF and positioned ventrally and laterally to the site of afferent stimulation, within the dBNST. To increase the percentage of usable experiments, baselines of 30-40 minutes were established prior to the onset of tetanus or drug application in order to ensure the stability of a response. All drugs were bath applied. Clampex 8.2 and Clampfit 9.0 were used to record and analyze all experiments (Molecular Devices, Sunnydale, CA).

Drug Application

All drug were bath applied. (S)-3,4-Dicarboxyphenylglycine (DCPG), L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4), (E)-1,1a,7,7a-Tetrahydro-7-(hydroxyimino)-N-phencyclopropa[b]chromene-1a-carboxamide (PHCCC), and (1R,4R,5S,6R)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268) were obtained from Ascent Scientific (Princeton, NJ). DCPG and (S)-3,5-dihydroxyphenylglycine (DHPG) were obtained from Tocris Bioscience (Ellisville, MO). Methoxamine hydrochloride and picrotoxin were from Sigma-Aldrich (St. Louis, MO). AZ12216052 was generously provided by AstraZeneca (Wilmington, DE). Cocaine hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO) and the National Institute of Drug Abuse (Bethesda, MD).

DCPG, L-AP4, methoxamine, LY379268, DHPG, and cocaine were dissolved in water. PHCCC, AZ12216052, and picrotoxin were dissolved in DMSO at a final concentration of ≤ 0.1% DMSO (final concentration, v/v). For *in vivo* injections, cocaine was dissolved in saline.

Single-train LTP

Previous work in our laboratory (Weitlauf, *et al* 2004) has demonstrated that 2 trains of 100 Hz stimulation, 20 seconds apart, will induce LTD in the dBNST. For the purposes of these studies, all LTP experiments used the subthreshold protocol of a single 100 Hz train. Additionally, a 40-50% maximum response amplitude was used in these experiments to allow for the increase in N2 amplitude.

Submerged Chamber Drug Experiments

Immediately after slicing, hemisected slices of 300 μm thickness were placed in submerged tissue incubation chambers containing warm, oxygenated ACSF (25-27°C). Slices were allowed to recover for 90 minutes, at which time picrotoxin was added (25μM). After 30 more minutes, a 5-minute application of 100μM DHPG was applied to the slices. After incubation with drug, the slices were removed and immediately placed on a glass petri dish sitting on a metal block in dry ice, where they are flash frozen. Tissue punches were then taken of the BNST and any other brain region of interest (usually the dorsal striatum) using 0.41 mm diameter metal punch (VWR Scientific, West Chester, PA). For some experiments, punches from

400 µm slices (exposed to picrotoxin but not to other drugs) were used to obtain total protein levels. All tissue punches were stored at -80°C until homogenization.

Slice Biotinylation

Biotinylation

This procedure can be adapted to be performed over wet ice on the benchtop. However, in order to ensure a uniform temperature of 4°C throughout, all of the biotinylation experiments in this thesis were carried out in a cold room for the duration of the experiment. All solutions are ice-cold and perfused with oxygen unless otherwise noted.

At the end of the slice recovery period, slices were transferred from the tissue incubation chambers to 24-well plates containing ACSF, and washed twice, for 10 minutes each. Slices were then incubated in ACSF containing 1 mg/mL sulfo-NHS-SS-biotin (Pierce) for 45 minutes. After incubation with the biotin, slices were washed with ACSF twice, for 10 minutes each, to remove any unreacted biotin. Then, the slices were washed twice for 20 minutes each with ACSF containing 100mM glycine, in order to quench the remaining biotin reagent. At the end of the last wash, slices were flash-frozen in a petri dish sitting on a metal block in dry ice, and tissue punches were collected and stored at -80°C.

Pulldown

This procedure was done on ice as much as possible to hinder protease activity.

Punches were homogenized in homogenization lysis buffer (25mM HEPES, 150mM NaCl, 1% Triton X-100, 0.1mM benzanimide, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 2mM Na Orthovanadate, 2mM Na Fluoride) for 30 seconds with a motorized tissue homogenizer. The samples were then spun at 10,000 x *g* at 4°C for 10 minutes to remove debris. This created 2 fractions in the sample: a supernatant that was Triton X-soluble (the sample), and a pellet that was Triton X-insoluble. Because this pellet most likely contained the post-synaptic density (PSD) and related proteins, and can be run on a gel, it was saved for future analysis. The protein concentrations of the samples were then determined by BCA assay (Pierce). Streptavidin agarose beads (Pierce) were washed 3 times at 4°C in ice-cold lysis buffer (same as homogenization lysis buffer except only 0.1% Triton X) and once in ice-cold 50 mM Tris-HCl, pH = 7.4. These washes were all done in 10-fold excess volume of wash buffer to bead slurry to ensure thorough washing, and mixed by inverting or flicking the tube as vortexing will fracture the beads. The samples were then incubated overnight at 4°C with 50 µL of bead slurry and 500 µL lysis buffer, on a rotator to ensure thorough mixing. This is an approximate ratio of 5 µg protein : 1µL beads. It is important to note that bead slurry is 50% beads and 50% buffer. The next day, the samples were spun down to isolate the beads. In each sample, cytosolic proteins will be contained in the supernatant, and any membrane-bound biotinylated proteins will be in the pellet. The supernatant was removed and saved, although it was too dilute to run on the gel. The bead fraction was then diluted with 4X sample buffer that contained DTT and BME. The reducing agents in the buffer cleaved the biotin-protein bond, which released the surface proteins from the beads.

Finally, the sample buffer containing the proteins was extracted off of the bead pellet using a Hamilton syringe and loaded onto the gel. The extraction was done slowly and with the syringe tip facing the wall of the tube to prevent it from clogging with beads. The syringe was cleaned between samples with 1X running buffer to prevent contamination resulting from a possible clog.

Western Blotting

Tissue Punching

A fresh tissue slice was placed in a glass petri dish, resting on a metal block in dry ice. As the slice froze, excess solution was wicked away with a kimwipe to prevent sample dilution. Tissue from the brain region(s) of interest was gathered using a metal tissue punch 0.41 mm in diameter (VWR) that was resting in dry ice. Collected punches were deposited in tubes and frozen at -80°C until needed.

Homogenization

Punches were homogenized in simple homogenization buffer (2% SDS, 0.1mM benzanimide, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 2mM Na-Orthovanadate, 2mM Na-Fluoride) for 30 seconds with a motorized tissue homogenizer. The samples were then centrifuged for 20 minutes at 1000 x *g*. Protein concentrations were determined using a BCA assay (Pierce). Samples were diluted to desired concentration with homogenization buffer, and then all given an equal volume of reducing sample buffer containing 5% SDS and BME (2X or 4X, in most cases with

40 mM DTT). Depending on the experiment, samples were vortexed and heated to either 60°C for 5-10 minutes or 95°C for 4 minutes. After heating, samples were again vortexed and spun down to load onto the gel.

SDS-PAGE

Gels were poured between standing glass plates and allowed to polymerize for at least 90 minutes prior to electrophoresis. Each gel was comprised of an 8% acrylamide stacking layer and a 10% acrylamide resolving layer. In over 95% of experiments, a thin, triple-wide gel was run for 45 minutes at 200V. Occasionally, gels were run for 60 minutes to achieve greater resolution of high-molecular-weight proteins. Gels were transferred onto 2 layers of PVDF membrane in series using Tris-glycine transfer buffer. Transfers were done in ice for 2 hours (or in the cold room overnight) at 200 mA. After transfer, the membrane closest to the gel was used for western blotting, while the second membrane was stained with colloidal gold to reveal total protein levels. The gold stain membrane was later used to confirm even gel loading and transfer, and measured with densitometry to provide a total protein control in experiments where the protein concentration of the samples was not normalized.

Western Blots

For nearly all antibodies, milk was used in the blocking reagent and primary/secondary antibody solutions. For a few antibodies, BSA was preferred.

All western blotting solutions were based in Tris-buffered saline (TBS: 20mM Tris, 137mM NaCl, pH = 7.6) containing 0.1% Tween.

Immediately after transfer, the membrane was incubated with blocking reagent (5% milk, 2mM NaO, 2mM NaF, 0.02% Na-Azide) on an orbital shaker for 1 hour at room temperature, or overnight at 4°C. After being rinsed in TBST, the membrane was incubated with the primary antibody solution (same as blocking solution, with the addition of the primary antibody at the desired concentration) on an orbital shaker for 1 hour at room temperature or overnight at 4°C. The membrane was then rinsed several more times, depending on the strength and specificity of the primary antibody, and then incubated with a secondary antibody conjugated to horseradish peroxidase (5% milk, 2mM NaO, 2mM NaF plus the secondary Ab at the desired concentration) on an orbital shaker for 1 hour at room temperature or overnight at 4°C. The membrane was washed, on average, for 1-3 hours in TBST (with the wash changed several times), depending on the strength of the secondary antibody, sensitivity of the primary antibody, amount of protein loaded in your gel, etc. in order to optimize the antibody signal. Blots were then processed for enhanced chemiluminescence using Western Lightning (Perkin-Elmer), exposed to film in a dark room, and the film was developed. The films were then scanned for analysis, and the optical density of the resulting bands was measured using Scion Image (NIH). Blots within the linear range were quantified and normalized to control proteins or gold stain.

Immunohistochemistry (DAB)

Mice were anesthetized with a lethal dose of Nembutal (sodium pentobarbital) and perfused intracardially with cold PBS, followed by cold 4% paraformaldehyde (PFA, in PBS). The brains were removed and post-fixed for 12-48 hours in 4% PFA at 4 ºC, and then transferred to 30% sucrose (in PBS) where they incubated overnight at 4 ºC, until they sunk. Coronal sections 40 µm thick were made using a cryostat (Leica, Nussloch, Germany) and stored in cryoprotectant at -20 ºC.

Sections were washed three times in 0.01M PBS for 10 minutes each and then incubated in a hydrogen peroxide solution (0.6% in PBS) for 30 minutes. (All washes and incubation steps took place on shaker at room temperature unless otherwise noted.) Sections were again washed three times in PBS, for 10 minutes, after which the sections incubated in blocking solution (5% normal goat serum (NGS), 0.2% Triton X-100 in PBS) for 1 hour. The sections were then incubated in primary antibody (anti-phospho-ERK, 1:400; Cell Signaling Technologies, Beverly, MA) for 48 hours at 4 ºC. Then, the sections were washed three times for 10 minutes in PBS, followed by 3, 20-minute washes in a PBS/1% NGS solution. This was followed by a 90-minute incubation in secondary antibody (goat-anti-rabbit, 1:1000, Vectastain ABC Kit; Vector Labs, Burlingame, CA). After three more 10-minute washes in PBS, the sections were allowed to incubate in Avidin-Biotin complex (ABC) solution for 1 hour. The sections were then washed twice for five minutes in PBS, followed by 3, 10-minute washes in PBS. Then, the sections incubated in DAB (Vectastain kit) for five minutes, after which they were

immediately washed in 100μM sodium azide for 2-3 minutes to quench the staining reaction. After a final 2-minute rinse in PBS, the sections were mounted on slides using PolyAquamount (Polysciences Inc., Warrington, PA). The slides were then imaged on a light microscope and saved for analysis using QCapture software.
For quantification, DAB-positive cells were counted manually (from TIF images) by the blinded experimenter. Each hemisphere was counted three times to ensure accuracy. The main criteria considered for determining DAB-positive cells were size and shape of stained area. Intensity of staining varied proportionally to the location of the DAB-positive cell within the depth of the section, and was therefore a secondary criterion. The total counts for each full section (two hemispheres) were calculated and averaged per animal.

Restraint Stress

Acute (60-minute) Restraint Stress

All restraint experiments involved mice 5-7 weeks old and occurred between 9AM and 12PM (2-5 hours after lights-on). A mouse was placed inside of a 50 mL conical tube modified to have breathing holes. The tube was then placed on a rack inside of a sound- and light-attenuating chamber for 60 minutes. The mouse was anesthetized with isofluorane for decapitation immediately upon removal from the tube, and the brain was processed for electrophysiological recordings in the BNST, as described above.

Chronic Restraint Stress

Experiments were done on mice 5-7 weeks old and occurred between 9 AM and 1 PM (2-6 hours after lights on). A mouse was placed inside of a 50-mL conical tube modified to have breathing holes. The tube was then placed on a rack inside of a sound- and light-attenuating chamber for 2 hours, after which the mouse was returned to his home cage. This continued for 10 consecutive days. On the 11th day, the mouse was anesthetized for decapitation, and the brain was processed for electrophysiological recordings in the BNST, as described above.

In vivo Drug Exposure

C57Bl/6J mice (5 weeks of age) were acclimated to the animal facility for 1 week prior to the onset of experiments. For chronic experiments, on day 1, mice were handled, which includes tail striping, weighing, and scruffing. On days 2-11, mice received intraperitoneal (i.p.) injections of cocaine, in saline (20 mg/kg). On day 12, mice were sacrificed and used for either electrophysiological, immunohistochemical, or biochemical experiments. For acute experiments, mice were handled for 1 day, followed by 4 days of receiving i.p. injections of saline to habituate them to the injection procedure. On the final day, mice were given 20 mg/kg cocaine and sacrificed 30 minutes later.

CHAPTER III

MGLUR5 LTD, ERK, AND COCAINE IN THE BNST

Introduction

mGluR5 and Cocaine

A large literature has demonstrated that mGluR5 function is altered by cocaine (Kenny, *et al* 2005; Backstrom and Hyytia, 2006; Bellone and Luscher, 2006). Activation of mGluR5 has been shown to reverse cocaine-induced changes in synaptic strength, and several forms of mGluR5-dependent plasticity are disrupted by cocaine. mGluR5 is a $G_q\alpha$ -coupled receptor, and thus can signal via the endocannabinoid (eCB) system, a potent regulator of presynaptic neurotransmitter release. Interestingly, several forms of eCB-dependent synaptic plasticity are also disrupted by cocaine, further suggesting the importance of mGluR5 in mediating cocaine-related behaviors. Indeed, eCB-dependent LTD in the NAc, which is mediated by mGluR5, is disrupted in cocaine-treated animals (Fourgeaud, *et al* 2004). Further, mGluR5-deficient mice will not self-administer cocaine (Chiamulera, *et al* 2001).

ERK, and its Activation by Cocaine

Recent work in the hippocampus demonstrates that ERK activation is required for mGluR-LTD (Gallagher, *et al* 2004). As mentioned previously, ERK is a protein

kinase that plays an important role in several signal transduction pathways. When activated via double phosphorylation, ERK has the ability to in turn activate dozens of effector proteins, including transcription/translation factors, other signaling kinases, and histone proteins. In 2004, work by Valjent, *et al* demonstrated that distinct patterns of ERK activation occur in the brain in response to various classes of abusive substances. In the BNST (and several other regions important for reward), cocaine strongly induced ERK activation (Valjent, *et al* 2004). Taken together, these studies suggest mGluR5 signaling in the BNST could be ERK-dependent, and able to be disrupted by *in vivo* exposure to cocaine.

Results

mGluR5-LTD in the BNST is ERK1-Dependent

The first work examining the effects of mGluR5 activation on glutamatergic transmission in the BNST was done in our laboratory by Brad Grueter. Grueter, *et al* 2006, showed that activating mGluR5 in the BNST via bath application of DHPG induced LTD (Figure 2A). The early component of the DHPG-induced depression of transmission requires CB1Rs. However, the LTD generated by DHPG is independent of CB_1Rs and is mediated by G-protein signaling postsynaptically. I first examined whether mGluR5-LTD in the BNST was dependent on activation of ERK. A 5 minute application of DHPG to BNST slices (see methods) induced robust ERK activation, with ERK1 being activated to a greater degree than ERK2 (*ERK1*: 137 ± 9% of basal, $p < 0.05$, student's t-test; *ERK2*: 111 ± 4% of basal, $p < 0.05$, student's t-test; $n = 8$;

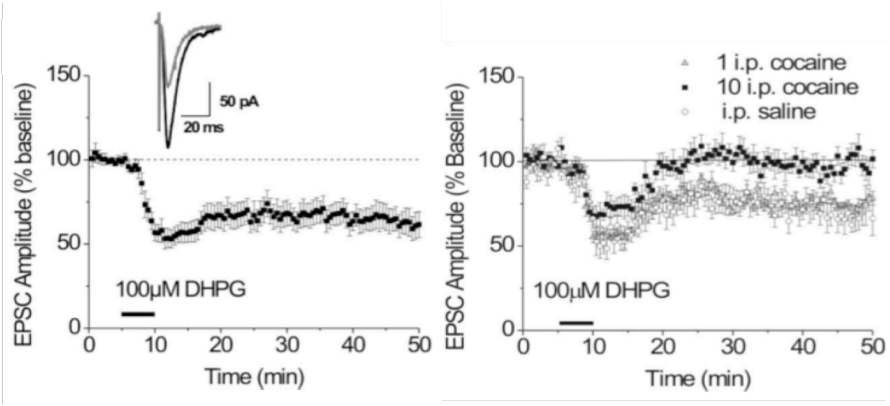


Figure 2. mGluR5-LTD in the BNST is Disrupted by Cocaine (from Grueter, *et al* 2006). (a) 100 μM DCPG induces a persistent long-term depression of excitatory transmission that is mediated by mGluR5. (b) mGluR5-LTD is disrupted 30 minutes after a single injection of cocaine (gray triangles) and 24 hours after 10 days of cocaine (closed circles), but not saline (open circles).

