ALLOSTERIC MODULATION OF METABOTROPIC GLUTAMATE RECEPTORS

By

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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>7TMR/GPCR</td>
<td>Seven Transmembrane Spanning/G Protein Coupled Receptor</td>
</tr>
<tr>
<td>ADX88178</td>
<td>5-methyl-N-(4-methylpyrimidin-2-yl)-4-(1H-pyrazol-4-yl)thiazol-2-amine</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>AMN082</td>
<td>N1,N2-dibenzhydrelthene-1,2-diamine</td>
</tr>
<tr>
<td>ASK</td>
<td>apoptosis signal-regulating kinase</td>
</tr>
<tr>
<td>BINA</td>
<td>biphenyl-2-indanone A</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBiPES</td>
<td>N-[4'-cyano-biphenyl-3-yl]-N-(3-pyridinylmethyl)-ethanesulfonamide hydrochloride</td>
</tr>
<tr>
<td>CDPPB</td>
<td>3-cyano-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CPCCOEt</td>
<td>7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxylate ethyl ester</td>
</tr>
<tr>
<td>CRD</td>
<td>cysteine-rich domain</td>
</tr>
<tr>
<td>CPPHA</td>
<td>N-[4-chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isindol-2-yl)methyl]phenyl]-2-hydroxybenzamide</td>
</tr>
<tr>
<td><strong>DCG-IV</strong></td>
<td>(2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)glycine</td>
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<td>---------------------------------------------------</td>
</tr>
<tr>
<td><strong>DFB</strong></td>
<td>[(3-fluorophenyl)methylene]hydrazone-3-fluorobenzaldehyde</td>
</tr>
<tr>
<td><strong>ERK</strong></td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td><strong>GABA</strong></td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td><strong>GIRK</strong></td>
<td>G protein coupled inwardly rectifying potassium channel</td>
</tr>
<tr>
<td><strong>JNK</strong></td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td><strong>L-AP4</strong></td>
<td>L-(+)-2-Amino-4-phosphonobutyric acid</td>
</tr>
<tr>
<td><strong>L-DOPA</strong></td>
<td>L-3,4-dihydroxyphenylalanine (levodopa)</td>
</tr>
<tr>
<td><strong>LSP1-2111</strong></td>
<td>((2S)-2-amino-4-[hydroxy[hydroxy(4-hydroxy-3-methoxy-5-nitrophenyl)methyl] phosphoryl]butanoic acid)</td>
</tr>
<tr>
<td><strong>LSP4-2022</strong></td>
<td>(2S)-2-amino-4-(((4-carboxymethoxy)phenyl)(hydroxy)methyl)(hydroxy)phosphoryl)butanoic acid</td>
</tr>
<tr>
<td><strong>Lu AF21934</strong></td>
<td>(1S,2S)-N1-(3,4-dichlorophenyl)cyclohexane-1,2-dicarboxamide</td>
</tr>
<tr>
<td><strong>LY379268</strong></td>
<td>(1R,4R,5S,6R)-4-Amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid</td>
</tr>
<tr>
<td><strong>LY487379</strong></td>
<td>2,2,2-trifluoro-N-[4-(2-methoxyphenoxo) phenyl]-N-(3-pyridinylmethyl)ethanesulfonamide</td>
</tr>
<tr>
<td><strong>MAPK</strong></td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>-------------</td>
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</tr>
<tr>
<td>mGlu</td>
<td>metabotropic glutamate receptor</td>
</tr>
<tr>
<td>MMPIP</td>
<td>6-(4-methoxyphenyl)-5-methyl-3-(4-pyridinyl)-isoxazolo [4,5-c]pyridine-4(5H)-one hydrochloride</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>NAM</td>
<td>negative allosteric modulator</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>PAM</td>
<td>positive allosteric modulator</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PHCCC</td>
<td>N-phenyl-7-(hydroxylimino)cyclopropa[b]chromen-1a-carboxamide</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIKE-L</td>
<td>phosphatidylinositol 3-kinase enhancer-long</td>
</tr>
<tr>
<td>SAM</td>
<td>silent allosteric modulator</td>
</tr>
<tr>
<td>VFD</td>
<td>Venus flytrap domain</td>
</tr>
<tr>
<td>VU0155041</td>
<td>(±)-cis-2-(3,5-Dichlorophenylcarbamoyl)cyclohexanecarboxylic acid</td>
</tr>
<tr>
<td>VU0364770</td>
<td>N-(3-chlorophenyl)picolinamide</td>
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CHAPTER 1

GENERAL INTRODUCTION

Metabotropic glutamate receptors

Glutamate is not only one of the 22 proteinogenic amino acids, but also the major excitatory neurotransmitter in the central nervous system (CNS). The glutamate receptors can be divided into two classes: the ionotropic glutamate receptors and the metabotropic glutamate receptors. While the ionotropic glutamate receptors (AMPA receptors, NMDA receptors and kainate receptors) mediate fast responses elicited by glutamate, the metabotropic glutamate (mGlu) receptors provide a mechanism by which glutamate can transduce environmental cues and modulate synaptic transmission via second messenger signaling pathways with slower time course. Because of their widespread distribution especially in the CNS, pharmacological manipulation of mGlus may represent ideal therapeutic interventions for a wide range of neurological and psychiatric disorders (Reviewed in (Gregory et al., 2013; Niswender and Conn, 2010)).

