

MODULATION OF BASAL GANGLIA FUNCTION BY METABOTROPIC  
GLUTAMATE RECEPTORS

By

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## LIST OF ABBREVIATIONS

2R,4R-APDC	(2R,4R)-4-Aminopyrrolidine-2,4-dicarboxylate
6-OHDA	6-hydroxydopamine
ACSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
AMPA	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMN082	N <sup>1</sup> ,N <sup>2</sup> -dibenzhydriethane-1,2-diamine
ANOVA	analysis of variance
APD	antipsychotic drug
BDNF	brain-derived neurotrophic factor
BHK	baby hamster kidney
BINA	biphenyl-indanone A
BLA	basolateral amygdala
BP	binding potential
cAMP	cyclic adenosine monophosphate
CDPPB	3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide
CHO	Chinese hamster ovary
CHPG	(RS)-2-chloro-5-hydroxyphenylglycine
CNS	central nervous system
COMT	catechol-O-methyl transferase
CRD	cysteine-rich domain
CPPG	(RS)- $\alpha$ -Cyclopropyl-4-phosphonophenylglycine
CPPHA	N-[4-chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)methyl]phenyl]-2-hydroxybenzamide
CRC	concentration response curve

CREB	cAMP-responsive element binding protein
DA	dopamine
DARPP-32	dopamine- and cAMP-regulated phosphoprotein
DCG-IV	(2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine
DCPG	(S)-3,4-dicarboxyphenylglycine
DHPG	(S)-3,5-dihydroxyphenylglycine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DOPAC	3,4-dihydroxyphenylacetic acid
EGFR	epidermal growth factor receptor
EPN	entopeduncular nucleus
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FLA	forelimb asymmetry
FMRP	fragile X mental retardation protein
GABA	$\gamma$ -aminobutyric acid
GIRK	G protein coupled inwardly rectifying potassium
GPCR	G protein-coupled receptor
GPe	globus pallidus, external segment
GPi	globus pallidus, internal segment
GSK-3	glycogen synthase kinase-3
HEK	human embryonic kidney
HPLC	high performance liquid chromatography
HTS	high-throughput screening

HVA	homovanillic acid
icv	intracerebroventricular
im	intramuscular
ip	intraperitoneal
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
L-AP4	L-(+)-2-Amino-4-phosphonobutyric acid
L-DOPA	L-3,4-dihydroxyphenylalanine (levodopa)
LID	L-DOPA-induced dyskinesia
L-SOP	L-serine-O-phosphate
LTD	long term depression
LTP	long term potentiation
LY341495	2S-2-amino-2-(1S,2S-2-carboxycyclopropyl-1-yl)-3-(xanth-9-yl)propanoic acid
LY354740	2-aminobicyclo[3.1.0]hexane 2,6-dicarboxylate
LY367385	(+)-2-Methyl-4-carboxyphenylglycine
LY379268	(1R,4R,5S,6R)-4-Amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid
MAO	monoamine oxidase
MAP kinase	mitogen activated protein kinase
MCPG	(S)- <i>a</i> -methyl-4-carboxyphenylglycine
mGluR	metabotropic glutamate receptor
MPEP	2-methyl-6-(phenylethynyl)-pyridine
mPFC	medial prefrontal cortex
MTEP	3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine
MPP+	1-methyl-4-phenyl-2,3,-dihydropyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MSN	medium spiny neuron
mTOR	mammalian target of rapamycin
NAM	negative allosteric modulator
NMDA	N-methyl-D-aspartate
PAM	positive allosteric modulator
PET	positron emission tomography
PD	Parkinson's disease
PHCCC	N-phenyl-7-(hydroxylimino)cyclopropa[b]chromen-1a-carboxamide
PI3K	phosphatidylinositol 3-kinase
PIKE	phosphatidylinositol 3-kinase enhancer
PKA	protein kinase A
PKC	protein kinase C
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PSP	progressive supranuclear palsy
Ro25-6981,	( $\alpha R, \beta S$ )- $\alpha$ -(4-hydroxyphenyl)- $\beta$ -methyl-4-(phenylmethyl)-1-piperidinepropanol
ROI	region of interest
RTK	receptor tyrosine kinase
sc	subcutaneous
SC-CA1	Schaffer collateral-CA1
SNC	substantia nigra pars compacta
SNr	substantia nigra pars reticulata
SSRI	selective serotonin reuptake inhibitor
STN	subthalamic nucleus
TAC	time-activity curve

TDZD	thiadiazolidine
TDZD-8	4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione
TGF $\beta$	transforming growth factor $\beta$
TVC	third ventricle cannulated
VFD	Venus flytrap domain
VMAT	vesicular monoamine transporter
VU0092273	1-{{4-(2-phenylethynyl)phenyl}carbonyl}piperidin-4-ol
VU0155041	( $\pm$ )- <i>cis</i> -2-(3,5-Dichlorophenylcarbamoyl)cyclohexanecarboxylic acid
VU0360172	<i>N</i> -cyclobutyl-6-{{3-fluorophenyl}ethynyl}nicotinamide hydrochloride

## CHAPTER I

### INTRODUCTION

#### Glutamate receptors

Fast excitatory neurotransmission in the mammalian central nervous system (CNS) is primarily mediated by ionotropic glutamate receptors, a class of multimeric ligand-gated cation channels that have been categorized into three groups based on their ligand sensitivity (see Dingledine et al., 1999 for comprehensive review) (Dingledine et al., 1999). The multimeric amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are composed of different combinations of four subunits, GluA1-4 (also known as GluR1-4). N-methyl-D-aspartate (NMDA) receptors are composed of combinations of subunits GluN1 (NR1), GluN2A-D (NR2A-D), and GluN3 (NR3); these receptors require glycine or D-serine as a coagonist. Finally, the kainate receptors are encoded by at least two gene families and are composed of multimers of the GluK1-5 (GluR5-7 and KA1-2) subunits.

While glutamate was originally thought to elicit all of its actions through activation of ionotropic glutamate receptors, the discovery that amino acid analogs such as L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4) could reduce synaptic transmission via a presynaptic mechanism (Harris and Cotman, 1983; Koerner and Cotman, 1981), and that glutamate and quisqualate could stimulate phosphoinositide hydrolysis in the striatum in a manner that depended on a coupling to heterotrimeric GTP-binding proteins (G proteins) (Sladeczek et al., 1985), led to the identification of another class of glutamate receptors called metabotropic glutamate receptors (mGluRs). This discovery marked a major shift in thinking regarding the roles of glutamate in the CNS; in addition



to mediating fast excitatory transmission, it could also serve as a neuromodulator and produce effects that more closely resembled those of monoamines and acetylcholine.

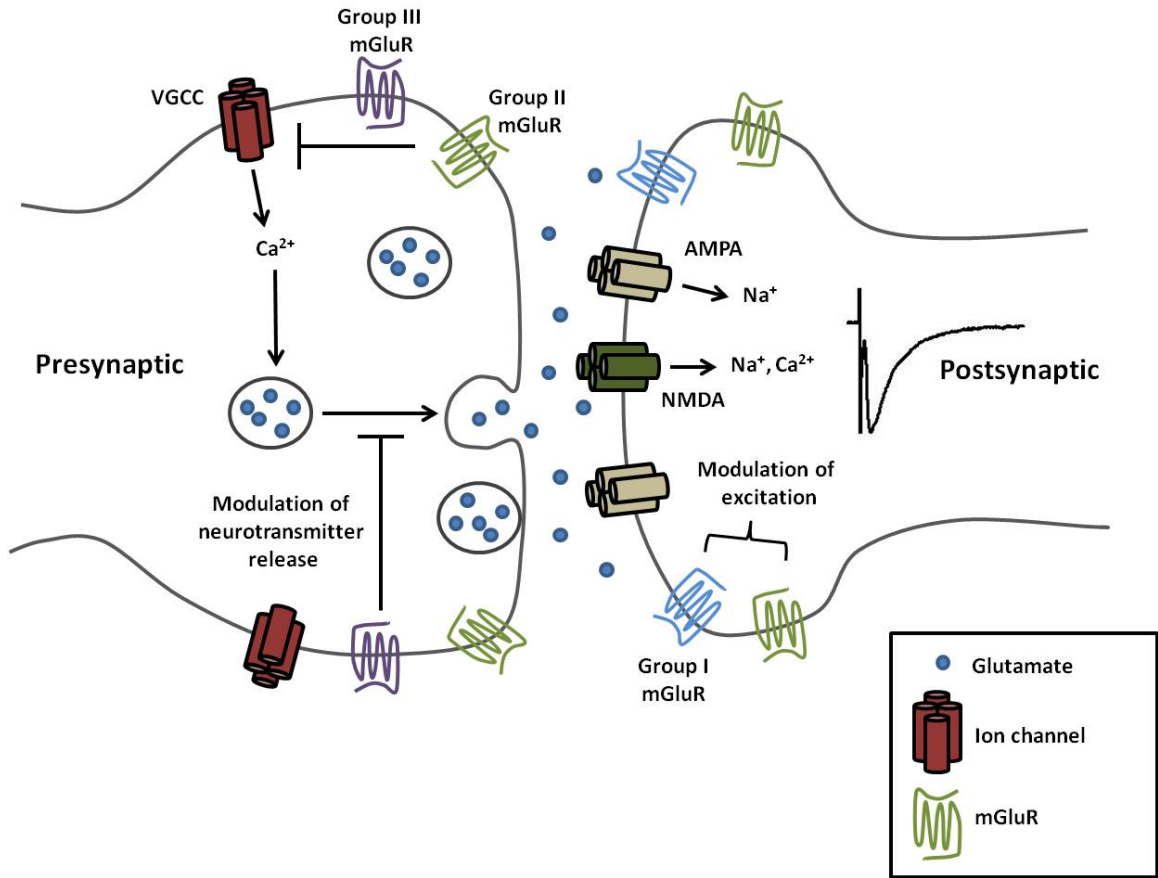
### **Classification of mGluRs**

In the 1990's, eight distinct mGluR subtypes were cloned (reviewed in (Conn and Pin, 1997) and classified into three groups based on sequence homology, signal transduction pathways, and sensitivity to synthetic ligands (Table 1). Group I mGluRs include mGluR1 and mGluR5, which predominantly couple to  $G\alpha_q$ -containing G proteins and stimulate phosphoinositide hydrolysis via phospholipase C activation, and can also stimulate mitogen-activated protein (MAP) kinase cascades and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway. Group II mGluRs include mGluR2 and mGluR3, which primarily couple to the pertussis toxin-sensitive  $G\alpha_{i/o}$  class of G proteins and inhibit adenylyl cyclase; group II mGluRs also modulate ion channel function via  $G\beta\gamma$  subunits. Group III mGluRs include mGluR4, mGluR6, mGluR7, and mGluR8, which have G protein-coupling and signal transduction profiles similar to those of group II mGluRs. In addition to differential G protein-coupling and signaling pathway activation, different groups of mGluRs also vary in their expression patterns (reviewed in (Niswender and Conn, 2010). With the exception of mGluR6 and mGluR8, mGluRs are widely expressed in many types of neurons, and mGluR1 and mGluR4 are particularly enriched in the cerebellum. mGluR8 expression is lower and more restricted than mGluR4, and mGluR7, and mGluR6 is exclusively expressed in ON-bipolar cells in the retina. In addition to expression in neurons, mGluR3 and mGluR5 are expressed in astrocytes. Group I mGluRs are primarily expressed postsynaptically, where they can modulate excitability, regulate ionotropic glutamate receptor function, and in some cases produce direct excitation of neurons (Figure 1). Group II mGluRs are historically considered presynaptic receptors, but increasing numbers of postsynaptic effects of

**Table 1. mGluR groups, splice variants, expression patterns, and common downstream effector proteins.**

<b>Group</b>	<b>Receptor, splice variants</b>	<b>CNS expression pattern</b>	<b>Synaptic localization</b>	<b>Common signaling pathways</b>
Group I	mGluR1 a-e	Widespread in neurons	Primarily postsynaptic	Phospholipase C stimulation; MAP kinase phosphorylation; Akt activation
	Taste mGluR1	Taste buds		
	mGluR5 a,b	Widespread in neurons and astrocytes		
Group II	mGluR2	Widespread in neurons	Presynaptic and postsynaptic	Inhibition of adenylyl cyclase; ion channel modulation
	mGluR3 GRM3Δ2 GRM3Δ4 GRM3Δ2Δ3	Widespread in neurons and astrocytes		
	mGluR4	Widespread in neurons; high in cerebellum		
	Taste mGluR4	Taste buds		
Group III	mGluR6 a-c	Retina	Postsynaptic in ON-biopolar retinal cells	Stimulation of cGMP phosphodiesterase (mGluR6)
	mGluR7 a-e	Widespread in neurons	Presynaptic active zone	
	mGluR8 a-c	Lower and more restricted expression in neurons (compared with mGluR4 and mGluR7)	Primarily presynaptic	

Adapted from Niswender and Conn, 2010.



**Figure 1. Schematic of mGluR localization and functions at glutamatergic synapses.** Group I mGluRs are predominantly postsynaptic, where they regulate neuronal excitability by modulating ion channel function. In addition, they activate signaling pathways that can regulate gene transcription, protein trafficking, and a variety of other cellular events, including production of retrograde signaling molecules. Group II and group III mGluRs are often found presynaptically, where they reduce neurotransmitter release through effects on ion channels and vesicle fusion machinery. In addition, group II mGluRs have been found to act postsynaptically as well.

group II mGluRs have been identified. Conversely, group III mGluR-mediated effects are typically presynaptic. When expressed presynaptically, group II and III mGluR activation inhibits neurotransmitter release via mechanisms including inhibition of voltage-gated calcium channels and G $\beta$  $\gamma$ -mediated inhibition of synaptic vesicle fusion machinery (Figure 1). Group II and III mGluRs can act as autoreceptors at glutamatergic synapses, but also act as heteroreceptors to modulate the release of  $\gamma$ -aminobutyric acid (GABA) and neuromodulators such as dopamine and acetylcholine.

### **Structure of mGluRs**

mGluRs belong to the class C group of G protein-coupled receptors (GPCRs) . Like all GPCRs, they contain seven transmembrane-spanning  $\alpha$ -helical domains (heptahelical domain) and an intracellular C terminus domain that is important for G protein coupling and is regulated by alternative splicing, protein-protein interactions, and phosphorylation (Niswender and Conn, 2010). The structure of mGluRs is similar to other class C GPCRs such as GABA<sub>B</sub> receptors, calcium-sensing receptors, pheromone receptors, and taste receptors (Pin et al., 2003). Class C GPCRs are distinguished by their large extracellular N-terminal domain; in mGluRs, this domain is commonly referred to as the Venus flytrap domain (VFD) and contains the highly conserved glutamate-binding site. Binding of glutamate to the VFD induces conformation changes that are propagated via the cysteine-rich domain (CRD) through the heptahelical domain to produce intracellular G protein activation (Niswender and Conn, 2010). mGluRs can also be regulated by binding of divalent cations (magnesium and calcium) to the VFD and phosphorylation of the second intracellular loop. mGluRs are known to function as constitutive homodimers (Pin et al., 2003), although recent evidence suggests that certain mGluR pairs can form functional heterodimers as well (Doumazane et al., 2011).

## Pharmacology of mGluRs

Identification of various ligands that act as agonists, antagonists, or allosteric modulators of specific subtypes of mGluRs has allowed major advances in our understanding of specific roles of mGluRs in various brain regions (for review, see Niswender and Conn, 2010). Group I mGluRs are selectively activated by the orthosteric agonist (S)-3,5-DHPG, which activates mGluR1 and mGluR5 with similar potencies (see Table 2 for a list of selected mGluR ligands with full chemical names). The closely related compound CHPG is a selective orthosteric agonist of mGluR5, and has demonstrated some utility for differentiation of mGluR5 vs. mGluR1-mediated effects despite its very low potency. An orthosteric antagonist of mGluR1 and mGluR5 called MCPG is also relatively weak but has been useful for confirming mGluR-mediated effects in brain slices. More recently, the mGluR1-selective orthosteric antagonist LY367385 was identified; this compound represents one of the few examples of an mGluR subtype-selective orthosteric ligand. A number of group II mGluR-selective orthosteric ligands including DCG-IV, LY354740, and LY379268 have been particularly useful for identifying synapses at which group II mGluRs act as presynaptic modulators of neurotransmitter release, and LY354740 and LY379268 are very potent and can also be administered systemically in animals. The orthosteric antagonist LY341495 is a group II mGluR-preferring antagonist, and can be used to selectively block group II mGluR-mediated effects at sub-micromolar concentrations; however, at higher concentrations, this compound also inhibits glutamate binding at group III mGluRs, and weakly at group I mGluRs. The classic orthosteric agonist used to evaluate group III mGluR-mediated effects is L-AP4. This compound activates mGluR4, 6, and 8, and at much higher (close

**Table 2. List of selected mGluR ligands.**

Nonselective ligands			
Compound	Chemical name	Target	Activity
L-glutamate	(S)-1-aminopropane-1,3,-dicarboxylic acid	Group I/Group II>Group III; ionotropic glutamate receptors	Endogenous agonist
(1S,3R)-ACPD	(1S,3R)-1-aminocyclopentane-1,3,-dicarboxylic acid	Group I/Group II>Group III	Orthosteric agonist
LY341495	(2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid	Group II>Group III>Group I	Orthosteric antagonist
Group I-preferring ligands			
(S)-3,5,-DHPG	(S)-3,5-dihydroxyphenylglycine	mGluR1/5	Orthosteric agonist
L-quisqualate	(L)-(+)-a-amino-3,5-dioxo-1,2,4-oxadiazolidine-2-propanoic acid	mGluR1/5; ionotropic glutamate receptors	Orthosteric agonist
CHPG	(RS)-2-chloro-5-hydroxyphenylglycine	mGluR5	Orthosteric agonist
(S)-MCPG	(S)-a-methyl-4-carboxyphenylglycine	Group I/Group II	Orthosteric antagonist
LY367385	(S)-(+)-a-amino-4-carboxy-2-methylbenzeneacetic acid	mGluR1	Orthosteric antagonist
CPCCOEt	7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester	mGluR1	NAM
Bay 36-7620	(3aS,6aS)-hexahydro-5-methylene-6a-(2-naphthalenylmethyl)-1H-cyclopenta[c]furan-1-one	mGluR1	NAM
MPEP	2-methyl-6-(phenylethynyl)pyridine	mGluR5/mGluR4	NAM/PAM
MTEP	3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine	mGluR5	NAM
Ro 67-7476	(S)-2-(4-fluorophenyl)-1-(toluene-4-sulfonyl)pyrrolidine	mGluR1	PAM
DFB	[(3-fluorophenyl)methylene]hydrazone-3-fluorobenzaldehyde	mGluR5	PAM
CPPHA	N-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide	mGluR5	PAM
CDPPB	3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide	mGluR5	PAM
VU29	4-nitro-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide	mGluR5	PAM
ADX47273	[S-(4-fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]oxadiazol-5-yl]-piperidin-1-yl]methanone]	mGluR5	PAM
VU0092273	1-[[4-(2-phenylethynyl)phenyl]carbonyl]piperidin-4-ol	mGluR5/mGluR3	PAM/NAM
VU0360172	N-cyclobutyl-6-[(3-fluorophenyl)ethynyl]nicotinamide hydrochloride	mGluR5	PAM
Group II-preferring ligands			
DCG-IV	(2S,2'R,3'R)-2-(2',3')-dicarboxycyclopropylglycine	mGluR2/3	Orthosteric agonist
(2R,4R)-APDC	(2R,4R)-4-aminopyrrolidine-2,4-dicarboxylate	mGluR2/3	Orthosteric agonist
LY354740	(1S,2S,5R,6S)-2-aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid	mGluR2/3	Orthosteric agonist
LY379268	(1R,4R,5S,6R)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid	mGluR2/3	Orthosteric agonist
BINA	Biphenyl-indanone A	mGluR2	PAM
Group III-preferring ligands			
L-AP4	L-(+)-2-amino-4-phosphonobutyric acid	mGluR4,6,8>mGluR7	Orthosteric agonist
(S)-3,4-DCPG	(S)-3,4-dicarboxyphenylglycine	mGluR8>mGluR4/6	Orthosteric agonist
CPPG	(RS)-a-cyclopropyl-4-phosphonophenylglycine	Group III	Orthosteric antagonist
MAP4	(S)-2-amino-2-methyl-4-phosphonobutanoic acid	Group III	Orthosteric antagonist
PHCCC	N-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide	mGluR4	PAM
AMN082	N,N'-Bis(diphenylmethyl)-1,2-ethanediamine	mGluR7	Allosteric agonist

Adapted from Niswender and Conn, 2010.

to millimolar) concentrations, mGluR7. (S)-3,4-DCPG is an mGluR8-preferring agonist that has been useful for identifying some effects of mGluR8 (Thomas et al., 2001), although it does activate mGluR4 and mGluR7 at high concentrations, so care must be used in the interpretation of results obtained with this compound. While this list is by no means exhaustive, it provides examples of key compounds that have been used to identify various groups of mGluRs as key modulators of synaptic transmission in various brain regions, and has allowed preliminary evaluation of the therapeutic potential of targeting various groups of mGluRs.

While orthosteric agonists and antagonists have allowed some elucidation of the mGluR subtypes that mediate various physiological effects, the high level of conservation of the glutamate binding site among the various mGluR subtypes has made identification of highly subtype-selective compounds very difficult. In addition, because many of them are amino acid analogs, they have polar groups that often exclude them from crossing the blood-brain barrier, limiting their utility for *in vivo* studies. For this reason, more recent drug discovery efforts have been focused on allosteric modulators of mGluRs, which bind to sites on the receptor other than the glutamate binding site (typically somewhere within the heptahelical domain) and modulate the ability of the receptor to respond to glutamate or other orthosteric agonists. Positive allosteric modulators (PAMs) enhance the ability of the endogenous agonist, in this case glutamate, to activate the receptor. This can be achieved by either increasing the affinity of the endogenous ligand or increasing agonist-induced coupling to G protein activation; the latter is thought to be the case for most mGluR PAMs, although a lack of high-affinity radioligands that bind to the glutamate site of mGluRs has made thorough evaluation of these mechanisms difficult. In some cases, PAMs also exhibit allosteric agonist activity, meaning that they have some intrinsic efficacy in the absence of glutamate binding. Negative allosteric modulators (NAMs) bind to sites on the receptor other than the

binding site for the endogenous ligand and noncompetitively inhibit the ability of the endogenous ligand or other orthosteric agonists to activate the receptor. In contrast to the lack of success seen with efforts to identify subtype-selective orthosteric compounds, a growing number of subtype-selective allosteric ligands are being reported; the ability to achieve selectivity with allosteric compounds is due to the fact that the binding sites that these compounds target are in less conserved regions of the receptor.

In recent years, major advances have been made in the development of subtype-selective PAMs and NAMs (for a list of selected compounds, see Table 2). Discovery and pharmacological characterization of a novel mGluR4 PAM, VU0155041, is described in Chapter II. While efforts to discover selective allosteric modulators of mGluR1, mGluR2, mGluR4, and mGluR5 have been very successful, allosteric modulators of mGluR3, mGluR7, and mGluR8 that are highly selective and useful for *in vivo* studies are still lacking, and represent areas of ongoing medicinal chemistry efforts. The discovery of highly selective PAMs of mGluR4 and mGluR5 has allowed major advances in our understanding of these receptors as therapeutic targets for CNS disorders (see the following sections for further discussion). Future identification of allosteric modulators of mGluR3, mGluR7, and mGluR8 will provide powerful tools to elucidate previously unidentified roles of these receptor subtypes, as well as their therapeutic potential for treating various neurological and psychiatric disorders.

### **Regulation of basal ganglia motor circuit function by mGluRs: relevance to Parkinson's disease**

Parkinson's disease (PD) is a chronic neurodegenerative disorder affecting up to three percent of people aged sixty-five and over worldwide (Lang and Lozano, 1998; Moghal et al., 1994; Zhang and Roman, 1993). PD is characterized by motor symptoms including bradykinesia, tremor, rigidity, postural instability, and gait disturbances, as well



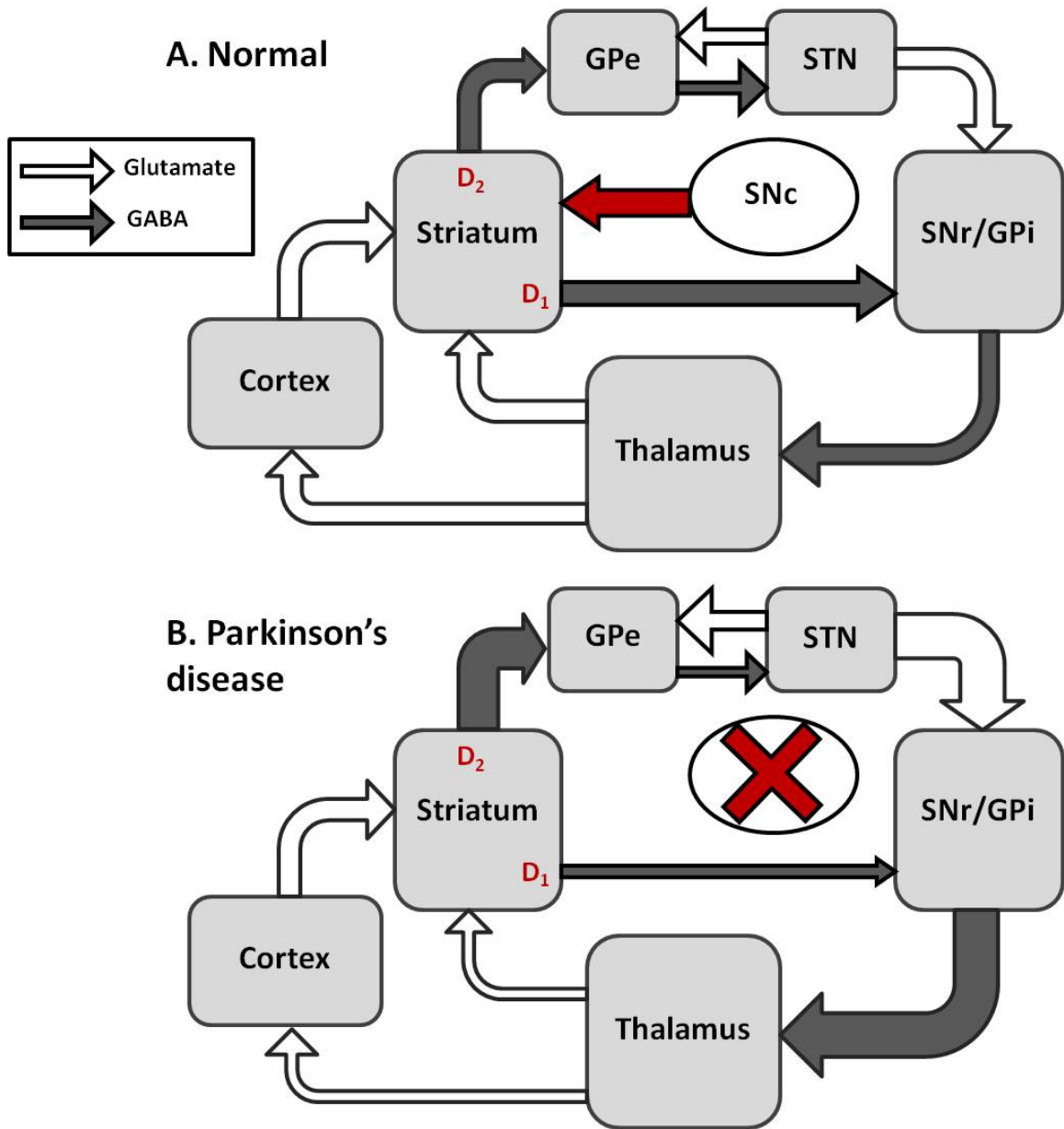
as nonmotor symptoms such as sleep disturbance, lower urinary tract symptoms, and cognitive impairment (Blackett et al., 2009; Jankovic, 2008). The primary pathology giving rise to motor impairments is the degeneration of midbrain dopaminergic neurons in the substantia nigra pars compacta (SNc), which provide dopaminergic input to the striatum and other basal ganglia nuclei under normal conditions. The loss of dopaminergic innervation of the striatum causes major physiological disruptions in the basal ganglia-thalamocortical motor circuit, which plays a critical role in controlling motor activity (DeLong and Wichmann, 2007). Dopamine replacement therapies, including the dopamine precursor levodopa (L-3,4-dihydroxyphenylalanine, L-DOPA) and dopamine receptor agonists are the primary pharmacological agents used for symptomatic treatment of PD (Chen and Swope, 2007). While these treatments provide relief of motor symptoms for several years in most patients, motor complications and psychiatric side effects develop in a subset of individuals, and end-of-dose “wearing off” of the drug effects limits their long-term efficacy (Chen and Swope, 2007; Stacy and Galbreath, 2008). Adjunct therapies such as monoamine oxidase (MAO) inhibitors, catechol-O-methyl transferase (COMT) inhibitors, and anticholinergic drugs modestly improve the efficacy and tolerability of levodopa therapy, but highly effective and tolerable pharmacological treatments for the late-stage motor symptoms of PD have not yet been identified (Chen and Swope, 2007). Moreover, no currently employed pharmacological agents slow the progressive neurodegeneration that ultimately worsens PD symptoms. The lack of disease-modifying therapeutics for PD is largely due to the current deficit of knowledge of the cellular events underlying SNc degeneration. The limitations of current pharmacological treatments for PD highlight the need for nondopaminergic therapeutic strategies for both symptom treatment and disease modification. Fortunately, advances in our understanding of the anatomy and function of the basal ganglia circuitry have opened the door for identification of novel therapeutic strategies for treating PD and

slowing its progression (for review, see DeLong and Wichmann, 2007). Because metabotropic glutamate receptors modulate synaptic transmission throughout the basal ganglia motor circuit, and pharmacological manipulation of these receptors can alter both normal and aberrant neurotransmission such as that observed in the parkinsonian brain, glutamate receptors have been suggested as promising therapeutic targets for treating PD.

Because the primary pathology underlying Parkinson's disease is the selective loss of dopamine neurons of the substantia nigra pars compacta, the significance of dopaminergic modulation of mGluR function in the basal ganglia has become increasingly appreciated. Importantly, changes in mGluR function in the absence of dopamine could impact the therapeutic potential of drugs targeting these receptors. In addition, aberrant mGluR function in the absence of dopamine could contribute to the changes in neurotransmission underlying the pathogenesis of PD symptoms. This section will explore the physiological roles of mGluRs in the basal ganglia motor circuit, changes in mGluR expression and function in the absence of dopaminergic modulation, regulation of dopaminergic transmission by mGluRs, and the significance of mGluR-dopamine interactions to Parkinson's disease pathophysiology and therapeutics.

### **Neurotransmission in the basal ganglia motor circuit is altered in PD**

The flow of information through the basal ganglia involves two major pathways that have opposing effects of basal ganglia output (Wichmann and DeLong, 1996). The primary input nucleus of the basal ganglia is the striatum, which receives excitatory projections from cortical regions including the primary motor cortex, as well as the thalamus (Figure 2). The striatal neuron population primarily consists of medium spiny neurons (MSNs), which are projection neurons that use GABA as their neurotransmitter.



**Figure 2. Schematic of basal ganglia circuitry.** A simplified, schematic diagram of basal ganglia circuitry under normal circumstances (A) and following loss of dopaminergic projections in Parkinson's disease (B). White arrows indicate glutamatergic projections, gray arrows indicate GABAergic projections, and the red arrow indicates dopaminergic projections. The relative size of arrows in panel B represents increases and decreases in neurotransmission at various synapses in Parkinson's disease. For clarity, some known, less prominent connections between basal ganglia nuclei are not depicted here.

These projection neurons relay information to the output nuclei through direct projections as well as indirectly through the globus pallidus external segment (GPe; GP in rodents) and the subthalamic nucleus (STN). The substantia nigra pars reticulata (SNr) and the internal globus pallidus (GPi, entopeduncular nucleus (EPN) in rodents) are the major output nuclei of the basal ganglia. The direct pathway exerts inhibitory control over the activity of these nuclei, whereas activity in the indirect pathway disinhibits excitatory STN neurons, providing increased excitation of the SNr and GPi. The output nuclei in turn provide information to the cortex via inhibitory projections to the thalamus. The balance of inhibitory control exerted by the direct pathway and excitatory control provided by the indirect pathway are thought to be crucial for maintaining appropriate control of motor activity. Although this is a highly simplified model of basal ganglia connectivity, it provides a valuable model for understanding the neuromodulatory roles of mGluRs and dopamine, as well as the changes in neurotransmission that are thought to underlie motor symptoms of PD such as tremor, bradykinesia, and muscle rigidity.

Dopaminergic neurons of the SNc primarily project to the striatum, but also modulate the activity of other key nuclei of the basal ganglia (Smith and Kieval, 2000). Simplistically, MSNs that project directly to the output nuclei primarily express D1 receptors, whereas MSNs that give rise to the indirect pathway primarily express D2 receptors, allowing nigrostriatal dopamine to increase activity through the direct pathway and decrease activity of the indirect pathway. In PD, the loss of dopaminergic modulation of striatal MSNs therefore causes increased activity of the indirect pathway relative to the direct pathway (Figure 2). The increased activity of the indirect pathway causes disinhibition of the STN, resulting in increased burst firing of STN neurons and an increase in synaptic excitation of projection neurons in the output nuclei (Bergman et al., 1994; Liu et al., 2002b; Ni et al., 2001a; Ni et al., 2001b; Vila et al., 2000). This

pathological increase in activity and concurrent changes in firing patterns in the indirect pathway are thought to contribute to the motor symptoms observed in PD patients.

While this simplified model of the basal ganglia has proven useful for making predictions about the therapeutic potential of novel targets for treating PD symptoms, recent studies have shown that the interconnections between basal ganglia nuclei are more complicated. For example, information flows through the indirect pathway via routes other than the major route described above (Parent et al., 2001; Smith et al., 1998; Yelnik, 2002). In addition, the STN sends major excitatory projections to the output nuclei of the basal ganglia and also projects to the striatum and the GPe, creating a reciprocal circuit between the GPe and STN (Parent et al., 2001; Smith et al., 1998; Yelnik, 2002). While the SNc primarily sends dopaminergic projections to the striatum, it also innervates the other key nuclei of the basal ganglia to varying extents (Smith and Kieval, 2000), and the loss of these projections in PD must also be considered. While much attention has been focused on the effects of removing dopaminergic modulation from the striatum, the loss of dopamine in other basal ganglia structures, particularly the STN, may contribute to PD symptoms as well (Blandini et al., 2000). In the context of this more complex view of basal ganglia organization and pathophysiology, it has been hypothesized that in addition to imbalance between activity of the direct and indirect pathways, complex changes in neuronal firing patterns and oscillatory activity may contribute to the pathogenesis of PD symptoms, particularly tremor (see Heimer et al., 2006, and Leblois et al., 2006) (Heimer et al., 2006; Leblois et al., 2006a; Leblois et al., 2006b). It is therefore possible that predictions about novel therapeutic targets based on the more simplified model may not be entirely valid, and unexpected results in preclinical studies may in some cases be attributed to these limitations. Nonetheless, the classical model of changes in basal ganglia function in PD has played a critical role in the identification of many types of receptors as novel targets for symptom treatment and

disease modification, and has led to many successful preclinical studies implicating glutamate receptors as promising targets for PD treatment.

### **Mechanisms underlying dopamine neuron degeneration**

Despite extensive investigation, the cellular mechanisms underlying the degeneration of midbrain dopaminergic neurons in PD are not well understood. Insight from genetic and toxin-based animal models suggests that oxidative stress, mitochondrial dysfunction, aberrant processing of proteins by the ubiquitin-proteasome system, inflammation, and activation of apoptotic pathways all play roles in dopaminergic cell death (reviewed in Betarbet et al., 2005; Gandhi and Wood, 2005; Olanow, 2007; Przedborski, 2005). However, there is also evidence that the process of dopaminergic cell death may not be entirely cell autonomous, and that increased glutamatergic transmission may contribute an excitotoxic component to the barrage of cellular insults that lead to degeneration in the SNc (Przedborski, 2005). Increased STN activity may lead to excessive excitation of remaining SNc neurons, leading to NMDA receptor-mediated excitotoxicity. This putative excitotoxic insult may contribute to the progressive loss of these neurons and concurrent worsening of motor symptoms. In agreement with this model, lesions of the STN reduce 6-OHDA-induced degeneration of SNc neurons in rats (Carvalho and Nikkhah, 2001). In addition, intranigral infusion or systemic administration of NMDA receptor antagonists such as MK-801 provides protection against the SNc degeneration induced by intranigral or intrastriatal MPP<sup>+</sup> administration in rats (Srivastava et al., 1993; Turski et al., 1991). Similarly, NMDA receptor antagonists have been shown to reduce nigrostriatal toxicity caused by systemic 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) administration in mice and primates (Chan et al., 1993; Lange et al., 1993; Zuddas et al., 1992), although some investigators have failed to observe this effect (Chan et al., 1997; Sonsalla et al., 1992). In the 6-

hydroxydopamine (6-OHDA) model of nigrostriatal degeneration in rats, continuous systemic administration of MK-801 reduces cell death in the SNc (Armentero et al., 2006). Taken together, these results support the hypothesis that NMDA receptor activation in the SNc contributes to neurodegeneration in PD, and that reducing increased excitatory transmission at STN-SNc synapses could be a useful strategy for slowing disease progression.

### **Animal models of PD provide tools for assessing the therapeutic potential of novel targets**

Animal models of PD provide important tools for evaluating the ability of novel drugs to reverse motor symptoms and slow the progressive neurodegeneration associated with the disease. The current discussion of animal models is focused on those used for studies reviewed in this chapter and subsequently used in the studies that are described in Chapters II and III, and is not an exhaustive overview of all animal models of PD. While there are several genetic models that are based upon specific gene mutations observed in familial cases of PD, these models often fail to recapitulate aspects of the human disease such as dopamine neuron degeneration and severe motor deficits, and have proven more useful for studying the pathogenesis of familial PD on a cellular level rather than for evaluating novel therapeutic strategies (Orth and Tabrizi, 2003; Terzioglu and Galter, 2008). Due to the drawbacks of these models, they are not particularly useful for assessing the therapeutic potential of novel drugs targeting glutamate receptors.

Evaluation of novel targets for symptomatic treatment of PD often relies on pharmacological and toxin-based models of PD in mice, rats, and nonhuman primates, which are generally reliable for producing parkinsonian motor symptoms (Betarbet et al., 2002). Pharmacological models are commonly used for studying the therapeutic

potential of compounds targeting glutamate receptors because they are convenient and relatively inexpensive. Pharmacological agents used to induce PD symptoms include neuroleptics such as the mixed D1/D2 dopamine receptor antagonist haloperidol, which induces aspects of parkinsonism such as muscle rigidity, akinesia, and catalepsy (Gerlach and Riederer, 1996). Reversal of these effects can be measured using techniques such as electromyography, monitoring of locomotor activity, and measurement of catalepsy (Gerlach and Riederer, 1996). Another pharmacological tool widely used to assess novel antiparkinsonian agents is reserpine, which depletes catecholamines by inhibiting their uptake into presynaptic vesicles by the vesicular monoamine transporter (VMAT) (Hornykiewicz, 1966). Reserpine produces profound akinesia in rodents, and reversal of this akinesia, which is usually assessed by measuring locomotor activity, is thought by some to be predictive of antiparkinsonian efficacy (Betarbet et al., 2002; Gerlach and Riederer, 1996). Because there is a lack of standardization of the use of these models by different investigators, it is possible that similar pharmacological models will yield different results due to different methodologies. It is therefore important that careful attention be paid to methods when interpreting and comparing results from these models. In addition, it is important to bear in mind that pharmacological agents that induce parkinsonism in rodents are reversible and do not recapitulate the morphological aspects of the disease, and therefore may not accurately mimic all aspects of the disease caused by SNc degeneration.

Toxin-based models of PD are useful for studying therapeutic strategies for both motor symptom treatment and neuroprotection (for review, see Betarbet et al., 2002; Schober, 2004; Terzioglu and Galter, 2008), and have been used extensively for studying the disease-modifying potential of novel compounds that act on glutamate receptors. The dopaminergic neuron-selective neurotoxin MPTP is used in mice and primates to study the cellular mechanisms underlying dopaminergic cell death. MPTP



can be administered systemically, but must be converted in the brain to the active metabolite 1-methyl-4-phenyl-2,3-dihydropyridinium ion (MPP<sup>+</sup>) by MAO-B. MPP<sup>+</sup> is selectively transported into dopaminergic nerve terminals by the dopamine transporter (Javitch et al., 1985), and causes neurodegeneration by disrupting mitochondrial function (Tipton and Singer, 1993). In primates, MPTP administration closely recapitulates the behavioral features of PD, and is considered the most predictive model for antiparkinsonian efficacy of novel drugs in humans (Gerlach and Riederer, 1996). Protection against MPTP-induced nigrostriatal lesion in mice is also a widely used assay for possible disease-modifying therapeutics for PD (Betarbet et al., 2002). Another commonly used neurotoxin is 6-OHDA, which causes selective degeneration of midbrain dopamine neurons when directly infused into the striatum, nigrostriatal tract, or SNc of rats (Deumens et al., 2002; Simola et al., 2007). Unilateral 6-OHDA lesion causes lateralized motor behaviors in rats such as circling behavior and forelimb-use asymmetry, and novel therapeutics can be evaluated for their ability to reverse these effects (Lundblad et al., 2002). Partial bilateral 6-OHDA lesions cause akinetic deficits in reaction time tasks, and the reversal of these deficits may represent possible antiparkinsonian effects in earlier stages of PD (Amalric et al., 1995). Protection against 6-OHDA-induced nigrostriatal damage is also a method for evaluating possible disease-modifying agents. While there is no animal model of PD that perfectly recapitulates the human disease, these models provide useful tools for assessing the therapeutic potential of new targets and compounds.

### **Rationale for targeting mGluRs for PD treatment**

Current therapeutic strategies for treating the motor symptoms of PD primarily rely on dopamine replacement with drugs such as levodopa (L-DOPA) (Chen and Swope, 2007). Unfortunately, long-term use of these drugs eventually results in loss of

efficacy and severe adverse effects such as dyskinesias. These drawbacks have led to extensive efforts to identify alternative treatment options for PD, including highly successful surgical interventions such as deep brain stimulation of the STN or GPi (Walter and Vitek, 2004; Wichmann and DeLong, 2006).

Recent studies have shown that mGluRs modulate synaptic transmission at every major synapse in the basal ganglia motor circuit (Conn et al., 2005), suggesting that these GPCRs are important for normal basal ganglia function, and may provide therapeutic targets for PD. With the exception of mGluR6, expression of all subtypes of mGluRs has been detected in various basal ganglia nuclei, and recent studies have identified many physiological roles for these receptors. Because drugs targeting mGluRs can modulate neurotransmission at key basal ganglia synapses that are overactive in PD, many mGluR ligands have been evaluated in animal models of PD, and several subtypes have been identified as exciting therapeutic targets. Finally, the importance of dopamine in the basal ganglia and the relevance of mGluR function to PD have led to the evaluation of interactions between mGluRs and dopamine in the basal ganglia, revealing many ways in which dopaminergic signaling modulates the activity of mGluRs. The following overview of mGluR function in the basal ganglia highlights the roles of mGluRs in modulating neuronal excitation and synaptic transmission in key basal ganglia nuclei under normal circumstances, interactions between mGluRs and dopamine, and the therapeutic potential of targeting various mGluR subtypes for the treatment of PD.

### **Modulation of basal ganglia neurotransmission by group I mGluRs**

*Group I mGluRs in the striatum.* In the striatum, both mGluR1 and mGluR5 are expressed by all cell types, which include MSNs, cholinergic interneurons, fast-spiking parvalbumin-containing GABA interneurons, and burst firing somatostatin-positive GABA

interneurons (Bell et al., 2002; Tallaksen-Greene et al., 1998). Group I mGluRs can influence MSNs both directly and by modulating the activity of interneurons in the striatum, and mGluR1 and mGluR5 often have distinct roles despite being expressed in the same neurons and coupling to the same signal transduction cascades (reviewed in Bonsi et al., 2008).