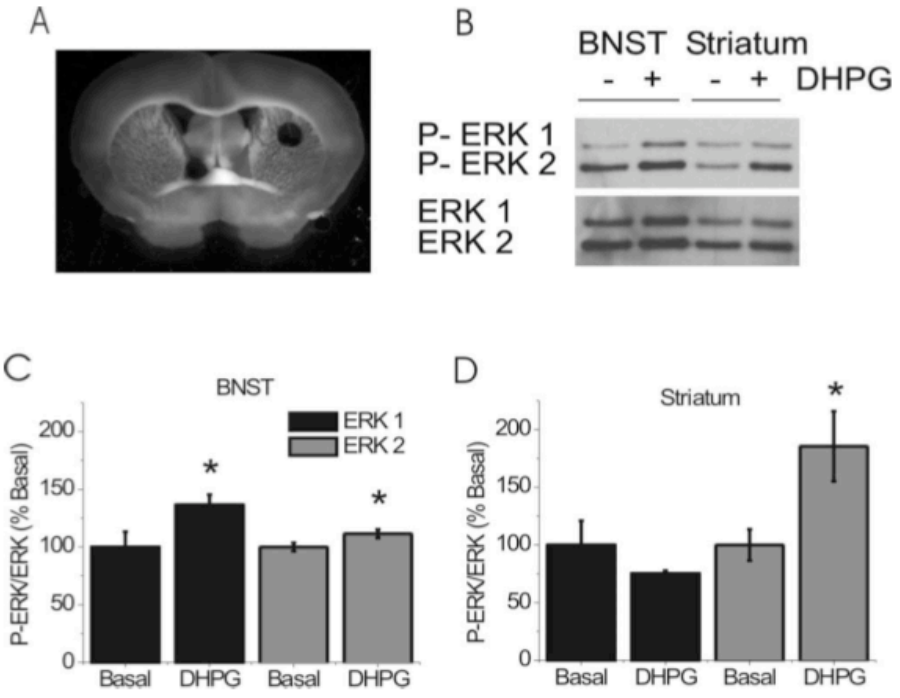


Figure 3. 100 μM DCPG Induces ERK Phosphorylation in BNST Slices. (a) A coronal brain slice with dorsal BNST and dorsal striatum punches taken. (b) Western blots showing DHPG induces ERK phosphorylation in BNST and striatal tissue. (c, d) Quantification of DHPG-induced ERK phosphorylation. In BNST, both isoforms of ERK were activated by DHPG, though ERK1 was activated to a greater extent than ERK2. In Striatum, ERK2 shows robust activation by DHPG. The level of each isoform was compared to its own control. (n = 8; * = $p < 0.05$)

Figure 3B,C). These findings were supported by the observation that mGluR5-LTD in the BNST is disrupted in ERK1 knockout mice (Grueter, *et al* 2006). In contrast, DHPG-induced ERK activation in the striatum was only observed for the ERK2 isoform (*ERK1*: 75 ± 3% of basal, $p > 0.05$, student's t-test; *ERK2*: 185 ± 35% of basal, $p < 0.05$, student's t-test; $n = 8$; Figure 3B,D).

Brad's initial study went on to show that mGluR5-LTD was also disrupted in mice chronically exposed to cocaine (both contingent and non-contingent), but intact in saline-treated controls (Figure 2B). To determine whether a cocaine-induced loss of mGluR5 or ERK protein was responsible for this disruption, I examined the levels of these proteins in mice chronically treated with either cocaine or saline. I observed no differences in total ERK1, ERK2, or mGluR5 protein levels after chronic cocaine (*ERK1*: 109 ± 4% versus 103 ± 4% optical density units, for saline and cocaine, respectively, $p > 0.05$ student's t-test; *ERK2*: 105 ± 5% versus 107 ± 5% optical density units, for saline and cocaine, respectively, $p > 0.05$, student's t-test; *mGluR5*: 100 ± 4% versus 99 ± 4% optical density units, for saline and cocaine, respectively, $p > 0.05$, student's t-test; $n = 12$-14; Figure 4).

Future work by Brad determined that mGluR5 was being activated by cocaine exposure. Mice were preinjected with the mGluR5 antagonist MPEP prior to cocaine, then recorded from as usual. Importantly, preinjection of MPEP prior to cocaine rescued mGluR5-LTD (Figure 5). Taken together, these data suggest mGluR5 signaling in the BNST is an *in vivo* target of cocaine, though the mechanism of cocaine-induced disruption of mGluR5-LTD is unknown.

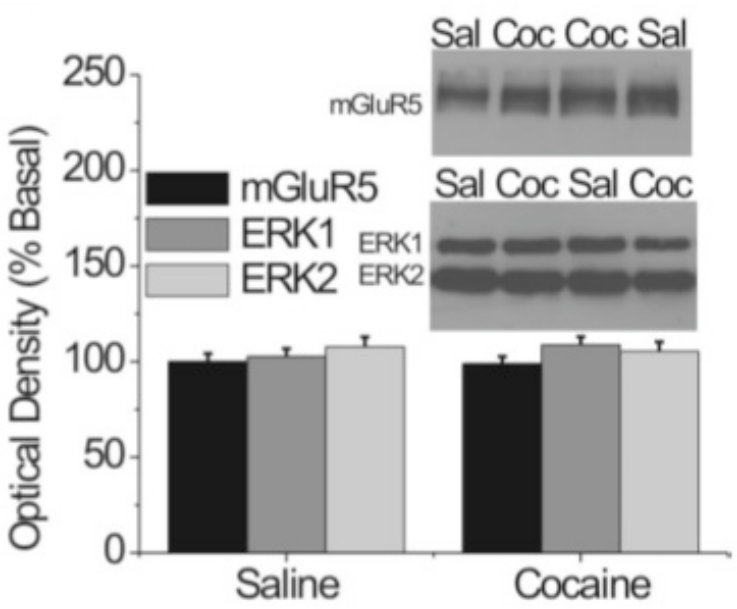


Figure 4. Total Protein Levels of ERK1, ERK2, or mGluR5 Were Unchanged After Chronic Cocaine. Optical density is plotted as percent control ± SEM. No significant difference was observed in ERK1, ERK2, or mGluR5 total protein levels between mice chronically treated with cocaine (n = 12) versus saline (n = 14). (*inset*) Western blot showing mGluR5 (upper) and total ERK (lower) protein levels in saline- and cocaine-treated mice.

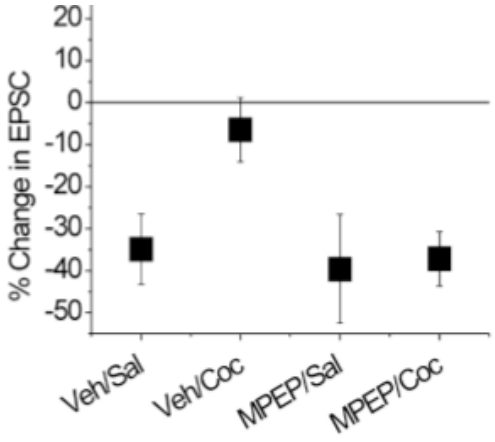


Figure 5. Preinjection of MPEP Prior to *in vivo* Cocaine Rescues *ex vivo* mGluR5-LTD (from Grueter, *et al* 2008). mGluR5-LTD is absent in slices from mice treated with vehicle prior to cocaine (Veh/Coc), but present in slices from mice given MPEP prior to cocaine (MPEP/Coc). Values represent the percent change in EPSC amplitude at 30 minutes post-DHPG ± SEM.

Acute Cocaine Induces ERK Activation in the BNST

One potential point of convergence between mGluR5 signaling in the BNST and cocaine-induced changes in the BNST is the ERK pathway. Because mGluR5-LTD is ERK1-dependent, and cocaine activates ERK in the BNST *in vivo*, I hypothesized that cocaine-induced ERK activation could be mediated by mGluR5. In order to examine this, it was critical to replicate the finding of cocaine-induced ERK activation in the BNST that was reported previously (Valjent, *et al* 2004). Mice were handled for several days and given i.p. saline injections for habituation. On the final day, mice were given either saline or 20 mg/kg cocaine and perfused 30 minutes later. Slices from cocaine-treated mice showed robust ERK activation compared to saline controls (see methods) (saline: 100 ± 23.4 percent control number of positive cells; cocaine: 197.8 ± 36.1 percent control number of positive cells; $n = 7\text{-}8$, $p < 0.05$, student's t-test; Figure 6).

MPEP Does Not Block Cocaine-Induced ERK Activation

I next attempted to determine whether mGluR5 is a mediator of cocaine-induced ERK activation in the BNST. Because the ERK phosphorylation produced by a single injection of cocaine was more robust than in chronically exposed mice (data not shown), this timepoint was used to examine mGluR5-dependent effects. Mice were handled and given habituating i.p. saline injections as before for several days. On the final day, mice were given an injection of either MPEP or vehicle, followed 30 minutes later by an injection of either saline or cocaine, then perfused 30 minutes

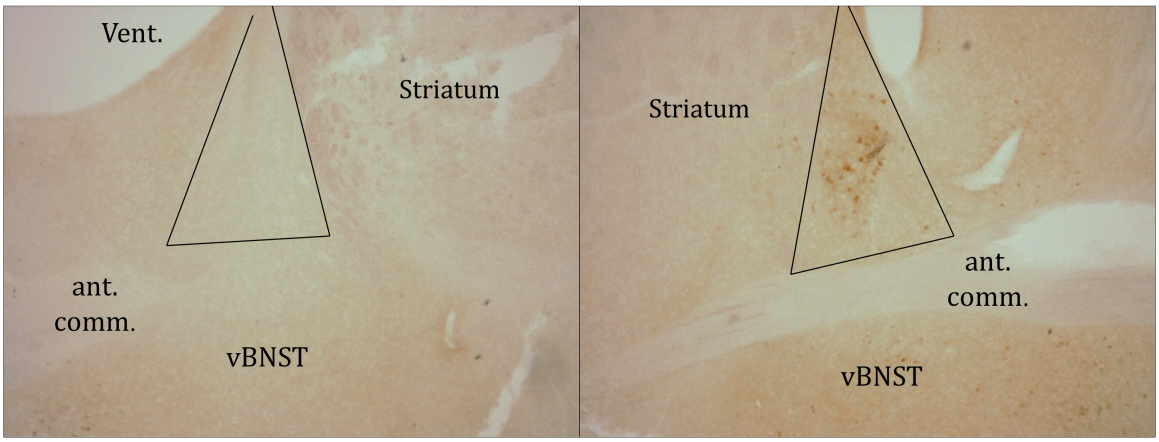


Figure 6. Cocaine Induces Significant ERK Phosphorylation in the BNST. Representative DAB-IHC images showing ERK avtivation by a single injection of saline (left) or cocaine (right). (n = 7-8; $p < 0.05$)

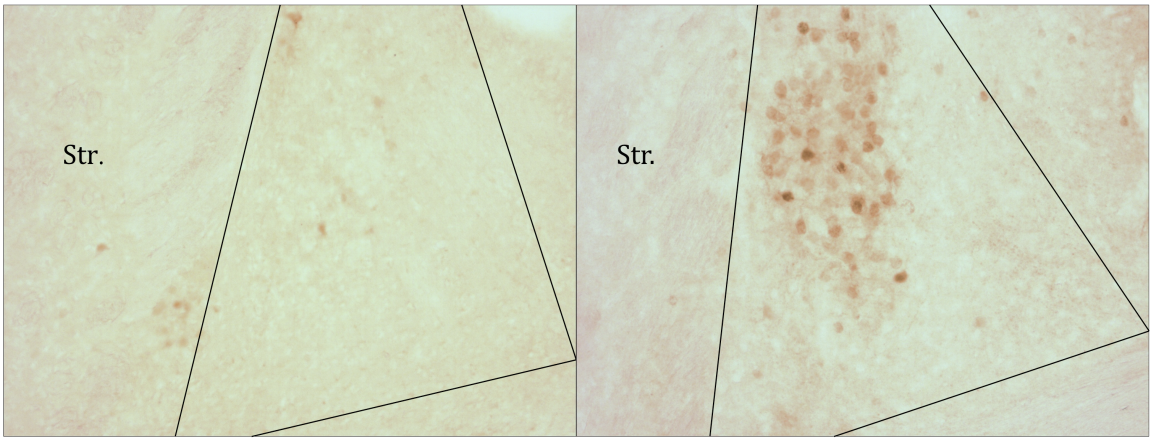


Figure 7. MPEP Preinjection Does Not Block Cocaine-Induced ERK Phosphorylation in BNST. Representative DAB-IHC images showing ERK phosphorylation in animals that received MPEP 30 minutes prior to a single injection of saline (left) or cocaine (right). Cocaine caused a significant amount of ERK phosphorylation even with MPEP pretreatment (n = 6-8; $p < 0.05$ MPEP/saline versus MPEP/cocaine).

afterward. A significant degree of cocaine-induced ERK activation was observed in mice preinjected with MPEP, suggesting mGluR5 is not necessary for cocaine-induced ERK activation in the BNST (vehicle cocaine: 192% of vehicle control ± 57.5; MPEP control: 112.5 ± 36.6; MPEP cocaine: 214% of vehicle control ± 26.9; n = 6-8; $p < 0.05$ MPEP control vs. MPEP cocaine, student's t-test; $p = 0.17$ vehicle control vs. vehicle cocaine, student's t-test) (Figure 7). Due to the finding that MPEP did not block cocaine-induced ERK activation, I shifted my focus from ERK activation to mGluR5 itself. Nonetheless, these data confirm in C57Bl/6J mice the earlier results in CD-1 mice that cocaine administration increases ERK activity (Valjent, *et al* 2004). Moreover, the data suggest that this activation may be independent of Group I mGluR activation.

Lack of Gross Changes in Surface mGluR5 After Cocaine

A single exposure to *in vivo* cocaine has been reported to produce a decrease in surface mGluR5 in the NAc (Fourgeaud, *et al* 2004). Thus, one reason why mGluR5 LTD is lost in the BNST after chronic cocaine administration could be due to a reduction in the availability of functional receptor. To determine whether cocaine induces mGluR5 internalization in the BNST, I created a modified biotinylation protocol (derived from Fourgeaud, *et al* 2004 and Thomas-Crusells, *et al* 2003). Hypothesizing that a downregulation of mGluR5 would be more likely after chronic cocaine, I chose to examine surface mGluR5 in BNST from chronically-treated mice. After 10 days of cocaine treatment, slices were made and underwent the biotinylation procedure (for details, see methods) after which punches of BNST and

dorsal striatum (as a control region) were taken. The punches were homogenized and processed for pulldown, producing three samples- whole homogenate, diluted cytoplasmic fraction, and the membrane-bound pellet layer. These samples were subjected to SDS-PAGE, and the western blots were probed for mGluR5 immunoreactivity. I observed no gross changes in the ratio of surface mGluR5 to total mGluR5 after chronic cocaine, suggesting the percentage of total mGluR5 on the cell surface was unchanged. Total mGluR5 protein was likewise unchanged (BNST saline: 100 ± 11.3% versus 90.4 ± 8.6% for cocaine, $p > 0.05$, student's t-test; Striatum saline: 100 ± 17.3% versus 72.7 ± 8.2% for cocaine, $p > 0.05$, student's t-test; n = 8-11; Figure 8).

DHPG-Induced Depression in Field Potential Recordings is Not Disrupted by Cocaine

After biochemical methods failed to illuminate the mechanism for cocaine-induced disruption of mGluR5-LTD, I next examined cocaine-induced disruption of mGluR5-LTD using field potential recordings paired with afferent stimulation. Our laboratory has previously demonstrated cocaine-induced changes in glutamatergic transmission in the BNST that were mediated by dopamine (Kash, Nobis, *et al* 2008). Further, we have previously reported that *ex vivo* cocaine application could disrupt the effects of DHPG on spontaneous excitatory transmission. Taking these together, I hypothesized that a cocaine-induced increase in glutamatergic transmission could activate mGluR5, occluding any subsequent activation of mGluR5, thus blocking mGluR5-LTD. To test this, I bath-applied 100 μM DHPG for

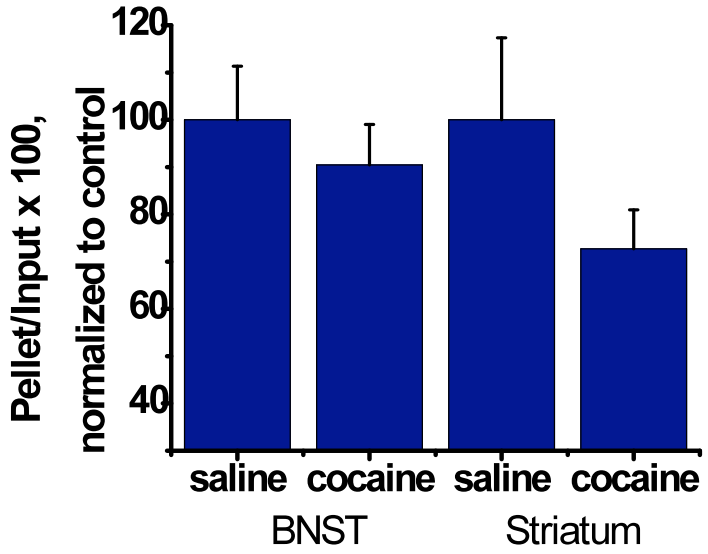


Figure 8. No Gross Change in Surface mGluR5 Levels was Observed After Chronic Cocaine in BNST or Striatum. Bars show mean ± SEM of the qualitative ratio of surface:total mGluR5 protein (Pellet/Input x 100, normalized to control) in BNST and striatum following chronic treatment of saline or cocaine (n = 8-11; $p > 0.05$).