Activation of group I mGluRs by the nonselective agonist DHPG potentiates NMDA receptor-mediated currents in MSNs through a mechanism that is dependent on protein kinase C activation (Pisani et al., 1997b; Pisani et al., 2001b). This effect is present in mGluR1 knockout mice but absent in mGluR5 knockout mice, suggesting that mGluR5 exclusively mediates the enhancement of NMDA receptor currents. In cholinergic interneurons, activation of mGluR1 or mGluR5 raises intracellular calcium levels and induces membrane depolarization by inhibiting potassium conductance (Bonsi et al., 2005; Calabresi et al., 1999; Pisani et al., 2001a; Takeshita et al., 1996), resulting in an increase in acetylcholine release (Marti et al., 2001). Acetylcholine can then activate M1 muscarinic receptors on MSNs, which can also potentiate NMDA receptor currents (Calabresi et al., 1998), indicating that group I mGluRs can enhance NMDA receptor activity both directly and indirectly. Fast-spiking parvalbumin-containing interneurons also express mGluR1 and mGluR5, and activation of mGluR1 mediates direct excitation of these neurons (Bonsi et al., 2007b).

mGluR1 is also present on dopaminergic terminals in the striatum (Zhang and Sulzer, 2003). Activation of these receptors by glutamate spillover from corticostriatal terminals inhibits striatal dopamine release. Conversely, extrasynaptic dopamine activates dopamine receptors on glutamatergic terminals in the striatum, and activation of these receptors inhibits glutamate release (Cepeda et al., 2001; Wang and Pickel, 2002). Corticostriatal transmission increases in response to nigrostriatal lesions, raising the possibility that following nigrostriatal damage, increased striatal glutamate could

decrease dopamine release from the remaining functional nigrostriatal terminals and worsen the effects of nigrostriatal lesion on basal ganglia neurotransmission.

mGluR1 activation directly mediates glutamatergic transmission in the SNc, resulting in complex effects on membrane properties. Although glutamate receptors are typically thought to mediate excitation of neurons, brief activation of mGluR1 hyperpolarizes SNc neurons (Fiorillo and Williams, 1998). This effect involves increased potassium conductance and depends on intracellular calcium stores. Prolonged activation of mGluR1 depolarizes SNc neurons and activates burst firing (Mercuri et al., 1992; Mercuri et al., 1993).

Interestingly, group I mGluRs cooperate with dopamine receptors to participate in the induction of long-term synaptic plasticity at glutamatergic corticostriatal synapses (reviewed in Gubellini et al., 2004). Plasticity at this synapse is thought to be important for motor learning and habit formation, and is likely disrupted in pathological states such as PD (Calabresi et al., 2009). Induction of long-term depression (LTD) at corticostriatal synapses by high frequency stimulation of cortical afferents requires mGluR1 as well as D1-like and D2-like dopamine receptor activation, dopamine- and cyclic adenosine monophosphate (cAMP)-regulated phosphoprotein (DARPP-32) phosphorylation, and mGluR-mediated retrograde endocannabinoid signaling (Calabresi et al., 2000; Calabresi et al., 1992a; Calabresi et al., 1992b; Calabresi et al., 2007; Calabresi et al., 1994; Choi and Lovinger, 1997; Gerdeman et al., 2002; Gubellini et al., 2001; Sergeeva et al., 2007; Sung et al., 2001). High frequency stimulation fails to induce corticostriatal LTD in slices obtained from 6-OHDA-lesioned rats (Calabresi et al., 1992b), highlighting the critical role of dopamine receptor coactivation in this form of mGluR-dependent synaptic plasticity, and the potential relevance of impaired corticostriatal plasticity to PD.

Under certain conditions, high frequency stimulation can also induce NMDA receptor-dependent long-term potentiation (LTP) of corticostriatal transmission

(Calabresi et al., 1992c; Partridge et al., 2000). While there is evidence that only mGluR1 activation is necessary for LTD induction, both mGluR1 and mGluR5 are involved in the induction of LTP (Gubellini et al., 2003). In addition, D1-like receptor activation is necessary for LTP induction, whereas D2-like receptors negatively modulate LTP (Calabresi et al., 2000; Calabresi et al., 2007). These findings highlight the importance of mGluR-dopamine interactions to multiple forms of synaptic plasticity.

Activation of group I mGluRs also modulates basal and dopamine-induced gene expression in MSNs (reviewed in Mao et al., 2008). Co-activation of mGluR5 and NMDA receptors leads to calcium-dependent downstream activation of MAP kinase cascades and phosphorylation of transcription factors such as ELK1 and cAMP-responsive element-binding protein (CREB) (Choe and McGinty, 2001; Choe and Wang, 2001a, b; Mao et al., 2005; Voulalas et al., 2005; Yang et al., 2004). This signal transduction pathway leads to transcription of the immediate early gene c-Fos as well as prodynorphin and proenkephalin in MSNs (Mao et al., 2002; Mao and Wang, 2001; Parelkar and Wang, 2003; Yang et al., 2004). The efficiency of signaling downstream of group I mGluRs is improved by formation of protein complexes including Homer proteins (Mao et al., 2005). Interestingly, group I mGluR blockade impairs the ability of amphetamine to stimulate CREB phosphorylation or induce c-Fos or prodynorphin expression (Choe et al., 2002), indicating considerable cross-talk between group I mGluRs and dopamine receptors in the control of inducible gene expression in MSNs. Amphetamine or D1 agonist-induced c-Fos and prodynorphin expression are also attenuated in mGluR1 knockout mice (Mao et al., 2001, 2002), supporting the hypothesis that coactivation of group I mGluRs and dopamine receptors plays an important role in mediating dopamine receptor-induced gene expression in striatal neurons.

Coactivation of dopamine receptors and group I mGluRs can have opposing effects on downstream signaling events. In MSNs that give rise to the indirect pathway,

mGluR5 and A2A adenosine receptors physically interact and promote downstream events such as activation of the MAP kinase cascade, whereas D2 receptors have an inhibitory effect on the same downstream effectors (Ferre et al., 2002; Nishi et al., 2003). In D1-expressing neurons, group I mGluRs can also oppose the effects of dopamine by modulating the activity of DARPP-32. D1 activation leads to the protein kinase A (PKA)-dependent phosphorylation of DARPP-32 at Thr34, which in turn inhibits the activity of protein phosphatase I (PP1) and results in amplification of D1-mediated downstream signaling (Svenningsson et al., 2004). Conversely, activation of group I mGluRs leads to phosphorylation of DARPP-32 on Thr75 and serine 137 by casein kinase 1 and cyclin-dependent kinase 5 (Liu et al., 2001; Liu et al., 2002a; Nishi et al., 2005). This form of DARPP-32 negatively regulates PKA activity, thereby reducing the downstream effects of D1 receptor activation. Thus, for some signaling pathways, the overall effects of group I mGluR activation in MSNs tend to oppose the effects of dopamine receptor activation.

*Dopaminergic modulation of group I mGluRs.* Dopamine depletion and nigrostriatal lesion have been shown to regulate the expression of group I mGluRs in the striatum. In reserpinized rats, a significant increase in mGluR5 mRNA and receptor density has been found in the striatum (Ismayilova et al., 2006). Similarly, positron emission tomography (PET) studies in 6-OHDA-lesioned rats and MPTP-treated primates have demonstrated an increase in striatal mGluR5 PET tracer binding when compared with tracer binding in normal animals (Pellegrino et al., 2007; Sanchez-Pernaute et al., 2008). In addition, expression levels of mGluR1a are reduced in MPTP-treated mice, and mGluR1a and mGluR5 undergo complex changes in trafficking resulting in abnormal ultrastructural localization in response to nigrostriatal lesion (Kuwajima et al., 2007). Taken together, these findings indicate that dopaminergic tone regulates both the expression levels and trafficking of group I mGluRs. It is possible that

increases in striatal group I mGluR expression could contribute to the pathogenesis of PD symptoms by increasing the activity of the indirect pathway.

*Group I mGluRs in the globus pallidus external segment.* Both group I mGluR subtypes are expressed in the rodent GP and the primate GPe (Hanson and Smith, 1999; Kuwajima et al., 2007; Poisik et al., 2003; Rouse et al., 2000; Testa et al., 1998; Testa et al., 1994), and several physiological consequences have been identified. Activation of group I mGluRs by the nonselective agonist DHPG inhibits calcium currents in GP neurons, most likely by inhibiting calcium influx through N- and P-type calcium channels (Stefani et al., 1998). In addition, DHPG directly depolarizes GP neurons in rat brain slices, and this effect is blocked by the mGluR1-selective antagonist LY367385, but not the mGluR5-selective antagonist MPEP (Poisik et al., 2003). Interestingly, repetitive activation of the internal capsule results in a long-lasting excitation of GP neurons that is mediated by mGluR1 (Kaneda et al., 2007). In addition, *in vivo* electrophysiology recordings in nonhuman primates have demonstrated that mGluR1 blockade decreases firing rates in GPe neurons (Kaneda et al., 2005). Together, these findings suggest that mGluR1 plays a role in mediating glutamatergic excitation of GPe neurons. In rat brain slices, mGluR5 blockade potentiates the DHPG-induced membrane depolarization (Poisik et al., 2003). Further, repeated application of DHPG results in desensitization of mGluR1-mediated depolarization, but blockade of mGluR5 during repeated DHPG application prevents mGluR1 desensitization. These findings suggest that mGluR5 activation causes cross-desensitization of mGluR1 in GP neurons, and highlight the fact that mGluR1 and mGluR5 can perform distinct functions in the same population of neurons. Pharmacological inhibition of protein kinase C (PKC) activity mimics the effect of mGluR5 blockade, so it is likely that mGluR5-induced PKC activity mediates the desensitization of mGluR1 in GP neurons.

The physiological effects of mGluR1 and mGluR5 in GP neurons are significantly altered in dopamine-depleted brain slices, suggesting that dopaminergic signaling plays a critical role in the normal function of GP group I mGluRs (Poisik et al., 2007). Following reserpine treatment, mGluR1 activation no longer induces depolarization of GP neurons, whereas mGluR5 gains the ability to depolarize neurons. Activation of D1-like and D2-like receptors by exogenous dopamine application or selective agonists of these receptors partially restores the ability of mGluR1, while simultaneously reversing the ability of mGluR5, to depolarize GP neurons. Blockade of PKA activity mimics the ability of dopamine to restore the normal function of the group I mGluRs in the GP of reserpinized animals, suggesting that dopamine receptor-mediated inhibition of PKA may play a role in determining the normal functions of mGluR1 and mGluR5 in GP neurons. In addition, mGluR1 is down-regulated in the GPe of MPTP-treated monkeys, whereas mGluR5 protein levels are not altered, suggesting that dopaminergic tone can differentially influence group I mGluR expression levels in this nucleus (Kaneda et al., 2005).

*Group I mGluRs in the subthalamic nucleus.* Both mGluR1 and mGluR5 are expressed in STN neurons (Awad et al., 2000; Testa et al., 1994). Similar to the effects of group I mGluRs in the rodent GP, these receptors have distinct roles in STN neurons that are regulated by dopamine receptor signaling (Valenti et al., 2002). Activation of group I mGluRs by DHPG increases intracellular calcium levels, and both mGluR1 and mGluR5 contribute to this effect (Marino et al., 2002). DHPG also directly depolarizes STN neurons and increases firing frequency and bursting activity (Awad et al., 2000; Beurrier et al., 1999), but in contrast to GP neurons, this is mediated by mGluR5, whereas mGluR1 activation reduces evoked excitatory transmission in the STN by a presynaptic mechanism (Awad-Granko and Conn, 2001). Interestingly, prolonged treatment of rats with haloperidol prior to brain slice preparation produces a significant



change in the roles of group I mGluRs in STN neurons; mGluR1 contributes to STN depolarization in haloperidol-treated animals. This finding provides another intriguing example of regulation of group I mGluR function by intact dopamine signaling.

*Group I mGluRs in the substantia nigra pars reticulata and globus pallidus internal segment.* Group I mGluRs are also expressed in the output nuclei of rodents and primates (Kaneda et al., 2005; Marino et al., 2001; Messenger et al., 2002; Testa et al., 1998; Testa et al., 1994; Wittmann et al., 2001a), suggesting that they may play an important role in modulating basal ganglia output. Activation of group I mGluRs by DHPG reduces both excitatory and inhibitory transmission in rat SNr GABAergic projection neurons (Marino et al., 2001; Wittmann et al., 2001a). The ability of DHPG to inhibit excitatory transmission is presynaptically mediated and involves mGluR1 but not mGluR5 activation (Wittmann et al., 2001a). While most group I mGluR immunoreactivity in the rat SNr is detected postsynaptically, some group I mGluR labeling has also been detected in pre-terminal axons at both asymmetric and symmetric SNr synapses (Marino et al., 2001; Wittmann et al., 2001a), suggesting that group I mGluR agonists may reduce both excitatory and inhibitory transmission by activation of presynaptic receptors. Activation of group I mGluRs by DHPG also directly excites SNr neurons. Application of DHPG to rat midbrain slices depolarizes SNr neurons, and this effect is exclusively mediated via mGluR1 (Marino et al., 2002; Marino et al., 2001; Valenti et al., 2002). Interestingly, *in vivo* electrophysiological recordings from GPi neurons in monkeys have demonstrated that the mGluR1-selective antagonist LY367385 reduces the firing rate of GPi neurons, providing direct evidence that mGluR1 plays a role in mediating glutamatergic excitation of neurons in the basal ganglia output nuclei (Kaneda et al., 2005).

mGluR1 expression levels are reduced in the GPi of MPTP-treated monkeys, and the effect of LY367385 on the firing rate of individual GPi neurons is reduced

(Kaneda et al., 2005). Disruption of dopaminergic signaling also disrupts the normal function of group I mGluRs in the rat SNr; while mGluR1 exclusively mediates DHPG-induced depolarization of SNr neurons in normal animals, mGluR5 gains the ability to mediate depolarization after prolonged haloperidol treatment (Marino et al., 2002), similar to the effect observed in the STN, and providing further evidence that dopamine receptor signaling is important for the segregation of physiological roles of group I mGluRs in the basal ganglia.

### **Effects of group I mGluR antagonists in animal models of PD**

Because the overall effects of group I mGluR activation lead to increased transmission through the indirect pathway and direct excitation of nuclei that are overactive in the parkinsonian brain, antagonists of these receptors may be targets for treating the motor symptoms of PD. In agreement with this hypothesis, several studies have demonstrated that systemic administration of negative allosteric modulators of mGluR5 such as MPEP and MTEP yields antiparkinsonian effects in animal models of PD (Breysse et al., 2003; Breysse et al., 2002; Coccorello et al., 2004; De Leonibus et al., 2009; Ossowska et al., 2005; Ossowska et al., 2001; Spooren et al., 2000; Turle-Lorenzo et al., 2005). Interestingly, combined blockade of mGluR5 and A2A adenosine receptors produces very robust antiparkinsonian effects (Coccorello et al., 2004; Kachroo et al., 2005), possibly due to the interaction of these receptors in the striatal neurons that give rise to the indirect pathway.

Several mechanisms could mediate the antiparkinsonian effects of mGluR5 antagonism. Direct-site infusion of group I mGluR agonists into the rat striatum increases activity of the indirect pathway while reducing motor activity (Kearney et al., 1998; Kearney et al., 1997), providing evidence that mGluR5 antagonists may reverse PD-like motor impairments by reducing striatopallidal transmission. mGluR5 antagonists may

also increase excitatory transmission in the GPe by relieving mGluR1 desensitization (Poisik et al., 2003), although dopamine depletion may diminish this effect (Poisik et al., 2007). Because mGluR5 activation depolarizes STN neurons and promotes burst firing (Awad et al., 2000; Beurrier et al., 1999), reducing mGluR5 activity in the STN represents another putative mechanism of action of mGluR5 antagonists. Consistent with this prediction, infusion of MPEP into the subthalamic nucleus reverses motor asymmetries caused by unilateral 6-OHDA lesion (Phillips et al., 2006). Finally, blockade of mGluR1 or mGluR5 in striatal cholinergic interneurons may reduce the release of acetylcholine, which would also be predicted to have antiparkinsonian effects (Pisani et al., 2003).

Although the mechanisms underlying the generation of levodopa-induced dyskinesias (LIDs) are not clear, recent studies have associated upregulation of mGluR5 in the primate striatal complex with the development of LIDs, suggesting that blockade of mGluR5 may prevent the development of LIDs or reduce their severity (Samadi et al., 2007). In support of this hypothesis, mGluR5 blockade reduces dyskinetic behaviors in rat models of LID (Dekundy et al., 2006; Levandis et al., 2008; Mela et al., 2007). Excitingly, negative allosteric modulators of mGluR5 are currently being investigated in humans for the treatment of LIDs, and the results of these studies may lead to improvements in the treatment of PD by combining dopamine-replacement therapies with mGluR5 antagonists to alleviate the adverse effects of levodopa. Further studies aimed at discovering the mechanism responsible for mGluR5 antagonist-mediated attenuation of LIDs will likely increase our understanding of interactions between group I mGluRs and dopaminergic transmission in the basal ganglia.

### **Group I mGluR-mediated protection against nigrostriatal degeneration**

The possibility that antagonists of group I mGluRs can reduce activity through the indirect pathway suggests that blockade of group I mGluRs may provide protection against progressive nigrostriatal degeneration. An interesting finding in support of this hypothesis is that mGluR5 knockout mice are more resistant to MPTP-induced nigrostriatal toxicity than their wild type counterparts (Battaglia et al., 2004), suggesting that endogenous activation of mGluR5 enhances MPTP-induced nigrostriatal degeneration. Furthermore, mGluR5 antagonists reduce nigrostriatal damage in MPTP-treated mice (Aguirre et al., 2005; Battaglia et al., 2004), methamphetamine-treated mice (Battaglia et al., 2002), and 6-OHDA-treated rats (Armentero et al., 2006; Vernon et al., 2005; Vernon et al., 2007). Intranigral infusion of the mGluR5 antagonist MPEP protects against 6-OHDA-induced SNc degeneration in rats, suggesting that decreasing endogenous mGluR5 activation in the SNc is a potential neuroprotective mechanism. Antagonists of mGluR5 may also act in the STN by reducing mGluR5-mediated increases in neuronal excitability and burst firing. Because activation of mGluR1 has been shown to mediate direct excitation of SNc neurons (Mercuri et al., 1992; Mercuri et al., 1993), it is also possible that antagonists of mGluR1 could protect against excitotoxicity in the SNc; intranigral infusion of the mGluR1-selective antagonist LY367385 reduces the extent of 6-OHDA-induced nigrostriatal lesion in rats, providing support for this hypothesis. Antagonists of both of the group I mGluRs may therefore be useful for slowing the loss of dopaminergic neurons in PD.

### **Modulation of basal ganglia neurotransmission by group II mGluRs**

*Group II mGluRs in the striatum.* Group II mGluR expression has been detected in several basal ganglia nuclei (Bradley et al., 2000; Kahn et al., 2001; Pisani et al., 2002; Testa et al., 1998), and multiple physiological effects of these receptors have been

identified, many of which are sensitive to alterations in dopaminergic transmission. In the rat striatum, activation of presynaptic group II mGluRs reversibly reduces glutamatergic corticostriatal transmission onto medium spiny neurons by inhibiting glutamate release (Battaglia et al., 1997; Cozzi et al., 1997; Lovinger and McCool, 1995; Picconi et al., 2002). *In vivo* microdialysis studies have shown that local administration of group II mGluR antagonists in the striatum increases extracellular glutamate levels, suggesting that these receptors tonically inhibit glutamate release (Cozzi et al., 1997). Further studies have revealed that extracellular glutamate derived from cysteine-glutamate antiporter activity is the main source of glutamate responsible for the tonic activation of striatal group II mGluRs (Baker et al., 2002). In contrast to the reversible inhibition of excitatory transmission in rat brain slices, activation of group II mGluRs at the mouse corticostriatal synapse causes a long-term depression of excitatory transmission that persists after drug-washout (Kahn et al., 2001), although a more recent study reported a reversible depression of excitatory transmission at the corticostriatal synapse in mice (Martella et al., 2009).

The potency of group II agonists for inhibiting corticostriatal transmission is enhanced in brain slices obtained from 6-OHDA-lesioned rats, and this enhancement is associated with an increase in receptor density following dopamine denervation (Picconi et al., 2002); this upregulation could represent a mechanism for reducing excessive corticostriatal transmission resulting from nigrostriatal degeneration by increasing autoreceptor activity (Picconi et al., 2002). Chronic administration of L-DOPA restores the potency of group II agonists and the expression level of group II mGluRs to normal levels, indicating that L-DOPA treatment can reverse changes in mGluR function that are caused by dopaminergic denervation. In MPTP-treated monkeys, there is no change in group II mGluR receptor density in the striatum (Samadi et al., 2008); however, in MPTP-treated monkeys a combination of L-DOPA and the D2 receptor agonist

cabergoline reduces specific binding of the group II mGluR antagonist [<sup>3</sup>H]LY341495 in the neostriatum, whereas treatment with L-DOPA alone has no effect, suggesting an interaction between group II mGluRs and D2 receptors (Samadi et al., 2008). Recent studies evaluating the effects of group II mGluRs on corticostriatal transmission in genetic models of PD have also found alterations in group II mGluR function. In mice lacking the familial PD-linked genes *PINK1* or *Parkin*, the potency of the group II mGluR agonist LY379268 for reducing excitatory corticostriatal transmission is increased (Martella et al., 2009). This increase in potency is not reversed by acute L-DOPA administration, so a possible role of the dopaminergic system in mediating this change in group II mGluR function has not been delineated.

Electrophysiological recordings from striatal cholinergic interneurons have revealed that activation of group II mGluRs directly modulates the excitability of these cells (Pisani et al., 2002). In rat striatal slices, bath application of a group II mGluR agonist such as LY379268 or DCG-IV reduces both excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) evoked by intrastriatal stimulation, and reduces calcium-dependent plateau potentials, possibly by modulating the activity of P-type calcium channels. In addition, group II mGluR agonists reduce electrically-evoked acetylcholine release from these neurons, possibly by acting as heteroreceptors at cholinergic terminals. *In situ* hybridization studies demonstrate that mGluR2 mRNA can be detected in cholinergic interneurons, whereas mGluR3 mRNA is absent, suggesting that these effects are mediated exclusively by mGluR2 activation. In contrast to the effect of nigrostriatal lesion on group II mGluR function at the corticostriatal synapse, the potency of the group II mGluR-selective agonist LY354740 for depressing evoked acetylcholine release is reduced following 6-OHDA lesion (Marti et al., 2003). As a consequence, reduction in mGluR2-mediated inhibition of striatal acetylcholine release following nigrostriatal degeneration could contribute to pathological

increases in striatal acetylcholine levels (Marti et al., 2003). Treatment of 6-OHDA-lesioned rats with L-DOPA restores the sensitivity of mGluR2, suggesting that the ability of mGluR2 to reduce acetylcholine release is indeed dependent upon dopaminergic transmission, and supporting the idea that L-DOPA treatment can reverse some changes in mGluR function that are caused by dopaminergic denervation.

A series of microdialysis studies have identified several mechanisms by which group II mGluRs regulate the release of dopamine in the striatum. Group II mGluR agonists decrease whilst group II/III mGluR antagonists increase extracellular dopamine levels (Hu et al., 1999). Decreasing extrasynaptic glutamate levels in the striatum by inhibiting glial cysteine-glutamate exchange increases extracellular dopamine levels and this is reversed by the group II mGluR agonist APDC (Baker et al., 2002). These data suggest that under normal conditions striatal dopamine release is decreased by tonic activation of group II mGluRs on nigrostriatal terminals. In addition, activation of group II mGluRs at the STN-SNc synapse reduces excitatory transmission (Wang et al., 2005; Wigmore and Lacey, 1998), which would also be predicted to reduce dopamine release in the striatum by reducing the excitatory drive onto SNc neurons. However, a microdialysis study testing the effect of systemic administration of the group II mGluR-selective agonist LY379268 failed to find any effect on extracellular dopamine levels in the striatum (Cartmell et al., 2000). Further studies are therefore needed to fully elucidate the role of group II mGluRs in regulating striatal dopamine release *in vivo*.

*Group II mGluRs in the globus pallidus external segment and STN.* Group II mGluRs modulate excitatory transmission in the indirect pathway through actions in both the globus pallidus and the STN. The primary source of excitatory input to the globus pallidus is from the STN, and mGluR2 mRNA has been detected in STN neurons (Messenger et al., 2002; Testa et al., 1994), raising the possibility that mGluR2 may be expressed on STN terminals in the globus pallidus. In agreement with this prediction,

immunolocalization studies have detected group II mGluR reactivity in glutamatergic pre-terminal axons in the rat globus pallidus (Poisik et al., 2005). Activation of these receptors by a group II mGluR agonist reduces the amplitude of evoked excitatory postsynaptic currents (EPSCs), and this effect is potentiated by the mGluR2-selective positive allosteric modulator LY487379. Conversely, group II mGluR agonists have no effect on inhibitory transmission in the globus pallidus. Although the primary source of glutamatergic input to the globus pallidus is from the STN, effects on glutamatergic inputs from the cortex, brainstem, and thalamus may also contribute to this effect.

In the STN of adult rats, activation of group II mGluRs also reduces the amplitude of EPSCs by reducing glutamate release from presynaptic terminals, via PKC activation (Shen and Johnson, 2003), although this effect was not observed in young (15-18 day old) animals (Awad-Granko and Conn, 2001). Potential interactions between dopamine and group II mGluRs have not been evaluated in the globus pallidus or STN, but may be the subject of future study.

*Group II mGluRs in the substantia nigra pars reticulata.* Anatomical studies using specific antibodies have demonstrated the presence of group II mGluRs on presynaptic glutamatergic axon terminals in the SNr, which primarily receives glutamatergic inputs from the STN (Bradley et al., 2000). The presence of group II mGluRs on these terminals raises the possibility that these receptors modulate excitatory neurotransmission in the SNr. Consistent with this, brief application of group II mGluR-selective agonists such as LY354740 reversibly reduces the amplitude EPSCs recorded from SNr GABAergic neurons after stimulation of STN afferents (Bradley et al., 2000). LY354740 does not alter the frequency or amplitude of spontaneous miniature EPSCs recorded from these neurons, suggesting that the effect of LY354740 on excitatory transmission is mediated presynaptically. In addition, LY354740 reduces the frequency of EPSCs evoked by applying glutamate directly to the STN without affecting the



amplitude, confirming that group II mGluR activation inhibits excitatory transmission arising from STN neurons.

Interestingly, the ability of LY354740 to inhibit excitatory neurotransmission in the SNr is substantially reduced in dopamine-depleted animals, suggesting that dopaminergic tone is required for this effect (Wittmann et al., 2002). Bath application of dopamine to reserpinized slices rescues the effect of LY354740, confirming that the reduced effect of LY354740 is due to a loss of dopamine rather than other catecholamines or serotonin. In contrast to changes in expression levels of group II mGluRs at corticostriatal synapses following dopamine denervation, the alteration in group II mGluR effects in SNr seems to involve an acute interaction between dopamine receptor and mGluR signaling. The effect of reserpine is mimicked by bath application of the dopamine receptor antagonist haloperidol as well as the D1 receptor-selective antagonist SCH23390, but not by the D2 receptor-selective antagonist sulpiride, suggesting that tonic stimulation of D1 receptors in the substantia nigra by ambient dopamine is required for group II mGluR-mediated inhibition of excitatory transmission. In agreement with this interpretation, pharmacological activation of D1 receptors in dopamine-depleted slices is able to rescue the effects of LY354740, whereas D2 receptor activation does not rescue the effects of LY354740.

A potential consequence of the reduction in group II mGluR efficacy following dopamine depletion is that loss of group II mGluR autoreceptor activity at the STN-SNr synapse could contribute to pathological hyperactivity by removing feedback inhibition of glutamate release, and could therefore contribute to the symptoms of PD (Wittmann et al., 2002). Increasing evidence suggests that modulation of neurotransmission in the SNr by somatodendritically released dopamine plays a crucial role in basal ganglia output, raising the possibility that the loss of dopaminergic modulation at both striatal and extrastriatal sites in the basal ganglia may contribute to the symptoms of PD. For

example, local depletion of nigral dopamine by infusing tetrabenazine through a microdialysis probe causes an impairment of motor function in a rotarod performance test, despite the fact that striatal dopamine levels are unaffected (Andersson et al., 2006). Surprisingly, in the same study, local infusion of tetrabenazine into the striatum greatly reduced dopamine levels, but only produced a mild impairment of rotarod performance. These findings suggest that loss of local actions of dopamine in the basal ganglia output nuclei may play a more prominent role in the pathogenesis of PD symptoms than previously thought. If a loss of D1 activation by dopamine released by SNc neurons reduces the effectiveness of group II mGluR autoreceptor activity at the STN-SNr synapse, this dopamine-mGluR interaction could contribute to hyperactivity of this synapse and the generation of the motor symptoms characteristic of PD.

Group II mGluRs also regulate somatodendritic dopamine release from SNc neurons, and this effect could have a local influence on output nuclei function. In contrast to the decrease in dopamine release observed when group II mGluR agonists are applied to the striatum, addition of group II mGluR agonists to nigral brain slices significantly increases local dopamine release (Campusano et al., 2002). Interestingly, partial lesion of the SNc by 6-OHDA enhances the ability of a group II mGluR agonist to evoke nigral dopamine release.

### **Group II mGluR agonists in animal models of PD**

Several of the physiological effects of group II mGluR activation suggest that agonists could counteract the pathological changes in basal ganglia neurotransmission that contribute to the motor symptoms of Parkinson's disease. In particular, inhibition of excitatory transmission at the STN-SNr synapse could reduce excessive STN-SNr transmission, and would therefore be predicted to have antiparkinsonian effects. Consistent with this hypothesis, intranigral or intracerebroventricular administration of

group II mGluR agonists reverses reserpine-induced akinesia in rats (Dawson et al., 2000; Murray et al., 2002), and systemic administration of the group II agonist LY354740 reverses catalepsy and muscle rigidity induced by haloperidol (Bradley et al., 2000; Konieczny et al., 1998). The antiparkinsonian effect of intranigral group II mGluR agonists suggests that reduction of excitatory transmission at the STN-SNr synapse may be involved in mediating this effect. However, in light of the finding that group II mGluR activation increases nigral dopamine release, it is possible that an increase in extracellular dopamine levels in the substantia nigra could also contribute to the antiparkinsonian effects, possibly via activation of D1 receptors in the substantia nigra (Mayorga et al., 1999).

While the effect of intranigral group II mGluR agonists indicates that activation of group II mGluRs in the substantia nigra at least partially mediates the reversal of PD-like motor impairments, several other sites of action may also contribute to antiparkinsonian effects. For example, increased corticostriatal transmission has been implicated in the pathogenesis of PD symptoms, so reduced excitatory transmission at corticostriatal synapses by group II mGluRs may contribute (Bonsi et al., 2007a). Further, the upregulation of group II mGluRs at corticostriatal synapses following dopamine depletion may increase the therapeutic potential for these receptors in the Parkinsonian brain, whereas the reversal of this upregulation by chronic L-DOPA treatment suggests that combining group II mGluR agonists with L-DOPA may not be a viable therapeutic strategy (Picconi et al., 2002). Because increased striatal acetylcholine release relative to reduced dopamine levels is thought to contribute to the motor symptoms of PD, the ability of group II mGluR agonists to reduce acetylcholine release represents another possible mechanism by which activation of group II mGluRs could have antiparkinsonian effects (Bonsi et al., 2007a; Pisani et al., 2003). Finally, the finding that mGluR2 activation reduces excitatory transmission in the STN highlights an additional putative

site of action for the reversal of motor impairments by group II mGluR agonists, because inhibiting excitatory drive onto STN neurons could reduce the hyperactivity of this nucleus (Shen and Johnson, 2003). At this time, the contributions of striatal or subthalamic mGluRs to the antiparkinsonian effects of group II mGluR agonists have not been directly evaluated.

Although the antiparkinsonian actions of group II mGluR agonists have been demonstrated in certain behavioral models of PD, systemic administration of LY379268 fails to reverse motor deficits caused by chronic reserpine treatment or unilateral 6-OHDA lesion (Murray et al., 2002), raising concerns that this therapy may not be useful in a chronic state of dopamine depletion. The reduced ability of group II mGluR activation to inhibit excitatory transmission at the STN-SNr synapse in reserpinized brain slices suggests that pharmacotherapies for PD that target group II mGluR-mediated inhibition of excitatory transmission in the SNr may not be useful due to the loss of D1 receptor-mediated facilitation of group II mGluR function (Wittmann et al., 2002). In addition, because the potency of the group II mGluR-selective agonist LY354740 for depressing evoked acetylcholine release in the striatum is reduced following 6-OHDA lesion of the SNc (Marti et al., 2003), the therapeutic potential of mGluR2 activation in the context of cholinergic interneurons might be reduced. These findings highlight the importance of assessing the dependence of mGluR function on dopaminergic neurotransmission when evaluating novel therapeutic strategies for the treatment of PD.

### **Group II mGluR-mediated protection against nigrostriatal degeneration**

Excitotoxicity due to excessive glutamatergic transmission at the STN-SNc synapse may contribute to the progressive degeneration of SNc neurons in PD and thus pharmacological manipulations that reduce STN-SNc transmission may confer neuroprotective benefits. Because activation of group II mGluRs at this synapse reduces

excitatory transmission, the potential neuroprotective effects of group II mGluR agonists have been evaluated in multiple animal models of toxin-induced nigrostriatal degeneration. Several studies have demonstrated that systemic and intranigral administration of group II mGluR agonists (such as LY379268 and DCG-IV) reduce 6-OHDA-induced nigrostriatal degeneration in rats (Murray et al., 2002; Vernon et al., 2005) and MPTP-induced nigrostriatal degeneration in mice (Battaglia et al., 2003; Matarredona et al., 2001; Venero et al., 2002), suggesting that activation of group II mGluRs may provide protection against an excitotoxic component of SNc degeneration. However, other possible mechanisms for group II mGluR-mediated neuroprotection involving glial production of growth factors have also been identified; for example, recent *in vitro* studies demonstrated that group II mGluRs increase production of the neuroprotective factor brain-derived neurotrophic factor (BDNF) in rat microglia and transforming growth factor- $\beta$  (TGF $\beta$ ) in mouse astrocytes (Bruno et al., 1998; Bruno et al., 1997; D'Onofrio et al., 2001; Matarredona et al., 2001; Venero et al., 2002). Increased production of these growth factors may represent an alternative mechanism by which group II mGluR activation could confer neuroprotective benefits. Interestingly, *in vitro* and *in vivo* studies employing mice lacking mGluR2 and mGluR3 suggest that the neuroprotective effects of systemic LY379268 administration in MPTP-mice are specifically mediated by mGluR3, and that simultaneous activation of mGluR2 may counteract the neuroprotective effects of mGluR3 activation (Corti et al., 2007); thus, that drugs targeting mGluR3 may be particularly beneficial for protection of SNc neurons. Further studies will be necessary to evaluate the relative contributions of effects on growth factor production by glial cells and effects on STN-SNc neurotransmission to the protection of dopamine neurons observed in these animal models.

### **Modulation of basal ganglia neurotransmission by group III mGluRs**

Group III mGluR expression has been detected in multiple basal ganglia nuclei including the striatum, globus pallidus, and substantia nigra (Bradley et al., 1999a; Bradley et al., 1999b; Corti et al., 2002; Messenger et al., 2002; Testa et al., 1994). Similar to group II mGluRs, activation of group III mGluRs reduces corticostriatal transmission in the medium spiny neurons of the striatum by a presynaptic mechanism (Pisani et al., 1997a), although this effect was not observed in a prior study (Lovinger and McCool, 1995). However, unlike the increase in potency of group II mGluR agonists in corticostriatal slices from 6-OHDA-lesioned rats, the potency of group III mGluR agonists does not change in response to nigrostriatal lesion (Picconi et al., 2002), suggesting that there are different mechanisms responsible for regulating expression of various mGluR subtypes. Moreover, chronic L-DOPA treatment of rats with nigrostriatal lesions does not alter the effect of group III mGluR agonists on corticostriatal transmission (Picconi et al., 2002). These findings indicate that unlike group II mGluRs, striatal group III mGluR function is not regulated by dopaminergic transmission or the changes in corticostriatal glutamatergic transmission that result from nigrostriatal damage.

Immunohistochemical studies have detected high levels of mGluR4 expression in the rodent globus pallidus but not in the striatum (Bradley et al., 1999a; Bradley et al., 1999b), whereas mGluR4 mRNA has been detected in the striatum. These findings raise the possibility that mGluR4 activation could modulate striatopallidal neurotransmission by acting on presynaptic receptors. In agreement with this prediction, the group III mGluR agonist L-AP4 reduces the amplitude of evoked inhibitory postsynaptic currents (IPSCs) in rat brain slices by a presynaptic mechanism (Matsui and Kita, 2003; Valenti et al., 2003). This effect is not mimicked by the mGluR8-preferring agonist DCPG, and is absent in brain slices obtained from mice lacking mGluR4 (Valenti et al., 2003). In

addition, the mGluR4-selective positive allosteric modulator PHCCC potentiates the ability of a submaximal concentration of a group III agonist to reduce inhibitory transmission (Marino et al., 2003). Taken together, these results strongly suggest that mGluR4 is the receptor subtype that mediates the depression of inhibitory transmission in the GP. Because the ability of L-AP4 to reduce inhibitory transmission is not significantly altered by overnight reserpine treatment, it is unlikely that dopaminergic tone is required for mGluR4 function at the striatopallidal synapse. In addition to the effects of mGluR4 activation on inhibitory transmission in the rodent GP, L-AP4 also reduces evoked excitatory transmission in the GP by a presynaptic mechanism (Matsui and Kita, 2003), indicating that activation of these receptors can simultaneously influence both excitatory and inhibitory transmission in the GP.

Anatomical studies have detected mGluR4 and mGluR7 immunoreactivity in the STN, suggesting that group III mGluR activation may also modulate excitatory or inhibitory synaptic transmission in this nucleus (Bradley et al., 1999a; Bradley et al., 1999b). Indeed, L-AP4 inhibits evoked excitatory transmission in the STN by a presynaptic mechanism (Awad-Granko and Conn, 2001). Conversely, L-AP4 does not affect inhibitory transmission. Due to the lack of subtype-selective pharmacological tools at the time this study was performed, it is not known if one or both of these receptors contribute to the depression of excitatory transmission. In addition, the effect of disrupting dopaminergic transmission on group III mGluR function in the STN has not yet been determined.

Group III mGluRs are also expressed in the SNr (Bradley et al., 1999a; Bradley et al., 1999b; Messenger et al., 2002), and therefore have the potential to modulate basal ganglia output. Electrophysiological studies have demonstrated that activation of group III mGluRs reduces excitatory transmission at the STN-SNr synapse by a presynaptic mechanism (Wittmann et al., 2001b). In addition, recent microdialysis

studies show that group III mGluR agonists L-AP4 and L-serine-O-phosphate (L-SOP) reduce KCl-evoked GABA release in the rat globus pallidus (Macinnes and Duty, 2008). Group III mGluR agonists also reduce activity of the direct pathway by depressing inhibitory transmission in the SNr (Wittmann et al., 2001b). Because the SNr receives major GABAergic input from the striatum, it is likely that group III mGluRs reduce inhibitory transmission in the SNr at least in part by reducing GABA release from striatonigral projections. The relatively high concentrations of L-AP4 that are necessary to produce a robust reduction in inhibitory transmission suggest that mGluR7 is likely to mediate or contribute to this effect. Indeed, anatomical studies indicate that mGluR7 is expressed presynaptically on both striatonigral and striatopallidal terminals (Kosinski et al., 1999), supporting the hypothesis that mGluR7 mediates the suppression of inhibitory transmission. Immunohistochemical evidence also suggests that mGluR7 may be the receptor subtype responsible for reducing excitatory transmission in the SNr (Kosinski et al., 1999).

*Dopaminergic modulation of group III mGluRs.* Interestingly, electrophysiological recordings from brain slices of reserpinized animals suggest that dopamine differentially modulates the effects of mGluRs on excitatory and inhibitory transmission in the SNr. While dopamine depletion or dopamine receptor blockade impairs the ability of group II mGluRs to reduce excitatory transmission at the STN-SNr synapse, the effects of group III mGluRs on excitatory transmission in the SNr do not seem to be regulated by dopamine. In reserpinized brain slices, the ability of L-AP4 to reduce excitatory transmission is unaltered; conversely, the ability of L-AP4 to reduce inhibitory transmission is significantly diminished. Because selective inhibition of either D1 or D2 receptors is sufficient to impair the response to a group III mGluR agonist, the effect of group III mGluRs on inhibitory transmission is likely to depend on tonic activation of both D1 and D2 receptors. These findings highlight the complexity of dopaminergic regulation



of mGluR function in the SNr; the ability of group II mGluRs to inhibit excitatory transmission is dopamine-dependent, whereas the seemingly similar effect of group III mGluRs is not, suggesting differential mechanisms of regulation of these two subgroups of mGluRs in the same neuronal population. In addition, dopamine modulates the function of group III mGluRs at inhibitory but not excitatory synapses, suggesting that the mechanisms by which the group III mGluRs modulate neurotransmission at these two synapses are differentially regulated as well. Further studies will be required to determine the mechanisms by which dopaminergic tone acutely regulates the ability of presynaptic mGluRs to modulate neurotransmission in the SNr.

*Group III mGluR modulation of dopaminergic transmission.* Group III mGluRs may regulate dopamine release in the basal ganglia by modulating excitatory transmission in SNc neurons. In rat brain slices, activation of group III mGluRs with agonists that do not distinguish between individual subtypes reduces excitatory transmission by a presynaptic mechanism (Valenti et al., 2005; Wigmore and Lacey, 1998). Whilst the mGluR8-preferring agonist DCPG does not mimic this effect, the mGluR4-selective positive allosteric modulator PHCCC potentiates the reduction of excitatory transmission (Valenti et al., 2005), suggesting that this effect is at least partially mediated by mGluR4 but not by mGluR8. A notable species difference is that in slices obtained from mice, both mGluR4 and mGluR8 modulate excitatory transmission in the SNc (Valenti et al., 2005). Because the most prominent source of glutamatergic afferents in the SNc is STN neurons, and mGluR4 and mGluR8 mRNA has been detected in STN neurons (Messenger et al., 2002; Testa et al., 1994), it is likely that the group III mGluRs reduce excitatory transmission by reducing glutamate release from STN terminals. Interestingly, recent studies indicate that activation of presynaptic group III mGluRs also reduces inhibitory transmission in the SNc (Giustizieri et al., 2005).

However, the effect of group III mGluR activation on dopamine release in the striatum and other basal ganglia nuclei has not been evaluated.

### **Group III mGluR agonists in animal models of PD**

The ability of group III mGluRs to reduce transmission through the indirect pathway by reducing inhibitory striatopallidal transmission and reducing excitatory transmission in the STN and SNr suggests that activating one or more of the group III mGluRs may relieve the motor symptoms of PD. The finding that intracerebroventricular or systemic administration of group III mGluR agonists or mGluR4-selective positive allosteric modulators reverses motor deficits in both acute and chronic rodent models of PD provides strong support for activation of group III mGluRs as a therapeutic strategy (Battaglia et al., 2006; Lopez et al., 2008; MacInnes et al., 2004; Marino et al., 2003; Niswender et al., 2008b; Valenti et al., 2003). Impressively, L-AP4 reverses the forelimb use asymmetry caused by unilateral 6-OHDA lesion to the same extent as L-DOPA, providing compelling evidence that targeting group III mGluRs may be highly efficacious in treating PD symptoms (Valenti et al., 2003). Because increased GABAergic transmission at the striatopallidal synapse is thought to contribute to the pathogenesis of PD-related motor deficits, the ability of mGluR4 activation to reduce striatopallidal transmission in both normal and dopamine-depleted brain slices makes mGluR4 an intriguing target for novel PD therapeutics (Marino et al., 2003; Valenti et al., 2003). Excitingly, numerous studies have demonstrated that intrapallidal infusion of group III mGluR agonists reverses akinetic deficits in several models of PD (Konieczny et al., 2007; Lopez et al., 2007; MacInnes et al., 2004; Sibille et al., 2007). The results of these studies provide strong evidence that reducing striatopallidal transmission by activating mGluR4 may be a promising approach for alleviating the motor symptoms of PD.

In contrast to the clear antiparkinsonian effects of group III mGluR activation at striatopallidal synapses, the ability of group III mGluRs to reverse motor deficits in PD models by reducing excitatory transmission in the STN or SNr is not as well established. Intranigral infusion of group III mGluR agonists reverses reserpine-induced akinesia and haloperidol-induced catalepsy in rats, indicating that group III mGluR activation in the substantia nigra may confer some antiparkinsonian benefits (Konieczny et al., 2007; MacInnes et al., 2004). However, a more recent study in rats demonstrated that intranigral infusion of group III mGluR agonists worsened akinetic deficits in a reaction time task caused by 6-OHDA, and intranigral infusion of the group III mGluR agonist ACPT-I did not robustly reduce haloperidol-induced catalepsy (Lopez et al., 2007). These findings suggest that directly targeting increased STN activity may not yield the expected alleviation of motor deficits, despite the fact that group III mGluR function at STN-SNr synapses is not reduced in the absence of dopamine (Wittmann et al., 2002).

Although early studies evaluating the effects of intracerebroventricular administration of nonselective group III mGluRs in preclinical models of PD yielded promising evidence that targeting these receptors could provide a new therapeutic avenue, several important questions remained: which individual receptor subtypes were responsible for the alleviation of PD-like motor deficits, and could targeting a specific receptor subtype using drugs with increased selectivity have advantages such as enhanced efficacy or reduced side effect liability? Based on initial physiological experiments demonstrating that the ability of L-AP4 to reduce inhibitory transmission in the globus pallidus was lost in mice lacking mGluR4 (Valenti et al., 2003), the last decade has seen a tremendous effort focused on developing drugs that specifically target mGluR4. These novel drugs fall into multiple categories based on their pharmacological mechanisms of action (for review, see Flor and Acher, 2012). Orthosteric agonists targeting mGluR4 directly activate the receptor by binding to the

glutamate-binding site; because these drugs bind to the highly-conserved orthosteric site, they typically have enhanced selectivity for mGluR4, but still activate other mGluR subtypes at higher concentrations. PAMs represent the other class of mGluR4-selective drugs that have been developed; as discussed above, these drugs bind to less-conserved allosteric sites and enhance the ability of glutamate to activate the receptor while having little or no efficacy on their own (reviewed in Sheffler et al., 2011). The first mGluR4 PAM to allow direct evaluation of the hypothesis that selective activation of mGluR4 may have therapeutic potential in PD was PHCCC, which increased locomotor activity in reserpine-treated rats following intracerebroventricular administration, providing the first proof-of-concept that selectively targeting mGluR4 could have beneficial effects on PD-like akinetic deficits (Marino et al., 2003). Since the initial finding that an mGluR4 PAM could reverse motor deficits in a preclinical model of PD, many other reports have corroborated this finding using novel compounds with improved properties of *in vivo* testing (Bennouar et al., 2012; Beurrier et al., 2009; Broadstock et al., 2011; East et al., 2010; Goudet et al., 2012; Jones et al., 2011a; Jones et al., 2011b; Le Poul et al., 2012; Marino et al., 2003; Niswender et al., 2008b). This topic is further addressed in Chapter II, which describes the discovery and characterization of the novel mGluR4 PAM VU0155041, and Chapter VI, which discusses the tremendous advances in preclinical development of mGluR4 PAMs that have been made since performance of the work described in Chapter II.

While strong evidence exists supporting a role for mGluR4 activation in the antiparkinsonian effects of nonselective group III mGluR agonists, the contributions of mGluR7 and mGluR8 are far less understood, primarily owing to a lack of selective pharmacological tools for these receptor subtypes. To date, the only reports of potential beneficial effects of mGluR7 activation in preclinical models of PD have depended on the use of the allosteric agonist N,N'-dibenzhydrylethane-1,2-diamine dihydrochloride

(AMN082) (Mitsukawa et al., 2005), of which the major metabolite has known off-target activity at a variety of other receptors (Sukoff Rizzo et al., 2011). AMN082 was recently reported to have antiparkinsonian effects in a variety of animal models following oral administration (Greco et al., 2009). Specifically, AMN082 exhibited a narrow window for reversing haloperidol-induced catalepsy, as higher doses of AMN082 failed to alleviate catalepsy. Greco et al. reported a similar bell-shaped curve when assessing the ability of AMN082 to reverse abnormal motor behaviors in 6-OHDA-lesioned rats. It is important to note that the ability of AMN082 to reduce catalepsy was lost in mGluR7 knockout mice, suggesting that this effect of AMN082 is indeed mediated by activation of mGluR7. The fact that beneficial effects of AMN082 were lost at higher doses raises the possibility that there is a narrow range of mGluR7 activation that may have therapeutic benefit. Alternatively, the loss of activity at higher doses of this drug could be due to receptor desensitization or detrimental off-target activity. Interestingly, reversal of catalepsy was mimicked by intrastriatal infusion of AMN082, suggesting a potential contribution of striatal mGluR7 activation to the anticataleptic effects of this drug, perhaps by reducing corticostriatal transmission. A more recent report demonstrated that intranigral infusion of AMN082 ameliorated reserpine-induced akinesia in rats, and that this effect was blocked by the group III mGlu receptor antagonist CPPG, again supporting an mGluR7-specific effect (Broadstock et al., 2011). While these results suggest that mGluR7 warrants some attention as a possible target for treating the motor symptoms of PD, further studies employing mGluR7-selective compounds with improved pharmacological profiles will be necessary to evaluate the promise of this therapeutic strategy.

### **Group III mGluR-mediated protection against nigrostriatal degeneration**

Like the other mGluR subgroups, group III mGluRs have been implicated as targets for neuroprotection in toxin-based animal models of nigrostriatal degeneration.

The ability of group III mGluR activation to reduce excitatory transmission at the STN-SNc synapse while simultaneously reducing disinhibition of the STN by depressing inhibitory striatopallidal transmission could reduce the increased excitatory transmission in the SNc that may lead to excitotoxic cell death. Recent *in vitro* and *in vivo* studies have shown that mGluR4 activation protects against NMDA-mediated neurotoxicity (Bruno et al., 2000; Flor et al., 2002; Gasparini et al., 1999), suggesting that mGluR4 may be an important mediator of protection against excitotoxic cell death in various neuronal populations. Interestingly, acute or subchronic intranigral administration of L-AP4 reduces nigrostriatal degeneration caused by 6-OHDA lesion in rats (Vernon et al., 2005; Vernon et al., 2007), and combined treatment with L-AP4 and the mGluR5 antagonist MPEP confers more robust protection than either drug alone (Vernon et al., 2008). Further, systemic administration of the mGluR4-selective positive allosteric modulator PHCCC protects mice against MPTP-induced nigrostriatal degeneration (Battaglia et al., 2006). Taken together, these studies suggest that activation of group III mGluRs, particularly mGluR4, may be a promising therapeutic strategy for reducing SNc degeneration in the parkinsonian brain.

### **Metabotropic glutamate receptor 5 positive allosteric modulators as therapeutic targets for schizophrenia**

While mGluRs have been heavily investigated as regulators of basal ganglia function from the perspective of PD treatment, modulation of glutamatergic signaling also has important therapeutic possibilities in a variety of other CNS disorders. One such disorder is schizophrenia, a psychiatric disorder for which group II mGluRs and mGluR5 have been implicated as therapeutic targets. Schizophrenia is a disabling psychiatric disorder characterized by three symptom clusters: positive symptoms, including hallucinations, delusions, and thought disorder; negative symptoms, such as social

withdrawal an anhedonia; and cognitive symptoms, which include deficits in executive functions and attention. Schizophrenia was originally thought to be the consequence of aberrant dopamine receptor activation; this theory was largely based on the finding that current treatments for schizophrenia, which include typical antipsychotics such as haloperidol and atypical antipsychotics such as olanzapine and clozapine, all exhibit some degree of D2 receptor antagonism (Shin et al., 2011). While dopamine dysregulation is likely to contribute to the pathophysiology of schizophrenia, especially in terms of positive symptoms, D2 receptor antagonists do not improve negative or cognitive symptoms of the disorder, indicating the dopamine dysregulation only accounts for some aspects of schizophrenia. More recently, other neurotransmitter systems including glutamate, GABA, serotonin, and acetylcholine have all been implicated in the pathophysiology of schizophrenia as well, indicating that targeting other systems may be more beneficial for treating negative and cognitive symptoms.

Interestingly, NMDA receptor antagonists such as ketamine and phencyclidine (PCP) induce symptoms in healthy subjects that are quite similar to the positive, negative, and cognitive symptoms of schizophrenia (Kantrowitz and Javitt, 2012). In addition, symptoms are markedly enhanced in schizophrenic patients who take these drugs. These observations led to the hypothesis that NMDA receptor hypofunction could play a role in the pathophysiology of schizophrenia. Concordant with this hypothesis, treatment of schizophrenic patients with serine or glycine, which act as coagonists at NMDA receptors, provides some symptomatic benefit, suggesting that enhancing NMDA receptor activity could be a promising therapeutic approach for the treatment of schizophrenia. To this end, inhibitors of the glycine transporter GlyT1 are under development; this strategy indirectly enhances NMDA receptor function by increasing availability of glycine (reviewed in Noetzel et al., 2012a). Importantly, therapeutic strategies that indirectly enhance NMDA receptor function are more likely to be

successful, because direct activation of NMDA receptors is likely to lead to excitotoxic damage and epileptic seizures.

Several studies also indicate the mGluR5 and NMDA receptors are close signaling partners. mGluR5 and NMDA receptors are physically linked by binding to scaffolding proteins (Ehlers, 1999) and can each modulate the other's function. Activation of NMDA receptors has been shown to potentiate NMDA receptor-mediated currents in neurons in brain regions including the striatum, hippocampus, and STN (Attucci et al., 2001; Awad et al., 2000; Benquet et al., 2002; Doherty et al., 2000; Mannaioni et al., 2001; Marino and Conn, 2002; Pisani et al., 1997b; Pisani et al., 2001b; Ugolini et al., 1999). Conversely, NMDA receptor activation can also influence the function of mGluR5 (Alagarsamy et al., 1999; Alagarsamy et al., 2005). These findings suggest that activation of mGluR5 may improve symptoms of schizophrenia by enhancing NMDA receptor function. Supporting the role of mGluR5 in regulating NMDA receptor function, mGluR5 knockouts display behavioral phenotypes reminiscent of the effects of NMDA receptor antagonists, including disruption of pre-pulse inhibition (Brody et al., 2004). In addition, mGluR5 antagonists have been shown to potentiate the psychotomimetic effects of NMDA receptor antagonists such as PCP (Brody et al., 2004; Campbell et al., 2004; Kinney et al., 2003b). In light of these findings, mGluR5 PAMs have been evaluated as potential treatments for schizophrenia.

Preclinical studies have indicated that mGluR5 PAMs have promise for the treatment of schizophrenia, as they produce multiple behavioral effects that are predictive of antipsychotic activity in animal models (reviewed in Noetzel et al., 2012a; Vinson and Conn, 2012). For example, multiple studies have demonstrated that structurally distinct mGluR5 PAMs reverse amphetamine-induced hyperlocomotion and amphetamine-induced disruption of pre-pulse inhibition, suggesting that these drugs reverse hyperdopaminergia-associated behaviors and may alleviate the positive



symptoms of schizophrenia (Epping-Jordan et al., 2005; Kinney et al., 2005; Liu et al., 2008; Rodriguez et al., 2010; Schlumberger et al., 2009a; Vinson and Conn, 2012; Xiong et al., 2010). In addition, the mGluR5 PAM CDPPE has been shown to reverse MK-801-induced disruption of sucrose preference (Vardigan et al., 2010), which may suggest that mGluR5 PAMs have the ability to reverse anhedonia-like behaviors as well. Finally, several studies have demonstrated cognition-enhancing effects of mGluR5 PAMs; these findings include studies in both normal animals and animals in which cognition is disrupted with amphetamine or NMDA receptor antagonists (Ayala et al., 2009; Chan et al., 2008; Clifton et al., 2012; Darrah et al., 2008; Gastambide et al., 2012; Horio et al., 2012; Liu et al., 2008; Uslaner et al., 2009). Taken together, these findings suggest that mGluR5 represents an interesting target for developing novel, nondopaminergic therapeutic agents for the treatment of schizophrenia, and that these drugs could have significant advantages over currently available treatments because they have the potential to improve cognition.

### **Objectives of this study**

The combined use of physiological, biochemical, and behavioral experimental approaches has allowed great advances in our understanding of the roles of mGluRs in the basal ganglia, as well as the complexity of interactions between mGluRs and other neurotransmitter systems, particularly dopamine. While these studies have led to the identification of promising targets for the treatment of PD and schizophrenia, it is likely that we are just beginning to understand the roles of mGluRs and their interactions with dopamine signaling. As more pharmacological tools become available that selectively activate or inhibit individual mGluR subtypes, remaining questions regarding optimal therapeutic strategies will be further elucidated. The studies described in Chapters II and III of this dissertation take advantage of subtype-selective pharmacological tools

targeting mGluR4 and mGluR8, respectively, to evaluate these receptors as targets for treating the akinetic deficits associated with PD. In Chapter IV, brain slice electrophysiological techniques are employed to elucidate the effects of pharmacological activation of group II mGluRs at the STN-SNr synapse; interestingly, agonists of group II mGluRs were found to induce a robust form of chemical LTD at this synapse. Because the STN-SNr synapse is overactive in PD, this finding suggests that group II mGluR activation could have antiparkinsonian effects as well. Finally, the studies described in Chapter V evaluate the *in vivo* effects of mGluR5 PAMs on striatal signaling pathways that may be relevant for reversal of behaviors induced by a hyperdopaminergic state. Taken together, these studies increase our understanding of the physiological roles and therapeutic potential of various mGluR subtypes in the basal ganglia.

## CHAPTER II

### DISCOVERY AND PHARMACOLOGICAL CHARACTERIZATION OF NOVEL POSITIVE ALLOSTERIC MODULATORS OF MGLUR4

#### Introduction

Based on the ability of mGluR4 activation to reduce inhibitory transmission at the striatopallidal synapse, mGluR4 has been suggested as a potential target for novel pharmacological treatments of PD (see Chapter I for comprehensive background). Validating this concept has been difficult, however, due to the lack of subtype-selective pharmacological tools that modulate mGluR4. The development of PHCCC allowed the hypothesis that a PAM of mGluR4 could alleviate PD-like motor symptoms to be tested for the first time (Marino et al., 2003). Although the discovery of PHCCC was an important milestone as the first identified mGluR4 PAM, its utility was limited due to its low potency (approximately 4  $\mu$ M in cell-based assays), poor solubility in solutions suitable for *in vivo* administration, and off-target activity as an antagonist of mGluR1. Unfortunately, medicinal chemistry efforts around the PHCCC scaffold failed to yield any major improvements over PHCCC, so an alternative approach was necessary in order to identify novel mGluR4 PAMs with potency, selectivity, and physiochemical properties that would allow deeper exploration of the physiological roles and therapeutic potential of mGluR4 activation.

In order to discover novel compounds that enhanced the activity of mGluR4, we employed a high-throughput screening (HTS) approach. This effort yielded many previously unidentified mGluR4 PAMs belonging to several different chemical scaffolds. This chapter highlights the pharmacological characterization of one such scaffold that includes the lead compound VU0155041, which is highly selective for mGluR4, more

potent than PHCCC, and is soluble in solutions suitable for intracerebroventricular administration. In addition, VU0155041 has intrinsic allosteric agonist activity, a feature not observed in the pharmacological characterization of PHCCC. Interestingly, we show evidence that VU0155041 binds to a site on mGluR4 that is distinct from the binding site of PHCCC and another novel mGluR4 PAM, VU0047162. Finally, we found that VU0155041 alleviates motor deficits in two preclinical models of Parkinson's disease, supporting the idea that enhancing mGluR4 activity is a potential therapeutic strategy for the motor symptoms of Parkinson's disease. The work presented here is adapted from (Niswender et al., 2008b).

## **Methods**

### **Cell line creation and culture of the human mGluR4/ Gqi5/CHO line**

Human mGluR4 (hmGluR4)/Chinese hamster ovary (CHO) cells were stably transfected with the chimeric G protein  $G_{qi5}$  (Conklin et al., 1993) in pIRESneo3 (Invitrogen, Carlsbad, CA) and single neomycin-resistant clones were isolated and screened for mGluR4-mediated calcium mobilization using the method described below. hmGluR4/CHO cells were cultured in 90% Dulbecco's Modified Eagle Media (DMEM), 10% dialyzed fetal bovine serum (FBS), 100 units/ml penicillin/streptomycin, 20 mM HEPES (pH 7.3), 1 mM sodium pyruvate, 2 mM glutamine, 400  $\mu$ g/ml G418 sulfate (Mediatech, Inc., Herndon, VA) and 5 nM methotrexate (Calbiochem, EMD Chemicals, Gibbstown, NJ). Culture of Human Embryonic Kidney (HEK-293) cell lines co-expressing rat mGluR4 and the G protein-coupled inwardly rectifying potassium (GIRK) channel have been described in detail elsewhere (Niswender et al., 2008a). Culturing conditions for other mGluR cell lines are described below. All cell culture reagents were purchased from Invitrogen Corp. (Carlsbad, CA) unless otherwise noted.

## Primary high-throughput screening

Assays were performed within Vanderbilt University's High-Throughput Screening Center. Human mGluR4/Gqi5/CHO cells (30,000 cells/20  $\mu$ l/well) were plated in black-walled, clear-bottomed, TC treated, 384 well plates (Greiner Bio-One, Monroe, North Carolina) in DMEM containing 10% dialyzed FBS, 20 mM HEPES, 100 units/ml penicillin/streptomycin, and 1 mM sodium pyruvate (Plating Medium). The cells were grown overnight at 37 °C in the presence of 5% CO<sub>2</sub>. The next day, the medium was removed using a VSpin (Velocity 11, Menlo Park, CA) fitted with a modified bucket allowing the 384 well plate to be mounted inverted over a catch basin and spun at 80g for 10 sec with 40% acceleration and deceleration.

The medium was replaced, using a Thermo Fisher Combi (Thermo Fisher, Waltham, MA), with 20  $\mu$ L of 1  $\mu$ M Fluo-4, AM (Invitrogen, Carlsbad, CA) prepared as a 2.3 mM stock in dimethyl sulfoxide (DMSO) and mixed in a 1:1 ratio with 10% (w/v) pluronic acid F-127 and diluted in Assay Buffer (Hank's balanced salt solution, 20 mM HEPES and 2.5 mM Probenecid (Sigma-Aldrich, St. Louis, MO)) for 45 minutes at 37 °C. Dye was removed using the VSpin and replaced, using a Combi, with 20  $\mu$ L of Assay Buffer. Test compounds were transferred to daughter plates using an Echo acoustic plate reformatter (Labcyte, Sunnyvale, CA) and then diluted into Assay Buffer, using a Combi, to generate a 20  $\mu$ M stock. Ca<sup>2+</sup> flux was measured using the Functional Drug Screening System 6000 (FDSS6000, Hamamatsu, Japan). Baseline readings were taken (10 images at 1 Hz, excitation, 470 $\pm$ 20 nm, emission, 540 $\pm$ 30 nm) and then 20  $\mu$ l/well test compounds were added using the FDSS's integrated pipettor. For the primary screen, cells were incubated with test compounds (final concentration 10  $\mu$ M) for 2.5 minutes and then an EC<sub>20</sub> concentration of glutamate was applied; 2 minutes later an EC<sub>80</sub> concentration of glutamate was added. The overall assay protocol was automated using the instruments noted above integrated with a Thermo Fisher F3 robotic arm

(Thermo Fisher, Waltham, MA) under the control of a Polara scheduler (Thermo Fisher, Waltham, MA). All data were recorded to instruments' local drives and later migrated to a network drive. FDSS data were analyzed using a custom analysis application and were associated with unique compound identifiers based on liquid handler transfer logs and plate barcode readings captured by the Echo and by Polara. Potentiator "hits" were selected by comparing the amplitude of the responses at the time of EC<sub>20</sub> addition plus and minus test compounds. Wells with responses that differed from vehicle wells by 3 standard deviations were selected as hits for further study.

For initial concentration-response curve experiments, compounds were serially diluted 1:3 into 10 point concentration response curves and were transferred to daughter plates using the Echo. Test compounds were again applied and followed by EC<sub>20</sub> concentrations of glutamate. Curves were fitted using a four point logistical equation using Microsoft XLfit (IDBS, Bridgewater, NJ). Subsequent confirmations of concentration-response parameters were performed using independent serial dilutions of source compounds and data from multiple days experiments were integrated and fit using a four point logistical equation in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

### **Confirmation/Selectivity studies**

*Rat M1 muscarinic receptor.* CHO cells expressing the rat M1 muscarinic receptor were purchased from the ATCC (Manassas, VA) and cultured in HAM F-12 medium with 10% FBS, 20 mM HEPES, and 50 µg/mL G418. For calcium assays, cells were plated at 10,000 cells/well in Plating Medium and dye loading was as above for mGluR4. Compounds were added 2.5 minutes prior to an EC<sub>20</sub> concentration of the muscarinic agonist carbachol followed 2 minutes later by an EC<sub>80</sub> concentration of carbachol. Raw data from the FDSS were imported into Microsoft Excel. Maximum

change in fluorescence, compared to vehicle control wells, was calculated in the presence of the EC<sub>20</sub> agonist concentration.

*Rat mGluRs 1 and 5.* Rat mGluR1 and 5 cells were culture as described in (Hemstapat et al., 2007). Calcium fluorescence assays were employed for counterscreening rat mGluR1/Baby Hamster Kidney (mGluR1/BHK) and rat mGluR5/HEK cells using a similar triple-addition protocol employing appropriate EC<sub>20</sub> and EC<sub>80</sub> glutamate concentration for each receptor, the exceptions being that cells were plated at 15,000 cells/well and 20,000 cells/well in black walled, poly-D-lysine coated 384 well plates (Greiner Bio-One, Monroe, NC) in Plating Medium, respectively, and calcium assays proceeded as above. Maximum calcium fluorescence, compared to control, was calculated for the EC<sub>20</sub> and EC<sub>80</sub> peaks, respectively, after exporting raw FDSS data to Microsoft Excel.

*Human mGluR2.* Membrane preparation and GTPγS binding assays for mGluR2 were performed as described in (Hemstapat et al., 2007) and stored as frozen aliquots. Membranes were thawed and homogenized using a glass homogenizer in ice-cold binding buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub>, 150 mM NaCl, 1 mM EDTA, 10 μg/ml saponin, and 1 μM GDP. Assay mixtures contained 10 μg membrane protein, test compound, glutamate, 0.1 nM [<sup>35</sup>S] GTPγS and assay buffer to yield a total volume of 100 μl. Nonspecific binding was determined in the presence of 10 μM unlabeled GTPγS. Assay mixtures were incubated at room temperature with shaking for 60 min and the reaction was terminated by rapid filtration through Unifilter-96 GF/B filter plates (presoaked with ice-cold binding buffer) and the filter plates were washed three times with ice-cold binding buffer using a 96 well Brandel harvester (Brandel Inc., Gaithersburg, MD). Filter plates were dried and filled with 40 μl MicroScint-20 and radioactivity was counting using a TopCount NXT microplate scintillation counter (PerkinElmer Life and Analytical Sciences, Downers Grove, IL).

*Rat mGluRs 4, 7 and 8.* Compound activity at the rat group III mGluRs was assessed using thallium flux through GIRK channels, a method that has been described in detail in (Niswender et al., 2008a). These cell lines were grown in Growth Media containing 45% DMEM, 45% F-12, 10% FBS, 20 mM HEPES, 2 mM L-glutamine, antibiotic/antimycotic non-essential amino acids, 700 µg/ml G418, and 0.6 µg/ml puromycin at 37°C in the presence of 5% CO<sub>2</sub>. Briefly, mGluR4, 7 or 8 GIRK cells were plated into 384 well, black-walled, clear-bottom poly-D-lysine coated plates at a density of 15,000 cells/20 µl/well in Plating Medium and incubated overnight at 37°C in the presence of 5% CO<sub>2</sub>. The following day, the medium from the cells and 20 µl/well of 1.7 µM concentration of the indicator dye BTC-AM (Invitrogen, Carlsbad, CA) in Assay Buffer was added. Cells were incubated for 1 h at room temperature and the dye was replaced with 20 µl/well of Assay Buffer. For these assays, compounds were added at 2x final concentration and then 2.5 min later either an EC<sub>20</sub> or EC<sub>80</sub> concentration of glutamate (mGluR4, 8) or L-AP4 (mGluR7) was added using the FDSS 6000. Agonists were diluted in thallium buffer (125 mM sodium bicarbonate, 1 mM magnesium sulfate, 1.8 mM calcium sulfate, 5 mM glucose, 12 mM thallium sulfate, 10 mM HEPES) at 5x the final concentration to be assayed. Five frames of data were collected (excitation, 470±20 nm emission, 540±30 nm) at ½ Hz prior to compound addition. Data collection continued at ½ Hz until 10 seconds prior to agonist addition, when the rate was increased to 1 Hz for 2 min after agonist addition. Data were analyzed as described in (Niswender et al., 2008a).

### **Striatal Slice Electrophysiology**

Coronal striatal slices were prepared from Sprague Dawley rats (postnatal day 14-16). Rats were anesthetized with isoflurane and decapitated. The brain was rapidly removed from the skull and submerged in ice-cold modified artificial cerebrospinal fluid



(ACSF), which was oxygenated with 95% O<sub>2</sub> /5% CO<sub>2</sub> and composed of (in mM) 230 sucrose, 2.5 KCl, 0.5 CaCl<sub>2</sub>, 8 MgSO<sub>4</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 10 D-glucose. The brain was then blocked in the coronal plane, glued to the stage of a vibratome (Vibratome, St. Louis, MO, USA) that was filled with ice-cold modified ACSF, and cut at a thickness of 290 μm. Slices were then incubated in oxygenated normal ACSF (in mM, 126 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 1.5 MgSO<sub>4</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 10 D-glucose) at 31-32°C for 0.5 h and maintained at room temperature afterward until transferred individually to a fully submerged recording chamber, which was continuously perfused with oxygenated ACSF at ~30°C.

Whole-cell recordings were made from visually identified medium spiny neurons (MSNs) in the dorsolateral striatum under an Olympus BX50WI upright microscope equipped with a 40x water immersion objective, Hoffman optics and video system (Olympus, Lake Success, NY, USA). A MultiClamp amplifier (Molecular devices, Union City, CA) was used for voltage-clamp recordings. Patch pipettes were prepared from borosilicate glass (World Precision Instrument, Sarasota, FL, USA) using a Narashige vertical patch pipette puller (Narashige, Japan) and filled with the pipette solution containing (in mM) 125 Cs-methanesulfonate, 5 NaCl, 10 TEA-Cl, 10 HEPES, 0.1 EGTA, 5 QX-314, 4 Mg-ATP, 0.3 Na-GTP and 10 phosphocreatine. The pH of the pipette solution was adjusted to 7.3 with 1 M CsOH, and osmolality was adjusted to 290-295 mOsm. The patch pipette had resistance of 4-6 MΩ when filled with the above solution. NMDA receptor mediated currents were induced by pressure ejection of 1 mM NMDA to the soma of the recorded neurons through a patch pipette using a Picospritzer II (General Valve, Fairfield, NJ, USA). The neuron was typically voltage-clamped at -60 mV. Tetrodotoxin (1 μM) was routinely included in the perfusate to block voltage-gated sodium channels. Data were acquired using a Digidata 1322A interfaced to a PC computer equipped with pClamp 9.2 software (Molecular Devices, Union City, CA),

analyzed using Clampfit and Microsoft Excel and presented as percentage of control value.

### **Reversal of haloperidol-induced catalepsy and reserpine-induced akinesia**

*Animals.* Third ventricle cannulated (TVC) Male Sprague-Dawley rats weighing between 225-255 grams (Taconic Farms, Inc., Hudson, NY) were used for the behavioral studies and maintained in accordance with American Association for the Accreditation of Laboratory Animal Care (AALAC) guidelines under a 12-hour light/dark cycle (lights on: 6 AM; lights off: 6 PM) with free access to food and water. The experimental protocols, which were performed during the light cycle, were approved by the Institutional Animal Care and Use Committee of Vanderbilt University and conformed to the guidelines established by the National Research Council Guide for the Care and Use of Laboratory Animals.

*Induction and measurement of catalepsy.* Catalepsy was assessed using a horizontal bar placed 6 cm from the testing surface. The forepaws of each rat were placed gently on the bar with the body positioned at an angle of  $\sim 45^\circ$  to the testing surface. The latency in seconds required for the rat to remove one or both forepaws from the bar was manually measured. Any rat that remained on the bar between 45-60 seconds was considered to be cataleptic. TVC rats, randomly assigned to treatment groups, were injected with haloperidol (1.5 mg/kg, i.p., dissolved in 0.2% lactic acid) and monitored for catalepsy 2 h later. Cataleptic rats were subsequently reexamined 15, 30 and 60 min after intracerebroventricular administration of either L-AP4 (100-1000 nmol/10  $\mu$ l), VU0155041 (31 or 93 nmol/10  $\mu$ l), or vehicle. L-AP4 was prepared in artificial cerebrospinal fluid (Harvard Apparatus, Holliston, MA). VU0155041 was dissolved in 1 N sodium hydroxide, brought to 8 mls with double distilled water, pH adjusted to 7.4 with HCl, and then brought to final volume with double distilled water.

*Induction and measurement of akinesia.* TVC rats were injected with reserpine (5 mg/kg, subcutaneously, dissolved in 1% acetic acid) and kept in their home cages for 2 hr after injection. Activity was measured by placing rats in photocell activity cages (Hamilton-Kinder, Poway, CA) equipped with 16 x 16 infrared beams. After a 30 min baseline period, rats were given a single intracerebroventricular injection of either L-AP4 (100, 300 or 1000 nmol), VU0155041 (93 or 316 nmol), or corresponding vehicles, and motor activity was recorded for an additional 30 min.

## **Compounds**

L-glutamate, PHCCC, and [( $\alpha R, \beta S$ )- $\alpha$ -(4-hydroxyphenyl)- $\beta$ -methyl-4-(phenylmethyl)-1-piperidinepropanol] (Ro25-6981) were purchased from Tocris Bioscience (Ellisville, MO). L-AP4 was purchased from Ascent Scientific (Weston-Super-Mare, UK). Haloperidol lactate was purchased from Abraxis (Schaumburg, IL). NMDA and reserpine was purchased from Sigma (St. Louis, MO). The Vanderbilt High Throughput Screening Center compound collection was obtained from ChemBridge Corporation (San Diego, CA) and ChemDiv, Inc. (San Diego, CA) and stored in barcoded, 384 well, U-bottom, standard volume, polypropylene plates (Corning, Corning, NY). The plates were thermally sealed with peelable seals using a PlateLoc (Velocity 11). Groups of ten plates were vacuum packed in thermally sealed freezer bags (FoodSaver, Jarden Corp.) and stored frozen at -80° C. Primary hits identified in the screen were reordered from ChemBridge or ChemDiv as 10 mM DMSO stocks; these orders were accompanied by NMR spectra to confirm compound identity. Compounds in Table 3 were then also confirmed by LC-MS at Vanderbilt. Synthesis of PHCCC, VU0155040, VU0155041, and chiral resolution of VU0155041 are described in (Niswender et al., 2008b).

## Results

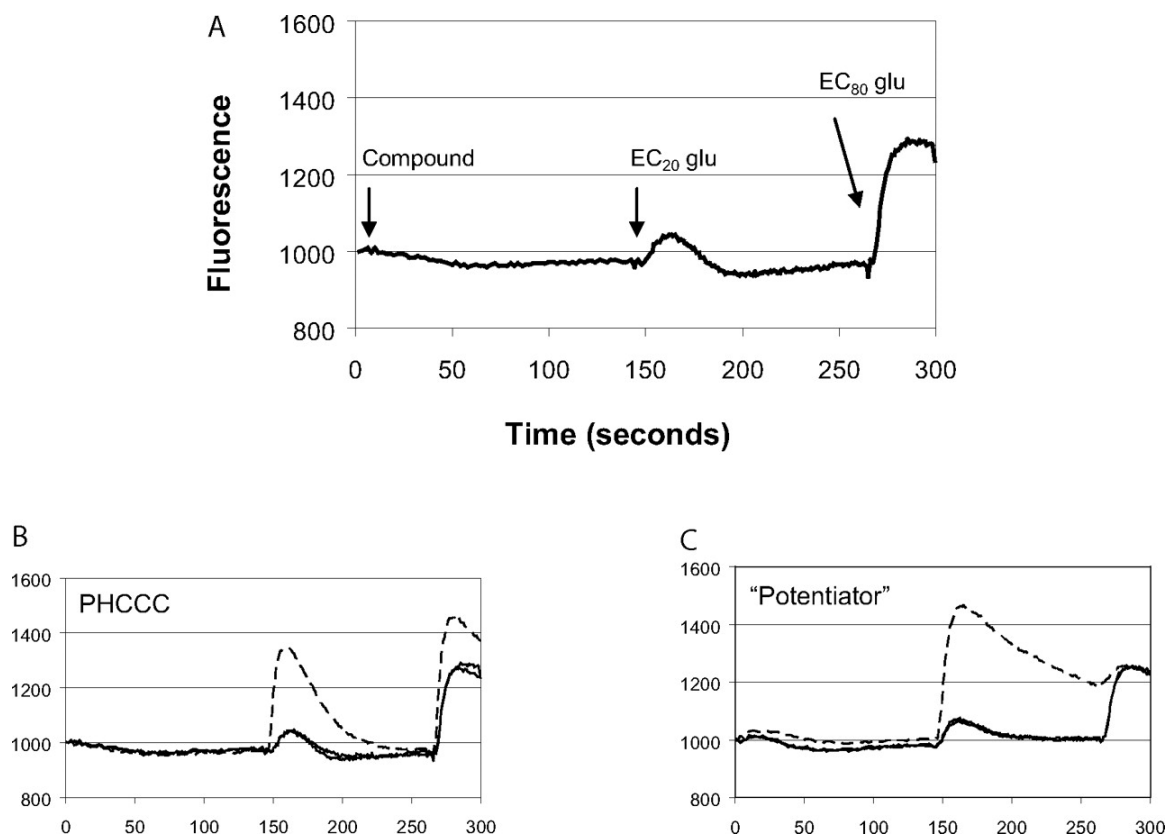
### Novel modulators of mGluR4 were identified via high throughput screening

HTS was carried out using a hmGluR4/G<sub>q15</sub>/CHO cell line described above and measuring receptor-induced intracellular calcium mobilization using a kinetic imaging plate reader that simultaneously monitors changes in fluorescence in each well of a 384 well microplate. The assay was performed by first taking a baseline measurement, then adding a test compound or DMSO-matched vehicle control (final concentration 10  $\mu$ M). After 2.5 min incubation with test compound or vehicle, a low (EC<sub>20</sub>) concentration of glutamate was added, followed 2 min later by an EC<sub>80</sub> concentration of glutamate. This “triple add” protocol allowed detection of agonists (measured immediately following test compound addition), PAMs (compounds that potentiate the response to an EC<sub>20</sub> concentration of glutamate) and antagonists, including negative allosteric modulators (NAMs; compounds that inhibit the response to an EC<sub>80</sub> concentration of glutamate in a noncompetitive manner). EC<sub>20</sub> and EC<sub>80</sub> concentrations were determined by performing a glutamate concentration-response curve assay at the beginning of each screening day. Raw kinetic data from the screen were normalized by dividing all of the fluorescence readings of the trace by the minimum data point occurring two to five seconds prior to the EC<sub>20</sub> glutamate addition. This step corrected for well-to-well differences in cell number, dye loading, non-uniform illumination/imaging, and to permit retention of information for compounds that were slightly fluorescent or that induced subtle changes in the baseline trace. Vehicle, EC<sub>20</sub>, and EC<sub>80</sub> controls were included on each plate and data were analyzed on a plate-by-plate basis. As an example of the uniformity of the data for the EC<sub>20</sub> window, control values (mean  $\pm$  CV) for eight random plates, taken on different days throughout the screen, were 1.6 $\pm$ 0.3%, indicating that the signal was very uniform across plates and among wells. At least 20% of the plates were visually spot checked to assure the quality of the data and validate these set points for

hit picking, prevent the loss of compounds with weak activity, and eliminate compounds giving apparent nonspecific or spurious signals such as compounds with obvious saturating fluorescence. Figure 3 shows example traces for either screening controls (Figure 3A), a trace obtained in the presence of the control PAM PHCCC (Figure 3B) or a trace obtained in the presence of a compound identified during the primary HTS (Figure 3C). Approximately 160,000 compounds were screened in the primary screen and 1490 “potentiator” hits were identified (0.9% hit rate). Of the primary hits, 1355 PAM hits (the remainder being unavailable for commercial reorder) were formatted into ten point concentration-response curves and tested for concentration-dependent activity on mGluR4. Compounds were also screened against a CHO cell line expressing the M1 muscarinic receptor to determine if their action was via a non-specific mechanism. A total of 434 compounds were confirmed as having concentration-dependent PAM activity, producing a retest rate of approximately 32%. Sixty five (15%) of the compounds potentiated (9 compounds) or antagonized (56 compounds) the response of M1-expressing cells to ACh, indicating that approximately 85% of the compounds were selective for mGluR4 over M1. Of these 434 PAMs, initial concentration-response curves indicated that 179 compounds exhibited a potency of under approximately 5  $\mu$ M, and 23 compounds were under 1  $\mu$ M in potency. An assessment of confirmed PAM hits from the screen quickly revealed that many of the compounds shared common chemical scaffolds; one of these scaffolds will be highlighted here.

### **A newly identified mGluR4 PAM cluster from the HTS revealed a robust structure-activity relationship**

The cluster chosen for further exploration, comprised of 8 HTS hits, was represented by a cyclohexyl amide moiety joined to a substituted phenyl ring (Table 3); the majority of the compounds also contained a carboxylic acid at position 1 of the

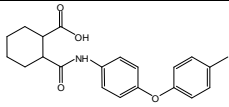
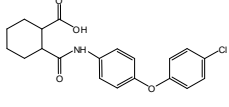
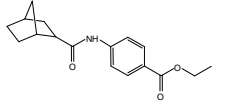
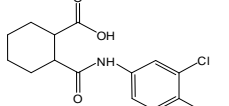
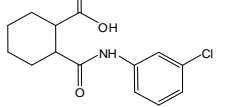
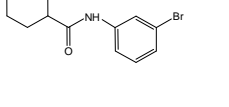
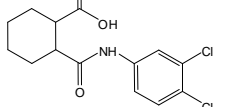
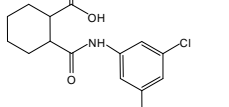


**Figure 3. HTS assay design and fluorescence traces of potential mGluR4 PAMs measured during the HTS campaign.** A, HTS assay design. Human mGluR4/Gqi5 cells were loaded with Fluo-4 calcium-indicator dye as described in Materials and Methods. A baseline fluorescence measurement was taken for 3s and then either vehicle or a 10  $\mu$ M concentration of compound was added. Approximately 2.5 minutes later (time 146s), an  $EC_{20}$  concentration of glutamate (2  $\mu$ M final) was added followed at 266s by an  $EC_{80}$  concentration of glutamate (20  $\mu$ M final). B and C, Representative traces of control compounds or compounds identified during HTS. B, Control trace measured in the presence (hatched line) or absence (solid line) of 10  $\mu$ M PHCCC. C, Trace observed in the presence of a novel compound (10  $\mu$ M, hatched line) that potentiates the response of glutamate at mGluR4.

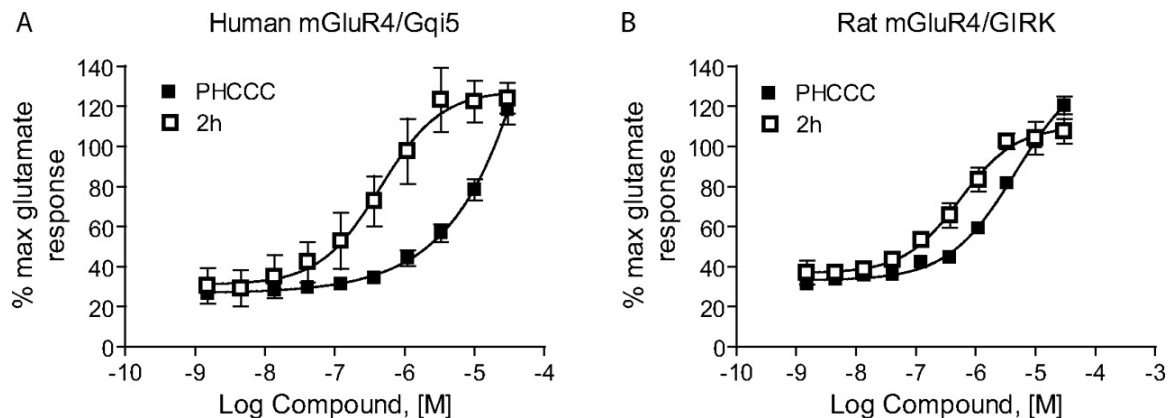
cyclohexane. The potencies of compounds in this cluster were assessed at both human mGluR4 and rat mGluR4. For human mGluR4, the assay employed was the chimeric G protein approach used for HTS. For the rat receptor, a cell line was used in which rat mGluR4 was co-expressed with the G protein-Regulated Inwardly Rectifying K<sup>+</sup> channel, GIRK. This assay relies upon activation of GIRK 1/2 channels via the Gβγ subunits of G<sub>i/o</sub> G proteins and exploits the ability of the GIRK channel to conduct ions of thallium through the channel pore in response to agonist activation of a GPCR (Niswender et al., 2008a). One advantage of the assay is that it does not require co-transfection of a chimeric or promiscuous G protein to induce coupling to a non-native signaling pathway. Since the group III mGluRs have been shown to regulate GIRK in electrophysiological studies (Saugstad et al., 1997; Saugstad et al., 1996) and ion channels in neurons (Bertaso et al., 2006; Guo and Ikeda, 2005; Pelkey et al., 2006), presumably via Gβγ subunits, this technique represents a mechanism to screen or confirm activity of compounds using an assay that monitors activity through G proteins representative of those normally used by the receptor. This technique is also an easy and efficient method to examine an alternate signaling pathway downstream of mGluR4 and confirm activity of compounds as general mGluR4 PAMs.