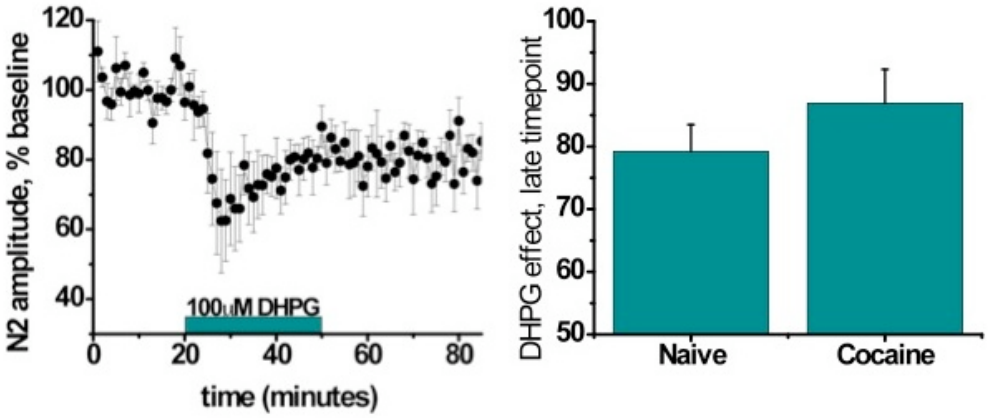


Figure 9. DHPG-Induced Depression in Field Potential Recordings is Not Disrupted by Bath-Applied Cocaine. (left panel) A 30-minute application of 100 μM DHPG produces a persistent depression in field potential recordings. (right panel) The late timepoint of DHPG-induced depression, measured 40-45 minutes post DHPG, is not altered by preapplication of 3 μM cocaine ($n = 6\text{-}9$; $p > 0.05$).

30 minutes and observed a gradual ~21% depression in transmission in BNST (Figure 9A). To examine whether cocaine could disrupt this effect, 3 μM cocaine was applied and the response allowed to stabilize for 1 hour, after which DHPG was applied. Bath-applied cocaine failed to disrupt the DHPG-induced depression of transmission at a late timepoint 40-45 minutes post DHPG (naïve DHPG: 79.2 ± 4.3% of baseline, n = 6; cocaine + DHPG: 86.9 ± 5.4% of baseline, n = 9; $p > 0.05$, student's t-test; Figure 9B).

Discussion

ERK Activation in the BNST by mGluR5 and Cocaine

This study demonstrated that mGluR5 activation leads to activation of ERK in the BNST. The preferential activation of the ERK1 isoform is unusual (Gallagher, *et al* 2004; Fourgeaud, *et al* 2004), and implies ERK1 signaling may be important for the expression or maintenance of mGluR5-LTD in this region. Alternatively, the shared mechanism of ERK activation in the BNST could suggest cocaine-induced disruption of mGluR5-LTD may be mediated via the ERK pathway. However, we found chronic cocaine had no effect on total protein levels or ERK1 or ERK2, which could mean that changes in cocaine-induced ERK activation are not reflected in total protein levels.

I report here that acute cocaine induces ERK activation in the BNST of C57Bl/6J mice. This is consistent with the previous finding in CD-1 mice (Valjent, *et al* 2004). In the previous study, cocaine-induced ERK activation was found to be blocked by

the D1 dopamine receptor antagonist SCH23390, suggesting dopamine may mediate this effect in CD-1 mice. This study also demonstrates that the cocaine-induced activation of ERK in the BNST is not dependent on mGluR5. It should be noted, however, that we used a protocol with a dose of MPEP known to rescue mGluR5-LTD (Grueter, *et al* 2008). We therefore recognize the possibility that a more prolonged blockade of mGluR5 could affect cocaine-induced ERK activation by shutting down mGluR5 signaling completely. Taken together, these findings point to a potential role for dopamine in cocaine-induced ERK activation in the BNST, and suggest mGluR5 is not mediating this phenomenon, despite the shared ability to activate ERK. This work also raises the question of whether mGluR5-mediated ERK activation could involve dopaminergic mechanisms.

<u>mGluR5 Itself May Not be Disrupted by Cocaine in the BNST</u>

Lastly, cocaine-induced disruption of mGluR5 signaling in the BNST also does not appear to be occurring at the level of the receptor itself. mGluR5 antagonism before chronic cocaine exposure rescued mGluR5-LTD, indicating mGluR5 itself may be targeted by cocaine (Grueter, *et al* 2008). However, similar to ERK, I found that chronic cocaine has no effect on total mGluR5 protein levels in the BNST, and furthermore, has no gross effects on surface levels of mGluR5. Indeed, these data suggested that mGluR5 itself is not disrupted by *in vivo* cocaine. Taken together, the work of this project suggests that mGluR5 signaling, downstream of the receptor itself but possibly upstream of ERK activation, may be targeted by *in vivo* cocaine and cause subsequent disruption of *ex vivo* mGluR5-LTD.

Limitations of Westerns With *in vivo* Changes

The BNST is an extremely heterogeneous brain region made up of many subnuclei, all extensively connected to other parts of the brain. This high level of variability makes it an interesting yet frustrating region to study. It is also a comparably small area within a coronal slice, yielding very little protein. Taken together, these factors make biochemical changes difficult to detect in the BNST, unless the changes are large in magnitude and/or occur region-wide. It is even more difficult to detect changes in phosphorylated proteins in such conditions, due to the relative instability of the protein's phosphorylation state.

Experimental manipulations done *ex vivo* help to counteract this. Mimicking mGluR5-LTD in submerged chambers helped elucidate the signal transduction mechanisms required for that plasticity (Grueter, *et al* 2006) (Figure 3). This approach was probably successful largely due to the widespread nature of mGluR5 expression (and subsequent DHPG-induced ERK activation) in the dBNST. However, the ability to use within-animal controls afforded by the *ex vivo* experiments (one hemisphere treated with drug, the other with vehicle) is also very important. Using ERK activation as an assay (for Western blotting and Immunohistochemistry) made *in vivo* experiments very difficult to control, because ERK is activated by a myriad of external factors. With increased ERK phosphorylation in control animals, experiment-induced increases are blunted or lost completely. For these reasons, pursuing *in vivo* changes in ERK phosphorylation in the BNST with Western blots is untenable.

Possibility of Stress-Induced ERK Activation in IHC

In addition to the acute effects of cocaine on ERK activation in the BNST, I also examined cocaine-induced ERK activation after chronic exposure to cocaine, using the same protocol as Grueter et al, 2006. I was unable to observe a difference in phospho-ERK levels between slices from chronic saline and chronic cocaine-treated mice, although a trend for increased ERK activation in chronic cocaine slices was observed in the first cohort (data not shown). Chronic saline mice appeared to have higher levels of basal ERK activation than acute saline mice, a major confound of these studies. Furthermore, later trials of the MPEP + acute cocaine experiments also exhibited a high degree of basal ERK phosphorylation in the saline controls (data not shown). The high degree of phospho-ERK in the saline controls, coupled with the failure of the dopamine receptor antagonist flupenthixol (subcutaneous preinjection) to block cocaine-induced ERK activation (data not shown), made continuing these experiment untenable.

The confounding factor of high basal ERK phosphorylation in saline-treated control mice could be due to stress. Various stressors have been shown to induce ERK activation in the brain (Wang, *et al* 2010). For several of these cohorts, the mice underwent cage changes the day before, or the morning of, the experiment, a procedure somewhat stressful to mice. Because the mice receiving cocaine were housed in the same conditions as the potentially-stressed control mice, there is also the possibility of stress-induced ERK activation in the cocaine-treated group. If stress-induced ERK activation did occur in the cocaine-treated mice, this could have led to a "ceiling effect" of ERK activation in cocaine-treated mice, making the

cocaine-induced increase in ERK phosphorylation difficult to detect. If such an occlusion was occurring, it could also explain the failure of the negative controls SCH23390 and flupenthixol, both of which would be predicted to block cocaine-induced ERK activation (see Valjent, *et al* 2004). Ultimately, the high levels of ERK activation in the saline controls caused cocaine-induced ERK phosphorylation to lose significance, and clouds the interpretation of these data.

Limitations of Fractionation, Quantification in Biotinylation Procedure

While ultimately this study showed no gross change in surface levels of mGluR5 after cocaine treatment (Figure 8), cocaine may be inducing more subtle changes in receptor expression, such as a shift in the relative synaptic vs. perisynaptic mGluR5 populations. A more stringent fractionation protocol such as a synaptosomal preparation might have been able to detect these more subtle differences because it removes all but the synaptic membranes from the sample. However, generating synaptosomes requires an enormous amount of tissue, and is not practical due to the small size of the BNST. Alternatively, performing a protein precipitation would have allowed mGluR5 protein to be detected in the dilute supernatant ("cytosolic") fraction of this protocol. Comparing the ratio of surface mGluR5 to total mGluR5 (pellet fraction to whole homogenate) was an experimentally valid measurement, but not an ideal one. However, if a gross change in surface mGluR5 (such as the greater than 50% loss observed in Fourgeaud, *et al* 2004) had occurred, this method would most likely have been able to detect it. This negative result was confirmed in a 2008 study looking at mGluR5 localization in the NAc of cocaine-treated rats using

electron microscopy, which also failed to replicate the huge loss of surface mGluR5 reported by Fourgeaud, *et al* (Mitrano, *et al* 2008).

Homer Proteins, and Their Regulation by Drugs of Abuse

Since cocaine does not seem to be disrupting mGluR5-LTD by inducing internalization of the receptor itself, this suggests cocaine may instead be disrupting mGluR5 signaling. Besides the ERK cascade, another potential mechanism for this is an alteration in the expression or localization of Homer proteins (Swanson, *et al* 2001). Homers are scaffolding proteins that link Group I mGluRs to intracellular calcium stores, thus serving a vital role in signal transduction. Under normal conditions, long Homer isoforms (expressing coiled-coil domains for protein-protein interactions) dominate, serving their intended structural purpose. However, under special circumstances, such as stress or exposure to drugs of abuse, a short Homer isoform, Homer1a, can be induced as an immediate-early gene product. Homer1a is thought to serve a dominant-negative function *in vivo*. When induced, Homer1a binds to Homer binding partners, preventing the binding of long Homer isoforms and thus disrupting the coupling of Group I mGluRs to their intracellular effectors. Because *in vivo* cocaine can lead to Homer1a induction and thus potentially disrupt subsequent mGluR5 signaling, these proteins could mediate cocaine-induced disruption of mGluR5-LTD in the BNST.

Limitations of pan-Homer Antibody

At the time of these experiments, no isoform-specific Homer antibodies were commercially available, but a pan-Homer antibody was. Unfortunately, it proved to be untrustworthy. Several cohorts of animals were treated and significant differences between groups were observed, but these westerns were never conclusive, because I could not identify the components of each band on the pan-Homer blots (Figure 10). Suspected artificial bands could never be ruled out, even with Homer1 KO tissue and the positive controls of acute cocaine-exposed rat and mouse samples. Another band believed to represent a Homer dimer was never disrupted by changes in the homogenization protocol that should have disrupted a protein-protein interaction such as a dimerization. The presence of a "mouse specific" band further suggested the antibody was not entirely trustworthy. Finally, anecdotal consultation with Paul Worley (an expert in Homer proteins, *pers. comm.*) confirmed this was a poor antibody. However, induction of Homer1a and subsequent disruption of mGluR5 signaling remains a credible hypothetical mechanism for cocaine-induced disruption of mGluR5-LTD.

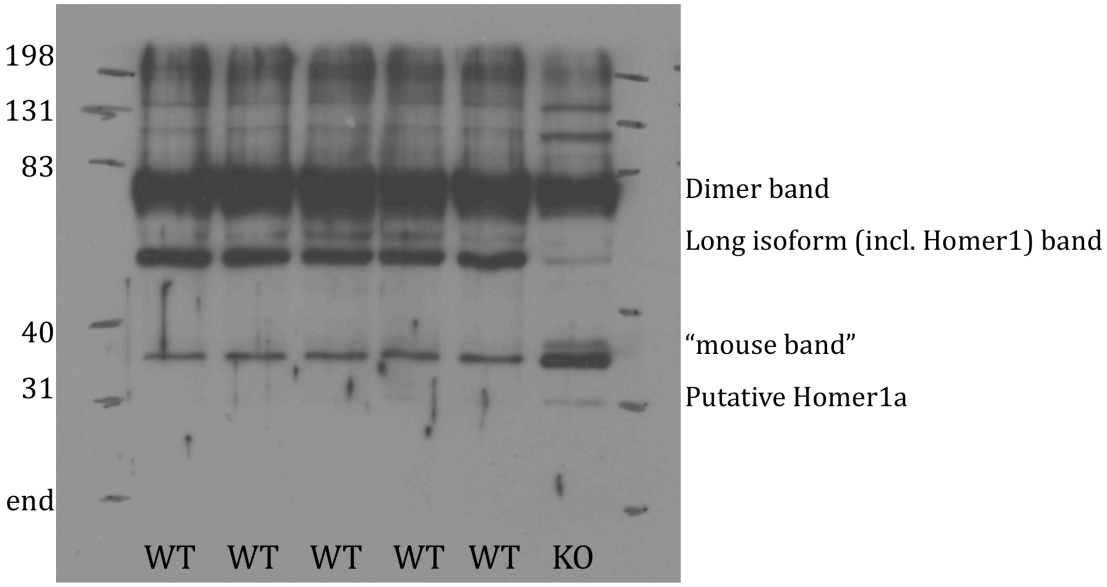


Figure 10. Scanned Film of a pan-Homer Western Blot. On this blot are samples from 5 cocaine-treated WT mice, run with tissue from a Homer1 KO mouse (negative control) in the far right lane; note the presence of the putative Homer1a band in this sample, and the lack of cocaine-induced Homer1a expression in the WT lanes.

DHPG-Induced Depression in Field Potential Recordings is Not a Model For Whole-Cell mGluR5-LTD

Finally, using field potential recordings, I find that DHPG-LTD is intact after bath-applied cocaine, a paradigm that disrupts whole-cell mGluR5-LTD (Grueter, *et al* 2008). This could be suggesting several things. First, the regulation of spontaneous EPSCs by DHPG (as measured by Brad's voltage clamp recordings) is not equivalent to the regulation of evoked transmission in my field potential recordings, a recurring theme in our electrophyisiological recordings (Kash, Nobis, *et al* 2008). Additionally, there are several reasons why the degree of LTD observed at part of one neuron's synapses may not be detectable in a population-level recording. As mentioned above, the heterogeneity of the BNST and its many subnuclei naturally precludes a scenario in which population level responses would be identical to those of individual neurons. Further, whole cell recordings are in submerged chambers, where drug delivery allows for quick saturation and optimal receptor occupancy of the agonist. In contrast, interface chambers (which were used for these field recordings) are notorious for their slow drug-on rates. In the 30 minutes that DHPG is washing over the slice, mGluR5 is certainly being activated, and subsequently undergoing agonist-induced internalization, throughout the slice on a continuous basis. Even if every neuron in the BNST responded identically to DHPG, the agonist may not be occupying enough receptors at once to produce a measurable population effect in an interface setting. Lastly, the differing drug-on rates and application durations may be producing different forms of plasticity. Activating the majority of a receptor population all at once will almost certainly have different consequences than mildly activating a subset of that population.

mGluR5, like many GPCRs, can activate a variety of intracellular signaling cascades, depending on the magnitude and duration of the signal being received, as well as the history of the synapse. It is possible, therefore, that the DHPG-induced depression observed in these field potential recordings would not exhibit the same characteristics as mGluR5-LTD, and would thus be a poor model for it.