Table 3 shows the structures, potencies, and efficacies of compounds in the cyclohexyl amide cluster identified via HTS. Structurally, compounds **2e**, **2g**, and **2h** were very similar, with the positions of the dichloro- substitutions of **2h** being preferred for both potency and efficacy. Compound **2f**, which lacks the carboxylic acid present in every other member of this series, was very similar in activity to **2e**, suggesting that the carboxylic acid group is not absolutely required for activity as an mGluR4 PAM. In Figure 4, concentration-response curves for the most potent compound in this cluster (**2h**, corresponding to ChemBridge compound 7307507) are shown in comparison to PHCCC; Figure 4 shows that this compound is more potent than PHCCC in both the

**Table 3. Structures and activity summary of HTS hits from a single cluster.**

ID	Structure	hmGluR4/Gqi5			rmGluR4/GIRK		
		EC50 ( $\mu\text{M}$ )	% Glu max	Fold shift	EC50 ( $\mu\text{M}$ )	% Glu max	Fold shift
2a		>10	128.4 $\pm$ 9.6	4.0 $\pm$ 0.7	>10	76.7 $\pm$ 7.1	2.7 $\pm$ 0.1
2b		2.1 $\pm$ 0.9	109.4 $\pm$ 9.8	5.5 $\pm$ 1.8	2.0 $\pm$ 0.3	83.1 $\pm$ 10.5	4.3 $\pm$ 0.2
2c		>10	91.4 $\pm$ 14.4	2.2 $\pm$ 0.4	>5	71.3 $\pm$ 11.1	1.9 $\pm$ 0.2
2d		>10	106.8 $\pm$ 20.1	3.8 $\pm$ 0.3	>10	64.5 $\pm$ 4.8	2.1 $\pm$ 0.1
2e		3.7 $\pm$ 0.8	126.8 $\pm$ 21.2	3.7 $\pm$ 1.3	>5	78.4 $\pm$ 5.4	2.8 $\pm$ 0.1
2f		>5	118.7 $\pm$ 15.3	3.5 $\pm$ 0.4	>5	128.1 $\pm$ 5.9	7.8 $\pm$ 0.8
2g		>5	117.2 $\pm$ 18.3	4.0 $\pm$ 0.8	>10	78.6 $\pm$ 6.8	3.0 $\pm$ 0.1
2h		0.75 $\pm$ 0.2	120.0 $\pm$ 6.7	6.5 $\pm$ 1.0	0.56 $\pm$ 0.1	107.6 $\pm$ 6.3	7.7 $\pm$ 0.6





**Figure 4. Novel PAM identified via HTS was significantly more potent than PHCCC.**

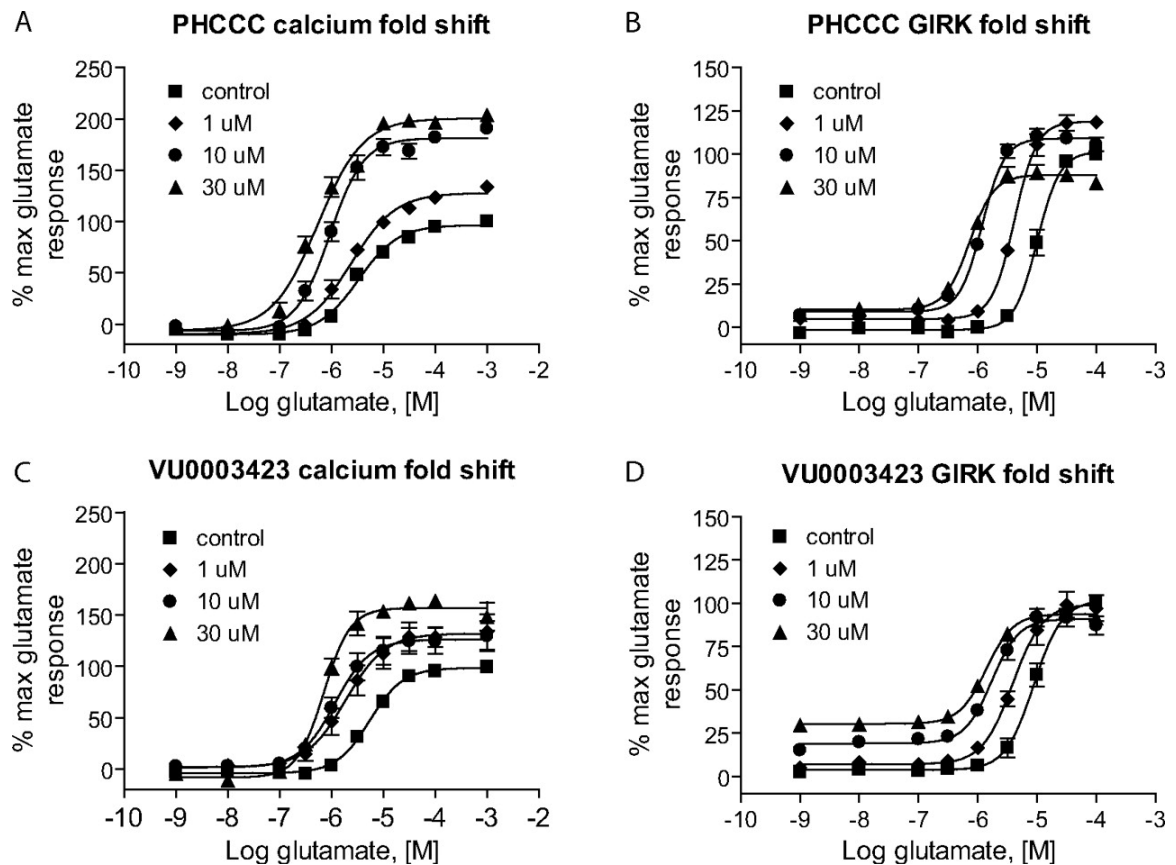
A and B, Increasing concentrations of compound **2h** or PHCCC were pre-applied to either human mGluR4/Gqi5 cells (A) or rat mGluR4/GIRK cells (C); an EC<sub>20</sub> concentration of glutamate was added approximately 2 1/2 minutes later and responses were measured as described in Materials and Methods. Potencies in the calcium assay (mean ± S.E.M.) were: PHCCC, >10 μM; **2h**, 750 ± 200 nM; GIRK assay, PHCCC 4.9 ± 1.3 μM; **2h**, 560 ± 100 nM. Results represent the mean ± S.E.M. of 3-4 independent experiments performed in triplicate or quadruplicate.

calcium and thallium flux/GIRK assays. This compound was subsequently reordered and confirmed for mGluR4 PAM activity; at this point the compound was given the identifier VU0003423 (**2h/7307507**). The above studies indicate that we have identified a new structural class of mGluR4 PAMs with the lead compound exhibiting improved potency (approximately 8-fold) compared to PHCCC.

### **Further examination of structure activity relationships using assessed by leftward shifts of glutamate concentration-response curves**

In order to evaluate the efficacy of PAMs, we performed concentration-response curves of glutamate in the absence or presence of a fixed concentration of PAM. I measured the efficacy of PAMs in shifting the glutamate concentration response curve by performing 10 point glutamate concentration-response curves in the presence of 30  $\mu\text{M}$  of each test compound (Table 3). In addition to having a higher potency than PHCCC and other compounds from this cluster, VU0003423 (30  $\mu\text{M}$ ) was also the most efficacious compound in this cluster as it shifted the glutamate concentration-response curve  $6.0 \pm 1.0$  and  $7.7 \pm 0.6$  fold (mean  $\pm$  S.E.M.,  $n=3-4$  independent experiments) in the hmGluR4/Gqi5 and rmGluR4/GIRK assays, respectively.

VU0003423 was further compared to PHCCC using various concentrations of each PAM for both the calcium (Figure 5, A and C) and thallium flux/GIRK (Figure 5, B and D) assays. In calcium assays, PAMs shifted the glutamate concentration-response to the left while simultaneously increasing the maximal response. In the thallium flux assay, the same compounds induced a leftward shift without an appreciable increase in maximal responses. These studies verify activity of VU0003423 at both the rat and human receptor and confirm PAM activity in two independent assays of mGluR4 function.



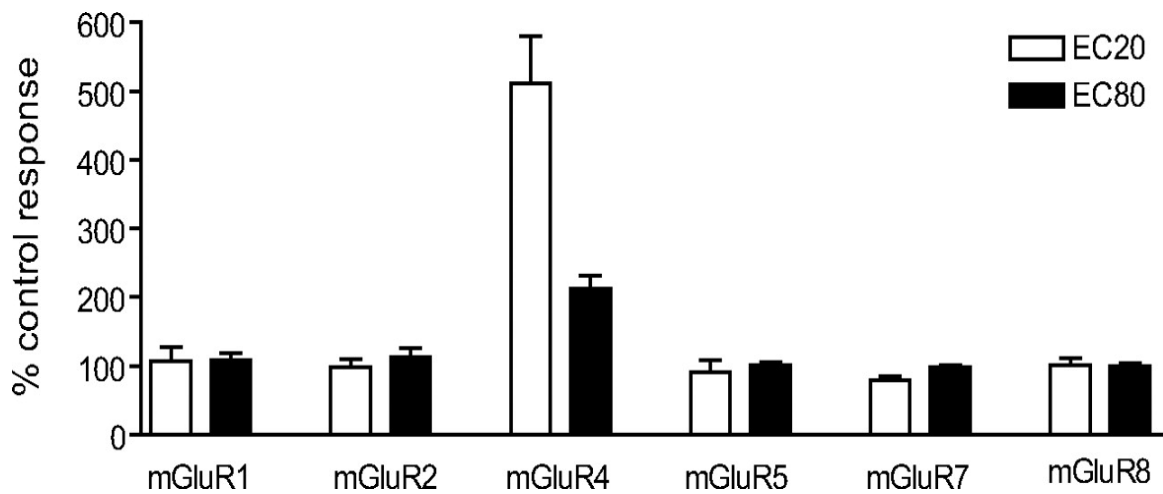
**Figure 5. VU0003423 was effective in shifting the glutamate-concentration response to the left in two independent assays of mGluR4 function.** Various concentrations of PHCCC (A and B) and VU0003423 (C and D) were applied to either human mGluR4/Gqi5 (A and C) or rat mGluR4/GIRK cells (B and D) approximately 2.5 minutes prior to the addition of increasing concentrations of glutamate and responses were measured as described in Materials and Methods. Each compound resulted in progressive leftward shifts of the glutamate concentration-response curve; shifts induced in the presence of a 30  $\mu$ M concentration of each compound for the calcium assay were (mean  $\pm$  S.E.M.): PHCCC,  $6.7 \pm 0.8$ -fold, and VU0003423,  $6.5 \pm 1.0$ -fold; and for the GIRK assay: PHCCC,  $13.7 \pm 1.6$ -fold, and VU0003423,  $7.7 \pm 0.6$ -fold. Results represent the mean  $\pm$  S.E.M. of 3-4 independent experiments performed in triplicate or quadruplicate.

### **Novel mGluR4 PAM was selective for mGluR4 relative to other mGluR subtypes**

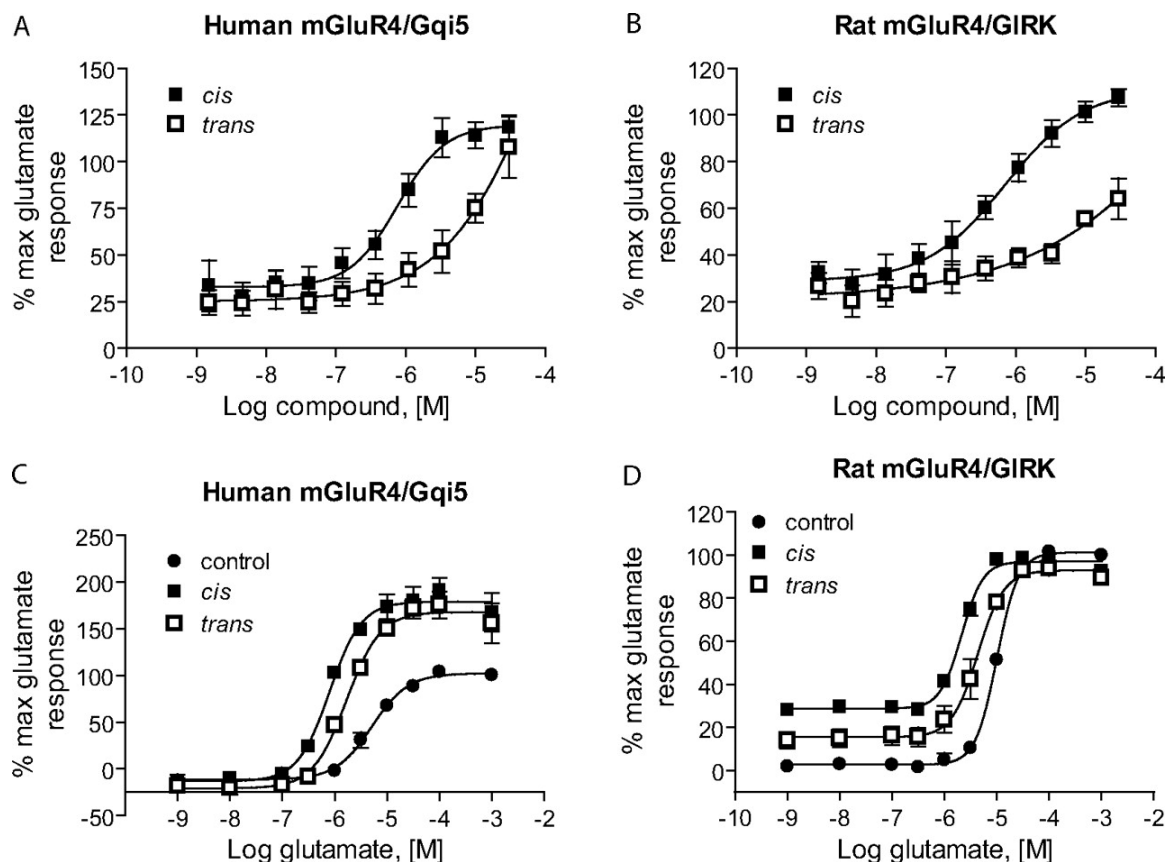
One primary goal in our efforts to discover an mGluR4 PAM that is more useful for *in vivo* studies than PHCCC is a high degree of selectivity against other mGluRs. As mentioned previously, PHCCC is an antagonist of mGluR1 (Marino et al., 2003), which complicates interpretations of effects of PHCCC in native systems. In order to evaluate selectivity, I tested the ability of VU0003423 to either potentiate responses to low ( $EC_{20}$ ) concentrations of glutamate or inhibit responses to high ( $EC_{80}$ ) concentrations of glutamate in assays for mGluR subtypes 1, 2, 4, 5, 7, and 8. Data were normalized to the response for each receptor obtained in the absence of VU0003423. No effect of VU0003423 (30  $\mu$ M) was observed at any of the tested mGluRs other than mGluR4 (Figure 6), indicating that this novel mGluR4 PAM is selective among the tested mGluRs and unlike PHCCC, does not have known off-target activity. Unfortunately, selectivity vs. mGluR3 and mGluR6 were not included in this experiment due to a lack of reliable cell-based assays for these receptors at the time these studies were performed.

### **Separation of VU0003423 into *cis* and *trans* regioisomers**

The compounds in this cluster, including VU0003423, have unknown stereochemistry. To determine if one regioisomer preferentially exhibits mGluR4 PAM activity, in collaboration with Dr. Craig Lindsley, I synthesized both the *cis* (VU0155041) and *trans* (VU0155040) regioisomers of VU0003423 and evaluated them for potency and efficacy at human and rat mGluR4. These studies revealed that at both human and rat receptors, the *cis* regioisomer of VU0003423 (VU0155041) was similar in potency to the lead compound ( $798 \pm 58$  nM at human mGluR4 and  $693 \pm 140$  nM at rat mGluR4; Figure 7, A and B). Conversely, the concentration-response curve for the *trans* regioisomer (VU0155040) did not plateau at the maximum concentration tested (Figure



**Figure 6. VU0003423 selectively potentiated the response of mGluR4 to glutamate when compared with other mGluRs.** A 30  $\mu$ M final concentration of VU0003423, followed by an EC<sub>20</sub> or EC<sub>80</sub> concentration of agonist, was applied to the following cell lines to test for selectivity among the mGluRs: rat mGluR1/BHK, human mGluR2/CHO, human mGluR4/Gqi5, rat mGluR5/HEK, rat mGluR7/GIRK, and rat mGluR8/GIRK. In these assays, effects on EC<sub>20</sub> and EC<sub>80</sub> responses to agonist were assessed independently rather than using sequential agonist adds. Assays for each receptor were carried out as described in Materials and Methods. For mGluR1, 2, 4, 5, and 8, the agonist used was glutamate, whereas the agonist for mGluR7 was L-AP4. Data were normalized to the corresponding response observed in the absence of VU0003423. Results represent the mean  $\pm$  S.E.M. of 3-4 independent experiments performed in triplicate or quadruplicate.



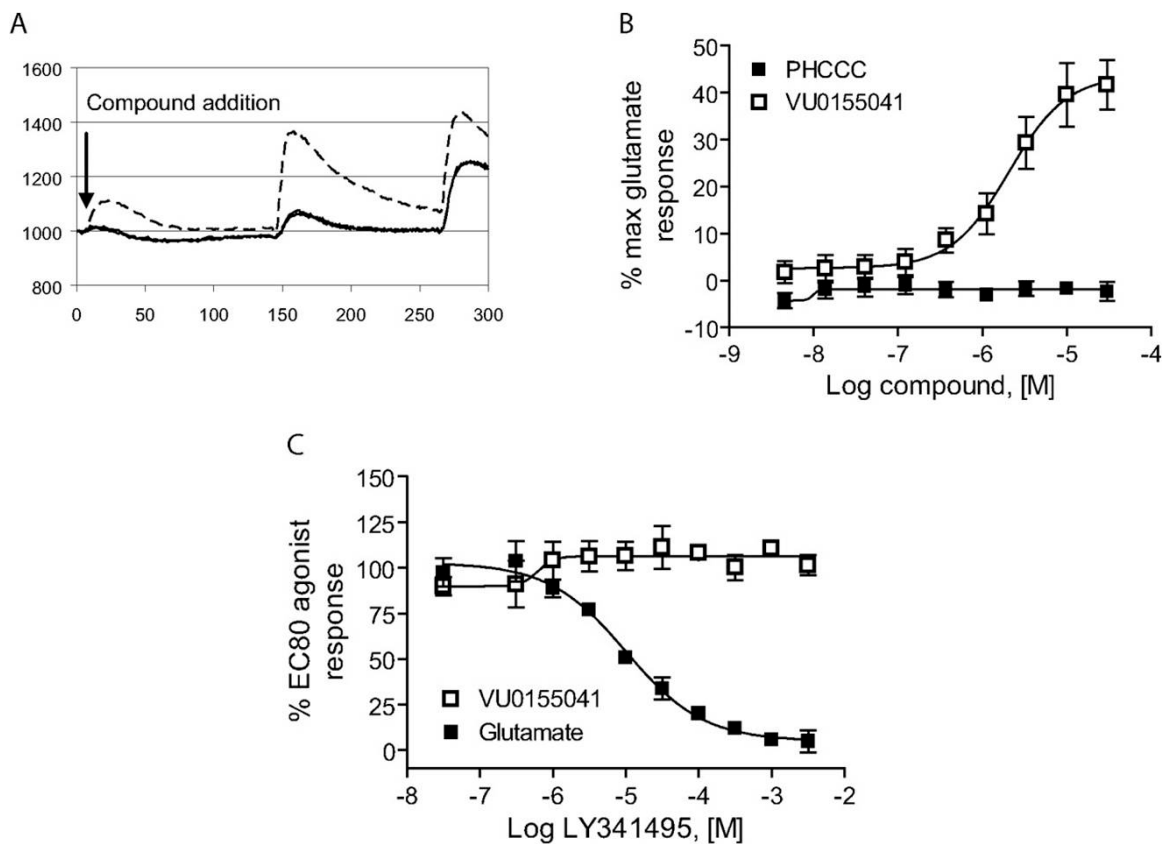
**Figure 7. The *cis* regioisomer (VU0155041) of VU0003423 was more potent and efficacious than the *trans* regioisomer (VU0155040) in two assays of mGluR4 function.** The *cis* (VU0155041) and *trans* (VU0155040) regioisomers of VU0003423 were synthesized as described in Niswender et al., 2008. (A) and (B), potencies of VU0155041 and VU0155040 were determined by adding increasing concentrations of each compound to cells, followed after 2.5 minutes by a submaximal ( $EC_{20}$ ) concentration of glutamate. At human mGluR4 (A), potencies were: VU0155041,  $798 \pm 58$  nM, and VU0155040,  $>10$   $\mu$ M. At rat mGluR4 (B), potencies were: VU0155041,  $693 \pm 140$  nM, and VU0155040,  $>10$   $\mu$ M. (C) and (D), A 30  $\mu$ M final concentration of compound, followed after 2.5 minutes by increasing concentrations of glutamate, was applied and responses were measured. At human mGluR4 (C), VU0155041 and VU0155040 caused  $6.4 \pm 0.7$ -fold and  $3.0 \pm 0.3$ -fold leftward shifts in the glutamate concentration-response curve (CRC), respectively. At rat mGluR4 (D), VU0155041 and VU0155040 caused  $4.7 \pm 0.4$ - and  $2.5 \pm 0.5$ -fold leftward shifts in the glutamate CRC, respectively. Results represent the mean  $\pm$  S.E.M. of 3-4 independent experiments performed in triplicate.

7, A and B). Fold-shift experiments at 30  $\mu$ M of each compound also showed that the *cis* regioisomer was more effective at this concentration on both human and rat mGluR4 (Figure 7, C and D). Further resolution by preparative chiral liquid chromatography of the pure *cis*-regioisomer into the two single *cis*-enantiomers revealed that both the (1*R*, 2*S*) and (1*S*, 2*R*) enantiomers were of equal potency and efficacy (data not shown).

### **Partial agonist activity of VU0155041 as revealed using the GIRK-mediated thallium flux assay**

Interestingly, in fold shift experiments performed using the thallium flux assay, we observed agonist activity of VU0003423 as well as the resolved regioisomers VU0155040 and VU0155041 (Figures 5 and 7). This agonist activity can be inferred from responses to ineffective concentrations of glutamate in the presence of the mGluR4 PAMs. Note that in the thallium flux assay, PAMs are added to cells prior to coapplication of thallium and glutamate, so agonist activity immediately following the PAM addition cannot be directly observed using our standard PAM evaluation protocol. In calcium assay experiments we also observed that compounds related to the VU0003423 scaffold induced a modest response when added alone. For instance, figure 8A is a calcium trace generated in the presence of 10  $\mu$ M ChemBridge compound 7307507 (VU0003423) from the original HTS; a small calcium response can be observed upon addition of the compound in the absence of glutamate. These effects were not observed with PHCCC (Figure 3B). This suggested that these new compounds might possess some intrinsic agonist activity at mGluR4.

To directly interrogate the agonist activity of VU0155041 in the thallium flux assay, I used a “single addition” protocol in which the PAM was applied to cells with thallium-containing buffer in order to measure responses immediately following PAM



**Figure 8. VU0155041 exhibited partial agonist activity at a site on rat mGluR4 that is distinct from the glutamate binding site.** A, Fluorescence trace of compound VU0003423 from the primary HTS revealing an increase in calcium mobilization upon addition of a 10  $\mu$ M concentration of compound (hatched line). B, Increasing concentrations of PHCCC or VU0155041 were added to rat mGluR4/GIRK cells directly in thallium buffer and responses were measured. Maximal agonist responses observed at 30  $\mu$ M, expressed as percent of the maximal glutamate response, were: VU0155041,  $41.6 \pm 5.3$ , and PHCCC,  $-2.3 \pm 2.0$ . The  $EC_{50}$  value for the partial agonist activity of VU0155041 was  $2.5 \pm 0.5$   $\mu$ M. C, Increasing concentrations of LY341495 were added to rat mGluR4/GIRK cells, followed 2.5 minutes later by an  $EC_{80}$  concentration of glutamate or VU0155041. LY341495 inhibited the glutamate response with an  $IC_{50}$  of  $14.5 \pm 4.3$   $\mu$ M; LY341495 failed to inhibit the VU0155041 response. Data were normalized to the percent of the relevant  $EC_{80}$  agonist response. Results represent the mean  $\pm$  S.E.M. of 3-5 independent experiments performed in triplicate or quadruplicate.



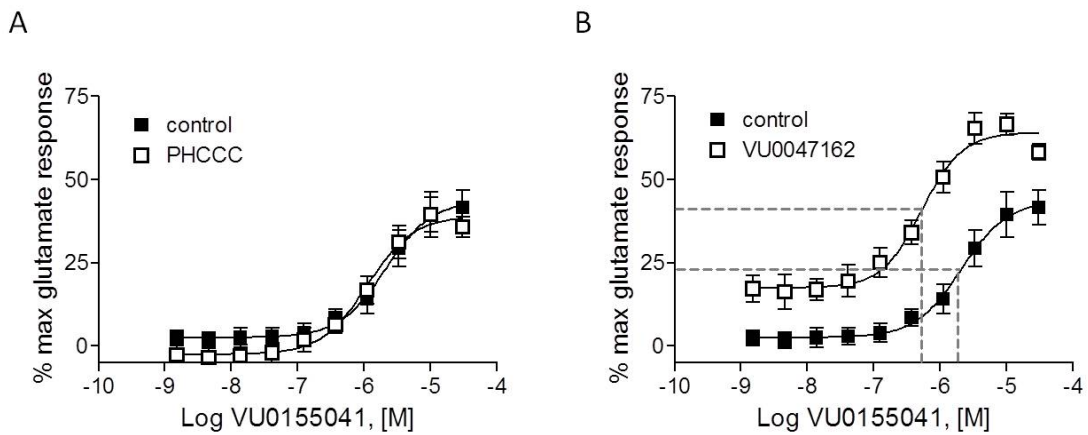
addition. Interestingly, VU0155041 alone induced a concentration-dependent response that reached approximately 45% of the maximal glutamate response, whereas PHCCC (concentrations up to 30  $\mu$ M) exhibited no agonist-like activity (Fig 2.6B). There are two possible reasons that agonist activity could be observed in a cell-based assay such as the thallium flux assay. First, the compound could possess intrinsic allosteric agonist activity, meaning that it directly activates the receptor without a requirement for glutamate binding. Alternatively, the response could be due to potentiation of ambient glutamate released from cells into the assay media. To distinguish between these possibilities, I tested the ability of the mGluR orthosteric antagonist LY341495 to block the apparent agonist activity of VU0155041. LY341495 completely blocked the response to an EC<sub>80</sub> concentration of glutamate, whereas it had no effect on the response to an EC<sub>80</sub> concentration of VU0155041 (Figure 8C). This finding suggests that the apparent agonist activity is not the result of potentiation of glutamate produced by the cells, but rather that VU0155041 and related compounds are partial allosteric agonists of mGluR4. Interestingly, this phenomenon has been observed previously with the mGluR5 PAM CDPPB (Kinney et al., 2005), which has intrinsic agonist activity that is likewise not blocked by an orthosteric antagonist of the receptor. It is important to note that if LY341495 did block the apparent agonist response to VU0155041, the agonist activity could not be definitively attributed to potentiation of “endogenous” glutamate, because it is possible that an orthosteric antagonist could noncompetitively block true allosteric agonist activity as well.

Discovery of the allosteric agonist activity of VU0155041 provides an exciting advance and suggests that it is possible to develop both pure allosteric potentiators as well as compounds with allosteric agonist activity at this receptor. It was interesting to find that VU0155041 had robust agonist activity whereas PHCCC did not exhibit this property in either assay. It is possible that these compounds act at distinct sites on

mGluR4, which may allow differential effects on receptor activation. Intriguingly, this possibility also suggests that there could be functional allosteric interactions between the mGluR4 PAMs that bind to different sites. To begin to address this question, I assessed the ability of PHCCC to potentiate the agonist activity of VU0155041. A fixed concentration of PHCCC (30  $\mu$ M) or DMSO-matched control buffer was added to cells 2.5 minutes prior to addition of increasing concentrations of VU0155041. Interestingly, PHCCC had no appreciable effect on the VU0155041 agonist curve, suggesting that these two compounds do not compete with each other, nor does PHCCC allosterically enhance the agonist activity of VU0155041 (Figure 9A). Conversely, another novel PAM discovered during our HTS campaign, VU0047162 (Chembridge compound 7637085, 30  $\mu$ M), increased both the potency and maximal efficacy of VU0155041 (Figure 9B). Interestingly, VU0047162 also possesses modest agonist activity in the thallium flux assay, and the maximal response elicited by coapplication of VU0155041 is more than additive with respect to the individual efficacies of these compounds. Taken together, these results suggest that VU0155041 and VU0047162 bind to distinct sites on mGluR4, and unlike PHCCC, these drugs are able to allosterically interact with one another to modulate mGluR4 function. While further studies are needed to determine the binding sites of these compounds, these data are interesting from a drug discovery perspective in the sense that there seem to be at least two allosteric modulator binding sites on mGluR4, and binding to these sites may differentially impact mGluR4 function.

**VU0155041 exhibited selectivity for mGluR4 relative to 67 different targets and did not affect the function of striatal NMDA receptors**

As discussed above, mGluR4 has been postulated to be a target for novel therapeutic agents used for the treatment of PD. Identification of novel structures with PAM activity at mGluR4 now allows us to further address the therapeutic potential of



**Figure 9. PHCCC and VU0047162 differentially affect the agonist activity of VU0155041.** A, A 30 μM final concentration of PHCCC was added to rat mGluR4/GIRK cells, followed after 2.5 minutes by various concentrations of VU0155041. PHCCC did not significantly alter the potency or efficacy of VU0155041. The EC<sub>50</sub> values for VU0155041 in the absence and presence of PHCCC were 2.3 ± 0.5 μM and 1.1 ± 0.1 μM, respectively ( $P=0.082$ , unpaired t test). B, A 30 μM final concentration of VU0047162 was added to rat mGluR4/GIRK cells, followed after 2.5 minutes by various concentrations of VU0155041. VU0047162 increased the maximal response and significantly altered the potency or efficacy of VU0155041. The EC<sub>50</sub> values for VU0155041 in the absence and presence of VU0047162 were 2.3 ± 0.5 μM and 0.53 ± 0.03 μM, respectively ( $P=0.0246$ , unpaired t test). Results represent the mean ± S.E.M. of 3 independent experiments performed in quadruplicate.

these drugs in alleviating motor deficits in preclinical models of PD. Previous studies have shown that icv administration of the general group III agonist L-AP4, as well as the mGluR4 PAM/mGluR1 antagonist PHCCC, are effective in reversing reserpine-induced akinesia, a preclinical model of PD (Marino et al., 2003; Valenti et al., 2003). The development of alternate mGluR4 PAMs with better selectivity for mGluR4 has now allowed us to further test the hypothesis that compounds with the ability to increase mGluR4 activity can have beneficial effects on akinetic deficits in these models. Although we had confirmed the selectivity of our lead PAM relative to other mGluR subtypes, prior to animal studies we sought to determine if this compound might exhibit ancillary activity at other GPCRs, transporters, or ion channels that could complicate implementation or interpretation of *in vivo* experiments. VU0155041 was evaluated at MDS-Pharma Services for effects on radioligand binding at 67 different targets including GPCRs, ion channels, and transporters. As reported in Table 4, VU0155041 had no effect on binding at any target examined. Despite the lack of binding activity at a large number of targets, it is possible that VU0155041 could have functional effects at other targets that could be responsible for, or at least confounding to, the interpretation that mGluR4 PAMs have antiparkinsonian activity. In particular, antagonism of the NMDA receptor NR2B subtype has been shown to have antiparkinsonian effects in preclinical models of PD (reviewed in Johnson et al., 2009). To verify that VU0155041 did not antagonize NMDA receptors, Dr. Zixiu Xiang tested the ability of this mGluR4 PAM to functionally block NMDA receptor currents in striatal medium spiny neurons. These results were compared with the ability of a known NR2B antagonist, [( $\alpha R, \beta S$ )- $\alpha$ -(4-hydroxyphenyl)- $\beta$ -methyl-4-(phenylmethyl)-1-piperidinepropanol] (Ro25-6981) (Fischer et al., 1997), to regulate NMDA currents in these same neurons. VU0155041 (10  $\mu$ M) did not affect NMDA receptor currents in striatal medium spiny neurons whereas 1  $\mu$ M Ro25-

**Table 4. Compound VU0155041 was examined at MDS-Pharma for displacement of radioligand binding activity within the LeadProfilingScreen® series and the % inhibition of respective radioligand binding at each target is shown.**

Receptor/Target	Species	VU0155041 % inhibition
Adenosine A <sub>1</sub>	human	9
Adenosine A <sub>2A</sub>	human	3
Adenosine A <sub>3</sub>	human	-4
Adrenergic α <sub>1A</sub>	rat	7
Adrenergic α <sub>1B</sub>	rat	4
Adrenergic α <sub>1D</sub>	human	-4
Adrenergic α <sub>2A</sub>	human	0
Adrenergic β <sub>1</sub>	human	-8
Adrenergic β <sub>2</sub>	human	0
Androgen (Testosterone) AR	rat	6
Bradykinin B <sub>1</sub>	human	3
Bradykinin B <sub>2</sub>	human	-1
Calcium Channel L-Type, Benzothiazepine	rat	-11
Calcium Channel L-Type, Dihydropyridine	rat	-3
Calcium Channel N-Type	rat	1
Dopamine D <sub>1</sub>	human	6
Dopamine D <sub>2S</sub>	human	-1
Dopamine D <sub>3</sub>	human	0
Dopamine D <sub>4.2</sub>	human	13
Endothelin ET <sub>A</sub>	human	-2
Endothelin ET <sub>B</sub>	human	-4
Epidermal Growth Factor (EGF)	human	0
Estrogen ERα	human	-4
G Protein-coupled Receptor GPR103	human	-14
GABA <sub>A</sub> , Flunitrazepam, Central	rat	-3
GABA <sub>A</sub> , Muscimol, Central	rat	0
GABA <sub>B1A</sub>	human	2
Glucocorticoid	human	6
Glutamate, Kainate	rat	-3
Glutamate, NMDA, Agonism	rat	-10
Glutamate, NMDA, Glycine	rat	7

Receptor/Target	Species	VU0155041 % inhibition
Glutamate, NMDA, Phencyclidine	rat	9
Histamine H <sub>1</sub>	human	-10
Histamine H <sub>2</sub>	human	3
Histamine H <sub>3</sub>	human	13
Imidazoline I <sub>2</sub> , Central	rat	4
Interleukin IL-1	mouse	-3
Leukotriene, Cysteinyl CysLT <sub>1</sub>	human	1
Melatonin MT <sub>1</sub>	human	-6
Muscarinic M <sub>1</sub>	human	0
Muscarinic M <sub>2</sub>	human	1
Muscarinic M <sub>3</sub>	human	3
Neuropeptide Y Y <sub>1</sub>	human	-9
Neuropeptide Y Y <sub>2</sub>	human	5
Nicotinic Acetylcholine	human	3
Nicotinic Acetylcholine $\alpha$ 1, Bungarotoxin	human	-5
Opiate $\delta$ (OP1, DOP)	human	-2
Opiate $\kappa$ (OP2, KOP)	human	6
Opiate $\mu$ (OP3, MOP)	human	1
Phorbol Ester	mouse	-7
Platelet Activating Factor (PAF)	human	9
Potassium Channel [K <sub>ATP</sub> ]	hamster	8
Potassium Channel hERG	human	-6
Prostanoid EP <sub>4</sub>	human	-2
Purinergic P <sub>2X</sub>	rabbit	3
Purinergic P <sub>2Y</sub>	rat	-14
Rolipram	rat	0
Serotonin (5-Hydroxytryptamine) 5-HT <sub>1A</sub>	human	1
Serotonin (5-Hydroxytryptamine) 5-HT <sub>3</sub>	human	-3
Sigma $\sigma$ <sub>1</sub>	human	-11
Sigma $\sigma$ <sub>2</sub>	rat	-19
Sodium Channel, Site 2	rat	0
Tachykinin NK <sub>1</sub>	human	-2
Thyroid Hormone	rat	-1
Transporter, Dopamine (DAT)	human	2

<b>Receptor/Target</b>	<b>Species</b>	<b>VU0155041 % inhibition</b>
Transporter, GABA	rat	5
Transporter, Norepinephrine (NET)	human	-2
Transporter, Serotonin (5-Hydroxytryptamine) (SERT)	human	3

6981 induced significant blockade (data not shown). These results indicate that VU0155041 does not antagonize NMDA receptor activity and that direct NMDA receptor antagonism should not confound interpretation of the effects of VU0155041 in models of PD.

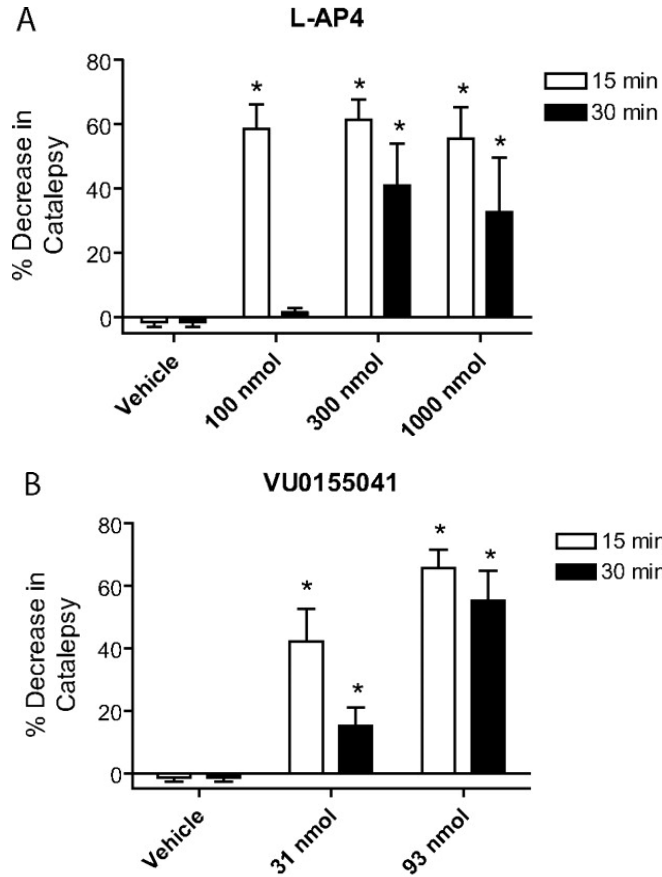
### **VU0155041 had antiparkinsonian effects in preclinical rodent models of PD**

Encouragingly, in addition to improvements in potency and selectivity over previously described mGluR4 PAMs, VU0155041 was also found to be soluble in an aqueous vehicle. In contrast, PHCCC is only soluble in vehicles containing high concentrations of DMSO or other relatively toxic vehicles; these vehicles can cause tissue damage when injected icv and can compromise the blood brain barrier when a compound is administered systemically. Due to the tolerance of VU0155041 for a more toxicology-friendly vehicle, the effects of this compound were compared to the general group III agonist, L-AP4, as the vehicles used for their preparation were both aqueous (Materials and Methods).

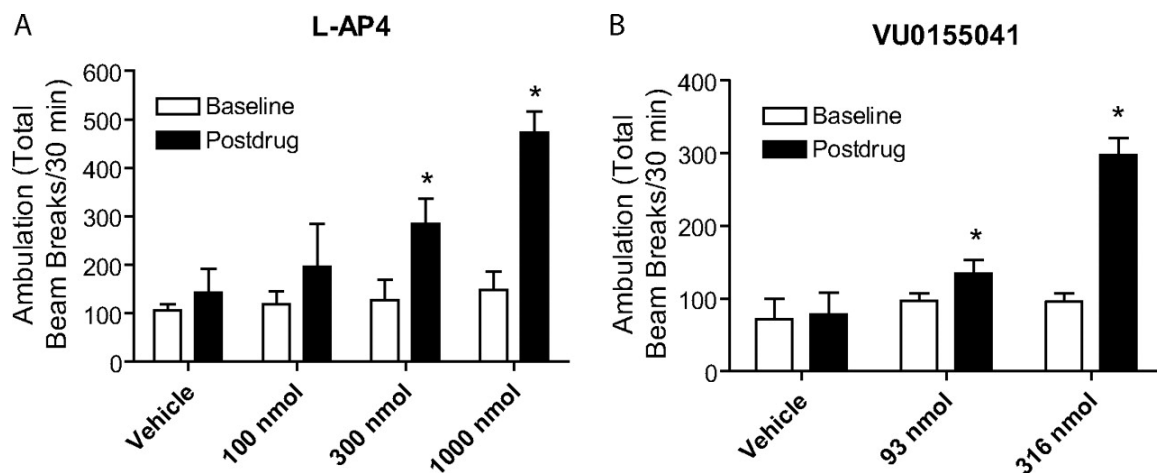
L-AP4 and VU0155041 were first assessed for their ability to decrease haloperidol-induced catalepsy. As previously demonstrated, L-AP4 significantly decreased haloperidol-induced catalepsy in a dose-dependent manner (Figure 10A). Encouragingly, VU0155041, at doses of 31 and 92 nmol, was also able to significantly decrease the cataleptic effects of haloperidol, and the effects of the compound were still present 30 min after infusion (Figure 10B).

Reversal of reserpine-induced akinesia is another preclinical model of PD used to assess the activity of compounds for antiparkinsonian effects, although the effects are much more difficult to reverse compared to the catalepsy model. Icv infusion of 300 and 1000 nmol doses of L-AP4 into ventricles of animals that had been pretreated for two hours with reserpine induced significant reversals of akinesia (Figure 11A). Icv infusion





**Figure 10. VU0155041 reversed catalepsy induced by the dopamine D2 receptor antagonist haloperidol in rats.** Rats were treated with 1.5 mg/kg of haloperidol as described in Materials and Methods. After 2h, animals were infused icv with the indicated doses of either L-AP4 or VU0155041 and catalepsy was measured 15, 30 and 60 min after injection (15 minute (white bars) and 30 minute, (black bars) results shown). Experiments represent data obtained for 6 rats per group. \* $p < 0.05$ , Dunnett's comparison with vehicle group. Data contributed by Drs. Carrie Jones and Analisa Thompson.



**Figure 11. VU0155041 reversed reserpine-induced akinesia in rats.** Rats were treated with 5 mg/kg reserpine as described in Materials and Methods. 2 hours later, animals were infused with the indicated doses of either L-AP4 or VU0155041 and a reversal of akinesia, monitored via locomotor activity, was measured. Data are plotted as baseline locomotor activity (white bars) and then the activity measured 15 minutes after compound infusion (black bars). Experiments represent data obtained for 7 rats per group. \* $p < 0.05$ , Dunnett's comparison with vehicle group. Data contributed by Drs. Carrie Jones and Analisa Thompson.

of a 316 nmol dose of VU0155041 also resulted in a significant reversal of akinesia (Figure 11B). VU0155041 represents only the second unique chemical scaffold with mGluR4 PAM activity that shows efficacy in rodent models of PD, further validating the role of this receptor as a therapeutic target in PD. Excitingly, the improvements in potency, selectivity, and solubility of VU0155041 compared to PHCCC suggest that this compound will serve as a valuable research tool to continue to define the role of mGluR4 in normal physiology and to further explore the therapeutic potential of mGluR4 in PD and other disease states.

## Discussion

Therapeutic strategies for alleviating the motor deficits of PD remain a major unmet medical need due to serious disadvantages of currently available treatments that rely on dopamine replacement (see Chapter I for further discussion). The success of surgical strategies for treating PD, such as lesion or deep brain stimulation of the STN or GPi, has led to the hypothesis that other manipulations aimed at reducing activity of the indirect pathway might restore the balance in basal ganglia circuitry that is lost following dopamine denervation and thereby reduce motor deficits. Several classes of pharmacological agents have been proposed as strategies to achieve the overarching goal of reducing excessive activity through the indirect pathway. These include NMDA receptor antagonists, adenosine A2A receptor antagonists, mGluR5 antagonists, and mGluR4 PAMs. The goal of the present study was to validate the concept that mGluR4 PAMs may represent a tractable target for treating the motor deficits of PD, both in terms of novel small molecule discovery and *in vivo* efficacy in preclinical models of PD.

Prior to the studies presented here, a lack of pharmacological tools that selectively enhance mGluR4 activation prevented thorough assessment of the viability of mGluR4 as a target for alleviating PD motor deficits. Previous efforts to achieve subtype

selectivity primarily focused on amino acid analogs (such as L-AP4) that are known to activate particular mGluR subtypes. However, the high level of conservation of the glutamate binding site among different mGluR subtypes has hampered the discovery of truly selective pharmacological tools; indeed, four years following the original publication of this work, extensive efforts to create mGluR4-selective orthosteric agonists have yielded a small number of compounds with improved selectivity over mGluR7 and mGluR8, but none that are truly selective for mGluR4 (Flor and Acher, 2012). In contrast, drug discovery efforts targeting allosteric sites on specific mGluR subtypes have provided major advances in the availability of subtype-selective tools and even potential clinical candidates. This is likely due to the fact that allosteric sites on different mGluRs are less conserved than the glutamate binding site, providing more feasible structural differences for achieving selectivity. However, prior to this study, the three previously identified mGluR4 PAMs (PHCCC, MPEP, and SIB-1893) all had off-target activity at another mGluR subtype and as a result were not ideal tool compounds for probing the normal physiological functions of mGluR4 or for assessing the therapeutic potential of this receptor.

In addition to the improved selectivity of VU0155041 over previously available mGluR4 PAMs, the discovery of this compound represents several additional advances in terms of determining the potential for developing a drug-like compound that enhances mGluR4 activity. Importantly, discovery of new mGluR4 PAMs from diverse chemical scaffolds provide many starting points for medicinal chemistry efforts to make compounds with improved drug-like properties suitable for systemic dosing and advancement to clinical testing. The lead compound described here, VU0155041, also exhibits several features that represent critical advances, including submicromolar potency, improved aqueous solubility, and excellent selectivity when tested at a large number of CNS-relevant targets. The improved properties of this compound have

allowed validation of the idea that mGluR4 PAMs have potential for the treatment of motor symptoms in PD, as suggested by its ability to reverse motor deficits in multiple models of PD.

The studies here also provide important new insights into the molecular pharmacology of allosteric modulators of mGluR4. It is interesting that both PHCCC and VU0155041 affected the concentration-response curves of glutamate differently in the two assays used for assessing mGluR4 activity. Both compounds increased the maximum response seen in the calcium assay but not the maximum response obtained using the thallium flux method. There are many possible explanations for this observation, including differences in signal amplification between the two pathways, possible species differences, or difference in receptor expression. Unfortunately, it is difficult to accurately measure expression of mGluR4 in cell lines due to the lack of a high affinity radiolabeled antagonist for the receptor (for example, see (Wright et al., 2000)); we should note, however, that the potency of glutamate in the two mGluR4 cell lines is similar ( $5.7 \pm 0.7 \mu\text{M}$ , calcium assay versus  $9.2 \pm 1.2 \mu\text{M}$ , thallium flux/GIRK assay, data obtained from Figure 6), suggesting that there may not be dramatic differences in receptor reserve between the two systems. Another possible explanation for the ability of compounds to potentiate maximal responses in the calcium assay may involve distinctions in the coupling efficiency of mGluR4 to  $G_{q15}$  versus  $G_{i/o}$ . This might manifest in an inability of glutamate alone to achieve the maximal response possible within the  $G_{q15}$ -mediated signaling pathway; the presence of a PAM might stabilize the receptor or enhance its function in such a way as to permit the efficacy as well as the potency of glutamate to be enhanced.

An additional distinction between VU0155041 and PHCCC was revealed by the ability of VU0155041 to activate mGluR4 in the absence of glutamate. When added alone in the GIRK assay, the concentration-dependent response induced by VU0155041

reached approximately 45% of the maximal glutamate response and was not affected by preincubation with the orthosteric antagonist LY341495. This suggests that the activity of VU0155041 is not due to a potentiation of glutamate present in the assay system, and further suggests that the agonist activity of VU0155041 is not mediated by actions at the orthosteric LY341495 binding site. Together, these data suggest that VU0155041 possesses allosteric agonist activity, which further differentiates the compound from PHCCC. Interestingly, a small degree of allosteric agonist activity may be beneficial for *in vivo* activity of an mGluR4 PAM, particularly when targeting mGluR4 activation at a GABAergic synapse, where the availability of glutamate to activate the receptor is unknown.

Among the most interesting of the molecular pharmacology results presented here was the finding that two allosteric modulators of mGluR4, VU0155041 and VU0047162, functionally interact with one another. This result suggests that at least two allosteric binding sites exist on mGluR4, and that in addition to exhibiting cooperativity with the glutamate binding site, they likewise display cooperativity with each other. The inability of PHCCC to alter the allosteric agonist activity of VU0155041 also suggests that this pair of drugs binds to two distinct sites on the receptor, because one would expect to see a competitive antagonism of VU0155041 receptor activation if PHCCC were competing for the same binding site without contributing any intrinsic agonist activity. Since the completion of these studies, the hypothesis that PHCCC and VU0155041 bind to different sites on mGluR4 has been validated through the use of a novel radioligand that is competitive with the PHCCC binding site and is not displaced by VU0155041 (Hess et al., unpublished data). Whether other mGluR4 PAMs such as VU0047162 bind to the same site as PHCCC remains unclear. However, it is possible that two PAMs could bind to the same site on the receptor but have a differential ability to allosterically modulate the activity of a second binding site, so the finding that

VU0047162 and PHCCC do not have the same effect on VU0155041-mediated receptor activation does not provide any substantial evidence that these two compounds do not share a binding site. Further studies using techniques such as competition binding and mutagenesis will be necessary to further characterize the binding sites of various mGluR4 PAMs.

In summary, we have identified a series of novel compounds with mGluR4 PAM activity. These compounds are active on both the rat and human receptors and function as PAMs in two different assays of mGluR4 function. The lead compound, VU0003423, is more potent than PHCCC and selective for mGluR4 among the mGluRs. The *cis*-regioisomer of VU0003423, VU0155041, does not exhibit binding activity at a large number of off-target receptors, transporters, and ion channels, does not inhibit NMDA receptor functional activity, is soluble in an aqueous vehicle, and exhibits activity in two different rodent behavioral models of PD. These compounds represent breakthrough new tools for the study of the role of mGluR4 in normal brain function as well as in pathophysiological states such as PD.

## CHAPTER III

### THE METABOTROPIC GLUTAMATE RECEPTOR 8 AGONIST (S)-3,4-DCPG REVERSES MOTOR DEFICITS IN PROLONGED BUT NOT ACUTE MODELS OF PARKINSON'S DISEASE

#### Introduction

Several recent studies using group III mGluR-selective agonists, such as L-AP4 and ACPT-I, have demonstrated that these compounds have antiparkinsonian effects in rodent PD models, including reserpine-induced akinesia, haloperidol-induced catalepsy, and 6-OHDA lesion-induced motor deficits (Konieczny et al., 2007; Lopez et al., 2012; Lopez et al., 2007; Lopez et al., 2008; MacInnes et al., 2004; Sibille et al., 2007; Valenti et al., 2003). To determine the specific mGluR subtypes that mediate the antiparkinsonian effects of group III mGluR agonists, recent studies have taken advantage of subtype-selective agonists and PAMs of mGluR4 (East et al., 2010; Goudet et al., 2012; Jones et al., 2011a; Jones et al., 2011b; Marino et al., 2003; Niswender et al., 2008b). In rats, these compounds reverse reserpine-induced akinesia, haloperidol-induced catalepsy, and motor deficits caused by unilateral 6-OHDA lesion, suggesting that mGluR4 activation is at least partially responsible for the antiparkinsonian effects of group III mGlu agonists in PD animal models. However, the potential contribution of other group III mGlu subtypes has not been fully elucidated. We now report that the mGluR8 agonist DCPG (Thomas et al., 2001) has behavioral effects predictive of antiparkinsonian actions in several rodent models of PD when administered via an intracerebroventricular route of administration. Interestingly, reversal of akinetic motor deficits by DCPG requires a prolonged state of dopamine depletion or dopamine receptor blockade, suggesting that mGluR8 function in the basal ganglia may differ in the intact versus dopamine-depleted states. These findings suggest that mGluR8



activation may partially mediate the anti-akinetic effects of group III mGlu agonists in prolonged dopamine depletion models. The work presented here is adapted from (Johnson et al., 2012). Dr. Carrie Jones contributed to the behavioral experiments described in this chapter.

## **Materials and Methods**

### **Animals**

Two hundred seventy-one third ventricle-cannulated (TVC) male Sprague-Dawley rats weighing 250 to 300 grams were purchased from Taconic Farms, Inc. (Hudson, NY). Cannula placement (AP= -0.8 mm, ML = 0.0 mm and DV= -8.0 mm, relative to bregma) allowed infusion of non-brain penetrant drugs into the third ventricle. Cannula placement was visually verified following sacrifice for all animals used in these studies. Animals that underwent forelimb asymmetry testing were lesioned by unilateral injection of 6-OHDA (6-OHDA) into the medial forebrain bundle prior to TVC surgery. 6-OHDA lesions were functionally verified using an apomorphine-induced rotation test (performed by Taconic Farms, Inc.). On day 21 post-lesion, apomorphine (0.05 mg/kg, sc) was administered and rotations contralateral to the lesion were measured in a rotometer for 6 consecutive 5 minute periods (30 minutes total). Only animals with greater than 180 rotations in 30 minutes or multiple 5 minute periods of more than 6 rotations per minute were used for the forelimb asymmetry study. For studies that did not require intracerebroventricular (icv) drug administration, thirty-eight male Sprague-Dawley rats (250 to 300 grams) that had not undergone TVC surgery were used (Harlan, Indianapolis, IN). Animals were maintained in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care under a 12 hour light/dark cycle (lights on 06:00 to 18:00) with free access to food and water. All experiments were performed during the light cycle, were approved by Vanderbilt

University's Institutional Animal Care and Use Committee, and conformed to guidelines established by the National Research Council *Guide for the Care and Use of Laboratory Animals*. All efforts were made to minimize animal suffering and the number of animals used.

## **Drugs**

(S)-3,4-DCPG and L-AP4 were purchased from Tocris Bioscience (Ellisville, MO). Haloperidol (free base) and reserpine were purchased from Sigma-Aldrich (St. Louis, MO). The decanoate salt of haloperidol was synthesized in-house. DCPG and L-AP4 were prepared in artificial cerebrospinal fluid (ACSF) and administered icv at a rate of 0.5 to 1  $\mu$ l/min in the indicated volume. Injection cannulae were left in place for an additional five minutes after completion of infusion. Haloperidol (1.5 mg/kg) was dissolved in 0.2% lactic acid, and pH was adjusted to ~6.5 with 1N NaOH. Haloperidol was administered intraperitoneally (ip) in a volume of 1 ml/kg. Reserpine (5 mg/kg, dissolved in 1% acetic acid) was prepared as described previously (Valenti et al., 2003), and administered subcutaneously (sc) in a volume of 1 ml/kg under light isoflurane anesthesia. Haloperidol decanoate (50-200 mg/kg) was dissolved in sesame oil using a mortar and pestle, and was administered intramuscularly (im) in a volume of 2 ml/kg; half of the dose was given in each femoral muscle. All drugs were prepared fresh on the day of the experiment.

## **Induction and measurement of catalepsy**

For acute catalepsy studies, haloperidol (1.5 mg/kg, ip) was administered two hours prior to baseline catalepsy measurement. For prolonged haloperidol-induced catalepsy studies, three doses of haloperidol (1.5 mg/kg, ip) were evenly spaced over 18-20 hours prior to baseline catalepsy measurement. For haloperidol decanoate-

induced catalepsy studies, animals were pretreated for the indicated time prior to measurement of catalepsy. Animals were returned to their home cages during the haloperidol pretreatment period. Catalepsy was assessed by placing each rat's forepaws on a horizontal bar positioned 6 cm above the testing surface and measuring the latency for the rat to remove one forepaw. Trials were ended after 60 seconds if no forepaw had been removed, and a score of 60 seconds was recorded for that trial. For reversal studies, rats were then given an icv infusion of either DCPG (2.5 or 10 nmol), L-AP4 (50 nmol or 100 nmol), or vehicle (ACSF). Rats were retested for catalepsy either five minutes after completion of drug infusion (haloperidol studies) or 10, 20, and 30 minutes after completion of infusion (haloperidol decanoate studies).

### **Induction and measurement of akinesia**

Rats were treated with reserpine (5 mg/kg, sc) and returned to their home cages for either 2 hours (acute treatment) or 18-20 hours (prolonged treatment) prior to measurement of baseline akinesia. Locomotor activity was measured for 30 minutes by placing rats in photocell activity cages (Hamilton-Kinder, Poway, CA) equipped with 16 x 16 infrared beams. Akinetic rats then received an icv infusion of DCPG (2.5, 10, or 30 nmol), L-AP4 (50 nmol), or vehicle (ACSF), and activity was measured for an additional 30 minutes. For haloperidol decanoate characterization, locomotor activity was measured for 30 minutes at the indicated times after haloperidol decanoate administration following baseline catalepsy measurement as described above.

### **Measurement of striatal dopamine levels**

Rats were treated with reserpine (5 mg/kg, sc) or vehicle for either 2 hours (acute treatment) or 18-20 hours (prolonged treatment) prior to sacrifice. Brains were removed rapidly and placed into ice-cold 0.9% NaCl solution. Tissue samples of caudate putamen

(CPu) were taken bilaterally, immediately weighed, and stored on dry ice. Samples were analyzed for striatal monoamine content using high performance liquid chromatography (HPLC) with electrochemical detection (Hackler et al., 2006). Briefly, samples were homogenized in 0.1M trichloroacetic acid containing 10 mM sodium acetate, 100  $\mu$ M EDTA, and 10.5% methanol (pH 3.8). Homogenized samples were centrifuged at 10,000 x g for 20 minutes, and the supernatant was removed and stored at -80°C prior to measurement of dopamine content. Total protein concentration was determined by assaying the pellet using the BCA Protein Assay Kit (Pierce Chemical Company, Rockford, IL).

### **Estimation of D2 receptor occupancy with PET imaging**

D2 dopamine receptor occupancy following haloperidol administration was measured using positron emission tomography (PET) as described previously (Jones et al., 2008; Tantawy et al., 2009). One week prior to the first day of testing, rats underwent surgery to install catheters in the jugular vein for radiotracer administration. On the first testing day, vehicle (0.2% lactic acid, ip) was administered two hours prior to injection of ~13 MBq/0.2 mL [ $^{18}$ F]fallypride [(S)-N-[(1-allyl-2-pyrrolidiny)methyl]-5-(3'-[ $^{18}$ F]fluoropropyl)-2,3-dimethoxybenzamide]. Rats were then returned to their home cages with free access to food and water. Sixty minutes after [ $^{18}$ F]fallypride injection, rats were anesthetized using isoflurane (~1.5%), placed in the microPET Focus 220 (Siemens, Knoxville, TN) and a 60 min dynamic acquisition was initiated. This delayed scan method was used in order to minimize the effects of anesthesia on binding potential estimates as previously described (Tantawy et al., 2011). One week later, the same rats were injected with haloperidol (free base, 1.5 mg/kg, ip) two hours prior to intravenous injection of [ $^{18}$ F]fallypride. After 60 minutes, rats were anesthetized with ~1.5% isoflurane, and images were acquired using the same protocol as in the control

session. Receptor occupancy for haloperidol decanoate was measured similarly, except that vehicle (sesame oil) or haloperidol decanoate (100 mg/kg, im) was administered 20 hours prior to radiotracer injection. Images were reconstructed after correcting for scatter and attenuation as described previously (Tantawy et al., 2009). Briefly, attenuation maps were created from a transmission image obtained using a  $^{57}\text{Co}$  source. Data were reconstructed on a 128 X 128 X 95 grid with a pixel size of 0.095 cm and a slice thickness of 0.080 cm. Dynamic images were reconstructed using an OSEM2D algorithm with a sequence of five 60s frames (5 X 60 s), 2 X 300 s, 2 X 600 s, 2 X 1200 s, 1 X 600 s, 6 X 300 s, 2 X 600 s, and 3 X 1200 s.

### **Measurement of 6-OHDA-induced forelimb use asymmetry**

Rats with unilateral 6-OHDA lesion of the medial forebrain bundle, whose dopamine depletion status was functionally validated by the ability of apomorphine to induce contralateral rotation (see above) were used to assess the ability of DCPG and L-AP4 to reverse lesion-induced forelimb use deficits (Schallert et al., 2000; Valenti et al., 2003). To this end, 6-OHDA-lesioned rats with TVC surgery were used to allow for the icv administration of DCPG and L-AP4. Rats were handled daily prior to testing. On the test day, rats were placed in a transparent plastic cylinder (20 cm in diameter and 30 cm high). Animals were not habituated to the cylinder prior to the first test session. Baseline limb-use asymmetry was measured over a 10 minute period, after which rats were infused with either L-AP4 (100 nmol/2.5  $\mu\text{l}$ ) or DCPG (2.5 or 10 nmol/2.5  $\mu\text{l}$ ). Five minutes after the end of the infusion, limb-use behavior was measured for an additional 10 minute period. Experimental sessions were monitored with a camera located beneath the cylinder for off-line analysis of limb-use. Video analysis of forelimb use was performed by two raters who were blinded to the treatment groups. Forelimb use was scored as described previously and data were expressed as Forelimb Asymmetry (FLA)

Index Score (Jones et al., 2011a; Lundblad et al., 2004). The FLA Index Score is a composite of the number of **W**all contacts of the unaffected ( $W_{unaffected}$ ) and affected ( $W_{impaired}$ ) forelimb and **L**andings of the unaffected ( $L_{unaffected}$ ) and affected ( $L_{impaired}$ ) forelimb (see section 2.8, Data analysis and statistics).

To verify the extent of 6-OHDA lesion-induced dopamine depletion a randomly selected cohort of rats ( $n = 24$ ) was sacrificed and striatal tissue was dissected from the ipsilateral (lesioned) and contralateral (intact) dorsal striatum. Samples were frozen on dry ice and tissue concentrations of dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were determined by HPLC with electrochemical detection as described above (Measurement of striatal dopamine levels). Correct placement of the guide cannula targeting the third ventricle was verified in all animals used for this study.

### **Data analysis and statistics**

All calculations associated with data analysis were performed using Microsoft Excel. Statistical tests were performed using JMP IN 5.1 or StatView. Graphs were created using SigmaPlot 9.0. Catalepsy and akinesia results are summarized as percent change between baseline and post-drug measurements. For most behavioral experiments, data were analyzed for a main effect of drug treatment using whole model one-way analysis of variance (ANOVA). When a statistically significant effect was found, *post hoc* analysis was performed using a Dunnett's test to compare drug treatment groups with the vehicle group. Data are expressed as mean  $\pm$  SEM. For behavioral characterization of haloperidol decanoate effects, data were analyzed using repeated measures ANOVA followed by pairwise comparisons using a Bonferroni correction. Lesion-induced changes in transmitter concentrations and reversal of haloperidol effects by DCPG were analyzed using an unpaired Student's *t* test. For D2 receptor occupancy

studies, the microPET images were coregistered to a rat brain template (Rubins et al., 2003) using AMIDE (Loening and Gambhir, 2003) and volumetric regions of interest (ROIs) were drawn around the striata and the cerebellum generating time-activity curves (TACs) over the duration of the scans. Binding potentials (BPs) were estimated via a Logan plot (Logan, 2000). D2 receptor occupancy was calculated as  $[1 - (\text{Drug BP}/\text{Vehicle BP})] \times 100$ . Forelimb asymmetry data were analyzed as described previously (Valenti et al., 2003). To assess forelimb use in 6-OHDA-lesioned rats the FLA Index Score was calculated as follows (Jones et al., 2011a):

$$\text{FLA Index Score} = 100 * [(L_{\text{impaired}} - L_{\text{unaffected}}) + (W_{\text{impaired}} - W_{\text{unaffected}})] / [(L_{\text{impaired}} + L_{\text{unaffected}}) + (W_{\text{impaired}} + W_{\text{unaffected}})]$$

Using this method, rats showing preferential use of the unaffected forelimb (ipsilateral to the lesion) have negative FLA Index Scores, whereas rats that do not show a forelimb bias have FLA Index Scores that are close to zero. Statistical comparison of drug effects on forelimb asymmetry was performed using two-way ANOVA followed by Bonferroni comparisons.

## Results

### **In contrast to the group III mGlu agonist L-AP4, the selective mGluR8 agonist DCPG does not reverse acute reserpine-induced akinesia and haloperidol-induced catalepsy**

Reserpine-induced akinesia and haloperidol-induced catalepsy are preclinical models of PD that are commonly used in rodents. For example, antiparkinsonian drugs such as L-DOPA increase locomotor activity in reserpine-treated rats (Colpaert, 1987), indicating that reversal of akinesia by pharmacological agents is consistent with antiparkinsonian activity. The nonselective group III mGluR agonist L-AP4 (50 nmol, icv) and the mGluR8-selective agonist DCPG (2.5, 10, or 30 nmol, icv) were evaluated for

their ability to reverse the akinesia induced by a two hour pretreatment with reserpine (5 mg/kg, sc; Figure 12A). In agreement with previous findings (Valenti et al., 2003), L-AP4 significantly increased locomotor activity in rats acutely treated with reserpine ( $P < 0.05$ , Dunnett's comparison with vehicle group;  $n = 10$  animals per treatment group).

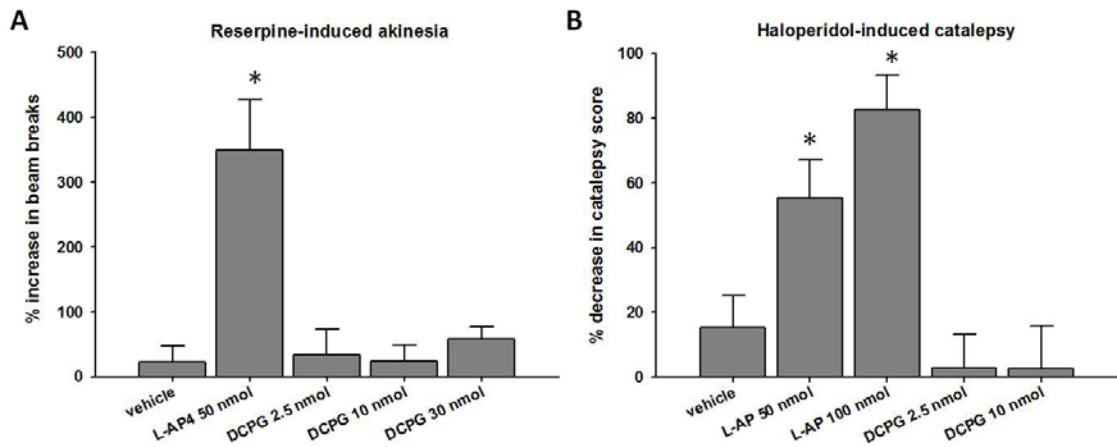
Conversely, DCPG did not increase locomotor activity at any dose tested (Figure 12A).

In addition to studies in the reserpine model, we evaluated the ability of L-AP4 (50 or 100 nmol) and DCPG (2.5 or 10 nmol) to reverse the catalepsy induced by a two hour pretreatment with haloperidol (1.5 mg/kg, ip). Similar to previously reported results (Valenti et al., 2003), L-AP4 significantly reversed haloperidol-induced catalepsy at both doses ( $P < 0.05$ , Dunnett's comparison with vehicle group;  $n = 10$  animals per treatment group). In contrast, DCPG had no effect on catalepsy induced by a two hour pretreatment with haloperidol (Figure 12B).

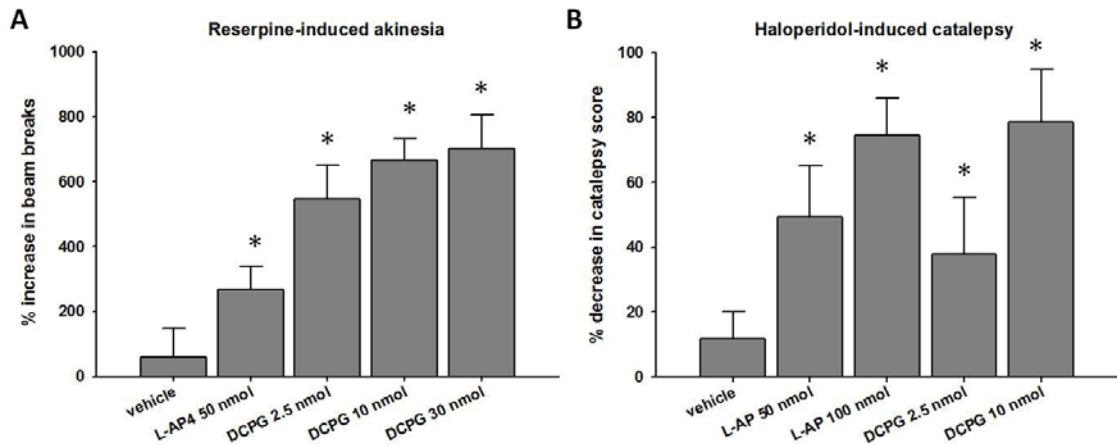
**In contrast to acute models of dopamine depletion or dopamine receptor blockade, DCPG reverses effects of prolonged reserpine-induced akinesia and haloperidol-induced catalepsy**

Because a more prolonged depletion of dopamine by reserpine or blockade of D2-like dopamine receptors by haloperidol has been shown to induce changes in mGluR function (Marino et al., 2002; Poisik et al., 2007), we also assessed the ability of L-AP4 (50 nmol, icv) and DCPG (2.5, 10, or 30 nmol, icv) to reverse the akinesia induced by an 18-20 hour pretreatment with reserpine (5 mg/kg, sc; Figure 13A). In contrast to the effects of DCPG following acute reserpine treatment, all tested doses of DCPG robustly increased locomotor activity in rats after more prolonged reserpine treatment ( $P < 0.05$ , Dunnett's comparison with vehicle group;  $n = 10$  animals per treatment group). L-AP4 also significantly increased locomotor activity in rats pretreated with reserpine for 18-20





**Figure 12. DCPG does not reverse acute reserpine-induced akinesia or haloperidol-induced catalepsy.** Animals were pretreated with reserpine (5 mg/kg, sc) for 2 hours prior to baseline locomotor activity measurement for 30 minutes (A), or with haloperidol (1.5 mg/kg, ip) for 2 hours prior to baseline catalepsy measurement (B). After baseline measurements, animals received an icv infusion of L-AP4 (50 or 100 nmol/2.5  $\mu$ l), DCPG (2.5, 10, or 30 nmol/2.5  $\mu$ l) or vehicle (ACSF). Five minutes after completion of infusion, locomotor activity (A) or catalepsy (B) was measured again, and the percent change from baseline was calculated. Data are shown as mean  $\pm$  SEM. Results were obtained from ten animals per treatment group. \* $P < 0.05$ , Dunnett's comparison with vehicle group.



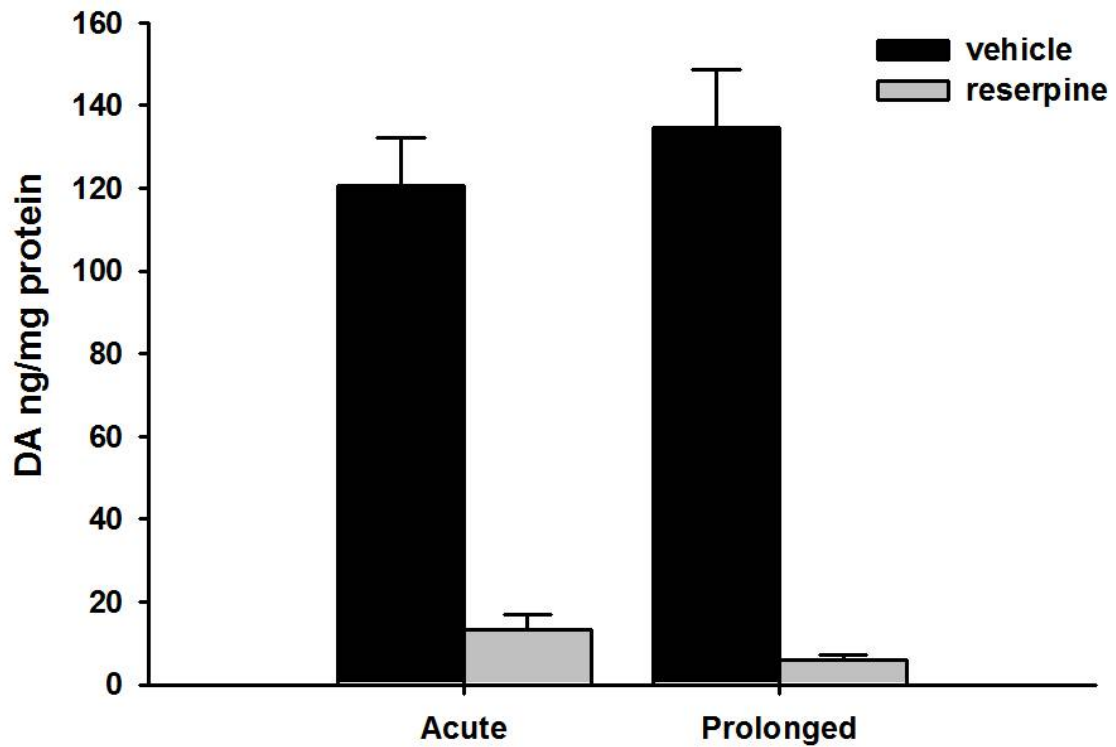
**Figure 13. DCPG reverses prolonged reserpine-induced akinesia and haloperidol-induced catalepsy.** Animals were pretreated with reserpine (5 mg/kg, sc) for 18-20 hours prior to baseline locomotor activity measurement for 30 minutes (A), or with three injections of haloperidol (1.5 mg/kg, ip) evenly spaced over 18-20 hours prior to baseline catalepsy measurement (B). After baseline measurements, animals received an icv infusion of L-AP4 (50 or 100 nmol/2.5  $\mu$ l), DCPG (2.5, 10, or 30 nmol/2.5  $\mu$ l) or vehicle (ACSF, 2.5  $\mu$ l). Five minutes after completion of infusion, locomotor activity (A) or catalepsy (B) was measured again, and the percent change from baseline measurement was calculated. Data are shown as mean  $\pm$  SEM. Results were obtained from ten animals per treatment group. \* $P$  < 0.05, Dunnett's comparison with vehicle group.

hours, and the magnitude of this effect was similar to that observed after acute reserpine treatment (Figure 13A; compare to Figure 12A).

Whereas a single dose of reserpine induces prolonged akinesia, the catalepsy produced by a single injection of haloperidol (1.5 mg/kg, ip) is no longer present 18-20 hours after administration. In order to produce a more prolonged dopamine receptor blockade, rats received three injections of haloperidol (1.5 mg/kg, ip) evenly spaced over 18-20 hours prior to baseline measurement of catalepsy. We then tested the ability of L-AP4 (50 or 100 nmol) and DCPG (2.5 or 10 nmol) to reverse the catalepsy induced by repeated haloperidol administration. In contrast to the lack of an effect of DCPG after acute haloperidol administration, catalepsy induced by repeated haloperidol administration was significantly reduced by 2.5 and 10 nmol DCPG (Figure 13B;  $P < 0.05$ , Dunnett's comparison with vehicle group;  $n = 10$  animals per treatment group). In addition, both doses of L-AP4 significantly reduced the catalepsy induced by this haloperidol dosing schedule. Again, the reversal of catalepsy by L-AP4 was similar after both acute and prolonged haloperidol administration (Figures 12B and 13B).

### **Striatal dopamine is reduced to a similar level after both acute and prolonged reserpine treatment**

In order to ensure that our acute and prolonged reserpine treatments produced similar levels of dopamine depletion, we determined tissue dopamine levels in the striatum two hours and 18-20 hours after treatment with vehicle or reserpine (5 mg/kg, sc) using HPLC. Both acute and prolonged reserpine treatments caused a significant reduction in striatal dopamine levels (Figure 14). Striatal dopamine content was reduced to 11.1% of control levels after two hour reserpine treatment ( $120 \pm 11.6$  ng/mg protein for vehicle-treated animals vs.  $13.4 \pm 3.7$  ng/mg protein for reserpine-treated animals; mean  $\pm$  SEM,  $n = 6$  animals per treatment group;  $t_{10} = 8.829$ ,  $P < 0.0001$ ). After 18-20



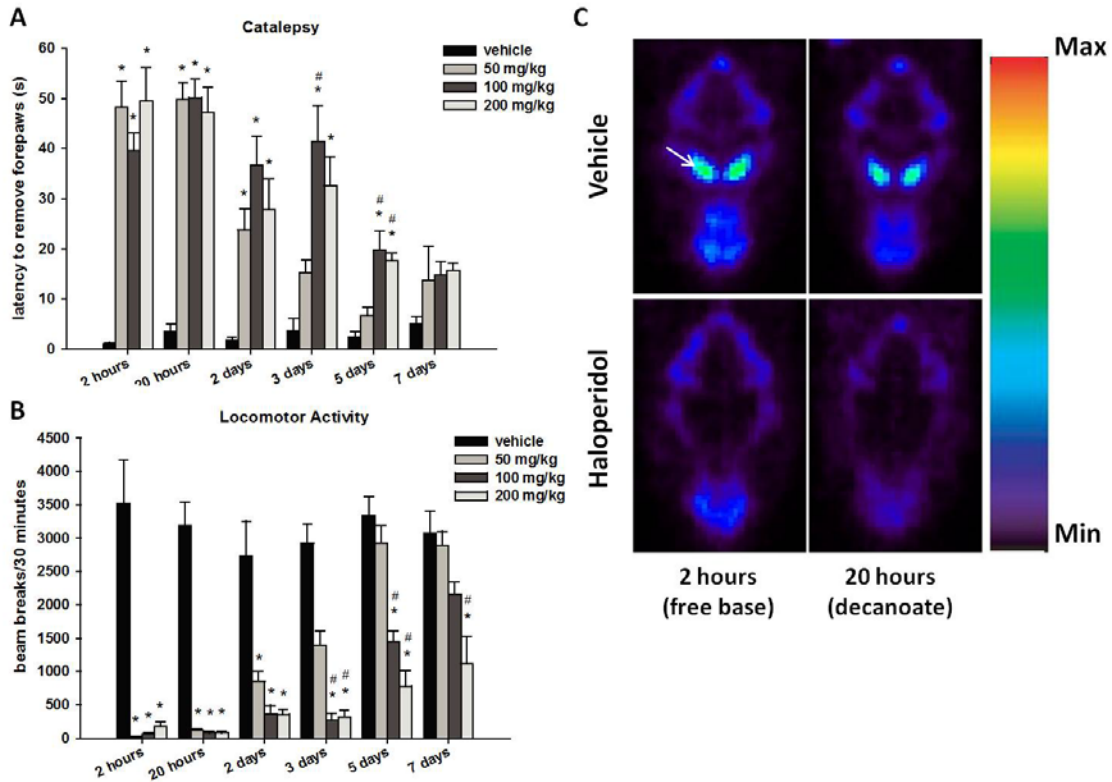
**Figure 14. Striatal dopamine levels are significantly reduced by both acute and prolonged reserpine treatments.** Rats were treated with reserpine (5 mg/kg, sc) or vehicle (1% acetic acid) for 2 hours (acute) or 20 hours (prolonged) prior to sacrifice. Dopamine (DA) levels from micropunches of the CPu were measured by HPLC and normalized to the total amount of protein in each sample. Normalized DA levels were reduced to 11.1% of control levels after acute reserpine treatment and 4.5% of control levels after prolonged reserpine treatment. Data were obtained from six animals per treatment group, and are shown as mean  $\pm$  SEM. \* $P < 0.0001$ , unpaired  $t$  test. Data contributed by Dr. Marketa Marvanova.

hour reserpine treatment, striatal dopamine content was reduced to 4.5% of control levels ( $134.6 \pm 13.1$  ng/mg protein for vehicle-treated animals vs.  $6.1 \pm 1.1$  ng/mg protein for reserpine-treated animals; mean  $\pm$  SEM,  $n = 6$  animals per treatment group;  $t_{10} = 9.113$ ,  $P < 0.0001$ ).

### **Haloperidol decanoate characterization**

Because the cataleptic behavior induced by 1.5 mg/kg haloperidol administered intraperitoneally decreases after 6-12 hours (data not shown), several doses of haloperidol were required to maintain catalepsy 18-20 hours after the first dose. In order to achieve a more prolonged effect with a single dose of haloperidol, I administered the decanoate salt of haloperidol intramuscularly (50 to 200 mg/kg). This treatment paradigm induced prolonged catalepsy and akinesia (Figures 15A and 15B, respectively). For catalepsy results (Fig. 3.4A), repeated measures ANOVA revealed a significant effect of drug treatment ( $F_{(3, 24)} = 29.8$ ,  $P < 0.0001$ ;  $n = 6-8$  animals per treatment group), a significant effect of time ( $F_{(5, 120)} = 30.8$ ,  $P < 0.0001$ ) and a significant interaction between dose and time ( $F_{(15, 120)} = 5.7$ ,  $P < 0.0001$ ). For haloperidol decanoate-induced akinesia results (Fig 3.4B), repeated measures ANOVA revealed a significant effect of drug treatment ( $F_{(3, 24)} = 60.0$ ,  $P < 0.0001$ ), a significant effect of time ( $F_{(5, 120)} = 37.7$ ,  $P < 0.0001$ ) and a significant interaction between drug treatment and time ( $F_{(15, 120)} = 8.8$ ,  $P < 0.0001$ ).

Previous studies in rats have demonstrated that greater than 80% of D2 receptors must be occupied by haloperidol to produce robust catalepsy (Natesan et al., 2006; Wadenberg et al., 2001). In our studies, rats were cataleptic for up to five days after haloperidol decanoate administration, suggesting high levels of D2 receptor occupancy. In order to confirm this, in collaboration with Dr. M. Noor Tantawy, I determined the *in vivo* D2 dopamine receptor



**Figure 15. A single dose of haloperidol decanoate induces prolonged catalepsy, akinesia, and D2 dopamine receptor occupancy.** Animals were treated with haloperidol decanoate (50, 100, or 200 mg/kg, im) or vehicle (sesame oil), and catalepsy (A) and locomotor activity (B) were measured at the indicated times. For both catalepsy and locomotor activity, repeated measures ANOVA revealed a significant effect of dose and time, as well as a significant interaction between dose and time. Catalepsy and locomotor activity measurements were obtained from six to eight rats per treatment group. For haloperidol decanoate-induced akinesia results (Fig 4B), repeated measures ANOVA revealed a significant effect of drug treatment ( $F_{(3, 24)} = 60.0$ ), a significant effect of time ( $F_{(5, 120)} = 37.7$ ) and a significant interaction between drug treatment and time ( $F_{(15, 120)} = 8.8$ ). Data are shown as mean  $\pm$  SEM. \* $P < 0.0083$  vs. vehicle group, pairwise comparison using Bonferroni correction. # $P < 0.0083$  vs. 50 mg/kg group, pairwise comparison using Bonferroni correction. Images showing [ $^{18}$ F]fallypride binding in the striatum were obtained using microPET (C). The striatum is indicated by an arrow in the top left panel. Images were captured following two hour treatment with 0.2% lactic acid (vehicle, top left panel) or 1.5 mg/kg haloperidol (free base, ip, bottom left panel), or 20 hour pretreatment with sesame oil (vehicle, top right panel) or 100 mg/kg haloperidol decanoate (im, bottom right panel). Images represent single horizontal slices (0.08 cm thick) from a representative animal summed over the duration of the sixty minute scan. Scans were performed on four rats for each treatment group.

occupancy 20 hours after 100 mg/kg haloperidol decanoate administration using microPET. The high affinity D2 receptor antagonist [<sup>18</sup>F]fallypride was used as a PET tracer to evaluate D2 receptor occupancy by haloperidol. In agreement with our prediction, 20 hour haloperidol decanoate treatment resulted in 97.9% D2 receptor occupancy (Figure 15C, right panels, and Table 5;  $n = 4$ ). This level of D2 receptor occupancy was comparable to that observed after 2 hour pretreatment with 1.5 mg/kg haloperidol (free base, 97.3%), which produced a level of catalepsy 2-4 hours post-administration similar to that observed 20 hours after haloperidol decanoate administration (Figure 15C, left panels, and Table 5;  $n = 4$ ).

#### **DCPG reverses haloperidol decanoate-induced catalepsy**

After characterization of time course of behavioral effects induced by haloperidol decanoate, I assessed the ability of DCPG (10 nmol, icv) to reverse the catalepsy induced by 20 hour treatment with haloperidol decanoate (100 mg/kg, im). DCPG significantly reversed catalepsy when measured ten minutes after completion of infusion (Figure 16;  $t_{16} = 5.157$ ,  $P < 0.05$ , unpaired  $t$  test;  $n = 10$  animals for vehicle group and 8 animals for DCPG group). Significant reversal of catalepsy was also observed 20 and 30 minutes after completion of DCPG infusion.

#### **L-AP4 and DCPG decrease forelimb use asymmetry following 6-OHDA lesion**

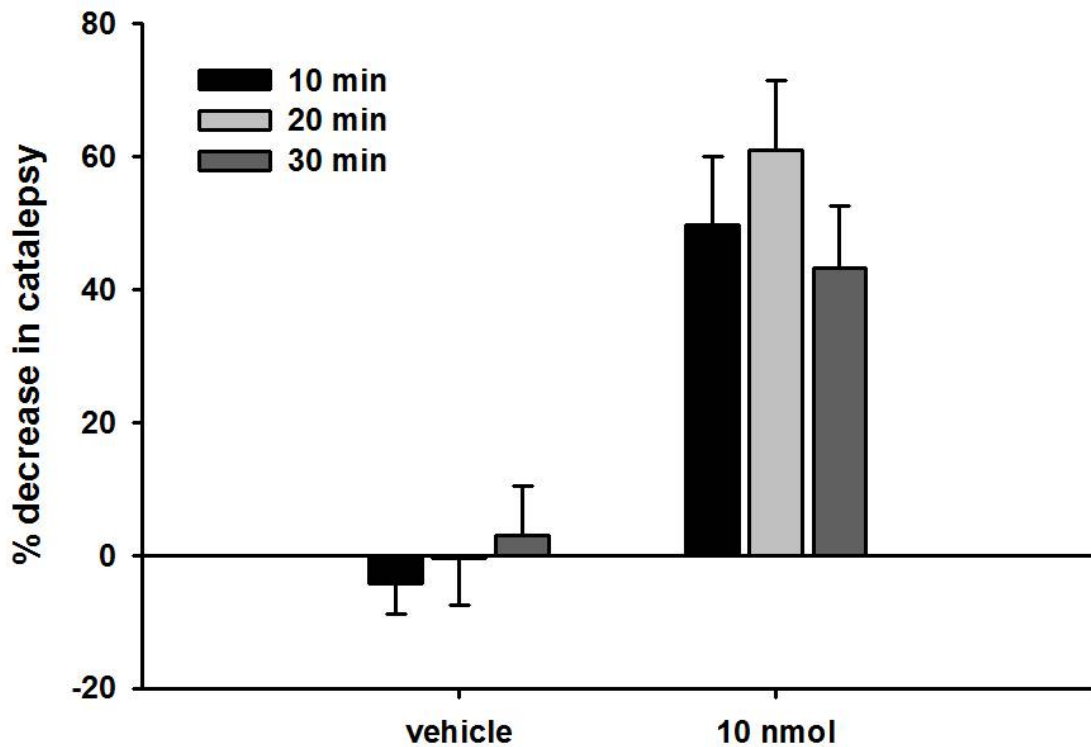
Unilateral 6-OHDA lesion represents an additional rat model of prolonged dopamine depletion. This model differs from reserpine-induced akinesia and haloperidol-induced catalepsy because it involves degeneration of the dopaminergic neurons of the SNc rather than pharmacological manipulation of intact dopamine systems. Rats with an extensive unilateral lesion of the SNc favor use of the forepaw that is ipsilateral to the

**Table 5. D2 receptor occupancy following two hour haloperidol or twenty hour haloperidol decanoate treatment.**

<b>Drug treatment</b>	<b>Vehicle BP<sup>1</sup></b>	<b>Drug BP</b>	<b>D2 receptor occupancy<sup>2</sup></b>
Haloperidol (free base)	18.4 ± 1.8	0.5 ± 0.2	97.3%
Haloperidol decanoate	19.1 ± 0.9	0.4 ± 0.2	97.9%

<sup>1</sup>Binding potential (BP) was calculated as Logan DVR – 1. <sup>2</sup>D2 dopamine receptor occupancy was calculated as  $[1 - (\text{Drug BP}/\text{Vehicle BP})] \times 100$ .