CHAPTER IV

MGLUR8, α1-ARS, AND STRESS IN THE BNST

Introduction

Group III mGluRs

mGluR8 is classified as a Group III receptor (mGluRs 4, 6, 7, and 8). These $G_{i/o}$-coupled receptors are the least well-studied group of mGluRs due to the relative lack of specific pharmacological tools targeting them. Group III mGluRs are generally thought to be expressed presynaptically and to serve as autoreceptors (Ayala, *et al* 2008; Valenti, *et al* 2005; Abitbol, *et al* 2008; Schmid and Fendt, 2006). In the BNST, the Group III mGluR agonist L-AP4 and the mGluR8-selective agonist DCPG have depressive effects on excitatory transmission, suggesting expression of mGluR8 and potentially other Group III mGluRs in this region (Grueter and Winder, 2005) (Figure 11).

mGluR8, and mGluR8 Knockout Mice

mGluR8 exhibits a diffuse pattern of expression during development that is limited to discrete pockets of expression in the adult mouse, a characteristic unique among presynaptic mGluRs (Duvoisin, *et al* 1995; Ayala, *et al* 2008). Interestingly, mGluR8 knockout mice exhibit an anxiety phenotype on the elevated plus and elevated zero mazes (Duvoisin, *et al* 2005, 2005; Linden, *et al* 2002). However, this phenotype

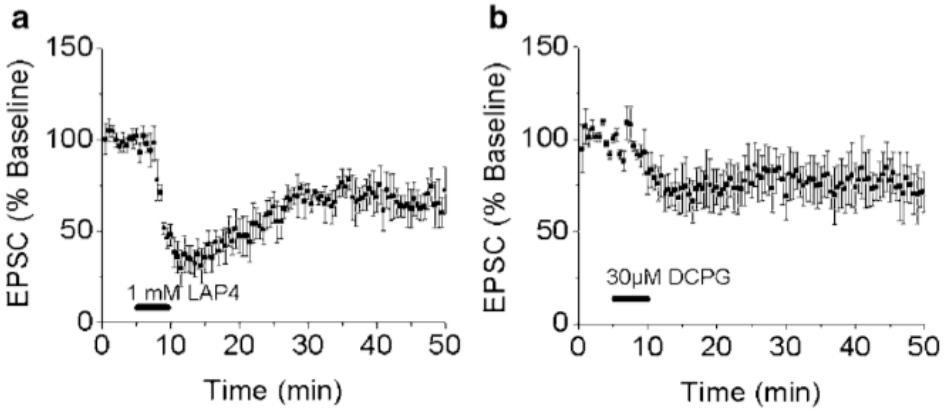


Figure 11. Group III Agonists Depress EPSCs in the BNST (from Grueter and Winder, 2005). (a) 1 mM L-AP4, the Group III agonist, produces a robust depression in submerged chamber whole-cell recordings. (b) 30 μM DCPG, the mGluR8-selective agonist, also produces a persistent depression of excitatory currents.

seems to be at least somewhat dependent on background strain and lighting conditions (Fendt, *et al* 2010). DCPG, the mGluR8-selective agonist, has been shown to produce anxiolytic effects in the elevated zero maze (Duvoisin, *et al* 2010b). Taken together, these studies hint at a potential role for mGluR8 in counteracting activation of anxiety-promoting circuitry.

α1 ARs and Stress/Anxiety

During a stressor, BNST extracellular levels of norepinephrine (NE) rise dramatically (Cecchi, *et al* 2002). Subsequent attempts to activate α1 adrenergic receptors (α1 ARs) in the BNST after exposure to a stressor fail, suggesting these receptors are activated by *in vivo* stress (McElligott, *et al* 2010) (Figure 12). In the PVN, pharmacological or stress-induced activation of α1 ARs leads to a functional inhibition of presynaptic Group III mGluRs, and loss of L-AP4 sensitivity (Kuzmiski, *et al* 2009; Gordon and Bains, 2003). With activity in the BNST being critical during stress exposure, presynaptic mGluRs are well positioned to regulate excitatory inputs coming into this region. My global hypothesis for this project was to determine which Group III receptors have measurable effects on excitatory transmission in the BNST, and whether these effects are altered by *in vivo* exposure to stress. I further hypothesized that stress-induced activation of α1 ARs in the BNST might regulate any mGluR8 effects on excitatory transmission.

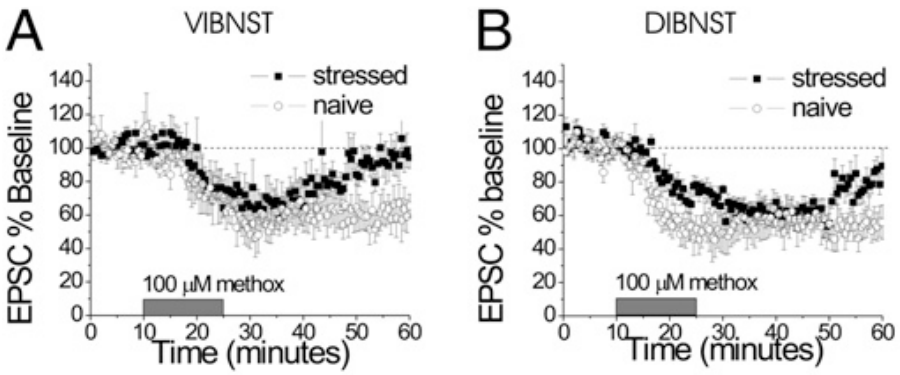


Figure 12. Chronic Restraint Stress Disrupts α1-LTD in the BNST (from McElligott et al, 2010). α1-LTD is partially disrupted after chronic restraint stress in both the ventral (a) and dorsal (b) BNST.

Results

Group III Agonist L-AP4 and mGluR8-Selective Agonist DCPG Depress Excitatory Transmission in BNST

Our laboratory has previously reported that the Group III mGluR agonist L-AP4 depresses excitatory transmission in the BNST (Grueter and Winder, 2005) (Figure 11). Here, I used field potential recordings and local afferent stimulation in the anterolateral dorsal BNST to further probe the specific Group III mGluR subtypes involved in mediating the effects of L-AP4 in the dorsal BNST (Figure 13A). Consistent with our previous finding, a 20-minute bath application of 200 μM L-AP4 produced a small yet significant depression of excitatory transmission in BNST-containing slices (8.8 ± 4.9 peak average percent depression, $p < 0.05$ versus baseline, paired student's t-test; Figure 13C,F).

We observe mGluR8-like immunoreactivity in the dorsal BNST, suggesting mGluR8 may be mediating L-AP4 effects in this region (Figure 13B). To more directly test whether mGluR8 activation has effects on excitatory transmission in the BNST, I utilized the mGluR8-selective agonist DCPG, which has also been previously shown to depress excitatory transmission in this region (Grueter and Winder, 2005) (Figure 11). Consistent with this, I found that a 20-minute application of DCPG depressed excitatory transmission in the BNST as examined by field potential recordings (Figure 14A). This effect of DCPG was concentration-dependent, with 10 μM and 30 μM DCPG inducing a depression which did not readily reverse (1 μM: 5.4

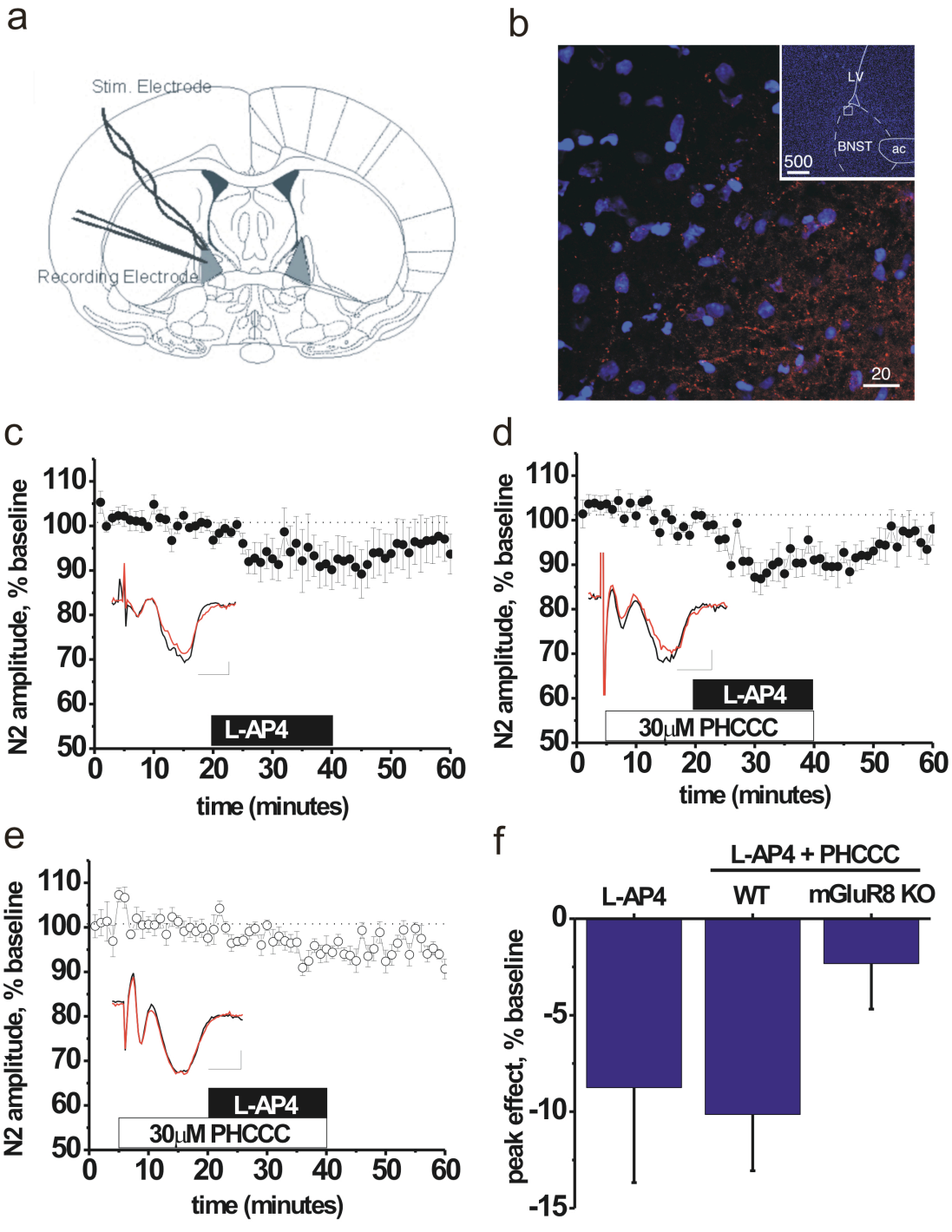

a
Stim. Electrode
Recording Electrode
b
LV
BNST
ac
500
20
c
N2 amplitude, % baseline
110
100
90
80
70
60
50
L-AP4
0
10
20
30
40
50
60
time (minutes)
d
N2 amplitude, % baseline
L-AP4
30μM PHCCC
time (minutes)
e
N2 amplitude, % baseline
L-AP4
30μM PHCCC
time (minutes)
f
L-AP4 + PHCCC
L-AP4
WT
mGluR8 KO
peak effect, % baseline
0
-5
-10
-15

Figure 13. mGluR8, Not mGluR4, Mediates the Effects of L-AP4 in the BNST. (a) Coronal brain slice showing the dorsal BNST (gray triangle) and the approximate locations of the stimulating and recording electrodes. (b) Confocal image showing a punctate distribution of mGluR8-like immunoreactivity (red) at the dorsal edge of the rat BNST. A Hoechst counterstain (blue) was used to label cell nuclei. (*inset*) anatomical schematic with a small box showing the photographed area. Scale bars in μm. (c) The Group III agonist L-AP4 (200 μM) causes a transient yet significant depression in excitatory transmission in BNST ($p < 0.05$ versus baseline, paired student's t-test; $n = 6$). (*inset*) representative trace showing the difference in N2 amplitude after L-AP4 (red trace) compared to baseline (black trace). (d) The mGluR4 potentiator PHCCC (30 μM) does not shift the L-AP4 effect in WT mice ($n = 7$). (*inset*) representative trace showing the difference in N2 amplitude after PHCCC + L-AP4 (red trace) compared to baseline (black trace). (e) The effect of PHCCC + L-AP4 is absent in mGluR8 knockout mice ($n = 6$). (*inset*) representative traces showing the lack of difference in N2 amplitude after PHCCC + L-AP4 (red trace) compared to baseline (black trace). (f) No difference in peak effect of average percent depression was observed between PHCCC + L-AP4 and L-AP4 alone, and no effect was present in slices from mGluR8 knockout mice ($n = 6$-7). Scale bars on traces represent 0.2 mV (y axis) and 2 msec (x axis).

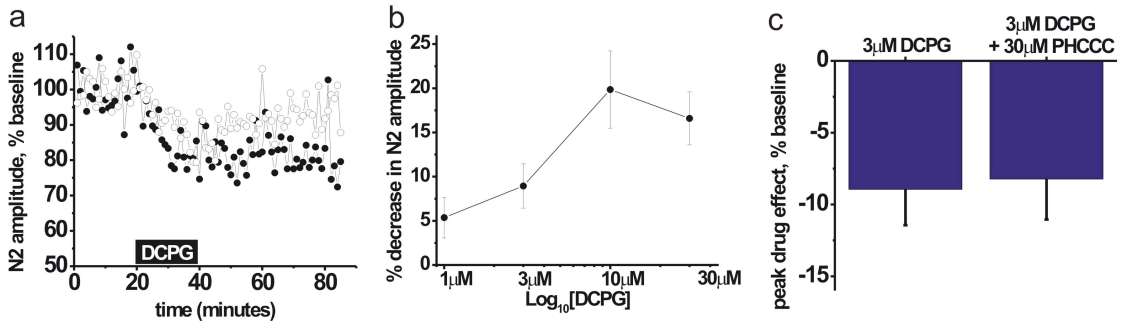


Figure 14. The mGluR8-Selective Agonist DCPG Depresses Excitatory Transmission in the dBNST. (a) Representative single experiments demonstrating the effect of 10 μM (closed circles) and 30 μM (open circles) DCPG on excitatory transmission in BNST. (b) DCPG produces a concentration-dependent depression in excitatory transmission (*n* = 6-8). (c) DCPG-induced depression is unaltered by co-application of the mGluR4 potentiator PHCCC (*n* = 5-8).

± 2.3, 3 μM: 8.9 ± 2.5, 10 μM: 19.9 ± 4.2, and 30 μM: 16.6 ± 3 peak average percent depression, n = 6-8; representative single experiments in Figure 14A,B).

DCPG and L-AP4 Effects Not Potentiated by PHCCC, and Absent in mGluR8 Knockout Mice

To investigate the contribution of mGluR4 to the L-AP4 effect on excitatory transmission in the BNST, I utilized the mGluR4-selective allosteric potentiator PHCCC (Maj, *et al* 2003). PHCCC was applied 15 minutes prior to a 20-minute co-application of L-AP4 and PHCCC (10.1 ± 2.9 peak average percent depression, Figure 13D,F). PHCCC failed to shift the peak effect of L-AP4, suggesting mGluR4 does not contribute to the effects of L-AP4 on excitatory transmission in this region (L-AP4: 8.8 ±4.9 peak average percent depression, versus L-AP4 + PHCCC: 10.1 ± 2.9, peak average percent depression, $p > 0.05$, student's t-test, Figure 13F).

To test the role of mGluR8 signaling in the actions of L-AP4 in the BNST, I repeated the PHCCC + L-AP4 co-application experiments using brain slices from mGluR8 knockout mice (Figure 13E,F). There was no significant effect of L-AP4 or PHCCC in mGluR8 KO mice (WT: 10.1 ± 2.9 peak average percent depression, versus mGluR8 KO: 2.6 ± 2.8 peak average percent depression, $p > 0.05$, student's t-test; drug effect in KO compared to baseline fails to reach significance with paired student's t-test; Figure 13F). These data suggest mGluR8, and not mGluR4, is the primary mediator of the effects of L-AP4 on excitatory transmission in the BNST.

At higher concentrations, DCPG loses selectivity for mGluR8 and can activate mGluR4 as well (Abitbol, *et al* 2008; Thomas, *et al* 2001). To determine whether

mGluR4 was involved in mediating the effect of DCPG in the BNST, I repeated these experiments with the addition of PHCCC. Adding PHCCC failed to enhance the effect of DCPG, suggesting that mGluR4 is not being recruited by DCPG to regulate excitatory transmission in the BNST (3 μM: 8.9 ± 2.5 peak average percent depression; 3 μM + PHCCC: 8.2 ± 2.8 peak average percent depression, $p > 0.05$, student's t-test, Figure 14C). The finding that PHCCC does not alter the actions of either DCPG or L-AP4 in BNST, coupled with the fact that DCPG has affinity for mGluRs 8 and 4, but not 7, suggests that mGluR8 is the primary receptor mediating the effects of Group III agonists on transmission in the BNST.