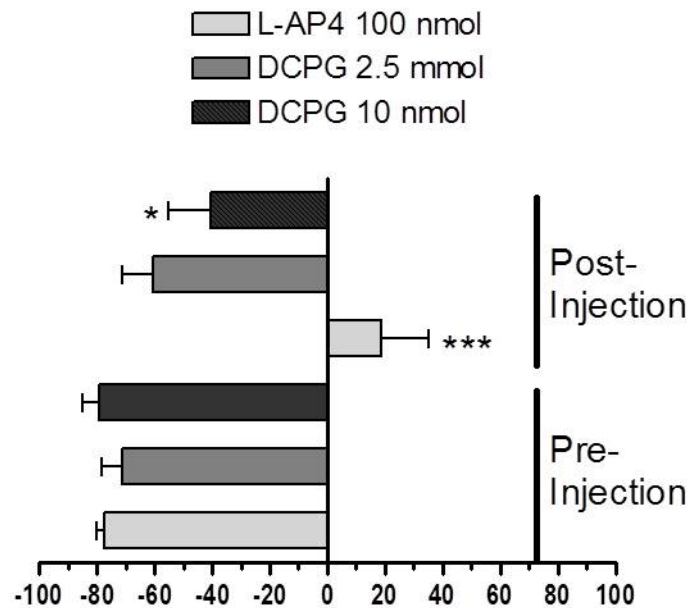


**Figure 16. DCPG reverses prolonged haloperidol decanoate-induced catalepsy.** Animals were pretreated with haloperidol decanoate (100 mg/kg, im) 20 hours prior to baseline catalepsy measurement. After baseline measurements, animals received an icv infusion of DCPG (10 nmol/5  $\mu$ l) or vehicle (ACSF, 5  $\mu$ l). Ten, twenty, and thirty minutes after completion of infusion, catalepsy was measured again, and the percent change from baseline measurement was calculated. Data are shown as mean  $\pm$  SEM. Results were obtained from eight to ten animals per treatment group. \* $P < 0.05$ , unpaired  $t$  test.

site of the lesion when supporting their weight during a wall contact following a rearing event. Drugs that effectively alleviate PD symptoms in humans, such as L-DOPA and dopamine receptor agonists, reduce forelimb-use asymmetry caused by unilateral 6-OHDA lesion (Lundblad et al., 2002). We therefore tested the ability of L-AP4 (100 nmol, icv) and DCPG (2.5 or 10 nmol, icv) to reduce forelimb use asymmetry in unilaterally 6-OHDA-lesioned animals. Two-way ANOVA revealed significant effects of Treatment (pre vs. post injection [ $F_{1,62} = 29.78$ ]), Drug ( $F_{2,62} = 6.43$ ) and a significant Treatment X Drug interaction ( $F_{2,62} = 8.07$ ). As shown in Figure 17, L-AP4 [ $t = 5.90$ ,  $p < 0.001$ ,  $n = 10$ ] and the high dose of DCPG (10 nmol [ $t = 2.60$ ,  $p < 0.05$ ,  $n = 12$ ]), but not the low dose of DCPG (2.5 nmol [ $t = 0.70$ ,  $p > 0.05$ ,  $n = 12$ ]) caused a significant reversal of the FLA Index Score. Tissue neurochemistry revealed that, compared to the unaffected hemisphere, 6-OHDA-lesioned rats exhibited a greater than 95% loss of striatal dopamine and the dopamine metabolites DOPAC and HVA in the lesioned hemisphere (Table 6).

## Discussion

Metabotropic glutamate receptors have recently been identified as promising new therapeutic targets for alleviating the primary motor symptoms of PD (Johnson et al., 2009). Recent studies have demonstrated that central or systemic administration of group III mGluR-selective agonists elicits behavioral effects consistent with antiparkinsonian actions in both acute and prolonged animal models of PD. For example, icv administration of L-AP4 reverses reserpine-induced akinesia and haloperidol-induced catalepsy after a short (1.5-2 hour) pretreatment with reserpine or haloperidol (Valenti et al., 2003). Similarly, icv administration of L-AP4 or the group III mGluR agonist L-SOP has been shown to alleviate akinesia following a longer (18 hour) pretreatment with reserpine (MacInnes et al., 2004), demonstrating that group III mGlu



**Figure 17. DCPG reduces forelimb use asymmetry in rats with unilateral 6-OHDA lesion.** Animals were evaluated for forelimb use asymmetry for 10 minutes prior to icv infusion of L-AP4 (100 nmol) or DCPG (2.5 or 10 nmol). Five minutes after completion of drug administration, forelimb use asymmetry was evaluated for an additional 10 minute period. The FLA Index Scores were calculated as described in the Methods. A negative FLA Index Score signifies preferential use of the unaffected forelimb, whereas FLA Index Scores close to zero indicate the absence of a forelimb use preference. Drug treatment resulted in significant reversal of forelimb asymmetry in rats treated with L-AP4 or 10 nmol DCPG. \*  $P < 0.05$ , \*\*\*  $P < 0.001$  vs. pretreatment (two-way ANOVA followed by Bonferroni comparison).

**Table 6. Concentrations of dopamine and its metabolites in the dorsal striatum of unilaterally 6-OHDA-lesioned rats.**

<b>Treatment</b>	<b>Dopamine</b>	<b>DOPAC</b>	<b>HVA</b>
		(ng/mg protein)	
<b>6-OHDA (n = 24)</b>			
contralateral	66.31 ± 13.10	18.10 ± 1.28	13.59 ± 1.51
ipsilateral	0.37 ± 0.061***	0.27 ± 0.04***	0.54 ± 0.08***
<b>% remaining</b>	<b>0.56</b>	<b>1.49</b>	<b>3.97</b>

\*\*\*  $P < 0.001$  vs. contralateral concentrations (Student's  $t$  test).

activation remains efficacious after a more prolonged state of dopamine depletion. In addition, icv or systemic administration of L-AP4 and ACPT-I, respectively, can reverse motor deficits caused by 6-OHDA lesion, demonstrating that group III mGluR activation is also efficacious in this model of chronic dopamine depletion (Lopez et al., 2012; Lopez et al., 2007; Lopez et al., 2008; Valenti et al., 2003). Previous efforts to identify the group III mGluR subtypes that mediate the antiparkinsonian actions of group III mGluR-selective agonists have demonstrated that mGluR4 activation is likely to play a major role (Beurrier et al., 2009; Broadstock et al., 2011; East et al., 2010; Goudet et al., 2012; Jones et al., 2011a; Jones et al., 2011b; Marino et al., 2003; Niswender et al., 2008b). However, expression of mGluR7 and mGluR8 has also been detected in the basal ganglia (Bradley et al., 1999b; Messenger et al., 2002), indicating that these receptor subtypes may also contribute to the antiparkinsonian actions of group III mGluR agonists.

In the present study, we used the mGluR8-selective agonist (S)-3,4-DCPG to explore the role of mGluR8 activation in mediating the antiparkinsonian effects of group III mGlu agonists in both acute and prolonged models of dopamine depletion. The only previous study to explore potential antiparkinsonian effects of mGluR8 activation in an acute model of PD evaluated the effects of direct infusion of DCPG into the globus pallidus (GP) and substantia nigra pars reticulata (SNr) of rats that had been acutely treated with haloperidol (1.5 hour pretreatment) (Lopez et al., 2007). This study demonstrated that DCPG did not reverse haloperidol-induced catalepsy when infused into either structure, a finding consistent with our observation that DCPG does not reverse the motor deficits induced by acute haloperidol or reserpine administration. Here we report that after prolonged (18-20 hour) treatment with haloperidol or reserpine, icv DCPG robustly reverses PD-like akinetic motor deficits. The differential ability of DCPG to exert antiparkinsonian effects following acute versus prolonged reserpine treatment is

unlikely to be due to differences in the extent of dopamine depletion, because both treatments produce a similar reduction of striatal dopamine levels. Similarly, the level of dopamine receptor occupancy produced by 2 hour haloperidol (free base) treatment versus 20 hour haloperidol decanoate treatment is virtually identical, indicating that differences in the extent of dopamine receptor antagonism between these two models cannot account for the differential ability of DCPG to reverse catalepsy. Finally, DCPG alleviates forelimb-use asymmetry in the unilateral 6-OHDA rat model of PD. Taken together, these results indicate that the antiparkinsonian actions of DCPG are only present after prolonged dopamine hypofunction.

Studies in heterologous expression systems have shown that DCPG has 50 to 100-fold selectivity for mGluR8 over mGluR4, and has little or no activity at other mGluR subtypes (Niswender et al., 2008a; Thomas et al., 2001). The ability of DCPG to activate mGluR4 raises the possibility that the antiparkinsonian effects observed in our studies are due to agonist activity at mGluR4 rather than mGluR8. However, selective positive allosteric modulators of mGluR4 and mGluR4-preferring agonists reverse akinetic deficits induced by acute (one to two hour) pretreatment with haloperidol or reserpine, suggesting that after a short period of dopamine receptor blockade or dopamine depletion, enhanced mGluR4 activation can reverse catalepsy and akinesia (Beurrier et al., 2009; Goudet et al., 2012; Marino et al., 2003; Niswender et al., 2008b). Based on these findings, if the doses of DCPG used in our studies were sufficient to activate mGluR4, we would predict that DCPG would also reverse catalepsy and akinesia induced by two hour haloperidol or reserpine treatment. In contrast, we have found that the doses of DCPG used in our study did not produce any reversal of akinetic behaviors in those acute experimental paradigms, suggesting that the doses of DCPG that used here are not sufficient to activate mGluR4. While we cannot exclude the possibility that other receptors may be involved in mediating the effects of DCPG, the results presented

here suggest that DCPG likely mediates antiparkinsonian effects in catalepsy, akinesia, and forelimb use asymmetry models that are due to selective activation of mGluR8.

The differential ability of DCPG to produce antiparkinsonian effects after acute vs. prolonged dopamine depletion or dopamine receptor blockade suggests that prolonged dopamine depletion leads to a change in the function or expression of mGluR8. *In situ* hybridization studies have detected mGluR8 mRNA in all basal ganglia structures (Messenger et al., 2002). Interestingly, chronic administration of amphetamine increases mGluR8 mRNA levels in the rat striatum (Parelkar and Wang, 2008), suggesting that dysregulation of dopaminergic signaling in the basal ganglia can alter the expression of mGluR8. However, no changes in mGluR8 mRNA levels have been observed following 6-OHDA lesion (Messenger et al., 2002), suggesting that changes in mGluR8 transcription may not underlie the differential effects of DCPG after acute vs. prolonged dopamine depletion. Alternatively, changes in the functional expression of mGluR8 at the protein level, such as increased translation, trafficking to the plasma membrane, or posttranslational modifications, represent possible explanations for this phenomenon.

Although there is a lack of anatomical evidence regarding changes in mGluR8 expression following dopamine depletion, previous studies using direct-site infusions of group III mGlu agonists in prolonged models of dopamine depletion suggest that several basal ganglia structures may be candidates for mediating the antiparkinsonian effects of DCPG. Following 18 hour reserpine treatment, direct infusion of the group III mGlu agonist L-SOP into the rat GP or SNr reverses akinesia (MacInnes et al., 2004), suggesting that the GP and the SNr are possible sites of action for an mGluR8-mediated antiparkinsonian effect. The ability of intrapallidal infusion of group III mGlu agonists to reverse akinetic deficits caused by bilateral 6-OHDA lesion also points to the GP as a possible site of action of DCPG (Lopez et al., 2007). However, other results suggest that

these structures are unlikely to be the sites of action for the antiparkinsonian effects of DCPG. For example, a reduction of inhibitory striatopallidal transmission is thought to underlie the antiparkinsonian effects of group III mGlu activation in the GP (Lopez et al., 2007; Valenti et al., 2003), and electrophysiological studies have shown that DCPG does not reduce inhibitory striatopallidal transmission in brain slices obtained from normal or reserpinized rats (Valenti et al., 2003). In agreement with this finding, intrapallidal DCPG administration fails to reverse akinetic deficits following bilateral 6-OHDA (Beurrier et al., 2009). These results suggest that activation of mGluR8 in the GP is unlikely to mediate the antiparkinsonian effects of DCPG. In the SNr, direct infusion of group III mGluR agonists worsens the impaired motor performance of rats with bilateral 6-OHDA lesion (Lopez et al., 2007), so it is not likely that the improvement in motor performance observed after icv administration of DCPG is mediated by activation of mGluR8 in the SNr. Interestingly, a recent study showed that direct infusion of very high doses of DCPG into the SNr reverses motor deficits following overnight reserpine treatment (Broadstock et al., 2011). However, this effect was not readily blocked by pretreatment with a group III mGlu antagonist, supporting the idea that activation of mGluR8 in the SNr may not be responsible for the alleviation of PD-like motor deficits observed following icv administration of DCPG. Alternatively, other basal ganglia structures, such as the striatum, as well as CNS structures outside of the basal ganglia, represent possible sites of action for the antiparkinsonian effects of DCPG.

In conclusion, we have found that the mGluR8 agonist DCPG produces anti-akinetic effects consistent with antiparkinsonian-like actions in prolonged but not acute models of PD. These findings suggest that mGluR8 activation may partially mediate the alleviation of motor deficits by group III mGlu-selective agonists in prolonged models of dopamine depletion and indicate that selective activation of mGluR8, or simultaneous activation of multiple group III mGlus, may represent a novel therapeutic strategy for the



treatment of PD. Further studies will be necessary to elucidate the mechanisms underlying the antiparkinsonian effects of DCPG.

## CHAPTER IV

### ACTIVATION OF GROUP II METABOTROPIC GLUTAMATE RECEPTORS INDUCES LONG-TERM DEPRESSION OF EXCITATORY SYNAPTIC TRANSMISSION IN THE SUBSTANTIA NIGRA PARS RETICULATA

#### Introduction

Recent studies suggest that the loss of dopaminergic modulation of striatal activity in PD patients leads to increased inhibitory tone at the striatopallidal synapse in the indirect pathway of the basal ganglia (DeLong and Wichmann, 2007). This leads to disinhibition of the STN, changes in STN firing patterns, and an increase in excitatory drive from the STN to the basal ganglia output nuclei, which include the GPi and the SNr (the major basal ganglia output nucleus in rodents) (Bergman et al., 1994; Liu et al., 2002b; Ni et al., 2001a; Ni et al., 2001b; Vila et al., 2000). Because aberrant glutamatergic transmission is associated with pathological alterations in basal ganglia function, glutamate receptors have been suggested as promising therapeutic targets for treating PD (Johnson et al., 2009). Activation of group II and group III mGluRs has been implicated as a therapeutic strategy for PD due to their potential to reduce neurotransmitter release at pathologically overactive synapses such as the excitatory synapse between the STN and the SNr (Bradley et al., 2000; Johnson et al., 2009; Wittmann et al., 2001b).

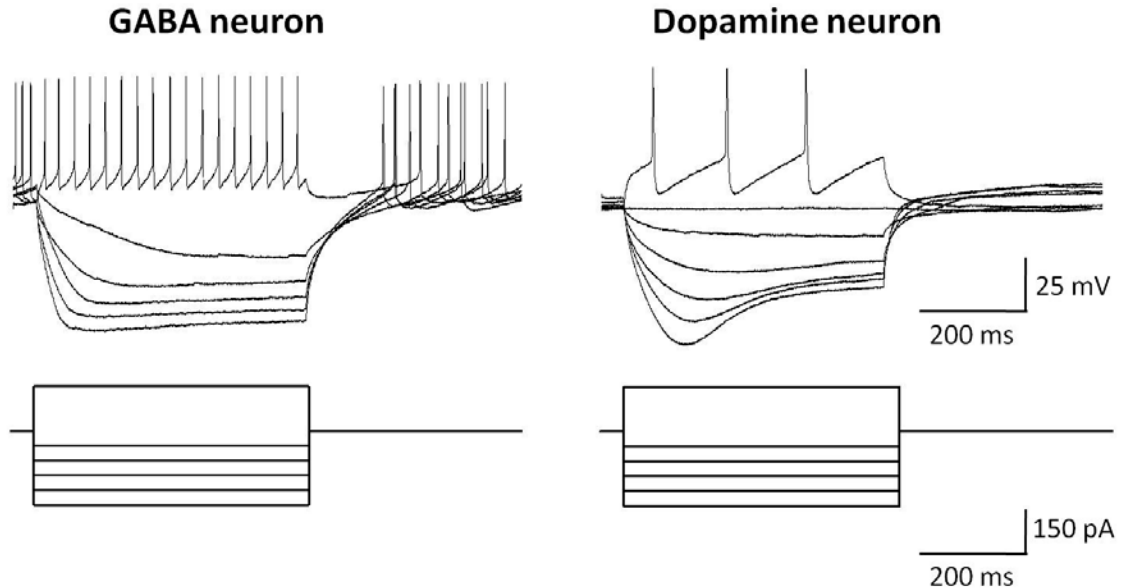
Anatomical studies using group II mGluR-specific antibodies have demonstrated that group II mGluRs are present on presynaptic glutamatergic axon terminals in the SNr (Bradley et al., 2000). Our laboratory has previously shown that brief application of group II mGluR agonists reversibly reduces the amplitude of synaptically evoked EPSCs recorded from GABAergic SNr neurons (Bradley et al., 2000). The antibodies and group II mGluR agonists used in these studies did not distinguish between mGluR2 and

mGluR3; therefore, it is not known if one or both group II mGluR subtypes are required to regulate excitatory transmission in the SNr. In addition, the effects of prolonged activation of group II mGluRs at this synapse, which may occur under both normal and pathological conditions, have not been fully elucidated. The work presented here is adapted from (Johnson et al., 2011).

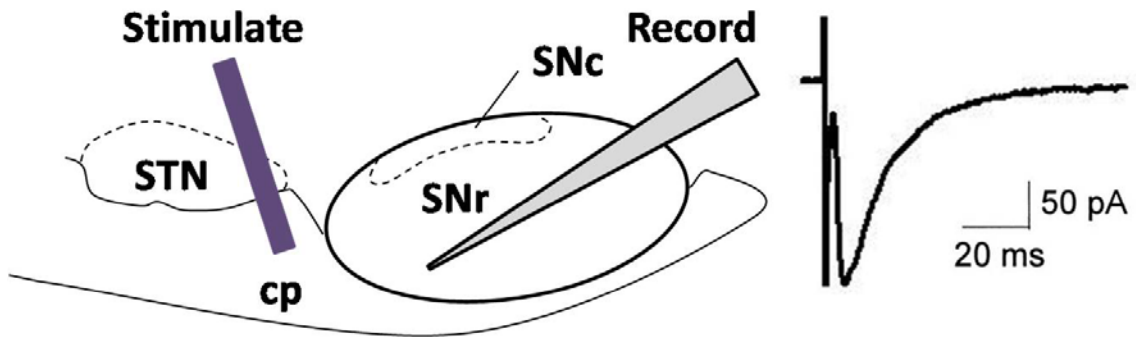
## Methods

To further explore these questions, I examined the regulation of synaptic transmission in Sprague-Dawley rats (14-18 days old, Charles River, Wilmington, MA) and mGluR2 and mGluR3 knockout mice and wild type littermates (21-30 days old, bred by Taconic Farms, Cambridge City, IN) (Linden et al., 2005). Animals were housed under a 12 hour light/dark cycle (lights on at 6 AM to 6 PM) with free access to food and water in accordance with American Association for the Accreditation of Laboratory Animal Care guidelines. Procedures were approved by the Institutional Animal Care and Use Committee of Vanderbilt University and conformed to the guidelines established by the National Research Council Guide for the Care and Use of Laboratory Animals. On the morning of the study, animals were decapitated under isoflurane anesthesia and brains were rapidly removed and submerged into ice-cold cutting solution (in mM: sucrose, 187; KCl, 3; CaCl<sub>2</sub>, 2; MgSO<sub>4</sub>, 1.9; KH<sub>2</sub>PO<sub>4</sub>, 1.2; glucose, 20; NaHCO<sub>3</sub>, 26; equilibrated with 95% O<sub>2</sub>/5% CO<sub>2</sub>). Parasagittal midbrain slices 275-300 μm thick were prepared using a Vibratome (Vibratome 3000, St. Louis, MO) and then transferred to ACSF (in mM: NaCl, 124; KCl, 2.5; MgSO<sub>4</sub>, 1.3; NaH<sub>2</sub>PO<sub>4</sub>, 1.0; CaCl<sub>2</sub>, 2.0; glucose, 20; NaHCO<sub>3</sub>, 26; glutathione, 0.005, and sodium pyruvate, 0.5; equilibrated with 95% O<sub>2</sub>/5% CO<sub>2</sub>) and allowed to recover at 32°C for 30 minutes. Slices were then incubated at room temperature in ACSF for at least 30 minutes prior to recordings.

During recordings, slices were submerged in a brain slice chamber and perfused with oxygenated room temperature ACSF at a rate of 1.5-2 ml/min, and glutathione and sodium pyruvate were excluded from perfusate. Neurons were visualized using an Olympus BX51WI upright microscope (Olympus, Lake Success, NY) coupled with a 40x water immersion objective and Hoffman optics. Borosilicate glass pipettes were pulled using a Flaming/Brown micropipette puller (model P-97; Sutter Instruments, Novato, CA) to produce patch electrode resistances of 3.0–7.0 M $\Omega$  when filled with intracellular solution (in mM: potassium gluconate, 140; HEPES, 10; NaCl, 10; EGTA, 0.6; GTP, 0.2; and ATP, 2; pH adjusted to 7.4 with 0.5N KOH). Whole cell patch clamp recordings were made from putative GABAergic neurons in the SNr using a Warner 505B amplifier (Warner Instruments) or an Axon Multiclamp 700B amplifier (Molecular Devices). GABAergic projection neurons were identified according to previously established electrophysiological characteristics (Figure 18) (Richards et al., 1997). EPSCs were recorded at a holding potential of -60 mV (unless otherwise noted) and evoked every 10 seconds using a concentric bipolar tungsten electrode placed in the cerebral peduncle rostral to and outside of the SNr (for a schematic representation of recording setup, see Figure 19). Experiments were performed in the presence of (-)-bicuculline methobromide (20  $\mu$ M, Tocris Bioscience, Ellisville, MO or Ascent Scientific, Weston-SuperMare, UK) to block GABA<sub>A</sub>-mediated inhibitory currents unless otherwise noted. The voltage-clamp signal was low-pass-filtered at 1 kHz, digitized at 10 kHz, and acquired using a Clampex9.2/DigiData 1332 system (Molecular Devices, Sunnyvale, CA). Holding current, input resistance, and access resistance were monitored throughout all experiments. Stable baseline EPSC amplitudes were recorded for at least 5 minutes prior to bath application of drugs. LY379268 was generously provided by Dr. James Monn (Eli Lilly, Indianapolis, IN).



**Figure 18. Representative current clamp experiments evaluating membrane properties of GABAergic and dopaminergic neurons in the SNr in response to hyperpolarizing and depolarizing current injections.** Whole cell current clamp recordings were used to distinguish between putative GABAergic and dopaminergic neurons in the substantia nigra pars reticulata (SNr). Recordings were performed at the resting potential of the neuron and changes in membrane potential and action potential firing were monitored after hyperpolarizing and depolarizing current injections (represented by the schematic in the lower part of the figure). GABAergic neurons typically exhibit spontaneous, repetitive firing, short-duration action potentials, little spike frequency adaption, and little or no hyperpolarization-induced current (mediated by  $I_h$ ), whereas dopaminergic neurons display lower frequency or no spontaneous firing, longer-duration action potentials, and pronounced sag potentials in response to hyperpolarizing current injections.



**Figure 19. Schematic diagram of evoked EPSC recordings from SNr neurons.** This figure shows a schematic representation of a parasagittal midbrain slice from a juvenile rat. Whole cell voltage clamp recordings of electrically evoked EPSCs were made from putative GABAergic neurons of the rostral portion of the substantia nigra pars reticulata (SNr). A bipolar stimulating electrode placed in the cerebral peduncle (cp) rostral to and outside of the SNr was used to evoke monosynaptic EPSCs. The subthalamic nucleus (STN) and substantia nigra pars compacta (SNc) are also shown for reference.

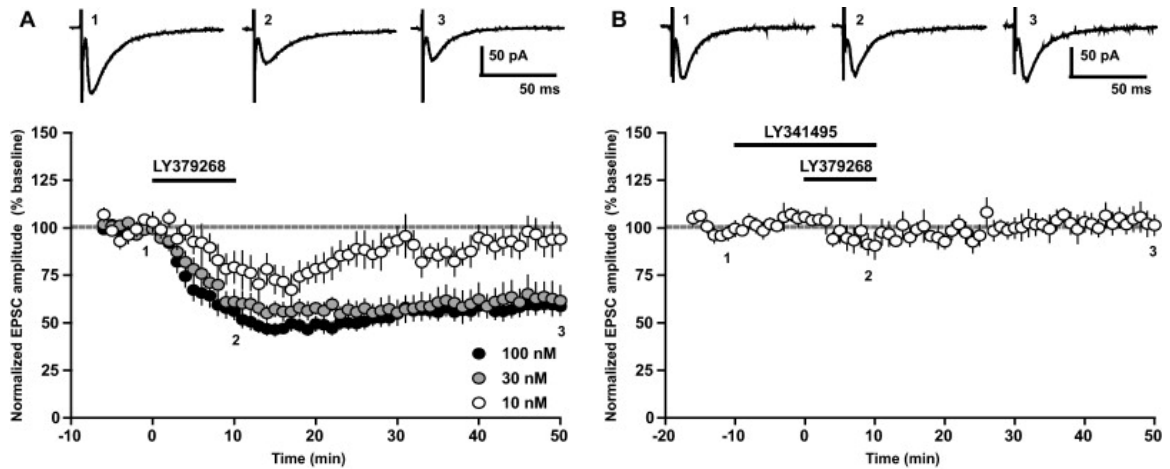
LY341495 was purchased from Tocris Bioscience. All drugs were prepared as frozen stocks and diluted in ACSF immediately prior to the experiment.

All data analysis was performed using Clampfit software (v9.2, Molecular Devices, Sunnyvale, DA), GraphPad Prism (GraphPad Software Inc, San Diego, CA), and Excel (Microsoft Corp., Redmond, WA). EPSC amplitudes were averaged every 6 sweeps and normalized to the average of the baseline EPSC amplitudes. Data are expressed as mean  $\pm$  SEM. Statistical comparisons were performed using a Student's unpaired *t*-test with significance level set to  $p < 0.05$ .

## Results

After stable baseline measurement, the group II mGluR agonist LY379268 (10, 30, or 100 nM) was bath applied to slices for 10 minutes, and recordings were continued for at least 40 minutes after drug washout. LY379268 (100 nM) induced a robust long-term depression (LTD) of EPSC amplitudes ( $58.7 \pm 4.5\%$  of baseline 40 minutes after LY379268 washout,  $n = 14$ ; Fig. 4.3A, black circles). A similar level of depression was observed after application of 30 nM LY379268 ( $61.7 \pm 7.8\%$  of baseline 40 minutes after LY379268 washout,  $n = 7$ ; Fig. 4.3A, gray circles). In contrast, 10 nM LY379268 produced a slightly less robust acute depression of EPSC amplitudes that peaked 17 minutes after onset of LY379268 application ( $67.3 \pm 8.1\%$  of baseline) but failed to induce LTD ( $94.0 \pm 6.1\%$  of baseline 40 minutes after LY379268 washout,  $n = 6$ ; Figure 20A, open circles). LY379268 did not alter holding current or input resistance at any concentration tested (data not shown).

To ensure that the effects of LY379268 were mediated by group II mGluRs, slices were pretreated with the group II mGluR-preferring antagonist LY341495 (500 nM) 10 minutes prior to application of LY379268 (100 nM). LY341495 completely blocked the ability of LY379268 to reduce EPSC amplitudes ( $101.4 \pm 5.4\%$  of baseline 40 minutes



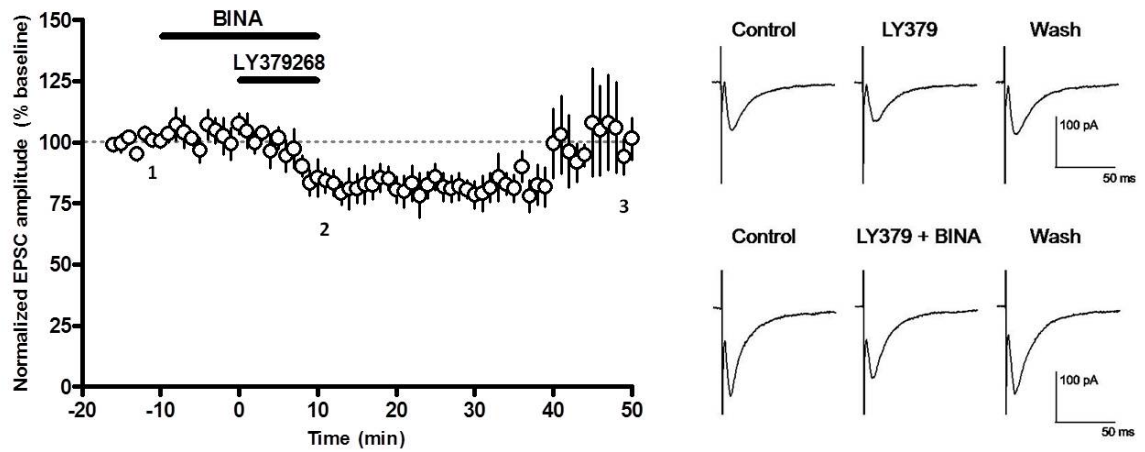
**Figure 20. Activation of group II mGluRs induces long-term depression of excitatory transmission in the SNr.** (A) Time course of the EPSC amplitude following 10 minute bath application of LY379268 (10-100 nM) shows that induction of LTD by LY379268 is concentration-dependent. Data represent mean  $\pm$  SEM for 6-14 cells per drug treatment. Representative sample traces from a single experiment with 100nM LY379268 were taken from time points indicated by numbers and are the average of 6 individual traces. (B) Pre-incubation of slices with the mGluR2/3 antagonist LY341495 (500 nM, 10 minutes prior to LY379268 application) blocks induction of LTD ( $n = 5$ ).



after drug washout,  $n = 5$ ; Figure 20B), confirming that the LTD induced by LY379268 depends upon group II mGluR activation.

Because inhibitory transmission mediated by GABA is a critical determinant of basal ganglia output, I performed an additional experiment in the absence of the GABA<sub>A</sub> receptor antagonist bicuculine to ensure that group II mGluR activation is able to induce LTD in the presence of normal GABA transmission. In order to accurately measure EPSC amplitudes in the absence of bicuculine, I voltage-clamped the recorded cells near the reversal potential of chloride (-75 mV). Similar to results obtained in the presence of bicuculine, I found that a 10 minute bath application of LY379268 (100 nM) induced robust LTD in all recorded neurons ( $46.8 \pm 11.4\%$  of baseline 40 minutes after LY379268 washout,  $n = 3$ ; data not shown).

In order to determine which of the group II mGluRs mediates LY379268-induced LTD in rat SNr neurons, we took advantage of two pharmacological tools that selectively modulate mGluR2 function. The first compound, biphenyl indanone-A (BINA), is a selective PAM of mGluR2 (Galici et al., 2006). Bath application of BINA (3  $\mu$ M) 10 minutes prior to a submaximal concentration of LY379268 (10 nM) failed to potentiate the ability of the low concentration of LY379268 to induce LTD (Figure 21; see Figure 20A, open circles, for comparison with 10 nM LY379268 alone). Because the ability of BINA to potentiate mGluR2 function in cell lines was determined using glutamate as the mGluR2 agonist, I performed cell-based calcium assays for mGluR2 to ensure that BINA also potentiates LY379268-mediated activation of mGluR2. Indeed, BINA increased the maximal response of rat mGluR2 to LY379268, and also shifted the LY379268 concentration-response curve to the left, indicating that BINA does have the ability to potentiate LY379268-mediated mGluR2 responses (data not shown). This result suggested that the lack of potentiation by BINA is not due to a lack of

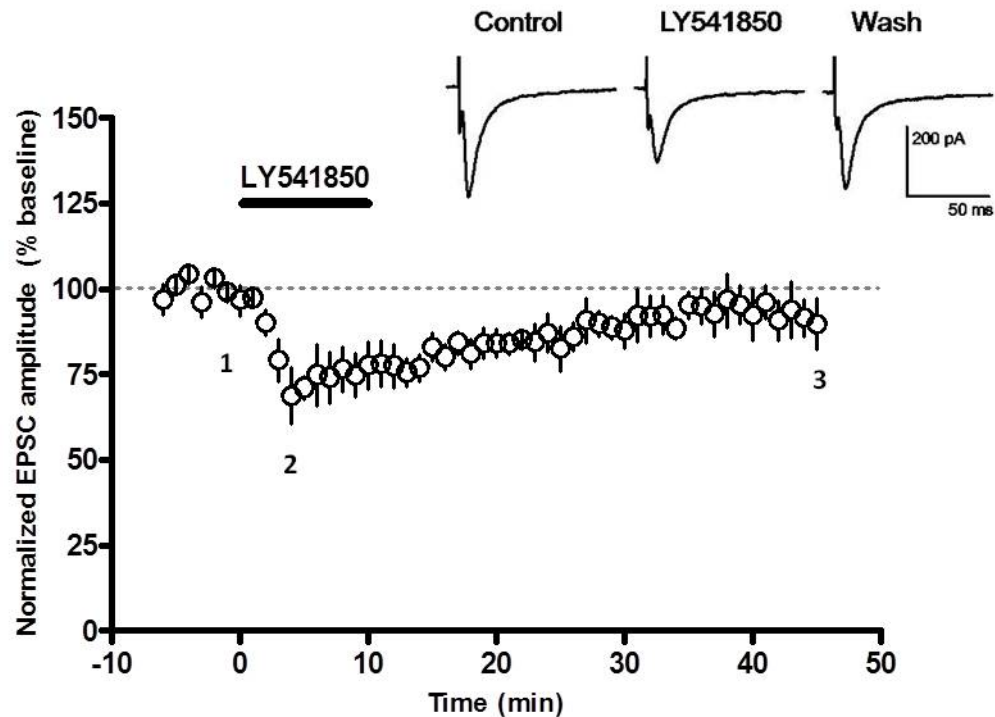


**Figure 21. BINA does not potentiate induction of LTD by a subthreshold concentration of LY379268.** Time course of the EPSC amplitude following 10 minute bath application of BINA (3  $\mu$ M) followed by 10 minute coapplication of BINA and LY379268 (10 nM) shows that BINA does not potentiate LTD induction by a subthreshold concentration of LY379268. Data represent mean  $\pm$  SEM for 4 cells. Sample traces represent the average of 6 traces from a single minute of a representative experiment at the time points indicated by 1 (control), 2 (LY379268 with or without BINA), and 3 (wash) on the time course graph.

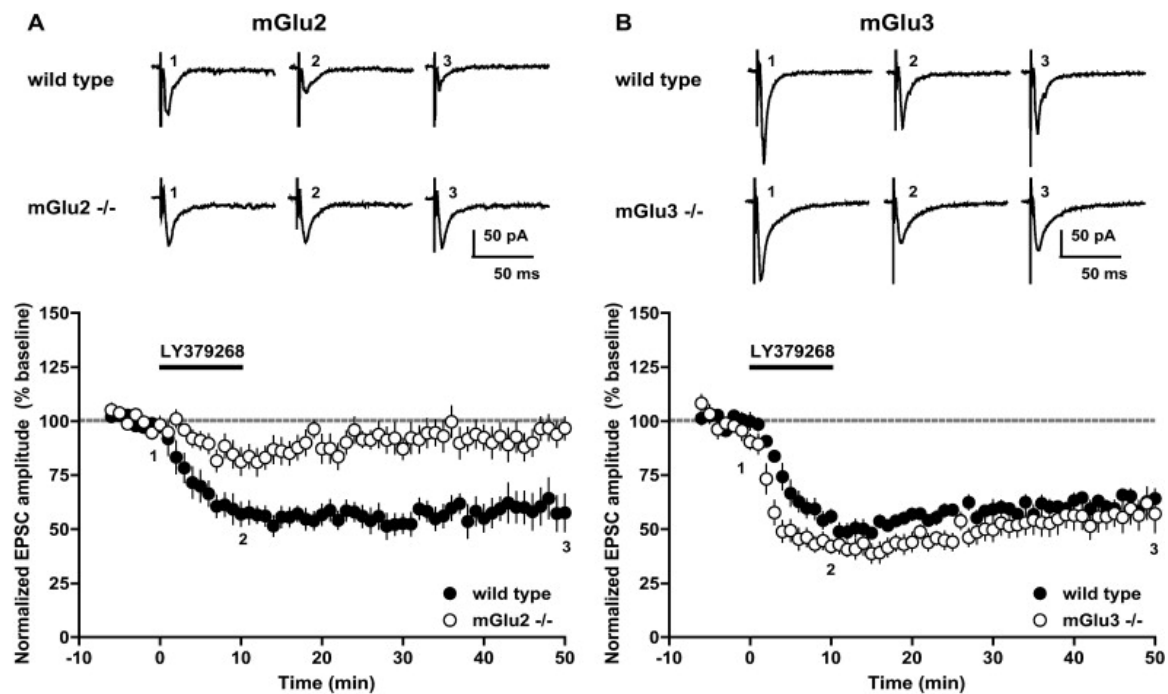
interaction between the two drugs, and raised the possibility that mGluR3 activation is required for induction of LTD by LY379268.

I also tested the ability of an orthosteric partial agonist of mGluR2, LY541850, to induce LTD of excitatory transmission in rat SNr neurons. LY541850 binds to both mGluR2 and mGluR3 with similar affinity, but is an agonist at mGluR2 and an orthosteric antagonist at mGluR3 (Dominguez et al., 2005). Results from independent evaluation in our laboratory indicate that LY541850 can elicit ~50-80% of the maximal glutamate response at mGluR2 depending on the cell line and assay used (Dr. Douglas Sheffler; data not shown). I tested the ability of LY541850 to modulate excitatory transmission in SNr neurons using a 10 minute bath application, which is identical to the protocol used for LY379268 application. In contrast to the LTD observed with LY379268 application, LY541850 (10  $\mu$ M) induced a modest, reversible depression of EPSC amplitude (Figure 22). These results further suggested that mGluR3 activation might be required for induction of LTD by LY379268.

Due to a lack of antagonists that distinguish between mGluR2 and mGluR3, I employed mGluR2 and mGluR3 knockout mice to further evaluate which of the receptor subtypes mediates LY379268-induced LTD. In slices obtained from wild type littermate controls, LY379268 (100 nM, 10 minutes) induced LTD that was not significantly different from the depression observed in slices obtained from rats ( $57.6 \pm 8.7\%$  of baseline 40 minutes after LY379268 washout for mGluR2 control animals,  $n = 7$ , vs.  $58.7 \pm 4.5\%$  for rats,  $n = 14$ ,  $p = 0.90$ ;  $64.2 \pm 4.4\%$  of baseline 40 minutes after LY379268 washout for mGluR3 control animals,  $n = 7$ , vs.  $58.7 \pm 4.5\%$  for rats,  $n = 14$ ,  $p = 0.45$ ; Figure 23A and 23B, closed circles). Likewise, no difference in the magnitude of depression of EPSC amplitude was observed between the two wild type control groups ( $p = 0.49$ ). In mice lacking mGluR2, LY379268 failed to induce LTD ( $96.7 \pm 5.3\%$  of baseline 40 minutes after LY379268 washout,  $n = 8$ , vs.



**Figure 22. The mGluR2-selective agonist LY541850 does not induce LTD in rat SNr neurons.** Time course of the EPSC amplitude following 10 minute bath application of LY541850 (10  $\mu$ M) shows that LY541850 induces a transient, reversible depression of excitatory transmission. Data represent mean  $\pm$  SEM for 4 cells. Sample traces represent the average of 6 traces from a single minute of a representative experiment at the time points indicated by 1 (control), 2 (peak LY541850 response), and 3 (wash) on the time course graph.



**Figure 23. LY379268-induced LTD requires mGluR2 activation.** (A) Time course of the EPSC amplitude following 10 minute bath application of LY379268 (100 nM) reveals that LY379268 induced LTD in wild type littermate controls (closed circles,  $n = 7$ ) but failed to induce LTD in mGluR2 knockout mice (open circles,  $n = 8$ ). Data represent mean  $\pm$  SEM. Representative sample traces from a single experiment were taken from time points indicated by numbers and are the average of 6 individual traces. (B) LY379268 (100 nM, 10 minutes) induced LTD in both wild type littermate controls (closed circles,  $n = 7$ ) and mGluR3 knockout mice (open circles,  $n = 6$ ).

57.6 ± 8.7% for wild type controls,  $n = 7$ ,  $p = 0.002$ ; Figure 23A, open circles).

Interestingly, a small reversible depression was observed in slices obtained from mGluR2 knockout mice (81.0 ± 6.3% of baseline 12 minutes after onset of LY379268 application). This depression may be mediated by mGluR3 activation, or may be due to unknown off-target activity. LY379268-induced LTD was unaffected in slices obtained from mice lacking mGluR3 (57.2 ± 8.7% of baseline 40 minutes after LY379268 washout,  $n = 6$ , vs. 64.2 ± 4.4% for wild type littermate controls,  $n = 7$ ,  $p = 0.47$ ; Figure 23B, open circles). However, a moderate increase in the rate of EPSC amplitude depression was observed in slices obtained from mGluR3 knockout mice when compared with wild type littermate controls ( $p < 0.05$  for minutes 2-4, 6, 8, and 10 of LY379268 application). This finding may suggest that in slices from wild type animals, mGluR3 activation moderately opposes the initial EPSC depression induced by LY379268 application. Alternatively, an upregulation of mGluR2 or change in mGluR2 function in slices obtained from mGluR3 knockout mice could be responsible for this phenomenon.

## Discussion

Although activation of mGluRs has been shown to induce LTD at synapses in other brain regions, our current findings represent the first demonstration of mGluR-mediated long term synaptic plasticity within the indirect pathway of the basal ganglia. While long-term depression induced by pharmacological activation of group II mGluRs has not been described previously at the STN-SNr synapse, this phenomenon has been observed at excitatory synapses in several other brain regions, including the basolateral amygdala (BLA) (Lin et al., 2000), the striatum (Kahn et al., 2001), the nucleus accumbens (Robbe et al., 2002a; Robbe et al., 2002b), the bed nucleus of the stria terminalis (Grueter and Winder, 2005), and the medial prefrontal cortex (Barbara et al.,

2003; Huang and Hsu, 2008; Otani et al., 1999; Otani et al., 2002). Depending on the synapse, group II mGluR-mediated LTD can be induced and expressed either presynaptically or postsynaptically. Previous studies evaluating mRNA and protein distribution of group II mGluRs in the basal ganglia indicate a presynaptic localization of group II mGluRs at the STN-SNr synapse, whereas no evidence of postsynaptic group II mGluR expression has been detected in SNr neurons (Bradley et al., 2000; Testa et al., 1994). In addition, application of the group II mGluR agonist LY354740 does not alter the frequency or amplitude of spontaneous miniature EPSCs recorded from GABAergic SNr neurons, nor does it affect the membrane properties of SNr neurons (Bradley et al., 2000), suggesting that the effect of group II mGluR agonists on excitatory transmission is mediated by activation of a presynaptic receptor. Thus, it is likely that the induction of LTD in SNr neurons is at least partially mediated by a presynaptic mechanism, although potential contributions of postsynaptic receptors cannot be ruled out. Because mGluR3 is also expressed in astrocytes (Testa et al., 1994), downstream effects of glial mGluR3 activation could also underlie alterations in neurotransmission induced by group II mGluR agonists. However, the finding that activation of mGluR2 receptors is sufficient to induce LTD in the mouse SNr suggests that glial mGluR3 receptors are not likely to play a role in this form of synaptic plasticity.

The inability of mGluR2-selective pharmacological tools to mimic or potentiate LY379268-induced long term depression may seem a bit puzzling in light of the findings from studies using knockout mice suggesting that mGluR2 activation is sufficient to induce LTD of excitatory transmission in SNr neurons. However, several plausible explanations exist that could account for this apparent discrepancy. First, it is possible that the requirements for LTD induction is different in rat SNr neurons than in mouse SNr neurons. The present findings suggest that activation of mGluR2 is sufficient to induce

LTD in the mouse SNr, whereas in the rat SNr activation of mGluR3 could be either sufficient or required in addition to mGluR2 activation.

Interestingly, it is also possible that mGluR2 activation is sufficient to induce LTD in the rat SNr, but that the pharmacological tools used here did not produce a strong enough activation of mGluR2 to induce LTD. As noted above, LY541850 is a partial agonist of mGluR2, so it is possible that it lacks the efficacy required to induce LTD. Interestingly, LY541850 (1  $\mu$ M) has been shown to induce LTD in the BLA (Lucas et al., 2012), suggesting that this drug can induce mGluR2-dependent LTD at other synapses, and that the concentration of drug used in the present study (10  $\mu$ M) was not insufficient to produce a response. Differences in expression levels in various brain regions could explain the ability of LY541850 to induce mGluR2-mediated LTD at some synapses but not others. For example, if there is higher mGluR2 receptor reserve in the BLA, LY541850 could mimic the effects of a full agonist, whereas it might only produce a level of receptor activation that is 50% of the maximal response of a full agonist at a synapse with lower expression levels. Alternatively, different requirements for the degree of mGluR2 activation could also explain the ability of LY541850 to induce mGluR2-dependent LTD at only a subset of synapses.

The inability of BINA to potentiate a subthreshold concentration of LY379268 also does not exclude the possibility that group II mGluR-LTD in the rat SNr is exclusively dependent on mGluR2 activation. The concentration of BINA chosen for the present study (3  $\mu$ M) has been shown to potentiate mGluR2 effects in the medial prefrontal cortex, suggesting that the concentration used is likely to be sufficient, although the possibility that a higher concentration of BINA would yield a different result cannot be ruled out. One intriguing explanation for the lack of BINA effect is that mGluR2 forms a presynaptic heterodimer at the STN-SNr synapse that prevents BINA from potentiating mGluR2 responses. Interestingly, group II and group III mGluRs have been



found to form heterodimers in cell lines (Doumazane et al., 2011), and the pharmacological consequences of mGluR heterodimerization are just beginning to be elucidated. Importantly, a recent report found that BINA is not able to potentiate the function of mGluR2/4 heterodimers (Kammermeier, 2012), and there is functional evidence that mGluR4 is also presynaptically expressed at excitatory synapses in the rat SNr (Broadstock et al., 2011), raising the possibility that the lack of effect of BINA is caused by mGluR2 forming a heterodimer with mGluR4 or another GPCR that causes a loss of BINA binding or cooperativity. Yet another explanation is that BINA and LY541850 exhibit signaling bias, and while they are able to activate or potentiate mGluR2-mediated effects on the signaling pathways utilized in our cell-based assays, they might not be sufficiently efficacious on the signaling pathways required for LTD induction. Unfortunately, without subtype-selective antagonists, it is not possible to distinguish between these possibilities, and the extent to which we understand the contributions of individual receptors to LY379268-induced LTD remains dependent on the use of knockout mice.

From a therapeutic perspective, it is possible that inducing LTD at the STN-SNr synapse could be beneficial in the treatment of the motor symptoms of PD by reducing excessive excitatory transmission to the basal ganglia output nuclei. Interestingly, previous studies have shown that intranigral or intracerebroventricular administration of group II mGluR agonists reverses reserpine-induced akinesia in rats (Dawson et al., 2000; Murray et al., 2002) and systemic administration of LY354740 reverses the catalepsy and muscle rigidity induced by the dopamine receptor antagonist haloperidol (Bradley et al., 2000; Konieczny et al., 1998). Induction of LTD in the SNr therefore represents a novel mechanism by which group II mGluR agonists may alleviate PD-like motor deficits in preclinical models, although several other potential mechanisms of action may also contribute to the antiparkinsonian effects observed after group II mGluR

administration (Johnson et al., 2009). While studies evaluating the behavioral effects of group II mGluR agonists in preclinical models of PD have used agonists that activate both mGluR2 and mGluR3, our finding that mGluR2 activation is sufficient to induce LTD in the mouse SNr suggests that selective activation of mGluR2 using a subtype-selective agonist or positive allosteric modulator may have therapeutic benefit for the primary motor symptoms of PD as well. However, recent studies demonstrating that mGluR3 activation slows nigrostriatal degeneration in toxin-based models of PD-like neurodegeneration suggest that coactivation of both mGluR2 and mGluR3 receptors may yield both symptomatic relief as well as disease modification (Battaglia et al., 2009; Corti et al., 2007).

In addition to the potential therapeutic benefits of group II mGluR activation for treating PD, synaptic plasticity within the indirect pathway of the basal ganglia could play a role in basal ganglia-dependent learning and memory processes. Types of action learning such as memorization of skilled movement sequences, goal-directed learning, and habit learning are known to be dependent on the basal ganglia, and particularly rely on alterations in transmission in the dorsal striatum (Lovinger, 2010). While much attention has been focused on the relevance of various forms of corticostriatal synaptic plasticity to basal ganglia-dependent forms of learning, the potential contribution of extrastriatal forms of plasticity has not been established. Future studies *in vivo* will therefore be necessary to further explore the potential physiological and therapeutic relevance of synaptic plasticity in the SNr.

## CHAPTER V

### POSITIVE ALLOSTERIC MODULATORS OF MGLUR5 MODULATE AKT AND GSK-3 $\beta$ SIGNALING IN VIVO

#### Introduction

As discussed in Chapter I, mGluR5 PAMs have behavioral effects that are predictive of antipsychotic activity in animal models including the reversal of amphetamine-induced hyperlocomotion (reviewed in Noetzel et al., 2012a; Vinson and Conn, 2012). The ability of mGluR5 PAMs to reverse behaviors induced by a hyperdopaminergic state in subcortical regions suggests that mGluR5 PAMs may have effects on cellular signaling that oppose the effects of dopamine receptor activation. However, the signaling cascades that may contribute to the ability of mGluR5 PAMs to reverse amphetamine-induced locomotion have not been elucidated.

Recently, Akt (also known as protein kinase B) and related signaling molecules have received increased attention for their potential involvement in schizophrenia. Akt is a serine-threonine protein kinase that is present in three isoforms (Akt1, Akt2, and Akt3) that are encoded by different genes. Akt is well-known for its roles in glucose metabolism, cell proliferation, migration, regulation of gene transcription, and apoptosis, and is a major target of cancer research. It has also become increasingly appreciated as an important signaling molecule in the CNS, where it plays roles in synaptogenesis, myelination, neuroprotection, and synaptic plasticity. Its activity is typically regulated by activation of PI3K activation, which increases phosphatidylinositol (3,4,5)-trisphosphate content in the plasma membrane and in turn recruits Akt to the plasma membrane via a pleckstrin homology domain. Once Akt translocates to the plasma membrane, it is activated by two phosphorylation steps: phosphorylation at Thr308 by phosphoinositide-

dependent kinase 1 (PDK1), which is mandatory for activity, and phosphorylation at Ser473 by mammalian target of rapamycin (mTOR) complex 2, which markedly enhances activity. Interestingly, multiple genetic studies have identified Akt1 polymorphisms associated with schizophrenia, and decreased levels of Akt have been found in postmortem brain samples from schizophrenic patients as well as in lymphocytes of living schizophrenic patients (reviewed in Freyberg et al., 2010). In addition, mice that have been genetically modified to prevent phosphorylation of Akt at Ser473 have neurochemical and behavioral phenotypes reminiscent of those observed in schizophrenic subjects (Siuta et al., 2010). Recent biochemical studies have found that a number of currently prescribed antipsychotic drugs (APDs) increase Akt phosphorylation (reviewed in Beaulieu et al., 2009), pointing to the Akt pathway as a potential target of APD action.

A major target of Akt kinase activity is glycogen synthase kinase-3 (GSK-3), which is a serine-threonine protein kinase that exists in two isoforms ( $\alpha$  and  $\beta$ ) that are encoded by distinct genes. GSK-3 is constitutively active and is primarily regulated through inhibitory phosphorylation at Ser9 by Akt and other protein kinases (Kaidanovich-Beilin and Woodgett, 2011). While originally discovered for its role in regulating glycogen synthesis, recent studies of GSK-3 function in the brain have led to an increased appreciation for its important roles in the CNS, including regulation of ion channel function and trafficking, synaptic plasticity, and neurodegeneration (reviewed in Bradley et al., 2012; Kaidanovich-Beilin and Woodgett, 2011; Wildburger and Laezza, 2012). Like Akt, GSK-3 $\beta$  activity is altered by treatment with APDs. In addition, amphetamine and apomorphine reduce Akt activity and GSK-3 $\beta$  phosphorylation via activation of D2 receptors, and pharmacological or genetic inhibition of GSK-3 $\beta$  markedly reduces amphetamine-induced hyperlocomotion (Beaulieu et al., 2004; Urs et al., 2012), suggesting that modulation of this signaling pathway is important for

behavioral responses to dopamine receptor activation. Supporting this hypothesis, another recent study found that transgenic mice expressing constitutively active forms of GSK-3 display enhanced responses to amphetamine and increased susceptibility to mood disturbances (Polter et al., 2010). Taken together, these findings imply that GSK-3 activity plays important roles in the regulation of both normal and pathological behaviors. We therefore hypothesized that modulation of the Akt/GSK-3 $\beta$  pathway could contribute to the antipsychotic-like effects of mGluR5 PAMs.

Here we report that *in vivo* administration of mGluR5 PAMs increases Akt and GSK-3 $\beta$  phosphorylation in the dorsolateral striatum. Furthermore, pretreatment with an mGluR5 PAM reverses the biochemical effects of amphetamine administration on Akt and GSK-3 $\beta$  signaling, suggesting that mGluR5 activation can directly oppose the cellular effects of dopamine receptor activation in the striatum. Using slice electrophysiology studies, we have found that pharmacological inhibition of GSK-3 $\beta$  mimics the ability of an mGluR5 agonist to enhance NMDA currents in striatal medium spiny neurons; this represents a potential physiological mechanism by which GSK-3 $\beta$  inhibition could reverse psychostimulant-induced behaviors. Finally, we provide evidence that mGluR5 PAM-induced Akt and GSK-3 $\beta$  phosphorylation is not restricted to the striatum, as similar effects were observed in the medial prefrontal cortex (mPFC). Taken together, these studies suggest that activation of Akt and subsequent inhibition of GSK-3 $\beta$  represent a cellular mechanism by which mGluR5 PAMs could reverse amphetamine-induced hyperlocomotion and potentially produce antipsychotic effects in schizophrenia patients.

## Methods

### Animals

For studies evaluating the effects of *in vivo* mGluR5 PAM administration on Akt and GSK-3 $\beta$  phosphorylation, male Sprague Dawley rats weighing 250-300 grams were used. For electrophysiology experiments, male and female Sprague Dawley rats 15-19 days old were used. Animals were maintained in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care under a 12 hour light/dark cycle (lights on 06:00 to 18:00) with free access to food and water. All experiments were performed during the light cycle, were approved by Vanderbilt University's Institutional Animal Care and Use Committee, and conformed to guidelines established by the National Research Council *Guide for the Care and Use of Laboratory Animals*. All efforts were made to minimize animal suffering and the number of animals used.

### Drug treatments for phosphorylation studies

VU0092273 and VU0360172 were synthesized in-house as previously described (Noetzel et al., 2012b). VU0092273 (30 mg/kg) and VU0360172 (56.6 mg/kg) were dissolved in 10% Tween 80, sonicated for 30-60 minutes at 37°C, and injected intraperitoneally (ip) as a microsuspension in a volume of 3 ml/kg. Doses of VU0092273 and VU0360172 were chosen based on previous studies demonstrating that these doses effectively reverse amphetamine-induced hyperlocomotion (Noetzel et al., 2012b) and unpublished data). Amphetamine hemisulfate (1 mg/kg, corrected for salt mass) was dissolved in saline and dosed subcutaneously (sc) in a volume of 1 ml/kg.

### **Sample preparation, western blotting, and analysis of protein phosphorylation**

Following the drug treatment time indicated in the text and in figure legends, rats were anesthetized under isoflurane anesthesia, decapitated, and brains were rapidly removed and placed in a chilled brain matrix. Coronal slices (1 mm thick) were cut using razor blades. Slices containing mPFC (prelimbic and infralimbic regions) and anterior dorsal striatum were frozen on a metal surface that was pre-chilled on dry ice. mPFC was dissected by hand using a scalpel blade. Micropunches of dorsolateral striatum were obtained using a blunted 13 gauge needle. Following dissection, samples were placed into a microcentrifuge tube on dry ice and then stored at -80°C prior to homogenization.

Samples were manually homogenized in 25-50  $\mu$ L buffer containing (in mM): Tris HCl, 50, pH 7.4; NaCl, 50; EGTA, 10; EDTA, 5; NaF, 2;  $\text{Na}_3\text{VO}_4$ , 1; supplemented with 1X Complete Mini protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich). Homogenized samples were centrifuged at 16,100xg in a table top microfuge for 10 minutes at 4°C. Supernatant fractions were removed, placed in a fresh tube, and protein assays were performed to determine protein concentration (*DC<sup>TM</sup>* Protein Assay, Bio-rad Laboratories, Inc.). 20-30  $\mu$ g of each sample was mixed with 2X Laemmli buffer (Bio-rad Laboratories, Inc.), and heated at 65°C for 5 minutes. Samples were then separated by SDS-PAGE, transferred to nitrocellulose membrane, blocked with Odyssey blocking buffer (LI-COR Biosciences), and incubated with primary antibodies recognizing phosphorylated or total levels of proteins overnight at 4°C with gentle agitation. The following primary antibodies were used: phospho-Akt Ser473 (Cell Signaling #4058), phospho-Akt Thr308 (Cell Signaling #2695), total Akt (Cell Signaling #2920), phospho-GSK3 $\beta$  Ser9 (Cell Signaling #9322), and total GSK3 $\alpha/\beta$  (Santa Cruz Biotechnology #sc-7291). Membranes were then incubated with appropriate IRDye-conjugated secondary antibodies (Rockland Immunochemicals) for one hour at room

temperature with gentle agitation. Signals were detected using an Odyssey Quantitative Fluorescence Imaging System (LI-COR Biosciences). This method allowed simultaneous detection of phosphorylated and total protein levels. Band intensities were quantified using LI-COR Image Studio software. For each sample, the ratio of phosphorylated protein to total protein was obtained. All phosphorylation ratios were then normalized to the average phosphorylation ratio of samples from vehicle-treated animals. Statistical analysis depended on the experimental design. In most cases, unpaired *t* tests were used to compare vehicle vs. drug-treated groups for individual time points. In time course graphs (Figures 25 and 29), statistical comparisons were made between values obtained from vehicle-treated and drug-treated animals for each time point, but only the values for drug-treated animals are shown. For the experiment testing reversal of amphetamine effects by VU0092273 pretreatment (Figure 26), whole model analysis using one-way analysis of variance (ANOVA) was performed. Planned comparisons between a subset of treatment groups (vehicle/saline vs. vehicle amphetamine, vehicle/saline vs. VU0092273/amphetamine, and vehicle/amphetamine vs. VU0092273/amphetamine) were then made using an unpaired *t* test. Graphpad Prism software was used to create graphs and perform indicated statistical analyses.

### **Brain slice preparation and NMDA current recordings**

Rats were anesthetized with isoflurane, decapitated, and brains were rapidly removed and submerged in ice-cold modified artificial cerebrospinal fluid (ACSF) containing (in mM): 220 sucrose, 2.5 KCl, 0.5 CaCl<sub>2</sub>, 8 MgSO<sub>4</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 10 D-glucose, oxygenated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. Coronal slices 280-300 μm thick were then prepared using a Compressstome (Precisionary Instruments). Slices were hemisected and then allowed to recover in ACSF containing (in mM): 126 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 1.5 MgSO<sub>4</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 10 D-glucose,



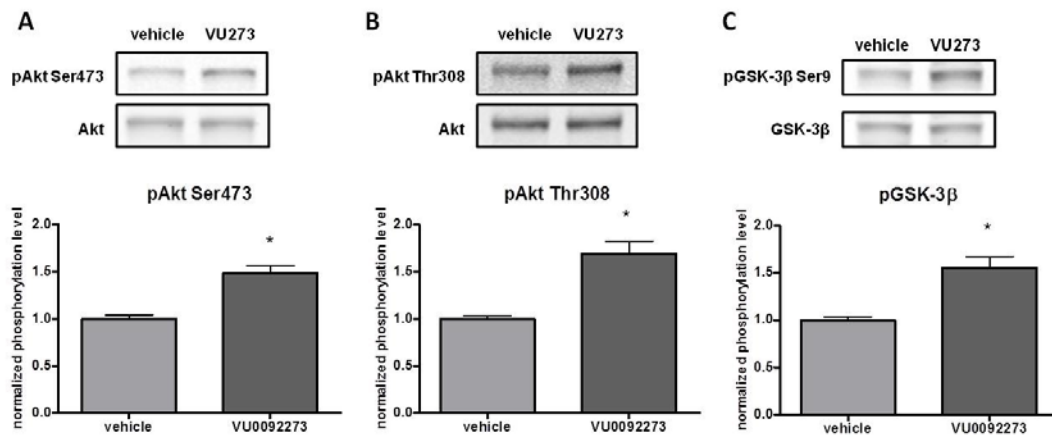
oxygenated with 95% O<sub>2</sub>/5% CO<sub>2</sub>, at 32°C for 0.5 h and maintained at room temperature afterward until transferred individually to a fully submerged recording chamber, which was continuously perfused with oxygenated ACSF at ~30°C. Glutathione (5 μM) was included in the cutting and holding solutions to increase slice viability, but was excluded from the recording solution.

Whole-cell recordings were made from visually identified medium spiny neurons (MSNs) in the dorsolateral striatum under an Olympus BX50WI upright microscope equipped with a 40x water immersion objective, Hoffman optics and video system (Olympus, Lake Success, NY, USA). A MultiClamp 700B amplifier (Molecular Devices, Union City, CA) was used for voltage-clamp recordings. Patch pipettes were prepared from borosilicate glass (World Precision Instrument, Sarasota, FL, USA) using a Flaming/Brown micropipette puller (model P-97; Sutter Instruments, Novato, CA) and filled with the pipette solution containing (in mM) 125 Cs-methanesulfonate, 5 NaCl, 10 TEA-Cl, 10 HEPES, 0.1 EGTA, 5 QX-314, 4 Mg-ATP, 0.3 Na-GTP and 10 phosphocreatine. The pH of the pipette solution was adjusted to 7.3 with 1 M CsOH, and osmolality was adjusted to 290-295 mOsm. The patch pipettes had resistances of 3-5 MΩ when filled with the above solution. NMDA receptor-mediated currents were evoked by pressure ejection of 1 mM NMDA (diluted in ACSF) to the soma of the recorded neurons through a low-resistance (1-2 MΩ) patch pipette using a Picospritzer III (General Valve, Fairfield, NJ, USA). The recorded neuron was voltage-clamped at -60 mV. Tetrodotoxin (1 μM) was routinely included in the perfusate to block voltage-gated sodium channels. Data were acquired using a Digidata 1322A interfaced to a PC computer equipped with pClamp 10.2 software (Molecular Devices, Union City, CA), analyzed using Clampfit and Microsoft Excel and presented as percentage of control value. Graphs were prepared using Graphpad Prism.

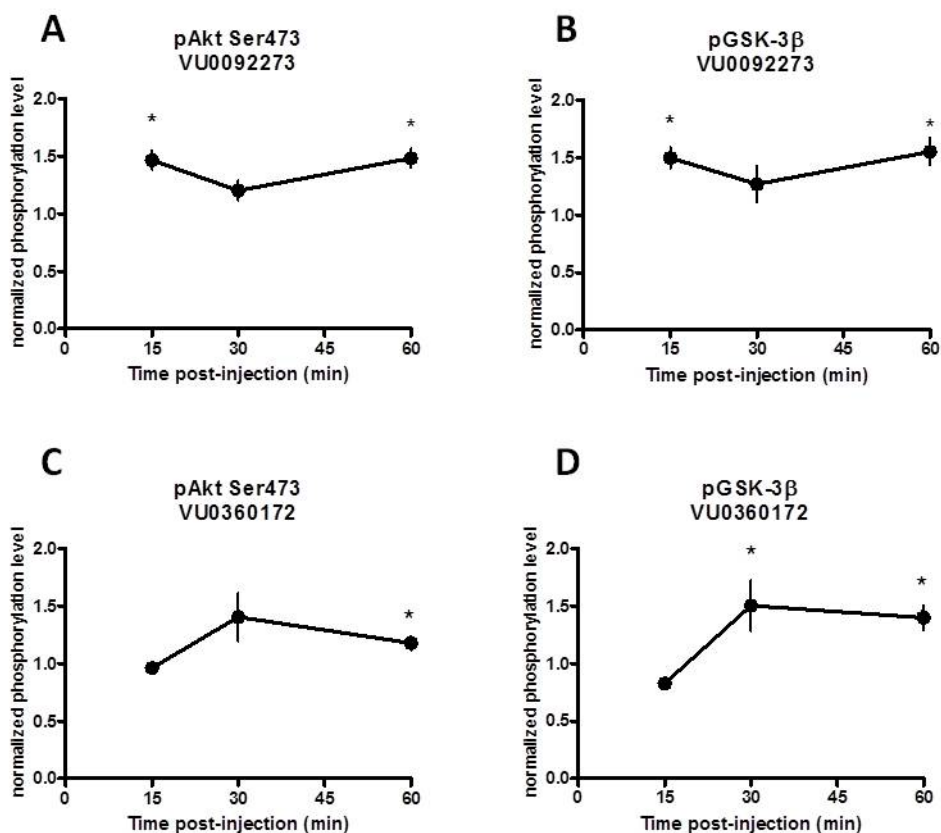
## Results

### **The mGluR5 PAMs VU0092273 and VU0360172 increase Akt and GSK-3 $\beta$ phosphorylation in the dorsolateral striatum**

The ability of mGluR5 PAMs to reverse amphetamine-induced hyperlocomotion has been well established (reviewed in Noetzel et al., 2012a; Vinson and Conn, 2012). However, the cellular mechanisms that underlie this effect are currently unknown. Because inhibition of Akt and subsequent activation of GSK-3 $\beta$  have been implicated in the behavioral effects of amphetamine, and pharmacological or genetic inhibition of GSK-3 $\beta$  attenuates amphetamine-induced hyperlocomotion (Beaulieu et al., 2004; Kalinichev and Dawson, 2011), I evaluated the ability of the mGluR5 PAM VU0092273 to modulate Akt and GSK-3 $\beta$  signaling *in vivo* by systemically administering VU0092273 and then measuring Akt and GSK-3 $\beta$  phosphorylation in protein extracts from dorsolateral striatum samples. Interestingly, following administration of the minimum dose that produces a maximal reversal of amphetamine-induced hyperlocomotion (30 mg/kg ip; Dr. Jerri Rook, unpublished data), a one hour treatment with VU0092273 significantly increased Akt phosphorylation at both Ser473 and Thr308 (Figures 24A and 24B). Concordantly, inhibitory phosphorylation of GSK-3 $\beta$  phosphorylation at Ser9, a site that is known to be phosphorylated by Akt, was also increased (Figure 24C). To further characterize the modulation of Akt and GSK-3 $\beta$  phosphorylation by mGluR5 PAMs, I evaluated phosphorylation of Akt (at Ser473) and GSK-3 $\beta$  in dorsolateral striatum samples at multiple times points following administration of VU0092273 and another mGluR5 PAM, VU0360172 (Figure 25). Akt and GSK-3 $\beta$  phosphorylation levels were significantly increased both 15 and 60 minutes following VU0092273 treatment (30 mg/kg, ip), but no increase was observed at 30 minutes. Similar to the effects of VU0092273, VU0360172 (56.6 mg/kg, ip) produced an increase in both Akt and GSK-3 $\beta$



**Figure 24. VU092273 modulates Akt and GSK-3 $\beta$  signaling in the dorsolateral striatum.** Representative western blots (top) and densitometric analysis (bottom) of phosphorylated protein levels in extracts from dorsolateral striatum samples from rats treated with VU092273 (30 mg/kg ip, 1 hour) or vehicle (10% Tween 80). Results are presented in arbitrary units normalized to phospho-protein levels measured in samples from vehicle-treated animals.  $n = 12-17$  rats per treatment group. Data in bar graphs are expressed as mean  $\pm$  SEM. \* $P < 0.0001$ , unpaired  $t$  test.

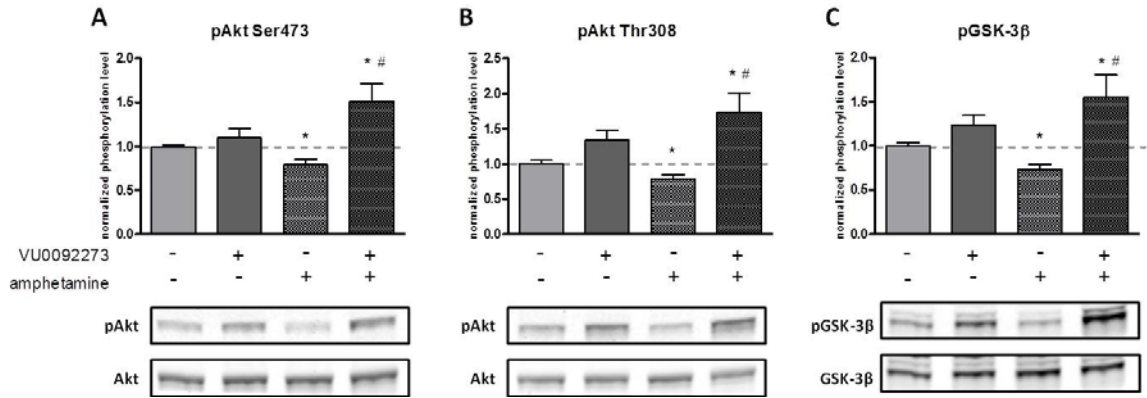


**Figure 25. Time course of Akt and GSK-3β phosphorylation in the dorsolateral striatum following mGluR5 PAM administration.** Data points represent quantification from densitometric analysis of phosphorylated protein levels in extracts from dorsolateral striatum samples from rats treated with VU092273 (30 mg/kg ip) or VU360172 (56.6 mg/kg ip) for the indicated times prior to sacrifice. Results are presented in arbitrary units normalized to phospho-protein levels measured in samples from animals treated with vehicle (10% Tween 80) for the same amount of time. Statistical comparisons were made between phospho-protein levels in samples from animals that were treated with vehicle or drug for the same amount of time (data from vehicle-treated animals are not shown). The 60 minute time point data in panels (A) and (B) are the same data for drug-treated animals that are represented in Figure 24A and 24C. Data in bar graphs are expressed as mean  $\pm$  SEM. (A, B)  $n = 6-17$  rats per treatment group. \* $P < 0.001$ , unpaired  $t$  test. (C, D)  $n = 7-14$  rats per treatment group. \* $P < 0.05$ , unpaired  $t$  test.

phosphorylation following a 60 minute treatment. GSK-3 $\beta$  phosphorylation levels were also increased following a 30 minute treatment, whereas the increase observed for phospho-Akt at 30 minutes was not significant ( $P=0.08$ ). Unlike VU0092273, VU0360172 did not increase phospho-protein levels following a 15 minute treatment. Differences in pharmacokinetic properties such as the time of peak brain concentrations of VU0092273 ( $t_{max} = 1$  hour) and VU0360172 ( $t_{max} = 30$  minutes) may provide an explanation for the differential time course of protein phosphorylation following administration of each drug (Noetzel et al., 2012b).

### **The mGluR5 PAM VU0092273 reverses the effects of amphetamine on Akt and GSK-3 $\beta$ signaling in the dorsolateral striatum**

Treatment of rats or mice with amphetamine has been shown to decrease striatal Akt phosphorylation at Thr308 and thus activate GSK-3 $\beta$  by reducing inhibitory phosphorylation at Ser9 (Beaulieu et al., 2005; Beaulieu et al., 2004; Beaulieu et al., 2007; Shi and McGinty, 2007). As discussed above, these biochemical events are thought to play a role in amphetamine-mediated behaviors including amphetamine-induced hyperlocomotion. I therefore sought to test the hypothesis that pretreatment with an mGluR5 PAM could reverse the previously reported biochemical effects of amphetamine on the Akt/GSK-3 $\beta$  pathway. Rats were pretreated with vehicle (10% Tween 80) or VU0092273 (30 mg/kg ip) 15 minutes prior to treatment with physiological saline or amphetamine (1 mg/kg, sc). As previously reported in mice, 90 minute treatment with amphetamine significantly reduced Akt phosphorylation at Thr308 as well as GSK-3 $\beta$  phosphorylation (Figure 26). Interestingly, we also observed a decrease in Akt phosphorylation at Ser473, an effect which has not been observed in previous studies (Beaulieu et al., 2005; Shi and McGinty, 2007). Samples from rats treated with VU0092273 prior to amphetamine treatment contained increased levels of phospho-Akt

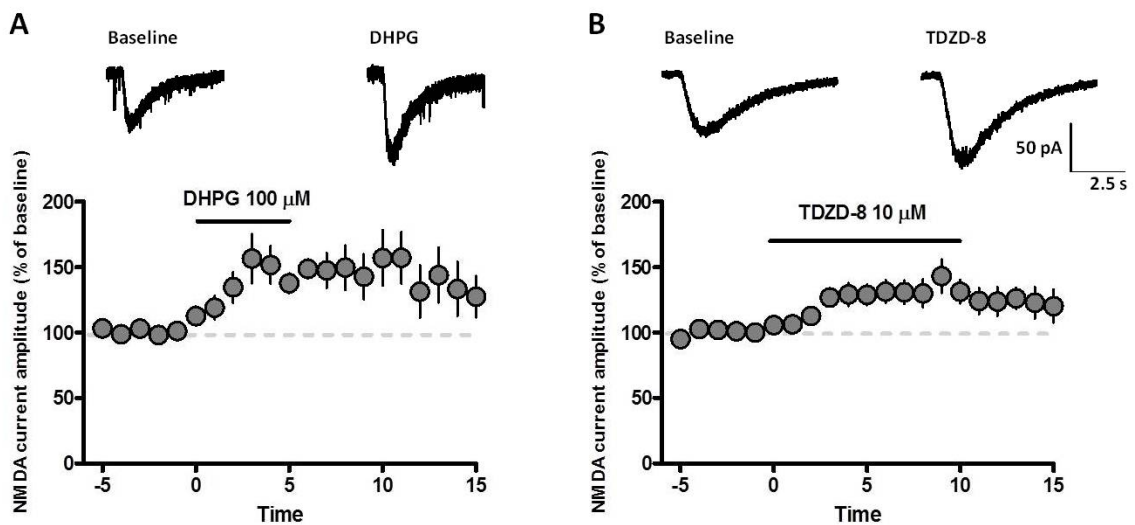


**Figure 26. VU0092273 reverses the effects of amphetamine on Akt and GSK-3β phosphorylation in the dorsolateral striatum.** Representative western blots (bottom) and densitometric analysis (top) of phosphorylated protein levels in extracts from dorsolateral striatum samples of rats treated with VU0092273 (30 mg/kg, ip) or vehicle (10% Tween 80) 15 minutes prior to treatment with amphetamine (1 mg/kg, sc, 90 minutes prior to sacrifice) or 0.9% saline. Results are presented in arbitrary units normalized to phospho-protein levels measured in samples from vehicle-treated animals.  $n = 7-8$  rats per treatment group. Data in bar graphs are expressed as mean  $\pm$  SEM. Whole model ANOVA revealed a significant effect of drug treatment for all three phospho-proteins that were evaluated. \* $P < 0.05$ , planned comparison vs. no drug treatment made by unpaired  $t$  test. # $P < 0.05$ , planned comparison vs. amphetamine treatment made by unpaired  $t$  test.

and phospho-GSK-3 $\beta$ , demonstrating that mGluR5 PAM treatment reversed the effects of amphetamine on this pathway (Figure 26).

### **Inhibition of GSK-3 $\beta$ mimics the ability of mGluR5 activation to enhance NMDA receptor-mediated currents in striatal medium spiny neurons**

A well-characterized physiological effect of mGluR5 activation in the striatum is the potentiation of NMDA receptor-mediated inward currents in medium spiny neurons (MSNs) (Pisani et al., 1997b; Pisani et al., 2001b). Potentiation of NMDA currents in MSNs could represent a mechanism by which mGluR5 PAMs could reverse the ability of a psychostimulant such as amphetamine to induce hyperlocomotion, as NMDA receptor inhibition downstream of D2 receptor activation has been implicated as a possible effect contributing to psychostimulant-induced locomotor activity (Liu et al., 2006). We have therefore begun to evaluate the potential involvement of the Akt/GSK-3 $\beta$  signaling pathway in the modulation of NMDA receptor function by mGluR5 by performing whole cell voltage clamp experiments in rat striatal slices. Consistent with previous studies (Pisani et al., 1997b; Pisani et al., 2001b), I found that activating mGluR5 with a 5 minute bath application of the group I mGluR agonist DHPG (100  $\mu$ M) markedly increased the amplitude of NMDA-receptor mediated currents evoked by pressure ejection of NMDA (1 mM) onto MSNs (peak potentiation of  $156.6 \pm 18.8\%$  of baseline,  $n=5$  cells, Figure 27A). Interestingly, I found that a 10 minute bath application of the selective GSK-3 $\beta$  inhibitor TDZD-8 (10  $\mu$ M) also increased the amplitude of NMDA-evoked inward currents in MSNs (peak potentiation of  $143.2 \pm 12.7\%$  of baseline,  $n=7$  cells, Figure 27B). The maximal enhancement of NMDA current amplitude by these two drug treatments were not significantly different ( $P=0.56$ , unpaired  $t$  test). While many



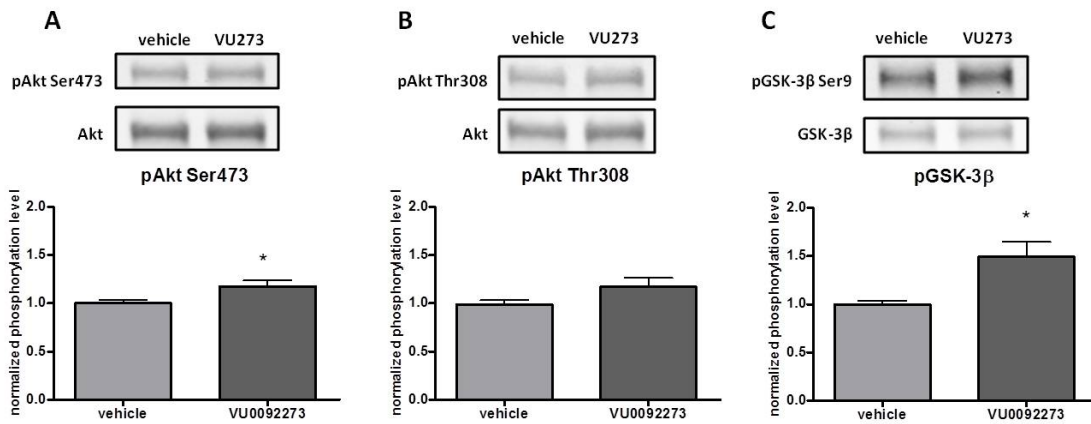
**Figure 27. A GSK-3 $\beta$  inhibitor mimics the effects of mGluR5 activation on NMDA currents in striatal medium spiny neurons.** Representative traces and summary time courses of NMDA current amplitudes recorded from putative medium spiny neurons in the dorsolateral region of striatal slices. (A) The group I mGluR agonist DHPG (100  $\mu$ M, 5 min bath application) increases the amplitude of NMDA currents evoked by pressure ejection of NMDA (1 mM) onto the cell ( $n=5$  cells). (B) The GSK-3 $\beta$  inhibitor TDZD-8 (10  $\mu$ M, 10 min bath application) also increases the amplitude of NMDA currents ( $n=7$  cells). Sample traces are single representative traces taken from the baseline or peak drug response in that cell. Summary data are normalized to the average baseline NMDA current amplitude for each cell and are shown as mean  $\pm$  SEM. Horizontal bars on time courses represent the time during which the drug was perfused into the recording chamber. The beginning of the drug add was designated as time zero, and only the 5 minutes of baseline recording prior to the drug add are shown here. Peak drug effect was not significantly different between the two drug treatments ( $P=0.56$ , unpaired t test).



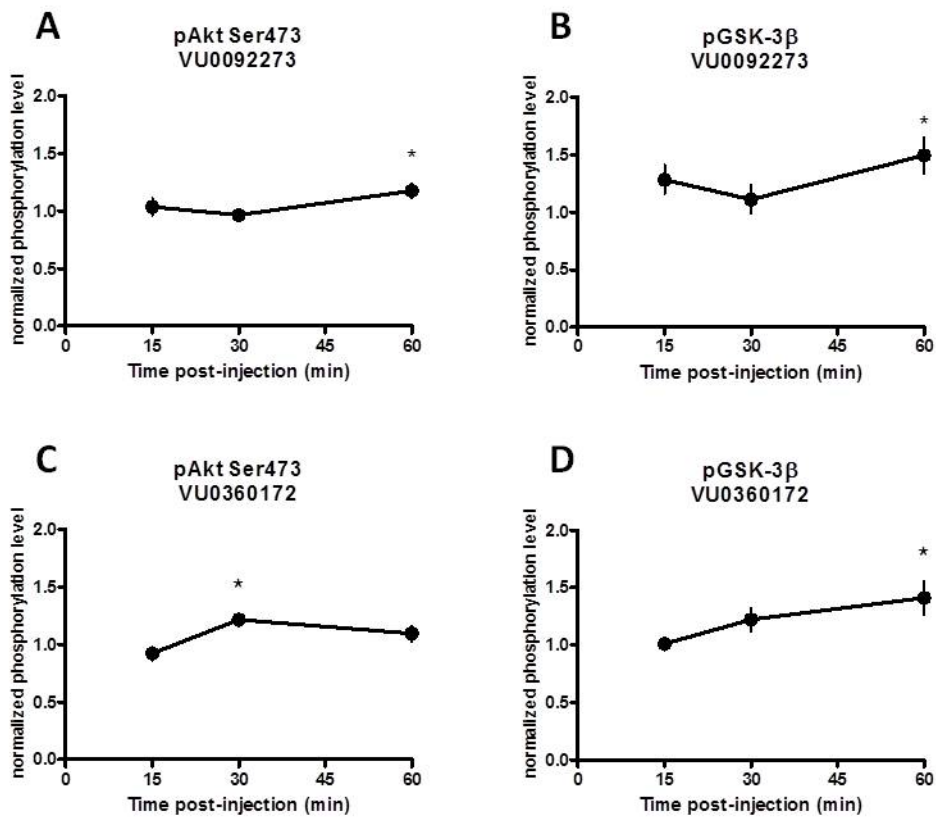
more studies will be necessary to firmly establish a role for GSK-3 $\beta$  inhibition in mGluR5-dependent modulation of NMDA receptor function, this finding provides preliminary evidence that GSK-3 $\beta$  can regulate NMDA receptors in striatal MSNs.

### **mGluR5 PAMs increase Akt and GSK-3 $\beta$ phosphorylation in the medial prefrontal cortex**

The results of our studies in the dorsolateral striatum implicate mGluR5 as a regulator of Akt and GSK-3 $\beta$  signaling in a brain region that is likely relevant to the effects of psychostimulants on locomotor behavior; however, we also wanted to determine the ability of mGluR5 to modulate this signaling pathway in other brain regions that may be relevant to the effects of APDs. To this end, I measured mGluR5 PAM-mediated changes in Akt and GSK-3 $\beta$  phosphorylation in the mPFC, another brain region that is thought to be dysfunctional in schizophrenia and a target of atypical APDs (Artigas, 2010). Interestingly, mGluR5 PAMs increased GSK-3 $\beta$  phosphorylation at Ser9 to a similar magnitude as observed in the dorsolateral striatum. However, more modest effects were observed on Akt signaling in the mPFC (Figure 28). Unlike the rapid time course of VU0092273-mediated increases in Akt and GSK-3 $\beta$  phosphorylation observed in the striatum, a significant increase in phospho-protein levels was only found in the mPFC following a one hour treatment (Figure 29A and 29B; compare with Figure 25A and 25B). Conversely, similar time courses of Akt and GSK-3 $\beta$  phosphorylation were observed in the dorsolateral striatum and mPFC following VU0360172 administration (Figure 29C and 29D; compare with Figure 25C and 25D).



**Figure 28. VU092273 modulates Akt and GSK-3 $\beta$  signaling in the medial prefrontal cortex.** Representative western blots (top) and densitometric analysis (bottom) of phosphorylated protein levels in extracts from medial prefrontal cortex samples from rats treated with VU092273 (30 mg/kg ip, 1 hour) or vehicle (10% Tween 80). Results are presented in arbitrary units normalized to phospho-protein levels measured in samples from vehicle-treated animals.  $n = 6-13$  rats per treatment group. Data in bar graphs are expressed as mean  $\pm$  SEM. \* $P < 0.05$ , unpaired  $t$  test.



**Figure 29. Time course of Akt and GSK-3β phosphorylation in the medial prefrontal cortex following mGluR5 PAM administration.** Data points represent quantification from densitometric analysis of phosphorylated protein levels in extracts from medial prefrontal cortex samples from rats treated with VU0092273 (30 mg/kg ip) or VU0360172 (56.6 mg/kg ip) for the indicated times prior to sacrifice. Results are presented in arbitrary units normalized to phospho-protein levels measured in samples from animals treated with vehicle (10% Tween 80) for the same amount of time. Statistical comparisons were made between phospho-protein levels in samples from animals that were treated with vehicle or drug for the same amount of time (data from vehicle-treated animals are not shown). The 60 minute time point data in panels (A) and (B) are the same data for drug-treated animals that are represented in Figure 28A and 28C. Data in bar graphs are expressed as mean  $\pm$  SEM.  $n = 4-17$  rats per treatment group. \* $P < 0.05$ , unpaired  $t$  test.

## Discussion

The present study demonstrates that modulation of Akt and GSK-3 $\beta$  signaling occurs in both the dorsolateral striatum and the mPFC following systemic administration of mGluR5 PAMs. In light of these findings, mGluR5 PAMs join a growing list of drugs with known or predicted antipsychotic or otherwise mood-stabilizing effects that inhibit GSK-3 $\beta$  via Akt-dependent (and possibly Akt-independent) mechanisms (reviewed in Beaulieu et al., 2009; Freyberg et al., 2010). These include typical antipsychotics such as haloperidol, which block D2 receptors in the striatal complex; atypical antipsychotics such as olanzapine, quetiapine, and clozapine, which display varying degrees of D2 antagonism and 5-HT<sub>2A</sub> receptor antagonism; lithium, a drug commonly used to treat bipolar disorder and known to inhibit GSK-3 via both direct and indirect mechanisms; valproate and lamotrigine, which are two mood stabilizers with poorly understood mechanisms of action that may include GSK-3 inhibition; and group II mGluR agonists, which have preclinical behavioral profiles predictive of antipsychotic effects (Noetzel et al., 2012a; Sutton and Rushlow, 2011; Xi et al., 2011). In addition, various drugs that have clinical utility as antidepressants (e.g. fluoxetine, ketamine) also lead to inhibition of GSK-3 $\beta$  (Beaulieu et al., 2009), indicating that GSK-3 $\beta$  inhibition is a common feature of a remarkable variety of psychotropic drugs that have demonstrated therapeutic efficacy in a broad range of psychiatric disorders.