DCPG Acts Presynaptically to Depress Excitatory Transmission in BNST

To test the likely site of action of L-AP4 and DCPG, paired pulse ratios (PPR) of EPCSs were examined. (PPR data was obtained by Brad Grueter, PhD.) There was an enhancement of PPR after DCPG application, consistent with group III mGluRs reducing glutamate release probability to depress transmission (1.25 ± 0.14 fold of basal paired-pulse ratio). To further confirm a presynaptic localization of mGluR8, spontaneous EPSC (sEPSC) frequency and amplitude were examined prior to and following application of 10 μM DCPG (Figure 15A-D). (sEPSC data was obtained by Yuval Silberman, PhD.) In agreement with the PPR data, the mean frequency of sEPSCs was significantly decreased following DCPG application (2.4 ± 0.6 Hz control, versus 1.5 ± 0.5 Hz post-DCPG, $p \leq 0.01$, paired student's t-test; Figure 15A,C,D). The mean amplitude of sEPSCs in the BNST was modestly but significantly decreased by DCPG application (-8.0 ± 0.5 pA control, versus -6.5 ± 0.5 pA post-DCPG, $p \leq 0.005$,

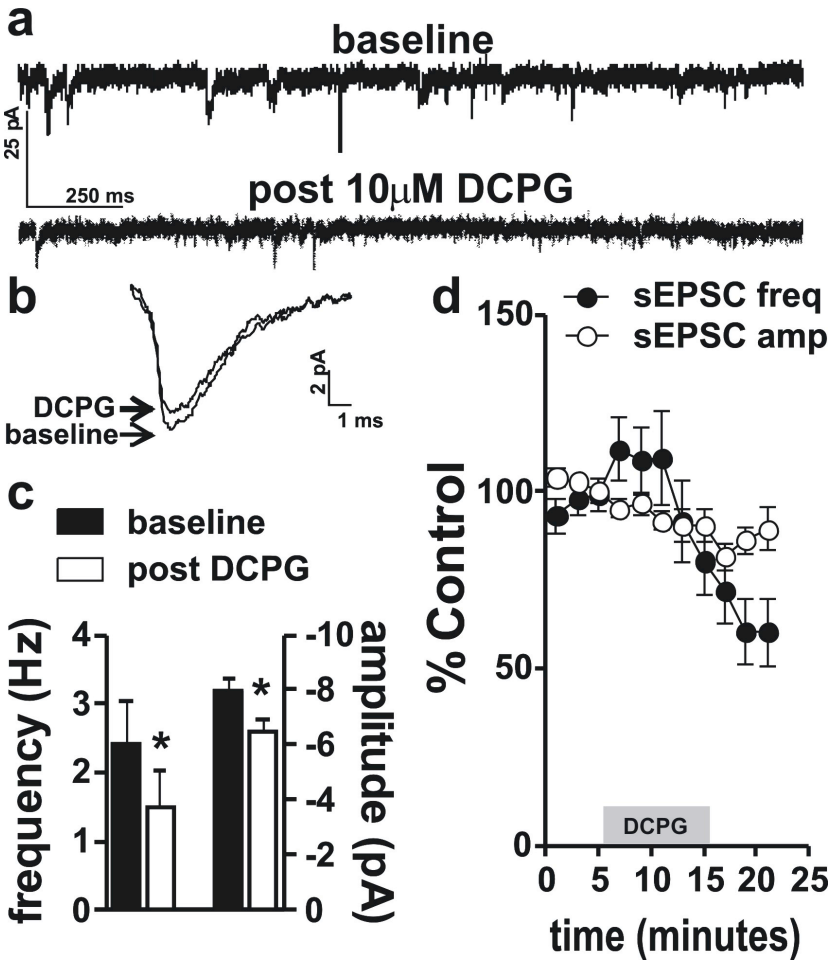


Figure 15. DCPG Inhibits Glutamatergic Transmission in the dBNST**.** (a) Representative recordings in the dBNST demonstrating the ability of 10 μM DCPG to inhibit the frequency of sEPSC events. (b) Representative sEPSC traces showing the effect of DCPG on sEPSC amplitude. (c) Mean effect of DCPG on sEPSC frequency (Hz, left Y axis) and amplitude (pA, right Y axis). (*frequency*: $p \leq 0.01$, paired t-test; *amplitude*: $p \leq 0.005$, paired t-test; $n = 8$). (d) DCPG reduces sEPSC frequency and amplitude over time, expressed as % control. Note the larger % inhibition caused by DCPG on sEPSC frequency as compared to sEPSC amplitude. (work done and figure generated by Yuval Silberman, PhD.)

paired t-test; Figure 15B,C,D). DCPG had no effect on sEPSC kinetics (Figure 15B). As shown in Figure 15D however, the effect on frequency was much more pronounced than that on amplitude. Taken together, these data suggest that mGluR8 in the BNST functions as a presynaptic autoreceptor, whose activation reduces glutamate release.

α1 AR Activation Abolishes mGluR8 Suppression of Transmission

To determine whether α1 AR activation could alter mGluR8 activity, I first took a pharmacological approach by activating both receptors in series. As previously reported, a 20-minute application of 100 μM methoxamine, an α1 AR agonist, produced a robust, persistent depression of excitatory transmission consistent with long-term depression (LTD) (McElligott and Winder, 2008; Figure 17E). After allowing 60 minutes for the methoxamine effect to be established, DCPG (10 μM) was applied for 20 minutes (Figure 16B). Consistent with what has been reported for L-AP4 in the PVN (Gordon and Bains, 2003), the effect of DCPG on excitatory transmission was abolished after methoxamine application when compared to the effect of DCPG alone (control DCPG: 19.9 ± 4.2 peak average percent depression, versus 1.9 ± 2.1 after methoxamine, $p \leq 0.005$, Friedman's non-parametric repeated measures ANOVA and Dunn's multiple comparison test; Figure 16A, B and inset).

mGluR8 Function After α1 AR Activation is Partially Recovered With a High Concentration of DCPG

To determine whether this lack of an effect of DCPG after methoxamine constituted a shift in potency or efficacy, I repeated the experiment using a high concentration

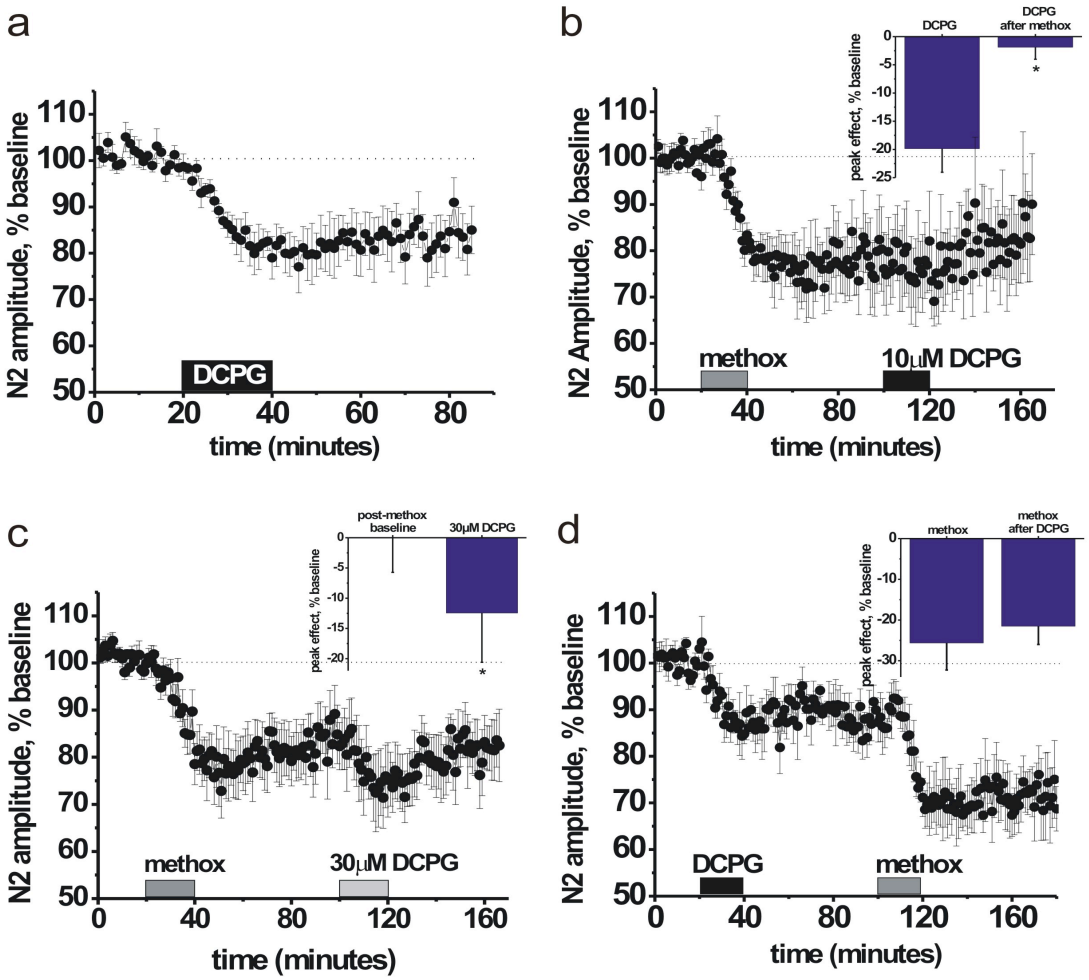


Figure 16. Activation of α1 Adrenergic Receptors Disrupts mGluR8 Effects on Excitatory Transmission. (a) The effect of 10 μM DCPG alone ($p \leq 0.001$ vs. baseline, $n = 8$). (b) 100 μM methoxamine-induced depression abolishes the effect of 10 μM DCPG ($n = 6$). (*inset*) DCPG-induced depression of transmission is lost after methoxamine ($p \leq 0.005$, Friedman's non-parametric repeated measures ANOVA and Dunn's multiple comparison test, $n = 6$-8). (c) A high concentration of DCPG (30 μM) is able to produce a depression after methoxamine, suggesting a shift in DCPG potency ($p < 0.05$ vs. post-methoxamine baseline, paired student's t-test, $n = 7$). (d) 10 μM DCPG does not disrupt subsequent methoxamine-induced depression ($n = 5$). (*inset*) 100 μM methoxamine effect is intact after 10 μM DCPG ($n = 5$-6).

of DCPG (30 μM). The 30 μM concentration of DCPG was able to produce a significant depression after methoxamine, suggesting a shift in the potency of this compound at mGluR8 had occurred (12.5 ± 8.1 peak average percent depression compared to post-methoxamine baseline, $p < 0.05$, paired student's t-test; Figure 16C and inset).

mGluR8 Activation Does Not Affect Subsequent α1 AR Activation

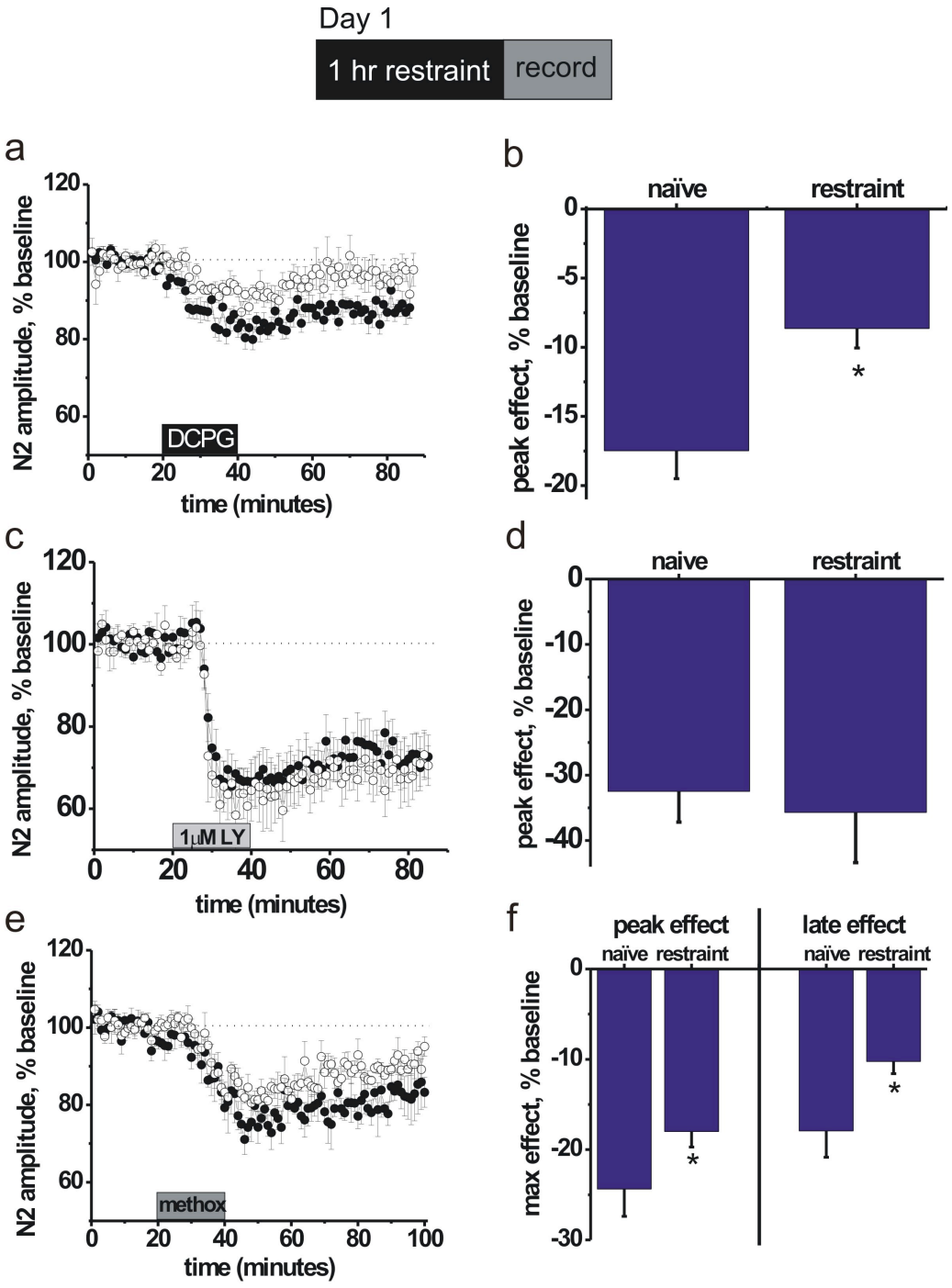
In contrast, when the order of drug application was reversed and slices were pretreated for 20 minutes with 10 μM DCPG, 100 μM methoxamine still depressed excitatory transmission (control methoxamine: 25.6 ± 6.6 peak average percent depression versus 21.5 ± 4.5 after DCPG, $p > 0.05$ for control versus after DCPG, student's t-test; Fig 16D and inset).

<u>mGluR8 Modulation of Transmission is Disrupted After Acute Restraint Stress</u>

Next, I examined whether mGluR8 function is disrupted by acute *in vivo* stress exposure. Mice underwent one 60-minute restraint session and were euthanized immediately afterwards (see Figure 17 schematic). In slices prepared from these mice, the effect of 10 μM DCPG on excitatory transmission was attenuated compared to pooled interleaved naïve controls (one-way ANOVA: $F(3, 29) = 4.802$, $p = 0.0078$; stressed: 8.6 ± 1.4 peak average percent depression, versus 17.5 ± 2.0 for pooled interleaved naïve controls; $p \leq 0.01$, Dunnett's post hoc comparison, Figure 17A,B).

α1 AR Function is Modestly Disrupted After Acute Restraint Stress

Our laboratory has previously reported that chronic restraint stress disrupts α1 AR-mediated LTD (α1-LTD) induction in the BNST (McElligott, *et al* 2010). Here I


Day 1
1 hr restraint
record
a
N2 amplitude, % baseline
120
100
80
60
DCPG
0
20
40
60
80
time (minutes)
b
naïve
restraint
peak effect, % baseline
0
-5
-10
-15
-20
*
c
N2 amplitude, % baseline
120
100
80
60
1μM LY
0
20
40
60
80
time (minutes)
d
naive
restraint
peak effect, % baseline
0
-10
-20
-30
-40
e
N2 amplitude, % baseline
120
100
80
60
methox
0
20
40
60
80
100
time (minutes)
f
peak effect
naïve restraint
late effect
naïve restraint
max effect, % baseline
0
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-20
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Figure 17. A Single Restraint Stress Exposure Disrupts mGluR8 Regulation of Transmission in the BNST. (*top*) schematic representing the acute restraint stress timeline. (a) 10 μM DCPG-induced depression (pooled interleaved naïve controls, closed circles) is disrupted in mice that underwent a single restraint stress (open circles) (*n* = 8-13). (b) peak average percent depression of 10 μM DCPG in pooled interleaved naïve control mice and acutely restrained mice ($p \leq 0.01$, Dunnett's post hoc comparison; *n* = 8-13). (c) Group II agonist LY379268-induced depression (1 μM) is intact after restraint (open circles) (*n* = 5-7). (d) peak average percent depression by 1 μM LY379268 in naïve and restrained mice (*n* = 5-7). (e) Methoxamine-induced depression (100 μM) is altered in acutely restrained mice (open circles) (*n* = 4-5). (f) (*left panel*) Peak average percent depression by methoxamine is modestly but significantly attenuated in naïve versus acutely restrained mice ($p < 0.05$, student's t-test, *n* = 4-5). (*right panel*) Maximal effect of methoxamine is still modestly but significantly attenuated at a late timepoint (45-52 minutes after drug removal) ($p < 0.05$, student's t-test, *n* = 4-5).