While GSK-3 $\beta$  inhibition has not been confirmed as a critical signaling event underlying the therapeutic effects of APDs or mood stabilizers, studies performed in rodents in which GSK-3 isoforms are genetically altered to increase or reduce their activity provide several lines of evidence that GSK-3 $\beta$  inhibition may play an important role in the mechanisms of action of these drugs. For example, Beaulieu et al. showed that mice carrying a heterozygous knockout of the gene encoding GSK-3 $\beta$  display a marked reduction in amphetamine-induced hyperlocomotion (Beaulieu et al., 2004).

Moreover, selective deletion of GSK-3 $\beta$  in D2-expressing neurons dramatically blunts amphetamine-induced hyperlocomotion and disruption of pre-pulse inhibition, a finding which highlights the importance of D2-mediated activation of GSK-3 $\beta$  to behavioral responses to hyperdopaminergia (Urs et al., 2012). In agreement with these findings, pharmacological experiments have demonstrated that multiple GSK-3 inhibitors, including lithium, reduce amphetamine-induced hyperlocomotion (Beaulieu et al., 2004; Kalinichev and Dawson, 2011). Conversely, transgenic mice that express constitutively active forms of GSK-3 $\alpha$  and GSK-3 $\beta$  exhibit enhanced hyperlocomotion in response to amphetamine, suggesting that GSK-3 activity plays a permissive role in the behavioral expression of amphetamine-induced hyperlocomotion (Polter et al., 2010). Taken together, these studies identify inhibition of GSK-3, and specifically GSK-3 $\beta$ , as a potential mechanism by which upstream GPCR activation could reverse behaviors elicited by hyperdopaminergic activity in the striatum.

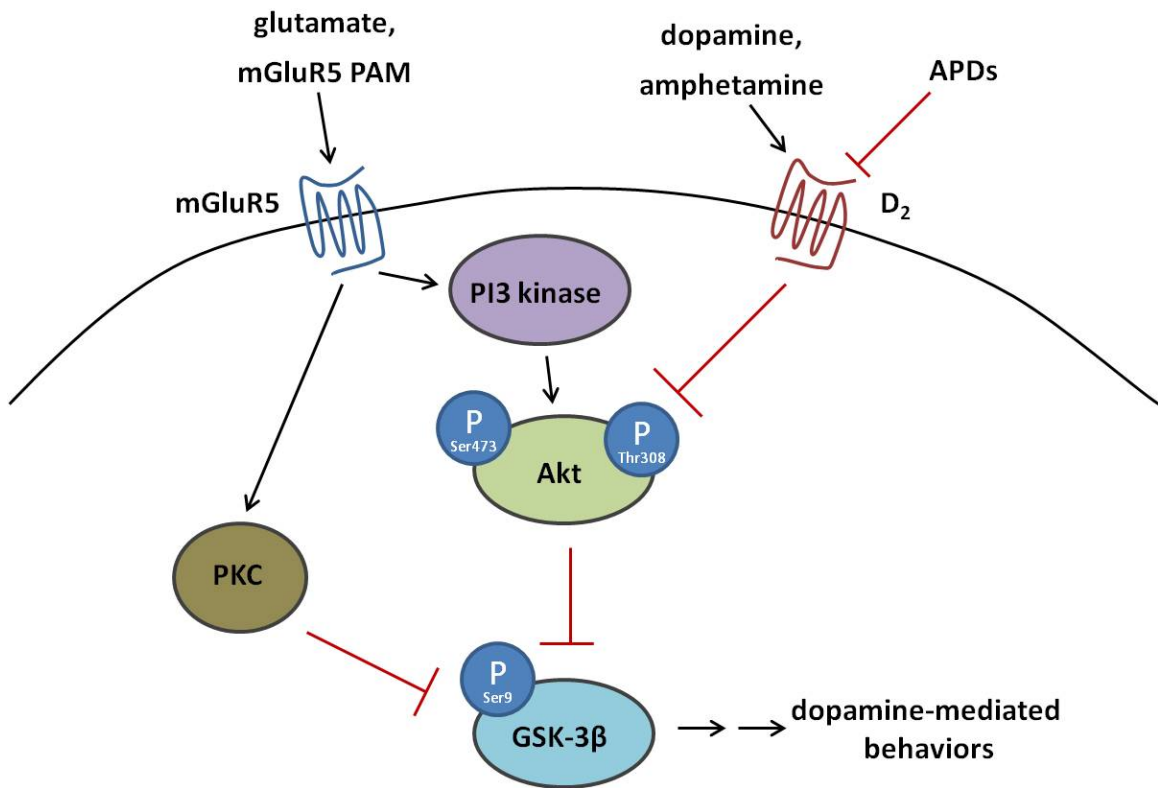
To date, very few studies have evaluated regulation of Akt or GSK-3 $\beta$  signaling by mGluR5 in the striatum. Two previous reports have evaluated the effects of the group I mGluR agonist DHPG on striatal Akt phosphorylation. In agreement with our findings that enhancing mGluR5 activation increases Akt phosphorylation, one study demonstrated that intracerebroventricular DHPG infusion caused an increase in striatal Akt phosphorylation (Pan et al., 2004). Conversely, the other study found that an intrastriatal infusion of DHPG reduced Akt phosphorylation at Thr308 (Schwendt et al., 2012). While the reasons for this discrepancy remain unclear, several possible explanations exist. First, DHPG activates both mGluR1 and mGluR5, which may yield a different effect on Akt signaling than selective enhancement of mGluR5 signaling. In fact, the finding that mGluR1 and mGluR5 have differential effects on striatal Akt function would not be surprising, as there are many examples of these receptors being expressed in the same population of neurons but having non-overlapping physiological

effects (Bonsi et al., 2008). Neither of these studies employed selective antagonists or knockout mice in order to test the contributions of mGluR1 vs. mGluR5 to the observed effects of DHPG, so the potential effect of mGluR1 activation on striatal Akt signaling has not been determined and may complicate the comparison of these results with studies of mGluR5 PAM effects. Second, a more broad activation of mGluR5 (as would be expected after systemic or intracerebroventricular administration) could produce different effects than an intrastriatal infusion, and it is possible that a circuit-level effect of mGluR5 activation is required for the increase in Akt phosphorylation that we observed. Finally, minor methodological differences such as pretreatment time and protein extraction method could also explain these discrepancies.

While the roles of mGluR5 modulation of Akt signaling in the striatum have not been well established, mGluR5 has been linked to this pathway in a number of other contexts related to CNS functions. For example, mGluR5-mediated regulation of Akt has been identified as a requirement for group I mGluR-induced synaptic plasticity in the hippocampus. Blockade of PI3K or mTOR prevents induction of LTD by DHPG at the Schaffer collateral-CA1 synapse, suggesting that this pathway is important for group I mGluR-mediated initiation of protein translation (Banko et al., 2006; Hou and Klann, 2004). Similarly, the PI3K/Akt pathway has been implicated in activity-induced protein synthesis in the dendrites of hippocampal neurons (Gong et al., 2006). Activation of mGluR5 can also protect hippocampal slices against  $\beta$ -amyloid toxicity by activating the PI3K/Akt pathway, and this phenomenon is also associated with an increase in GSK-3 $\beta$  phosphorylation (Liu et al., 2005). Other studies assessing forms of group I mGluR-mediated neuroprotection against oxidative stress, ischemic injury, and traumatic brain injury have also demonstrated that DHPG- or CHPG-induced neuroprotection requires PI3K/Akt pathway activation (Chen et al., 2012; Chong et al., 2006; Scartabelli et al., 2008), which is consistent with the well-established pro-survival role of Akt. Interestingly,

mGluR5 coupling to Akt signaling is altered in mouse models of neurological disorders such as Huntington's disease and Fragile X Syndrome (Ribeiro et al., 2010; Ronesi and Huber, 2008), although the consequences of changes in mGluR5-Akt coupling in these disorders are not fully understood.

While several physiological effects of mGluR5 activation have been functionally associated with modulation of the Akt pathway, the molecular events linking mGluR5 to Akt activation and GSK-3 $\beta$  inhibition are not clear, and may vary between cell populations. As discussed above, most known Akt-mediated effects of mGluR5 are dependent on PI3K activation. Figure 30 depicts a hypothetical signaling cascade in which mGluR5 activates PI3K, which in turn leads to Akt activation and inhibition of GSK-3 $\beta$ ; these effects of mGluR5 activation would oppose the effects of D2 activation in the striatum. However, questions remain regarding how mGluR5 activation induces PI3K activation. Previous work has shown that disruption of mGluR5 interaction with the scaffolding protein Homer uncouples mGluR5 from PI3K/Akt pathway activation in the hippocampus, whereas mGluR5-mediated activation of MAP kinase signaling remains intact when mGluR5-Homer interactions are blocked (Ronesi and Huber, 2008). This finding suggests that in hippocampal neurons, mGluR5-Homer complex formation is an important mediator of mGluR5 activation of PI3K, and that mGluR5 signaling through PI3K/Akt pathways and the MAP kinase pathway are mediated by different molecular mechanisms. To expand on this model, Rong et al. reported that a Homer-associated protein, phosphatidylinositol 3-kinase enhancer (PIKE), associates with mGluR-Homer complexes and links them to PI3K activation in hippocampal neurons (Rong et al., 2003). However, formation of an mGluR5-Homer-PIKE complex has not been demonstrated in the striatum, so it is unknown whether this complex could mediate striatal activation of PI3K by mGluR5.



**Figure 30. Hypothetical model of mGluR5 modulation of the Akt/GSK-3 $\beta$  pathway.** *In vivo* administration of PAMs increases phosphorylation of Akt and GSK-3 $\beta$ . PI3 kinase may mediate the mGluR5 PAM-induced increase in Akt phosphorylation, although this hypothesis has not been directly tested. Both Akt and PKC activity downstream of mGluR5 activation could contribute to the observed increase in GSK-3 $\beta$  phosphorylation. The effect of mGluR5 activation is opposite that of D<sub>2</sub> receptor activation, which causes a decrease in Akt phosphorylation at Thr308 and subsequent increase in GSK-3 $\beta$  activity that mediates some dopamine-induced behaviors. Conversely, antipsychotic drugs (APDs) such as haloperidol and clozapine are D<sub>2</sub> receptor antagonists, and similar to mGluR5 activation, APDs increase Akt and GSK-3 $\beta$  phosphorylation. Black arrows indicate activation whereas red lines indicate inhibition.



Another intriguing possibility is that mGluR5 activates PI3K/Akt signaling indirectly via transactivation of a receptor tyrosine kinase (RTK). GPCRs can transactivate RTKs through a variety of mechanisms, including G protein-mediated induction of RTK autophosphorylation and stimulation of RTK ligand production (Shah and Catt, 2004). Indeed, mGluR5-mediated increases in extracellular signal-regulated kinase (ERK) phosphorylation in astrocytes require transactivation of epidermal growth factor receptors (EGFRs) (Peavy et al., 2001), providing evidence that RTK transactivation is a potential mechanism by which mGluR5 signaling occurs in some cell populations.

While activation of PI3K seems to be the most parsimonious explanation for mGluR5-mediated modulation of Akt/GSK-3 $\beta$  signaling, other possible mechanisms could also play a role. One plausible example of a PI3K-independent mechanism by which mGluR5 PAMs could increase Akt and GSK-3 $\beta$  phosphorylation in the striatum is the direct disruption of signaling events responsible for D2-mediated inhibition of Akt activity. D2 receptor activation has been shown to reduce Akt phosphorylation at Thr308 by inducing formation of a  $\beta$ -arrestin2-protein phosphatase 2A (PP2A)-Akt protein complex that promotes PP2A-mediated dephosphorylation of Akt and subsequently disinhibits GSK-3 (Beaulieu et al., 2005). Tonic activation of D2 receptors *in vivo* is likely to regulate Akt signaling in the striatum under normal conditions, as basal Akt phosphorylation levels are markedly increased in mice lacking D2 receptors (Beaulieu et al., 2007). Interestingly, mGluR5 activation has been reported to suppress PP2A activity in cultured striatal and hippocampal neurons via both mGluR5-PP2A interactions and Src- and mTOR-mediated inhibition of PP2A, and this phenomenon contributes to the increase in phosphorylation of proteins such as ERK and fragile X mental retardation protein (FMRP) that occurs in response to mGluR5 activation (Mao et al., 2005; Narayanan et al., 2007), suggesting that a similar inhibition of PP2A-mediated

dephosphorylation of Akt is a potential alternative mechanism by which mGluR5 PAMs could enhance Akt activity. It is also possible that multiple, convergent signaling events contribute to the increase in Akt and GSK-3 $\beta$  phosphorylation induced by mGluR5 PAMs, or that different signaling events may produce similar effects on Akt and GSK-3 $\beta$  activity in distinct populations of neurons. Future studies may attempt to further elucidate the precise signaling events that are required for increases in Akt and GSK-3 $\beta$  phosphorylation following mGluR5 PAM administration.

The possible signaling events involved in mGluR5-mediated modulation of Akt and downstream effector signaling are very difficult to elucidate *in vivo* due to the great number of possibilities that require evaluation and the fact that many available inhibitors of specific components of the relevant signaling pathways do not have pharmacokinetic profiles that are amenable to *in vivo* studies, particularly in terms of CNS exposure. Unfortunately, extensive efforts to study this phenomenon in striatal slices, which would provide a system more suitable for pharmacological analysis of specific signaling events that occur downstream of mGluR5 activation, were unsuccessful (data not shown). The most obvious reason for a lack of mGluR5 agonist-induced modulation of Akt in striatal slices is that none of the tested experimental conditions were ideal for studying this phenomenon. However, we must keep in mind that the circuitry that mediates this effect *in vivo* may be disrupted in the slice preparation. Interestingly, the ability of haloperidol to increase Akt phosphorylation has only been observed *in vivo*, and has not been replicable in cultured neurons, providing support for the idea that intact circuitry may be required in order to observe effects on this signaling pathway in the striatum (Freyberg et al., 2010).

Our finding that the selective GSK-3 $\beta$  inhibitor TDZD-8 enhances the amplitude of NMDA-evoked inward currents in striatal MSNs provides preliminary evidence that mGluR5 activation may produce the same effect via downstream inhibition of GSK-3 $\beta$ .

However, many more experiments must be performed in order to fully elucidate the contribution of GSK-3 $\beta$  inhibition to the potentiation of NMDA currents by mGluR5. While kinase inhibitors that target the ATP binding site are notorious for off-target activity, TDZD-8 is a non-ATP-competitive inhibitor of GSK-3 $\beta$ , which tends to confer more selectivity because it targets a less-conserved region of the enzyme. However, we plan to test two additional GSK-3 inhibitors (CHIR99021 and AR-A014418) that have been tested for selectivity against large numbers of kinases and found to be very highly selective and have non-overlapping off-target activities (Pan et al., 2011); this experiment should increase our confidence that the potentiation of NMDA currents by TDZD-8 is mediated by GSK-3 $\beta$  inhibition. In conjunction with these experiments, we will also test the ability of a maximally effective concentration of one GSK-3 $\beta$  inhibitor to occlude the ability of DHPG to enhance NMDA currents. In addition, we plan to evaluate the effect of PI3K and Akt inhibition on DHPG-induced potentiation of NMDA currents. Interestingly, previous reports suggest that pharmacological inhibition of PKC with staurosporine or calphostin C, antagonists of PKC, prevents DHPG-induced potentiation of NMDA currents (Pisani et al., 1997b). While Akt-mediated phosphorylation of Ser9 is the best-characterized mechanism of GSK-3 $\beta$  inhibition, PKC has also been shown to phosphorylate GSK-3 $\beta$  at Ser9 (Espada et al., 2009), so PKC activation may also contribute to the modulatory effect of mGluR5 on GSK-3 $\beta$  phosphorylation (Figure 30). Alternatively, mGluR5-mediated transactivation of an RTK could require PKC activity. Both of these possibilities represent plausible explanations for a PKC inhibitor blocking an mGluR5 effect that also depends on GSK-3 $\beta$  inhibition, and future studies may further elucidate the relationship between these signaling cascades in the context of NMDA receptor regulation.

While the current study represents the first preliminary evidence that mGluR5-mediated potentiation of NMDA currents might depend on GSK-3 $\beta$  inhibition, studies of

modulation of NMDA function in layer V pyramidal cells of the mPFC have shown that both group II mGluRs and D2-like dopamine receptors can regulate NMDA receptor function, albeit in opposite directions, by modulating GSK-3 activity. D2 receptor activation in the prefrontal cortex reduces NMDA current amplitude, an effect which is reversed by a GSK-3 inhibitor (Li et al., 2009). Conversely, group II mGluR activation enhances the amplitude of NMDA currents recorded from the same neurons, and this effect is occluded when slices are incubated with the GSK-3 $\beta$  inhibitor TDZD-8 prior to the group II mGluR agonist (Xi et al., 2011). These findings suggest that GSK-3 activity can regulate NMDA receptors in the prefrontal cortex, possibly via changes in phosphorylation state or rapid effects on trafficking and surface expression (Li et al., 2009; Xi et al., 2011), suggesting that it is plausible that mGluR5 could potentiate NMDA currents in MSNs through similar mechanisms.

Our biochemical studies indicate that in addition to modulation of striatal Akt signaling, mGluR5 PAMs also increase Akt and GSK-3 $\beta$  phosphorylation in the mPFC. Recent studies using mice in which GSK-3 $\beta$  was selectively eliminated from forebrain pyramidal neurons have shed new light on the potential functions of GSK-3 $\beta$  in this region, and allow speculation about the potential behavioral consequences of mGluR5 PAM-mediated inhibition of GSK-3 $\beta$  in cortical regions. These mice display reduced anxiety-like behaviors as well as a pro-social phenotype (Latapy et al., 2012), suggesting that drugs that indirectly inhibit GSK-3 $\beta$  in the same neuronal populations may induce these behaviors as well. At this time, mGluR5 PAMs have not been evaluated in models of social behavior; however, it would be of interest to know if mGluR5 PAMs would promote social behavior, as social withdrawal is one of the negative symptoms associated with schizophrenia.

In conclusion, we have found that mGluR5 PAMs modulate Akt and GSK-3 $\beta$  signaling *in vivo*, which may contribute to the antipsychotic-like behavioral effects of

these drugs, particularly in terms of their ability to reverse amphetamine-induced hyperlocomotion. In addition, we present preliminary evidence that mGluR5-mediated modulation of this pathway may be important for well-known physiological effects of mGluR5 activation including enhancement of NMDA receptor function. Ultimately, behavioral studies will be necessary to determine the importance of Akt/GSK-3 $\beta$  pathway modulation to the ability of mGluR5 PAMs to reverse dopamine-mediated behaviors. This increased understanding of the influence of mGluR5 PAMs on signaling pathways *in vivo* could guide future drug development towards compounds that are optimized to affect critical downstream effector proteins and therefore provide maximal therapeutic benefit.

## CHAPTER VI

### GENERAL DISCUSSION AND FUTURE DIRECTIONS

#### **Metabotropic glutamate receptors as targets for Parkinson's disease: recent advances and therapeutic strategies**

Several mGluR subtypes have been identified as potential therapeutic targets for treating the motor symptoms of PD. Chapters II, III, and IV detailed studies aimed at evaluating various aspects of mGluR pharmacology and physiology with the overarching goal of elucidating promising therapeutic avenues and exploring possible mechanisms of action of various drugs that have antiparkinsonian effects in preclinical models of PD. Since the publication of the work described in Chapter II (Niswender et al., 2008b), major advances in mGluR4 PAM development have been made. The recent discovery of mGluR4-selective compounds with improved physiochemical properties for *in vivo* administration has lately allowed an essential question to be addressed: would systemic dosing of mGluR4 agonists or PAMs have the same beneficial effects on motor function that have been observed following intracerebroventricular administration? Initial evidence suggesting that systemic activation of mGluR4 is a viable therapeutic strategy came from the report of an mGluR4-preferring orthosteric agonist, LSP1-2111 (Beurrier et al., 2009). Although this compound activates mGluR7 and mGluR8 at higher concentrations, its 25 to 30-fold selectivity for mGluR4 over mGluR7 and mGluR8 allowed a preliminary evaluation of the ability of an mGluR4-preferring agonist to reverse PD-like motor deficits. Indeed, systemic administration of LSP1-2111 reversed haloperidol-induced catalepsy at relatively modest doses (1-15 mg/kg, ip). More recently, additional support for this concept has come from the development of several systemically active mGluR4 PAMs with true selectivity for mGluR4 vs. mGluR7 and

mGluR8. The first of these compounds to be reported, VU0364770, exhibited antiparkinsonian effects following subcutaneous dosing in multiple motor deficit models including haloperidol-induced catalepsy and forelimb use asymmetry induced by unilateral 6-OHDA lesion (Jones et al., 2011a). Another recently reported mGluR4-selective PAM termed Lu AF21934 also reduced haloperidol-induced catalepsy following subcutaneous administration (Bennouar et al., 2012), corroborating previous evidence that mGluR4 PAM treatment may be a viable therapeutic strategy for treating the motor symptoms of PD.

### **mGluR4 PAMs as a component of combination therapies**

An interesting feature of recent studies using systemically active mGluR4-selective PAMs is the evaluation of these drugs as potential adjunct therapies to L-DOPA treatment. When administered alone, the mGluR4 PAM VU0364770 had modest effects on forelimb use asymmetry induced by unilateral 6-OHDA lesion, but robustly reversed forelimb use asymmetry when administered in combination with a low, inactive dose of L-DOPA (Jones et al., 2011a). Interestingly, in the same preclinical model, the mGluR4-selective PAM Lu AF21934 did not reverse forelimb use asymmetry alone, but like VU0364770, it augmented the ability of a low dose of L-DOPA to restore forelimb use symmetry, and this beneficial effect was sustained following chronic co-dosing of Lu AF21934 and L-DOPA (Bennouar et al., 2012). Similarly, another systemically active mGluR4 PAM, ADX88178, did not improve stepping behavior deficits in rats with a bilateral 6-OHDA lesion, whereas ADX88178 robustly augmented the ability of an inactive dose of L-DOPA to alleviate akinesia in this model (Le Poul et al., 2012). Taken together, these studies suggest that mGluR4 PAMs or agonists may be particularly beneficial as a combination therapy with a low dose of L-DOPA, and in fact may require coadministration with L-DOPA to achieve robust antiparkinsonian efficacy. Indeed, the

potential synergistic effects between these drugs could limit the doses of each drug necessary to achieve the desired therapeutic effect, which could in turn reduce side effect liability or complications of long-term L-DOPA treatment such as development of L-DOPA-induced dyskinesia (LID). Intriguingly, chronic coadministration of Lu AF21934 and a low dose of L-DOPA reduced the percentage of L-DOPA-treated animals that developed dyskinesias, suggesting that mGluR4 activation may provide some protective benefit against adverse effects of chronic L-DOPA treatment (Bennouar et al., 2012). However, the same study also showed that Lu AF21934 did not reduce abnormal involuntary movement severity in those rats that did develop dyskinesias. This finding was corroborated by the report that ADX88178 also failed to reduce the severity of established LID (Le Poul et al., 2012). Overall, these results suggest that mGluR4 PAMs may prevent the development of LIDs by both a direct mechanism and by reducing the required dose of L-DOPA, but may not ameliorate the severity of established LIDs.

In addition to the potential for mGluR4 PAMs to augment the efficacy of L-DOPA, administering mGluR4 PAMs in combination with another experimental treatment for PD motor symptoms, adenosine A2A receptor antagonists, may also hold promise as a novel therapeutic strategy. Interestingly, mGluR4 and A2A receptors have overlapping expression patterns at both corticostriatal and striatopallidal synapses (Lopez et al., 2008). Whereas activating mGluR4 reduces neurotransmission at these important synapses, blocking A2A receptors has a similar effect, and depending on the mechanisms that underlie these effects on transmission, it is possible that simultaneously activating mGluR4 and inhibiting A2A receptors could have additive or even synergistic effects on motor deficits. The idea that group III mGluR activation could enhance the antiparkinsonian effects of adenosine A2A receptor antagonists was first explored by Lopez et al., who reported that the nonselective group III mGlu receptor agonist ACPT-I increased the reversal of haloperidol-induced catalepsy by A2A receptor



antagonists (Lopez et al., 2008). These findings have now been extended by the recent report that subcutaneous administration of the mGluR4 PAM VU0364770 synergistically augments the efficacy of preladenant (Jones et al., 2011a), an A2A receptor antagonist that recently showed promise for treating motor symptoms of PD in a Phase II clinical trial (Hauser et al., 2011). This exciting result suggests that combination therapy with low doses of preladenant and an mGluR4 PAM could be a promising clinical strategy to assess in future studies.

Another potential combination therapy could involve coadministration of multiple drugs that target different subtypes of mGluRs. Early indications that group III mGluRs represent novel targets for PD arose from studies employing nonselective group III mGluR agonists such as L-AP4. The finding that drugs that do not discriminate between individual mGluR subtypes are effective in preclinical models of PD, in combination with the fact that drugs targeting each individual group III mGluR subtype have now been demonstrated to alleviate PD-like motor deficits (Chapters II and III; (Greco et al., 2009)), suggests that simultaneously targeting multiple receptor subtypes could be a beneficial therapeutic strategy. This could be achieved by coadministration of multiple drugs targeting individual receptor subtypes, or by developing drugs that are optimized to potentiate the activity of multiple receptor subtypes. As discussed above in the context of combining mGluR4 PAMs with L-DOPA or preladenant, combination approaches could provide additive or synergistic therapeutic effects depending on the mechanisms of action that are targeted through each receptor, and could also allow lower doses of drug to be used to provide the same benefit, which in turn could limit side effect liability. Future studies may assess the potential benefits or drawbacks of simultaneously targeting multiple mGluR subtypes for the treatment of PD.

## **Group II mGluRs as therapeutic targets for PD**

As discussed in Chapter IV, group II mGluRs have also been implicated as therapeutic targets for both the motor symptoms and the progressive neurodegeneration associated with PD. While a few studies evaluating the effects of group II mGluR agonists in acute models of PD (such as haloperidol-induced catalepsy) support the idea that group II mGluR activation could reduce motor deficits, unpublished data from our laboratory suggests that in chronic models of PD-like neurodegeneration, group II mGluRs are not likely to alleviate motor symptoms; this finding casts doubt on the potential utility of these drugs for PD treatment. In agreement with our findings, Murray et al. reported that the group II mGluR agonist LY379268 failed to reverse akinesia induced by prolonged reserpine treatment, and also did not reduce rotational behavioral caused by a unilateral 6-OHDA lesion (Murray et al., 2002). While these drugs would be predicted to have antiparkinsonian effects in light of their ability to reduce excitatory transmission in the SNr, there are several possible explanations for their lack of effects following prolonged dopamine depletion. For example, group II mGluRs are expressed at many excitatory synapses in the basal ganglia and in other brain regions. While their inhibitory effects at excitatory corticostriatal and cortico-subthalamic synapses could also contribute to antiparkinsonian effects, inhibition of glutamate release at other synapses, such as thalamocortical synapses, could potentially have a harmful effect on PD-like motor symptoms. In addition, group II mGluR agonists reduce dopamine release in the striatum (discussed in Chapter I), and have been shown to reduce the efficacy of L-DOPA in 6-OHDA-lesioned rats (Rylander et al., 2009), suggesting that group II mGluR-mediated inhibition of dopamine release from remaining dopaminergic terminals could also counteract any beneficial effects of group II mGluR activation in PD.

Another possible reason for the lack of effect of group II mGluR agonists in chronic models of PD is that the beneficial effects of activating these receptors are lost

due to the loss of dopaminergic activity. As discussed in Chapter I, the ability of a brief application of a group II mGluR agonist to reduce excitatory transmission in the SNr is diminished following reserpine treatment or acute dopamine receptor blockade (Wittmann et al., 2002), so a lack of ambient dopamine in the SNr could diminish the ability of group II mGluRs to reduce excitatory transmission in more chronic models of PD. This possibility highlights the importance of assessing the effects of novel therapeutic agents in chronic models of PD, as a long-term loss of dopamine receptor function has been shown to lead to many changes in the function of several mGluR subtypes. Interestingly, in the case of the mGluR8 agonist DCPG, prolonged dopamine depletion has the opposite effect, in the sense that it actually confers therapeutic benefit to a drug that does not alleviate motor deficits in acute models of PD. Taken together, these findings emphasize the importance of evaluating potential therapeutic strategies using multiple types of preclinical models, and of keeping in mind the potential for the presence or absence of dopamine to alter receptor function.

### **Targeting mGluRs for protection against neurodegeneration**

The studies described in this dissertation focused on the potential for activation of various mGluR subtypes to alleviate the motor symptoms of PD. However, another critical aspect to managing this chronic neurodegenerative disease is the development of treatments that slow disease progression by protecting against the progressive loss of substantia nigra dopamine neurons. As discussed in Chapter I, several mGluR subtypes have been identified as potential targets for neuroprotection. These studies all rely on assessing protection against toxin-induced nigrostriatal degeneration. The commonly used models of PD-like neurodegeneration include 6-OHDA-induced lesions in rats and MPTP-induced lesions in mice. While these models may have limited utility for identifying treatments that target the cellular causes of PD, which are poorly understood

and may not be closely recapitulated by these toxins, they may be more useful for mimicking the circuit-level changes in basal ganglia function that occur following a critical level of dopamine neuron degeneration. Increased activity through the indirect pathway is predicted to increase excitatory input from the STN to the SNc, which could in turn add an NMDA receptor-mediated excitotoxic component to the progressive degeneration of dopamine neurons (see Chapter I). Toxin-based models of PD may therefore provide a tool for evaluation of potential disease-slowing therapeutic strategies that target the circuit-level changes in indirect pathway activity produced by nigrostriatal degeneration.

As discussed in Chapter I, group III mGluR agonists such as L-AP4 and the mGluR4 PAM PHCCC reduce nigrostriatal degeneration in both MPTP-treated mice and 6-OHDA-lesioned rats, suggesting that activating mGluR4, and potentially mGluR7 or mGluR8, could have neuroprotective benefits, at least from an anatomical perspective. Development of the mGluR4 PAM VU0155041 allowed further evaluation of this concept. Recently, Betts et al. reported that icv administration of VU0155041 protects against loss of nigrostriatal projections following unilateral 6-OHDA injection (Betts et al., 2012). More importantly, this anatomical protection correlated with protection against the development of motor deficits as well. These data provide evidence that mGluR4 PAMs may have protective benefit against progressive nigrostriatal degeneration, most likely by reducing activity of the indirect pathway. This study and others like it must be interpreted with caution, however, as they evaluate the ability of various treatments to prevent degeneration by administering test compounds at the same time as the toxin. In PD patients, this is not a practical strategy, as more than 80% of dopamine neurons are already lost when motor symptoms become apparent, so a more realistic experimental design would require assessing protection against progressive degeneration when drugs are administered after a significant lesion is already established. Unfortunately, the rapid and robust degeneration of dopamine neurons induced by 6-OHDA treatment does not

provide an ideal framework for this type of experimental design. Better models to assess neuroprotection in PD are needed in order to fully assess the potential disease-modifying effects of mGluR4 PAMs; however, preliminary results using the available tools provide hope that mGluR4 PAMs will be a useful strategy for treating both the motor symptoms and progressive degeneration associated with PD.

### **Modulation of Akt and GSK-3 $\beta$ by mGluR5 PAMs: Implications and future directions**

#### **mGluR5 PAMs as novel treatments for schizophrenia**

As discussed in Chapter I, mGluR5 PAMs have shown promising antipsychotic-like effects in behavioral assays such as amphetamine-induced hyperlocomotion and amphetamine-induced disruption of pre-pulse inhibition. The work presented in Chapter V, which focused on cellular signaling mechanisms that could contribute to the ability of mGluR5 PAMs to reverse behaviors induced by hyperdopaminergia, emphasized the similarities in the biochemical effects of mGluR5 PAMs and the typical and atypical antipsychotics that are currently used in the clinic. While these findings suggest that mGluR5 PAMs have the potential to alleviate the positive symptoms of schizophrenia, the major unmet medical need facing schizophrenic patients is effective treatments for negative and cognitive symptoms. It is therefore worth discussing the important ways in which mGluR5 PAMs could also diverge from currently used antipsychotics to provide additional benefits related to the other symptom clusters.

Although few studies have addressed the potential reversal of negative symptoms by mGluR5 PAMs, one study found that the mGluR5 PAM CDPBP reversed MK-801-induced deficits in sucrose preference (Vardigan et al., 2010), suggesting that mGluR5 PAMs could have some ability to relieve anhedonia. Much more effort has been focused on assessing mGluR5 PAMs in models of cognition, and many of these studies

have produced promising results. Several studies have demonstrated enhanced cognition in normal animals following mGluR5 PAM administration (Ayala et al., 2009), and mGluR5 PAMs are also able to reverse cognitive impairments in multiple models of schizophrenia-like deficits, including learning deficits observed in the neurodevelopmental E17 MAM model of schizophrenia (Gastambide et al., 2012), NMDA receptor antagonist-induced deficits in reversal learning and instrumental responding (Gastambide et al., 2013), MK-801 induced deficits in a set-shifting task (Stefani and Moghaddam, 2010), and several other cognitive tasks such as novel object recognition and social novelty discrimination (reviewed in Vinson and Conn, 2012). Taken together, these important findings indicate that mGluR5 PAMs have the potential to address the disabling cognitive deficits in schizophrenia that are not improved by any currently available treatments, and provide an important point of differentiation between mGluR5 PAMs and typical or atypical antipsychotics.

### **Potential physiological roles for mGluR5-mediated inhibition of GSK-3 $\beta$**

The preliminary results presented in Chapter V suggest that inhibition of GSK-3 $\beta$  could play role in mGluR5-induced potentiation of NMDA currents, at least in MSNs. One obvious question stemming from this question is whether or not mGluR5-induced potentiation in other brain regions could also be mediated by GSK-3 $\beta$  inhibition. For example, mGluR5 potentiates NMDA currents in CA1 pyramidal cells of the hippocampus (Mannaioni et al., 2001) and excitatory projection neurons of the subthalamic nucleus (Awad et al., 2000), so it would be interesting to further define the signaling pathways involved in these effects. In addition, mGluR1 has also been shown to potentiate NMDA currents in various cell populations, and in the hippocampus mGluR1-induced NMDA receptor potentiation has been shown to be G protein-independent (Benquet et al., 2002), so evaluation of the ability of mGluR1 to influence

this signaling pathway and modulate NMDA receptors might also produce interesting results. Interestingly, despite the fact that mGluR1 and mGluR5 are both categorized as  $G\alpha_q$ -coupled receptors, their effects on neurophysiology in various populations of neurons can be surprisingly divergent, so it would be equally interesting, and not entirely unexpected, to find that mGluR1 does not modulate the Akt/GSK-3 $\beta$  pathway.

Another interesting direction would be to evaluate the potential role of GSK-3 $\beta$  in corticostriatal plasticity. Antagonists of mGluR5 block an endocannabinoid-dependent form of LTD at corticostriatal synapses (Sung et al., 2001), raising the possibility that mGluR5-mediated modulation of Akt or GSK-3 $\beta$  could contribute to this form of plasticity. However, potential contributions of Akt or GSK-3 $\beta$  signaling to this type of plasticity have not been elucidated. Interestingly, chronic dosing of two drugs that have been shown to inhibit GSK-3 $\beta$  in the striatum, lithium and haloperidol, both abolish LTD induced by high frequency stimulation at corticostriatal synapses (Calabresi et al., 1994; Centonze et al., 2004). While this intriguing phenomenon has not been shown to be mediated by chronic GSK-3 $\beta$  inhibition, the fact that both of these drugs converge on GSK-3 $\beta$  inhibition and are not known to have other overlapping effects suggests that GSK-3 $\beta$  is a candidate for mediating this effect. It would therefore be interesting to determine the ability of chronic administration of mGluR5 PAMs to alter corticostriatal LTD as well, and to evaluate the importance of chronic GSK-3 $\beta$  inhibition to this effect.

### **Beyond schizophrenia treatment: alternate therapeutic indications for mGluR5 PAM-mediated inhibition of GSK-3 $\beta$**

Inhibition of GSK-3 $\beta$  has been implicated as a therapeutic strategy for multiple CNS disorders other than schizophrenia, suggesting that the ability of mGluR5 PAMs to inhibit GSK-3 $\beta$  may open the door to additional therapeutic indications for mGluR5 PAMs. For example, GSK-3 inhibition is of major interest for the treatment of Alzheimer's

disease (AD), a common and devastating neurodegenerative disorder characterized by progressive dementia. The pathology associated with AD includes significant atrophy of various brain regions, the presence of amyloid plaques, and neurofibrillary tangles containing hyperphosphorylated tau protein. GSK-3 $\beta$  has been identified as the kinase responsible for tau hyperphosphorylation, and this activity is enhanced by amyloid peptides, suggesting that GSK-3 $\beta$  activity is an important link between amyloid and tau-related neuropathology in AD. GSK-3 expression and activity-enhancing Tyr216 phosphorylation of GSK-3 $\beta$  is increased in postmortem cortex samples from AD patients (Kremer et al., 2011), suggesting that enhanced GSK-3 $\beta$  activity could exacerbate the tau-related pathology observed in AD. In addition to potential contributions to AD-associated neurodegeneration, GSK-3 $\beta$  has been shown to play interesting roles in synaptic plasticity, an important physiological correlate of learning and memory. Under normal conditions, GSK-3 inhibition has no effect on NMDA receptor-dependent LTP of excitatory transmission at the Schaffer collateral-CA1 (SC-CA1) synapse in the hippocampus. However, when GSK-3 $\beta$  is excessively active or overexpressed, as is thought to be the case in AD, it prevents LTP induction (Hooper et al., 2007; Zhu et al., 2007). Application of A $\beta$ <sub>1-42</sub> also impairs LTP induction at the SC-CA1 synapse, an effect that is rescued by GSK-3 $\beta$  inhibition (Jo et al., 2011). Interestingly, chronic lithium treatment enhances the magnitude of LTP at the SC-CA1 synapse, perhaps by inhibiting GSK-3 $\beta$  activity (Shim et al., 2012). Behavioral studies in mice overexpressing GSK-3 $\beta$  have demonstrated that increased GSK-3 $\beta$  activity impairs cognition in a variety of tasks, including the Morris water maze (Engel et al., 2006; Hernandez et al., 2002). Taken together, all of these findings point to GSK-3 $\beta$  inhibition as a potential therapeutic strategy for treating AD-related pathology as well as aspects of cognitive impairment associated with AD.



Recently, the thiazolidindione (TDZD) family GSK-3 $\beta$  inhibitor tideglusib entered phase IIb clinical trials for AD and a rare tau-related disorder called progressive supranuclear palsy (PSP) (Martinez et al., 2011). Despite promising results from previous smaller trials, effects of tideglusib on AD-associated cognitive decline were disappointing. However, tideglusib did effectively slow neurodegeneration in PSP, suggesting that GSK-3 $\beta$  inhibition is capable of modifying tau-related pathology in the human brain. It is possible that direct inhibition of GSK-3 $\beta$  will not be a good therapeutic strategy for treatment of AD symptoms due to side effects associated with ubiquitous expression of the enzyme and its important roles in many cellular functions. Moreover, its broad expression may lead to effects that counteract beneficial actions. However, a treatment that could indirectly inhibit GSK-3 $\beta$  in specific cell populations relevant to the pathophysiology of AD, including certain cortical regions and the hippocampus, may have the brightest prospects as a therapeutic strategy. Intriguingly, mGluR5 activation may provide a targeted inhibition of GSK-3 $\beta$  that could slow disease progression and improve symptoms without producing the adverse effects likely to be seen with global inhibition of GSK-3 $\beta$ .

As discussed in Chapter I, mGluR5 PAMs have been demonstrated to enhance cognition in a variety of animal models, and can reverse deficits in Morris water maze performance in aged rats (Jerri Rook, unpublished data). mGluR5 PAMs also enhance subthreshold levels of LTP at the SC-CA1 synapse (Ayala et al., 2009) and reverse LTP deficits in aged rats (Jerri Rook and Adam Walker, unpublished data). All of these findings suggest that mGluR5 PAMs could have significant potential for enhancing cognition, which could provide symptom relief in disorders such as AD. In addition, the antipsychotic-like effects of mGluR5 PAMs suggest that they could benefit AD patients suffering from associated psychiatric disturbances. Treatment with mGluR5 PAMs therefore has the potential to improve a variety of symptoms associated with AD, and

given the overlap between the behavioral effects of GSK-3 inhibitors and mGluR5 PAMs, some of these predicted effects could be associated with the ability of mGluR5 PAMs to inhibit GSK-3 $\beta$ .

Recently, GSK-3 inhibitors have also received attention as potential therapeutic agents for the treatment of bipolar disorder, which is a psychiatric disorder characterized by alternating periods of mania and depression. Lithium, a drug commonly used to reduce the manic symptoms of bipolar disorder (hyperactivity, increased hedonistic drive, pathologically elevated mood, and behavioral disinhibition), has been shown to inhibit GSK-3 through both direct and indirect mechanisms (reviewed in Beaulieu et al., 2009). Like lithium, a number of more selective GSK-3 inhibitors from multiple structural classes reduce amphetamine-induced hyperlocomotion in Swiss Black mice, a proposed animal model of bipolar disorder (Kalinichev and Dawson, 2011). Interestingly, commonly used antidepressants, including selective serotonin reuptake inhibitors (SSRIs) such as fluoxetine, increase inhibitory phosphorylation of GSK-3, and GSK-3 inhibition is critical for the antidepressant-like effects of both fluoxetine and ketamine in the forced swim test (Beurel et al., 2011; Polter et al., 2012), suggesting that GSK-3 inhibition may be an important common mechanism of diverse drugs with antidepressant properties. The ability of direct or indirect GSK-3 inhibitors to produce behavioral effects consistent with both antimanic and antidepressant effects suggests that targeting GSK-3 could be a good strategy for developing the next generation of drugs to treat bipolar disorder. While mGluR5 PAMs have not been shown to have antidepressant-like effects, their ability to reverse amphetamine-induced behaviors and mimic the biochemical effects of lithium in the striatum suggest that evaluation of mGluR5 PAMs for treatment of manic symptoms associated with bipolar disorder could be a worthwhile endeavor.

## Conclusion

Metabotropic glutamate receptors have received significant attention in recent years as therapeutic targets for a remarkable variety of neurological and psychiatric disorders. Given their differential distributions and physiological roles, activation or inhibition of each mGluR subtype has a unique array of physiological consequences and potential therapeutic indications. While the use of pharmacological agents and artificial manipulations of dopaminergic neurotransmission have provided insights into the roles of these receptors and their interactions with dopamine, our knowledge of the effects of mGluR activation by endogenous glutamate remains limited. In addition, currently available animal models of PD have yet to adequately recapitulate the human disease, placing another boundary on our ability to fully understand the changes in mGluR function caused by SNc degeneration. As our understanding of the cellular events leading to nigrostriatal degeneration advances, the ability of animal models to predict neuroprotective effects of mGluR ligands may improve. Furthermore, while many distinct roles of mGluR1 and mGluR5 have been determined, the previous lack of subtype-selective pharmacological tools that distinguish between group II and group III mGluR subtypes has restricted our ability to fully understand the roles of individual receptor subtypes. Therefore, much work remains to be done in order to fully elucidate the complex interactions of mGluRs and dopamine in the basal ganglia, and the most promising therapeutic strategies for treating basal ganglia-related disorders.

The studies described in this dissertation have all been directed at the common goal of increasing our understanding of the roles of individual mGluR subtypes in normal brain function and how we might take advantage of this knowledge to develop improved treatments for devastating CNS disorders such as Parkinson's disease and schizophrenia. Ongoing drug discovery efforts in our group have led to the development of mGluR4 and mGluR5 PAMs that are expected to enter clinical testing in the near

future. While an abundance of preclinical evidence suggests that targeting mGluRs could produce new therapeutic options for several CNS disorders that cannot be adequately treated with currently available medications, the most important and exciting results will ultimately arise from clinical testing of these novel drugs.

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