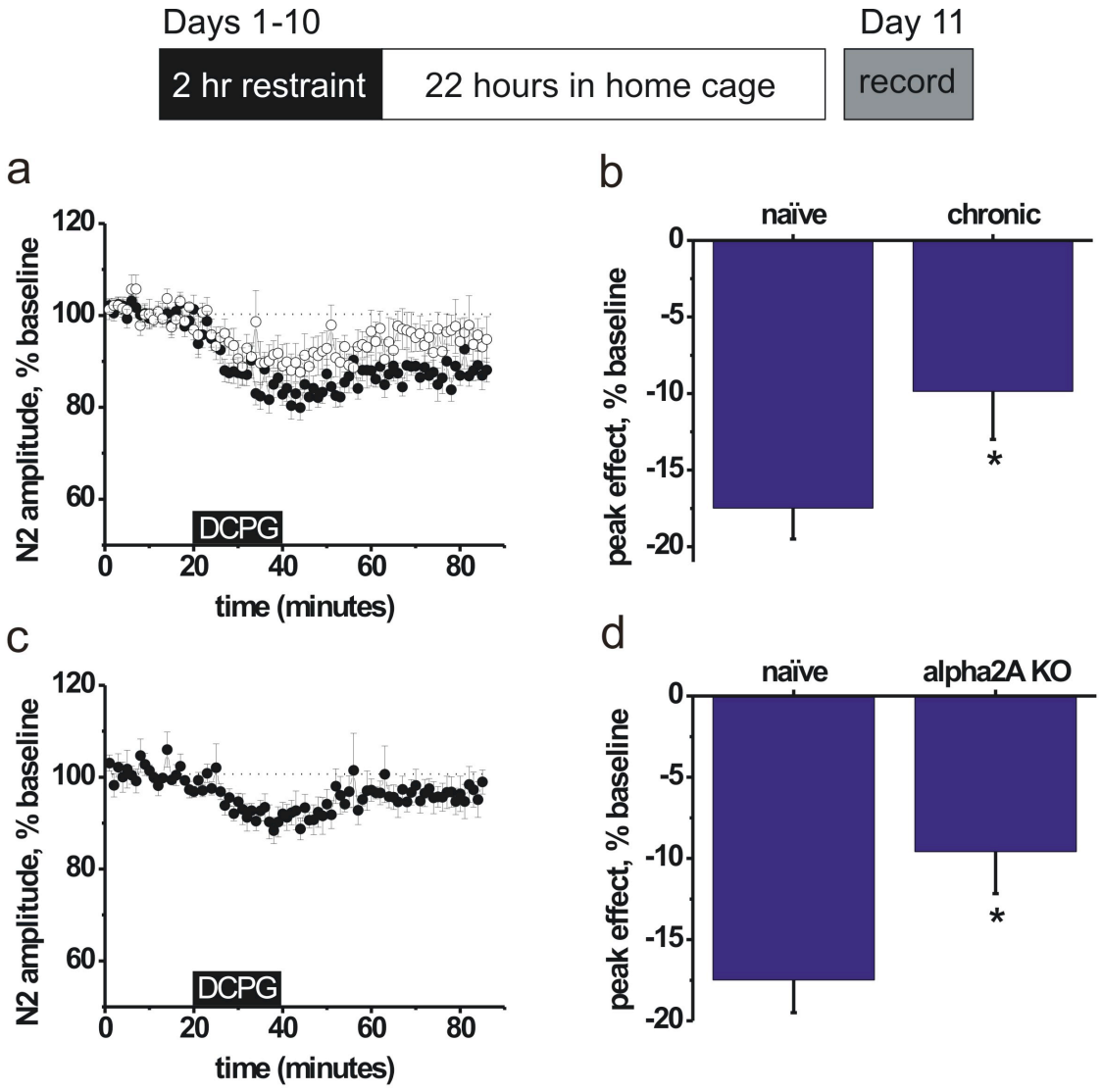
examined the impact of a single restraint stress on this plasticity. Both the peak and late effects of 100 μM methoxamine were modestly but significantly attenuated after a single restraint stress (*peak effect*: 18.0 ± 1.7 and 24.4 ± 3.0 peak average percent depression for single restraint versus naïve controls, respectively; $p < 0.05$, student's t-test; *late effect*: 10.2 ± 1.4 and 17.9 ± 2.9 average percent depression for single restraint versus naïve controls, respectively; $p < 0.05$, student's t-test; Figure 17E,F). Taken together with our previous study (McElligott, *et al* 2010), this suggests α1 ARs in the BNST were likely activated by the single restraint stress.

Group II mGluR Function is Intact After Acute Restraint Stress

Interestingly, sensitivity to the Group II agonist LY379268 was unaffected after acute stress, demonstrating that the stress-induced disruption is specific to mGluR8 (stressed: 35.7 ± 7.7 peak average percent depression, versus 32.5 ± 4.7 for interleaved controls; Figure 17C,D).

mGluR8 Function is Disrupted After Chronic Stress

Finally, I examined whether mGluR8 function is disrupted by a more chronic stressor. Using a protocol shown previously to disrupt α1-LTD in the BNST (McElligott, *et al* 2010), mice underwent 2 hours of restraint stress for 10 consecutive days, and were euthanized 24 hours after their last session, on day 11 (see Figure 18 schematic). As with a single *in vivo* stress, the effect of 10 μM DCPG was significantly attenuated following chronic restraint stress (chronic restraint: 9.9 ± 3.1 peak average percent depression, versus 17.5 ± 2.0 for pooled interleaved naïve controls, $p < 0.05$, Dunnett's post hoc comparison; Figure 18 A,B). I also


Days 1-10
Day 11
2 hr restraint
22 hours in home cage
record
a
N2 amplitude, % baseline
120
100
80
60
DCPG
0
20
40
60
80
time (minutes)
b
naïve
chronic
peak effect, % baseline
0
-5
-10
-15
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c
N2 amplitude, % baseline
120
100
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DCPG
0
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40
60
80
time (minutes)
d
naïve
alpha2A KO
peak effect, % baseline
0
-5
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-15
-20
*

Figure 18. mGluR8 Function Remains Disrupted After Chronic Stress. (*top*) schematic representing the chronic restraint stress timeline. (a) 10 μM DCPG-induced depression (pooled interleaved naïve controls, closed circles) is disrupted in mice that underwent chronic restraint stress (open circles) (n = 5-13). (b) peak average percent depression of 10 μM DCPG in pooled interleaved naïve control mice and chronic restraint mice ($p < 0.05$, Dunnett's post hoc comparison, n = 5-13). (c) DCPG-induced depression is also attenuated in α2A AR KO mice, a model of chronic stress. (d) peak average percent depression of 10 μM DCPG in pooled interleaved naïve control mice and α2A AR KO mice ($p < 0.05$, Dunnett's post hoc comparison, n = 6-13).

assessed mGluR8 function in α2A AR knockout mice, which exhibit chronic adrenergic system dysregulation and are considered a chronic stress model (Schramm, *et al* 2001). The effect of DCPG was also diminished in these mice as compared to pooled interleaved naïve controls (α2A AR KO: 9.6 ± 2.6 peak average percent depression, versus 17.5 ± 2.0 for pooled interleaved naïve controls, $p < 0.05$, Dunnett's post hoc comparison; Figure 18C,D). Taken together, these data reveal that mGluR8 regulation of excitatory transmission can be overridden by adrenergic signaling in the BNST and specifically disrupted by both acute and chronic *in vivo* stress.

Discussion

Group III ligand effects in BNST are mediated by presynaptic mGluR8

mGluRs have been shown to alter glutamatergic transmission, playing key roles in several forms of plasticity throughout the brain. In contrast to ionotropic glutamate receptors, the slower nature of mGluR signaling allows for potentially more long-lasting, adaptive changes in synaptic strength. Within the BNST, agonists to Groups I, II, and III mGluRs depress glutamatergic transmission (Muly, *et al* 2007; Grueter and Winder, 2005; Grueter, *et al* 2006). Consistent with previous studies, I report that activation of Group III mGluRs with either the general Group III agonist L-AP4 or the mGluR8-selective agonist DCPG decreases excitatory transmission in the dorsal BNST. The change in PPR induced by DCPG, coupled with a significant decrease in sEPSC frequency, suggests that presynaptic mGluR8 is depressing

transmission by decreasing glutamate release. As a small effect was also observed on sEPSC amplitude, we cannot exclude the possibility currently that mGluR8 may modulate postsynaptic function as well, though the effects on frequency were much more pronounced. These data are consistent with the autoreceptor function of mGluR8 seen in other brain regions (Ayala, *et al* 2008; Valenti, *et al* 2005; Abitbol, *et al* 2008; Schmid and Fendt, 2006).

I report converging evidence that mGluR8 is the primary mediator of Group III ligand effects on excitatory transmission in the BNST. First, the mGluR4 allosteric potentiator PHCCC failed to potentiate the effects of the mGluR8-selective agonist DCPG, suggesting DCPG is acting solely through mGluR8. Secondly, PHCCC failed to potentiate the effect of the Group III agonist L-AP4, the orthosteric ligand it is commonly paired with. Finally, there was no effect of PHCCC or L-AP4 in slices from mGluR8 KO mice, demonstrating that mGluR8 alone is necessary for the depressive effects of Group III ligands on excitatory transmission in this region.

Of the Group III receptors expressed in the brain, mGluRs 4 and 8 have a similar affinity for glutamate, and L-AP4 has similar high potency at these two receptors (Schoepp, *et al* 1999), making mGluR4 the most likely Group III receptor other than mGluR8 to be playing a role in regulating excitatory transmission in the BNST. The expression of mGluR6 is restricted to retina, ruling out a contribution in the BNST (Nakajima, *et al* 1993). It is, however, possible that mGluR7 could have effects on transmission in the BNST, as it is widely expressed in the adult brain. mGluR7 is unique among mGluRs in that is has a very low affinity for glutamate, and L-AP4 exhibits very low potency at this receptor (Schoepp, *et al* 1999). Due to both the

low potency of the compound at mGluR7, and also the particularly slow drug on-rate when L-AP4 is used in interface settings, it is unlikely that my concentration of 200 μM L-AP4, bath applied in an interface chamber, yielded any mGluR7 activation. Our own previous studies suggest that even in whole-cell configuration (when slices are submerged, allowing for more efficient drug delivery), near millimolar concentrations of L-AP4 may be required to activate receptors other than the higher affinity mGluRs 4 and 8 (Grueter, *et al* 2005). Therefore, while it is possible that mGluR7 is present in the BNST and able to regulate transmission, it is highly unlikely to be contributing to the drug effects observed in this study.

α1 ARs Can Regulate mGluR8-Sensitive Inputs

I have demonstrated here that mGluR8-expressing, stress-sensitive inputs in the dBNST are regulated by α1 ARs. Previous work has shown that bath application of the α1 AR agonist methoxamine (100 μM) for 20 minutes induces α1 AR-dependent long-term depression (α1-LTD) in the BNST that is modulated by chronic restraint stress (McElligott and Winder, 2008; McElligott, *et al* 2010). In this study, the same methoxamine application disrupted subsequent mGluR8-induced depression of excitatory transmission in the slice, as did both acute and chronic restraint stress. However, I observed some recovery of mGluR8 function after methoxamine when a high concentration of DCPG (30 μM) was applied. This indicates that the decreased response to 10 μM DCPG seen after methoxamine likely represents a shift in potency. One mechanism that could be underlying this shift is a decrease in mGluR8 signaling caused by desensitization or internalization of the receptor. Importantly,

in the reverse experiment, DCPG is unable to disrupt α1-LTD, suggesting that either α1 AR activation is upstream of mGluR8 activation, or α1 ARs regulate transmission at mGluR8-expressing and non-expressing inputs.

Stress Can Specifically Regulate mGluR8-Sensitive Inputs

Currently, the source of the stress-sensitive, mGluR8-expressing inputs into the dBNST is unknown. The BNST receives excitatory inputs from several limbic brain regions and projects to the PVN, a circuitry thought to be activated during anxiety-related behavior (Walker and Davis, 1997; McElligott, *et al* 2010). Both the medial infralimbic cortex (mPFC) and the BLA express mGluR8 mRNA, suggesting these regions could be sending stress-sensitive processes into the BNST (Allen Mouse Brain Atlas). Indeed, the BNST is a necessary intermediary in the recruitment of the PVN by the mPFC (Radley, *et al* 2009). Local afferent stimulation as performed in the present study likely indiscriminately activates excitatory inputs from many regions; thus, an interesting possibility is that the modest maximal effect of Group III agonists reported here reflects mGluR8 modulation of only a sub-population of afferents.

Importantly, the regulation of mGluR8 signaling by stress appears to be specific. I did not observe any obvious alteration in sensitivity to the Group II agonist LY379268 in acutely stressed animals. Group II receptors are known to be presynaptically expressed in BNST and activate $G_{i/o}$ signaling, similar to mGluR8 (Muly, *et al* 2007; Grueter and Winder, 2005). Their insensitivity to disruption by a single *in vivo* stressor suggests that mGluR2/3 do not regulate the same set of

excitatory inputs into the BNST regulated by mGluR8 and α1 ARs. Further, mGluR5-mediated LTD is intact after chronic restraint stress, suggesting postsynaptic mGluRs in the BNST may not be engaged during stress exposure (McElligott, *et al* 2010). While activation of any known mGluRs in the BNST depresses transmission, this selective disruption of mGluR8 signaling by stress highlights the potential importance of this receptor in regulating specific sets of inputs coming into the BNST.

mGluR8 Function Remains Disrupted After Chronic Stress

Importantly, mGluR8 function is disrupted in α2A AR KO mice, a chronic stress model (Scrhamm, *et al* 2001), and remains disrupted after chronic stress, conditions under which α1-LTD is also known to be disrupted in the BNST (McElligott, *et al* 2010). The lack of difference between acute and chronic stress effects on mGluR8 function suggests several things. First, the failure of these synapses to adapt to α1 AR-mediated loss of mGluR8 function may indicate an important regulatory role for mGluR8 on these inputs. Likewise, it suggests that these mGluR8-expressing, stress-sensitive inputs are bringing information into the BNST that should be overridden in situations of prolonged stress, thus mediating a valuable adaptive response to a stressor. Lastly, mGluRs throughout the brain play critical roles in gating metaplasticity. α1 AR activation and subsequent diminished mGluR8 function could be necessary in order to permit activation of other receptors and signaling cascades at these synapses, as part of the brain's stress response.

Potential Mechanisms of Stress-Induced Regulation of mGluR8

In addition to mGluR8, the function of BNST α1 ARs was also attenuated by a single restraint stress, consistent with the idea that α1 ARs in the BNST are activated by *in vivo* stress (McElligott, *et al* 2010). A link between α1 ARs, *in vivo* stress, and Group III mGluRs has previously been made in the PVN (Kuzmiski, *et al* 2009; Gordon and Bains, 2003). However, several factors differentiate those studies from the one reported here. First, the *in vivo* stressor used in the PVN experiments was hemorrhage- a profound, systemic physiological stressor. Instead, I used either a single, 60-minute session or a 10-day chronic protocol of restraint stress, a paradigm shown to increase NE in the BNST and cause activation of α1 ARs (Cecchi, *et al* 2002; McElligott, *et al* 2010). Restraint stress is considered to be a more processive stressor than hemorrhage, engaging brain regions such as the BNST that can oppose or allow further activation of stress circuitry via the HPA axis. Recent work from our laboratory demonstrated that α1-LTD in the BNST is occluded by chronic restraint stress, suggesting engagement of this form of plasticity during stress (McElligott, *et al* 2010). I report here that α1-LTD in the *ex vivo* BNST is reduced after a single restraint stress. By recapitulating the α1 AR-mediated disruption of mGluR8 signaling with acute and chronic restraint stress, as well as in the chronic stress model of α2A AR KO mice (which exhibit adrenergic disregulation), the current study further supports an occlusion hypothesis, and demonstrates that an acute stressor is sufficient to achieve this occlusion.

The studies carried out in the PVN also outline a mechanism by which presynaptic α1 ARs cause desensitization of presynaptic Group III mGluRs, measured by loss of

L-AP4 sensitivity. The location of the α1 ARs disrupting mGluR8 function in the BNST is, as yet, unknown. Our laboratory has shown previously that post-synaptic G-protein signaling is necessary for expression of α1-LTD, suggesting a postsynaptic locus for α1 ARs (McElligott and Winder, 2008). One possibility is that postsynaptic α1 ARs are disrupting presynaptic mGluR8 via a retrograde messenger. However, α1 ARs could exist anywhere at the synapse in BNST, including being present at multiple locations, allowing for other mechanisms of mGluR8 disruption by α1 ARs. Alternatively, in the hippocampus, presynaptic Group III mGluRs, including mGluR8 specifically, can be inhibited through phosphorylation by PKA (Cai, *et al* 2001). Thus, any receptor feeding into the cAMP-PKA pathway could desensitize mGluR8. Our lab has previously reported that the depressive effects of NE on excitatory transmission in the BNST are time-dependent (McElligott and Winder, 2008). A 10-minute application of NE transiently depresses excitatory transmission in the BNST, while a 20-minute application induces α1-LTD, which in turn disinhibits the PVN and allows for HPA axis activation (Cecchi, *et al* 2002). I propose that under weak stress, increased excitability in the BNST leads to mGluR8 activation, which in turn depresses excitatory transmission in the region, presumably towards restoring homeostatic norms. During a single, 60-minute restraint stress however, BNST levels of NE are elevated for prolonged periods, activating α1 ARs and inducing α1-LTD. In prolonging the decrease in glutamatergic transmission, α1-LTD overrides the signaling mechanisms of mGluR8, perhaps through endocannabinoid signaling. This LTD is then able to decrease the BNST's basal inhibition over the PVN, allowing for HPA axis activation in the face of more prolonged stress exposure. In summary,

my data indicate a unique role for mGluR8 among Group III mGluRs in the modulation of excitatory transmission in the BNST, and identify this regulation as a target of *in vivo* stress.

CHAPTER 5

GENERAL DISCUSSION/CONCLUSIONS

Implications of mGluR5/ERK/Cocaine Project Findings

Though ultimately unsuccessful, this project began to elucidate the mechanisms underlying cocaine-induced disruption of mGluR5-LTD in the BNST. Brad's finding that DHPG-LTD after cocaine is rescued by preinjection of MPEP suggested either mGluR5 signaling or the receptor itself was being targeted by cocaine *in vivo*. My work suggests that mGluR5 activation is not necessary for cocaine-induced ERK activation, implying that the disruption of mGluR5 signaling by cocaine-induced signaling may occur before the level of ERK activation. These studies also showed no gross difference in surface levels of mGluR5 after chronic cocaine, suggesting that expression of the receptor itself is not a target of cocaine. Taken together, these data suggest that another mediator of mGluR5 signaling may underlie the mechanism by which cocaine disrupts mGluR5-LTD. One possibility would be recruitment of distinct signaling cascades via altered homer expression (Patel and Winder, 2010).

Potential Mechanisms by Which Cocaine Could Regulate mGluR5 Function

There are still several potential mechanisms for cocaine-induced disruption of mGluR5 signaling. It is likely that cocaine is overriding the intracellular signaling cascades necessary for the induction and/or maintenance of mGluR5-LTD. As cocaine is known to increase dopamine levels in the BNST, this occlusion could be occurring downstream of D1 dopamine receptors, which are the receptors thought to be responsible for cocaine-induced ERK activation in many brain regions (Valjent, *et al* 2004). If occlusion is occurring, I would expect bath-applied D1 agonists to disrupt mGluR5-LTD *ex vivo*. Likewise, mGluR5-LTD should be disrupted in D1 DR knockout mice. Alternatively, cocaine may be disrupting eCB-dependent signaling, which has been reported in the striatum (Fourgeaud, *et al* 2004). Finally, cocaine is known to induce expression of the immediate-early gene Homer1a, a dominant negative Homer protein. Homer1a disrupts the function of long Homer isoforms, which are responsible for coupling Group I mGluRs to internal calcium stores under normal conditions. After cocaine, Homer1a could effectively halt mGluR5 signal transduction, preventing the release of internal calcium necessary for the expression of mGluR5-LTD (Grueter, *et al* 2006). Or, Homer1a could alter the subcellular localization of mGluR5, causing the receptor to signal through a pathway that may not permit LTD induction. Future studies with a better pan-Homer antibody, or subtype-specific antibodies, would resolve the question of whether cocaine-induced disruption of mGluR5-LTD occurs via induction of Homer1a.

Model of mGluR5 Regulation by Cocaine

Glutamatergic transmission is thought to be increased in the BNST after exposure to drugs of abuse. Therefore, I would hypothesize that this cocaine-mediated increase in glutamate would recruit the activation of mGluR5. Once activated, mGluR5 would act to counterbalance this increase in excitatory transmission by activating ERK and inducing LTD. This *in vivo* induction of mGluR5-LTD would thus occlude subsequent attempts to elicit it *ex vivo* (Figure 19). Such a model would allow for the receptor to regain its function after the effects of cocaine had subsided. Indeed, we now know that mGluR5-LTD is present 10 days after chronic cocaine treatment, and 24 hours after acute cocaine (Grueter et al, 2008). This hypothesis is further strengthened by the finding that cocaine applied to BNST slices mimics the effect of *in vivo* cocaine on mGluR5-LTD, suggesting increased dopamine levels within the BNST could be responsible for increased glutamatergic transmission (Grueter, *et al* 2008; Kash, Nobis, *et al* 2008). Interestingly, stress-induced ERK activation could occur by a similar mechanism, perhaps explaining why stressed cocaine-treated mice appeared to exhibit a "ceiling effect" of ERK phosphorylation. Finally, more direct disruption of mGluR5 signaling via Homer1a induction cannot be ruled out.

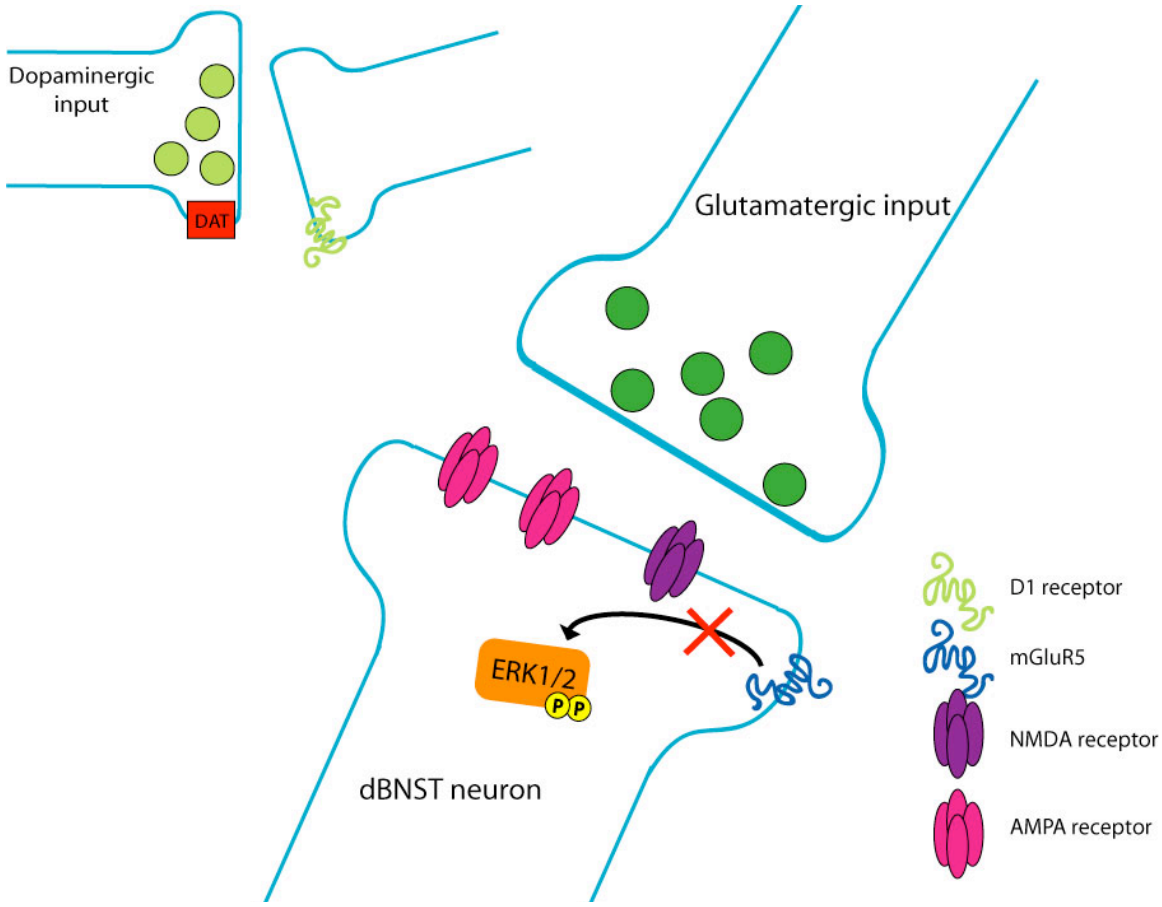

Figure 19. A Hypothesized Model of mGluR5 Regulation by *in vivo* Cocaine. Under normal conditions, mGluR5 functions normally to regulate glutamatergic transmission. However, cocaine exposure somehow disrupts mGluR5 signaling, possibly preventing ERK1 activation, which is required for mGluR5-LTD. Alternately, cocaine exposure may increase glutamatergic transmission in the BNST, leading to the activation and subsequent desensitization of mGluR5. Concurrently, cocaine activates D1 dopamine receptors, leading to ERK activation in the BNST and potentially occluding the ERK/MAPK pathway, preventing mGluR5 recruitment of ERK.

Outstanding Questions, Part I

As alluded to above, these data produced more questions than answers. The mechanism of cocaine-induced disruption of mGluR5-LTD is still unknown. The involvement of Homer1a has not yet been determined. Interplay between stress-induced changes and drug-induced changes have yet to be parsed out, though both may involve increased excitatory transmission in the BNST. A large outstanding question is what increased glutamatergic signaling and loss of mGluR5 means for the brain regions downstream of the BNST. As a "middle manager", the BNST translates incoming limbic information into a functional behavioral or physiological consequence by activating or inactivating downstream effector regions. Excitability in the BNST is directly related to the excitability of the midbrain DA neurons at the heart of the canonical reward circuitry (Caille, *et al* 2009). mGluR5 may therefore play an important role in regulating the excitability of this connection under normal conditions, but become overridden temporarily by drugs of abuse. Alternately, corticosterone has been shown to be required for acquisition of cocaine reinforcement (for review, see Goeders, 2002). Given the involvement of the BNST in stress as well as reward circuitry, mGluR5 activation in the BNST by cocaine could be mediating this HPA axis recruitment.

Finally, there is the intriguing observation that mGluR5-LTD is not altered by restraint stress (McElligott, *et al* 2010), a stimulus which may also increase glutamatergic signaling in the BNST. This specific disruption of mGluR5 by cocaine

rather than stress fits with the idea of occlusion mediated by DA signaling. Interestingly, recent work from our lab suggests NE may similarly increase glutamatergic signaling in the BNST (Nobis, Kash, *et al* 2011). An NE-dependent mechanism could therefore explain the specific disruption we find of mGluR8, but not mGluR2/3 or mGluR5, after stress.

Relationship Between α1 ARs, mGluR8, and Stress

α1 ARs in the BNST are critical regulators of the body's stress response (Cecchi, *et al* 2002). Due to their importance in stress circuitry, it is hard to image that BNST α1 ARs are not mediating the stress-induced disruption of mGluR8 observed here. Because stress-induced disruption did not affect mGluR2/3 function, and because mGluR8 activation did not alter α1 AR activation, mGluR8 signaling appears to be specifically regulated by α1 ARs. I attempted to address this hypothesis directly in a failed prazosin rescue experiment (data not shown), which yielded a negative result that was impossible to interpret. The exact mechanism of direct α1 AR-induced disruption of mGluR8 signaling in the BNST is also unknown, but work in the PVN suggests α1 ARs disrupt presynaptic Group III mGluRs via a PKC-dependent mechanism (Gordon and Bains, 2003).

Model of mGluR8 Regulation by Stress

Several studies have suggested that an increase in excitatory transmission in the BNST takes place during and after a stressor. Recent work from our own laboratory has shown that NE increases glutamatergic transmission in the BNST through a CRF-dependent mechanism (Nobis, Kash, *et al* 2011). I hypothesize that an initial increase in excitatory transmission occurs in the BNST immediately after the onset of stress, as NE is dumped into the BNST via the VNAB. This increased NE tone translates into an increase in excitability, followed by enhanced glutamatergic transmission via β ARs. Excitation of the BNST may initially oppose recruitment of the HPA axis, as a safeguard against triggering the body's stress response unnecessarily. It is during this early phase that mGluR8 is most likely activated by rising levels of glutamate in the BNST. mGluR8 activation therefore serves to shut off some of the glutamate sources entering the BNST during a stressor, and will consequently depress transmission at these synapses. As the duration of the stress progresses, however, α1-ARs are activated and α1-LTD is induced throughout the BNST, decreasing excitatory transmission and overriding mGluR8 signaling. Conversely, it is also possible that α1-ARs do not override mGluR8 signaling *in vivo* via a direct mechanism, but instead are activated after mGluR8 has already been desensitized/internalized, providing a second brake opposing excitatory transmission at mGluR8-expressing inputs. The decrease in overall BNST excitability would then produce a disinhibition of the PVN, allowing for activation of the HPA axis and a systemic stress response (Figure 20).

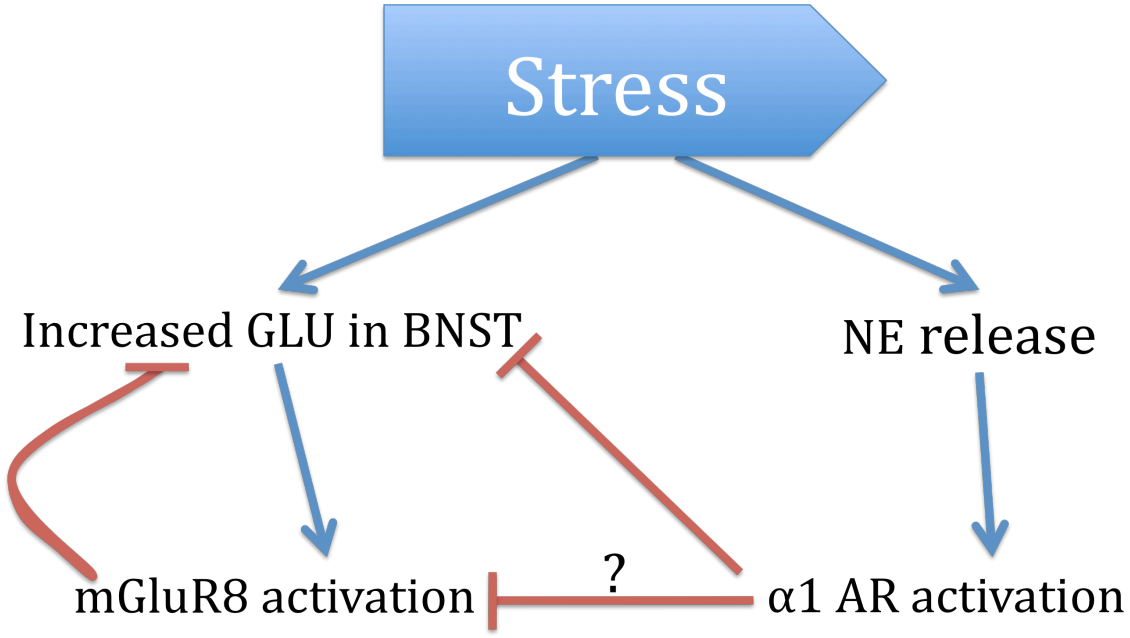

Figure 20. A Schematic Model Illustrating the Regulation of mGluR8 and α1 AR Signaling in the BNST by Stress. Restraint stress induces NE release into the BNST, and may also concurrently increase glutamatergic transmission. The limbic inputs to the BNST are also sending glutamate into the region, which results in mGluR8 activation. Once activated, mGluR8 opposes the increasing glutamatergic signaling by decreasing glutamate release from mGluR8-expressing inputs. As time passes and the stress persists, NE will induce α1 AR activation in the BNST, inducing α1-LTD. α1-LTD will depress excitatory transmission, opposing the stress-induced increase in glutamatergic signaling. Finally, based on methoxamine-induced disruption of mGluR8 signaling, α1 ARs may also directly regulate mGluR8-expressing synapses.

Outstanding Questions, Part II

Our data suggest that α1-ARs are regulating the same stress-sensitive inputs that express mGluR8. While occlusion of mGluR8 signaling by α1-ARs via eCBs or other signaling pathways is possible, during *in vivo* stress, it is likely that the receptors are activated in series, with mGluR8 being activated first and α1-AR activation occurring later. This fits with the time-dependence of α1-LTD in the BNST, and with a model of increase glutamatergic tone in the BNST during a stressor. Future studies should address whether α1-ARs regulate mGluR8 directly in the slice or during *in vivo* stress.

Another important future experiment will be to identify the source of the afferents expressing mGluR8 in the BNST. Because the infralimbic cortex and BLA both express mGluR8 mRNA and send excitatory projections to the BNST, I hypothesize that these inputs would be mGluR8-positive (Figure 21). The development of a mouse-compatible mGluR8 antibody would greatly aide these efforts. Identifying the origin of these stress-sensitive inputs will further illuminate the brain circuitry involved in the stress response.

Finally, it would be interesting to see whether different types of stressors (processive versus visceral) would have differing effects on mGluR8 function. Restraint stress is a fairly processive stressor, presumably activating brain regions like the BNST to decide whether the stressor warrants a physiological stress response. A more visceral stressor like forced swim test or hypothermia would perhaps not recruit this same set of mGluR8-inputs into the BNST, leaving mGluR8

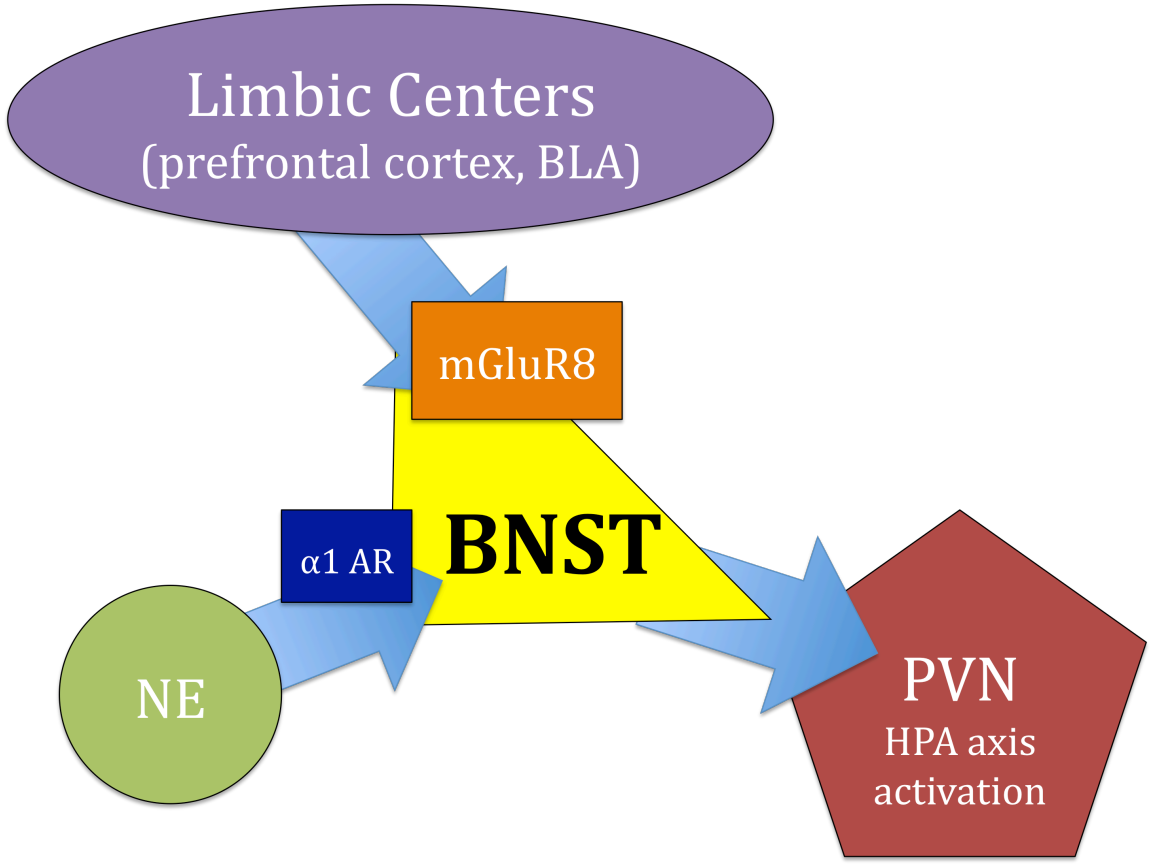

Figure 21. Circuit Diagram Illustrating the Hypothetical Role of mGluR8 in the Brain's Stress Response Circuitry. Because mGluR8 in the BNST is selectively sensitive to disruption by in vivo stress or α1 ARs, this suggests an important role for mGluR8 in stress signaling. I hypothesize that mGluR8 is expressed on limbic inputs from the BLA or mPFC, and that these inputs are selectively activated by stress. Alternately, NE-induced α1 AR activation in the BNST during stress may directly disrupt mGluR8 function. Overall, the tight regulation of excitatory transmission in the BNST supports the idea of the BNST as a critical relay station for the neural response to a stressor.

function intact. It is also unknown how long mGluR8 function remains disrupted after a stressor. I would predict a fairly rapid return to normal, based on the finding that the mGluR8 signaling pathway showed no adaptation to chronic stress.

Potential Benefit of This Work to the Study of Human Disease

In closing, the data presented in this thesis provide evidence for the importance of mGluRs in modulating excitatory transmission in the BNST during disease states. Modulatory compounds targeting mGluRs are currently being developed for the treatment of several diseases, including stress/anxiety. The finding that mGluR8 regulates stress-sensitive inputs entering the BNST, coupled with its discreet expression pattern, makes mGluR8 an attractive pharmacological target. Further, the finding that α1 ARs cause a shift in DCPG potency *ex vivo*, rather than a total loss of mGluR8 function, suggests a small population of functional mGluR8 could be present in the BNST after a stressor, and thus able to be targeted by anxiolytic compounds. Already, some studies have shown that the mGluR8-selective agonist DCPG has anxiolytic effects in mice, though in general the bioavailability of DCPG is thought to be poor (Duvoisin, *et al* 2010a). This work also further emphasizes the diversity of important functions managed by the BNST. Exposure to stress or drugs of abuse can disrupt the function of important mediators of plasticity in this region, possibly through similar mechanisms (a catecholamine-induced increase in excitatory transmission). Interestingly, my work also suggests Group II mGluR function in the BNST is stress-resistant. Taken together with the cocaine-specific

disruption of mGluR5 function, this thesis further delineates a role for each of the 3 mGluR subgroups in regulating excitatory transmission in the BNST. It is amazing how a class of similar receptors, with comparable functions, within the same brain region, could individually tune the BNST to the current external environment via its own set of synapses.

APPENDIX A

The Effect of DCPG may be Altered by Acute and Chronic Cocaine

A few studies have reported changes in mGluR8 mRNA or protein levels following exposure to psychostimulants. Acute cocaine administration causes a decrease in mGluR8 protein in the rat striatum that reverses after 6 hours (Zhang, *et al* 2009). Conversely, mGluR8 mRNA is upregulated in the rat forebrain after acute amphetamine (Parelkar and Wang, 2008). I hypothesized that mGluR8 function in the BNST might be disrupted by acute or chronic cocaine. However, my findings were mixed over several cohorts of animals. In some cohorts, the effect of DCPG appeared to be blunted in slices from cocaine-treated animals. However, in other cohorts, the effect of DCPG was also decreased in saline-treated mice (Figure 22). Stressful housing conditions were a potential confounding factor, as we now know *in vivo* stress can robustly disrupt mGluR8 function in the BNST. Ultimately, these studies caused us to alter how my animals were housed, and shifted my focus from drug-induced changes in mGluR8 function to stress.

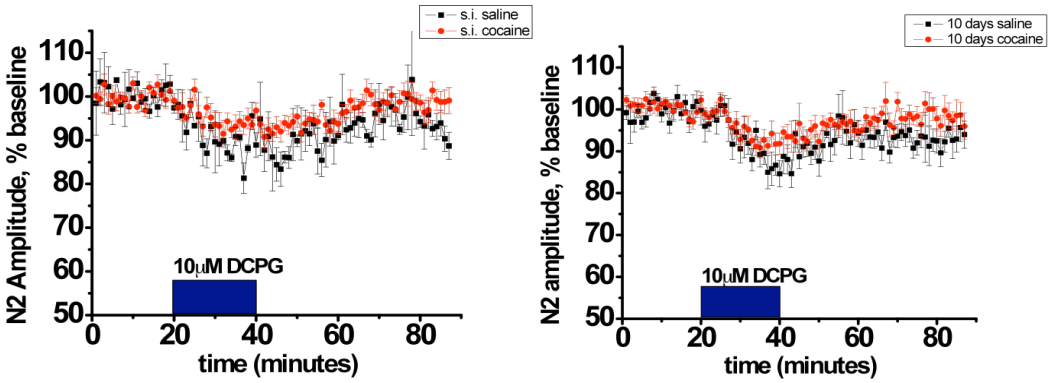

Figure 22. The Effect of DCPG May be Altered in Slices From Cocaine-Treated Mice. (a) The effect of a single injection of cocaine or saline on DCPG sensitivity. (n = 7 for cocaine, red circles; n = 3 for saline, black circles). (b) The effect of chronic cocaine or saline on DCPG sensitivity. (n = 7 for cocaine, red circles; n = 8 for saline, black circles).

APPENDIX B

DCPG May Facilitate Single-Train LTP

In the PVN, α1 ARs activated by stress inhibit presynaptic Group III mGluRs, which maintain a low level of glutamate release at these synapses under basal conditions (Gordon and Bains, 2003, Kuzmiski, *et al* 2009). This metaplastic disinhibition is permissive for a form of LTP, which can be induced via a subthreshold stimulation protocol that does not induce LTP under basal conditions. I hypothesized that mGluR8 activation (and subsequent inactivation/desensitization) in the BNST could be permissive for a similar form of subthreshold LTP in this region. Under normal conditions, LTP cannot be induced in the BNST using a single train 100 Hz protocol. However, application of DCPG for 20 minutes prior to the tetanus appeared to facilitate an LTP-like plasticity (Figure 23). Though preliminary, this data could suggest basal activation of excitatory transmission in the BNST by mGluR8. If this LTP-like plasticity is also present after stress, this would further support the idea of stress-induced metaplasticity, such as that seen in the PVN.

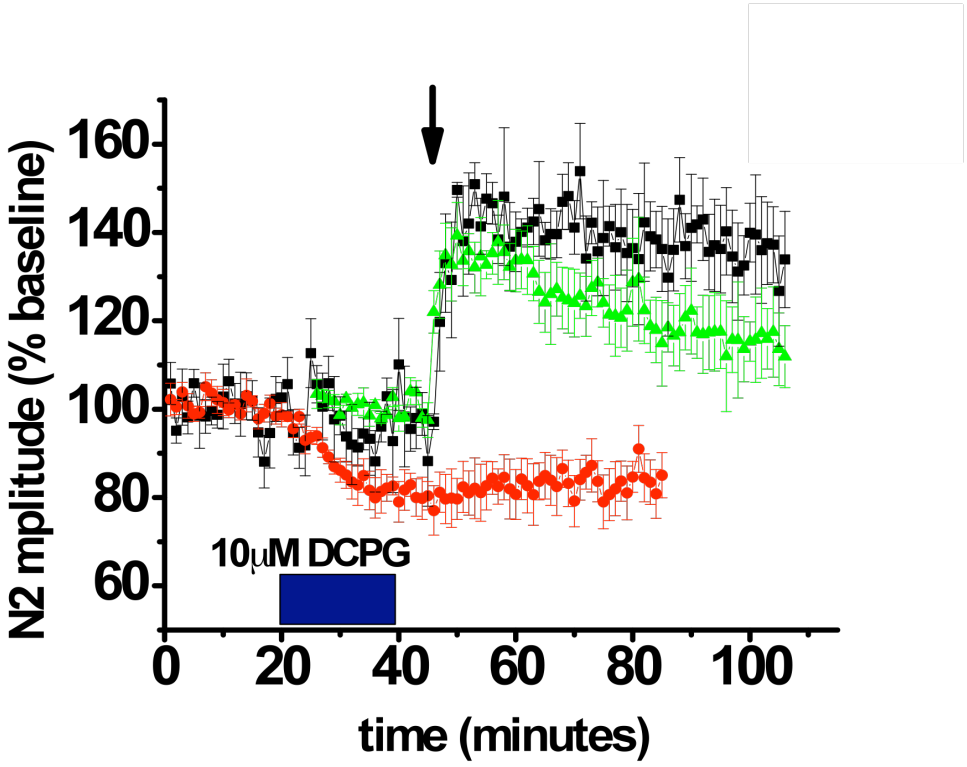

Figure 23. DCPG May Facilitate Sub-Threshold LTP in the BNST. 10 μM DCPG prior to a single tetanus appears to increase the size of the tetanus-induced plastcitiy. (DCPG control, n = 8, red circles; single train LTP, n = 4, green triangles; DCPG + single train LTP, n = 3, black squares).

APPENDIX C

Novel mGluR4/8 Potentiator AZ12216052 Depresses Transmission in BNST

In my initial attempts to characterize the effects of Group III mGluRs on excitatory transmission in the BNST, I utilized a compound that positively regulates mGluR8, AZ12216052 (Duvoisin, *et al* 2010). Slices were bathed in 10 μM AZ12216052 for 30 minutes, followed by a 20-minute co-application of AZ12216052 and 200 μM L-AP4 (16.7 ± 2.8 peak average percent depression vs. baseline, $p \leq 0.0001$, student's t-test; Figure 24A). A small but significant effect of AZ12216052 alone on excitatory transmission was observed (Figure 24A,C). The subsequent effect of L-AP4 in the presence of AZ12216052 was not significantly different from that of L-AP4 alone (16.7 ± 2.8 compared to 13.1 ± 2.7 peak average percent depression of L-AP4 alone in vehicle, Figure 24C). To determine whether the effects of AZ12216052 were mediated by mGluR8, these experiments were repeated using slices from mGluR8 knockout mice (Figure 24B). Neither AZ12216052 nor L-AP4 had any effect in mice lacking mGluR8, demonstrating that the effects of these compounds require mGluR8 (AZ effect in KO: 0.3 ± 2.3 peak average percent depression vs. 5.9 ± 1.7 in WT, $p < 0.05$, student's t-test; Figure 24D). Taken together, these data further confirmed the primary role of mGluR8 in mediating the effects of Group III ligands on transmission in the BNST.

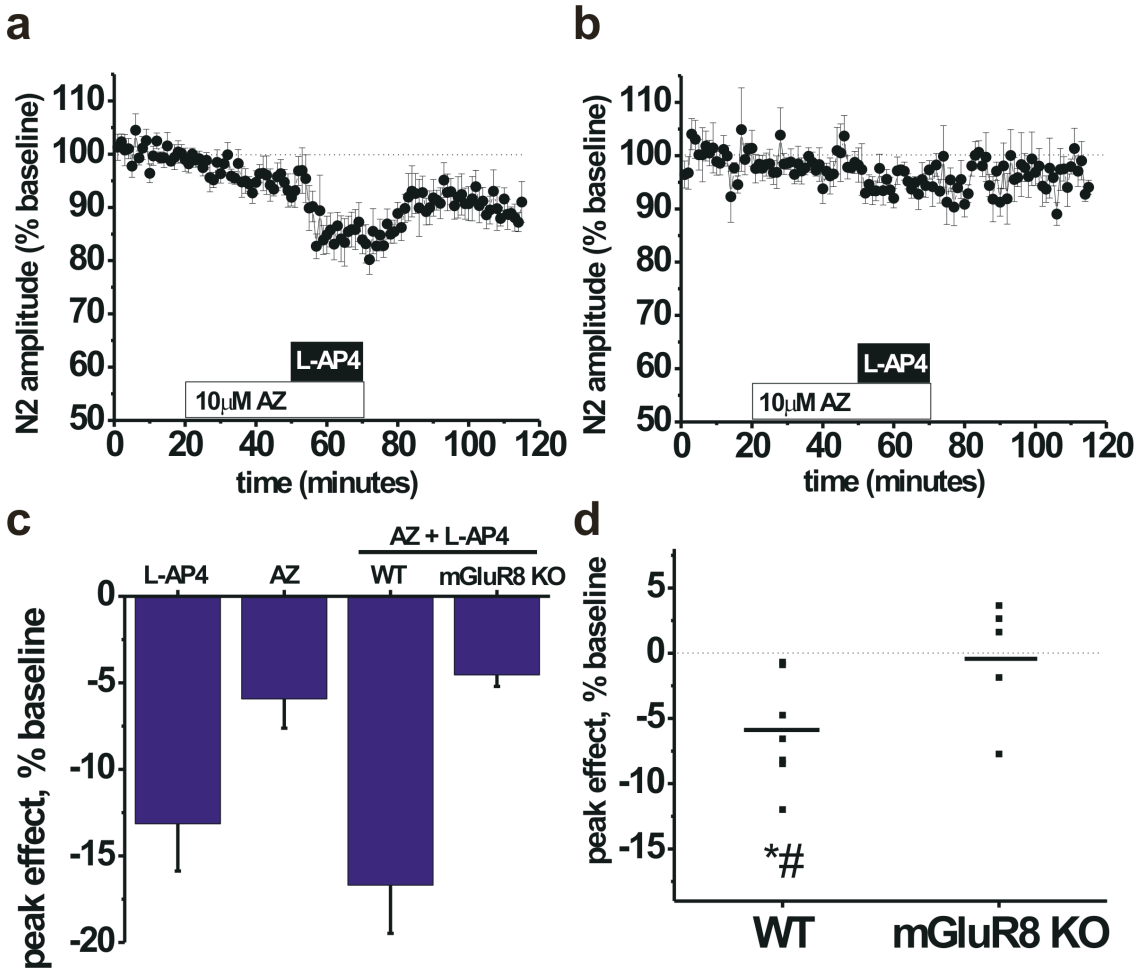
a
b
N2 amplitude (% baseline)
time (minutes)
L-AP4
10μM AZ
c
AZ + L-AP4
L-AP4
AZ
WT
mGluR8 KO
peak effect, % baseline
d
*#
WT
mGluR8 KO

Figure 24. The Effect of the mGluR8 Potentiator AZ12216052 on Transmission in BNST is Mediated by mGluR8. (a) 10 μM AZ12216052 depresses excitatory transmission in BNST ($p \leq 0.0001$ versus baseline, student's t-test, $n = 7$). (b) The effect of AZ12216052 + L-AP4 is absent in slices from mGluR8 knockout mice ($n = 5$). (c) Analysis of peak effect of average percent depression induced by AZ12216052, L-AP4, or in combination, on WT and mGluR8 knockout mouse slices (#$p \leq 0.005$ compared to WT, *$p \leq 0.01$ compared to baseline, student's t-test) . (d) Peak average percent depression of 10 μM AZ12216052 alone in WT and mGluR8 knockout mouse slices. 10 μM AZ12216052 causes a small but significant depression of transmission on its own that is absent in slices from mGluR8 knockout mice (*$p \leq 0.01$ compared to baseline, #$p = 0.05$, WT vs. KO, student's t-test, $n = 5$-7).

These studies were the first use of AZ12216052 on brain slices. Because the effect of AZ + L-AP4 was not significantly different from the peak effect of L-AP4 alone, this suggests AZ is at best a weak potentiator. Interestingly, I observed a small but significant depression in transmission with AZ12216052 alone. This depression could be explained several different ways. It is possible that AZ12216052 has intrinsic agonist activity at mGluR8, such that it is able to activate the receptor on its own, without an orthosteric ligand such as L-AP4 being present. Alternatively, it is possible that mGluR8 is basally activated by glutamate in the slices. If this second scenario were occurring, then addition of a potentiator such as AZ12216052 would increase this baseline mGluR8 activation, causing a depression in the presence of AZ12216052 alone. In either case, the depression caused by AZ12216052 is absent in mGluR8 knockout mice, demonstrating a requirement for mGluR8 in these effects. Overall, AZ12216052 was a poor compound for slice work due to its solubility issues, very mild potentiator effects, and lack of selectivity between mGluR4/8.

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