Classification of mGlus

The Seven Transmembrane Spanning/G Protein Coupled Receptors (7TMR/GPCR) account for 4% of the entire protein-coding genome (Bjarnadottir et al., 2006) and represent the targets of approximately 40-50% of medicinal drugs on the market (Thomsen et al., 2005). The core function of GPCRs is to serve as a transducer of signals from the extracellular environment to the intracellular machinery that aggregates to govern organismal responses. The GPCR superfamily can be classified into several classes: the Class A GPCRs (or Rhodopsin-like receptors) account for almost 85% of the GPCR genes, the Class B GPCRs (or secretin-like receptors) include 15 receptors and are regulated by peptide hormones, and the Class C GPCRs, which are characterized by a
large extracellular N-terminal domain and contain 22 distinct receptors. The Adhesion, Frizzled, Taste type-2 and other unclassified receptors comprise the rest of the superfamily (Bjarnadottir et al., 2006).

The mGlus belong to the Family C GPCRs, which also encompasses calcium sensing receptors, the GABA_B receptor, taste receptors and other orphan Class C receptors. Since the cloning of rat mGlu_1 in 1991, 8 mGlu subtypes have been cloned thus far, named mGlu_1 through mGlu_8. Within the family, the eight mGlu subtypes can be further classified into three groups (Table 1.1), with an intragroup sequence homology of about 70% and an intergroup sequence homology of about 45% (reviewed in (Conn and Pin, 1997)). The group I mGlus include mGlu_1 and mGlu_5, group II includes mGlu_2 and mGlu_3, whereas mGlu_4, 6, 7 and 8 comprise the group III mGlus. Such classification is further confirmed by the G-protein coupling and pharmacological profile of the three groups. While the group I mGluRs are coupled to G_q, the group II and group III are coupled to G_i/o G proteins.

In addition to the G protein-coupling specificity, the three groups of mGlus also differ in their expression patterns. With the exception of mGlu_6 which is expressed exclusively in ON-bipolar cells in the retina, all mGlus are widely expressed in many locations within the brain. Group I mGlus are primarily expressed postsynaptically to modulate neuronal excitability, whereas group III mGlus are typically located presynaptically. Both pre- and postsynaptic expression has been detected for group II mGlus. In addition, mGlu_3 and mGlu_5 are also expressed in astrocytes. Expression of each mGlu subtype has been mapped using immunohistochemistry. Specifically, high mGlu_1 expression was detected in the olfactory bulb, CA1 hippocampus, globus pallidus, thalamus and cerebellum (Petralia et al., 1997). Expression of mGlu_5 was found in the olfactory bulb, olfactory tubercle, cerebral cortex, hippocampus, striatum and nucleus accumbens (Shigemoto et al., 1993). For mGlu_2 and mGlu_3, significant staining was found in the cerebral cortex, hippocampus, thalamus and caudate-putamen (Petralia et al., 1996). mGlu_4 is highly enrich in the molecular layer of cerebellum, but is also expressed in globus pallidus substantia nigra and moderately in neocortex, hippocampus, striatum and thalamus.
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<th>Splice Variants</th>
<th>Selective Ligands</th>
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<tr>
<td>Group I</td>
<td>G&lt;sub&gt;q/11&lt;/sub&gt;</td>
<td>mGlu&lt;sub&gt;1&lt;/sub&gt;</td>
<td>mGlu&lt;sub&gt;1a-b&lt;/sub&gt;, mGlu&lt;sub&gt;1g393&lt;/sub&gt;, mGlu&lt;sub&gt;1g620&lt;/sub&gt;, Taste mGlu&lt;sub&gt;1&lt;/sub&gt;</td>
<td>LY367385 (orthosteric agonist) Bay 36–7620 (NAM) Ro 67–7476 (PAM) Ro 67–4853 (PAM) VU71 (PAM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mGlu&lt;sub&gt;5&lt;/sub&gt;</td>
<td>mGlu&lt;sub&gt;5a,b&lt;/sub&gt;</td>
<td>CHPG (orthosteric agonist) MPEP (NAM) MTEP (NAM) CDPPB (PAM) CPPHA (PAM) VU0365396 (SAM)</td>
</tr>
<tr>
<td>Group II</td>
<td>G&lt;sub&gt;i/o&lt;/sub&gt;</td>
<td>mGlu&lt;sub&gt;2&lt;/sub&gt;</td>
<td>mGlu&lt;sub&gt;2&lt;/sub&gt;</td>
<td>LY487379 (PAM) BINA (PAM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mGlu&lt;sub&gt;3&lt;/sub&gt;</td>
<td>GRM3Δ2 GRM3Δ4 GRM3Δ2Δ3</td>
<td>ML337 (NAM)</td>
</tr>
<tr>
<td>Group III</td>
<td>G&lt;sub&gt;i/o&lt;/sub&gt;</td>
<td>mGlu&lt;sub&gt;4&lt;/sub&gt;</td>
<td>mGlu&lt;sub&gt;4a,b&lt;/sub&gt;, Taste mGlu&lt;sub&gt;4&lt;/sub&gt;</td>
<td>LSP1-2111 (orthosteric agonist) LSP4-2022 (orthosteric agonist) PHCCC (PAM) VU0155041(PAM) VU0364770 (PAM) ADX88178 (PAM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mGlu&lt;sub&gt;6&lt;/sub&gt;</td>
<td>mGlu&lt;sub&gt;6a-c&lt;/sub&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mGlu&lt;sub&gt;7&lt;/sub&gt;</td>
<td>mGlu&lt;sub&gt;7a-c&lt;/sub&gt;</td>
<td>AMN 082 (allosteric agonist) MMPiP (NAM) ADX71743 (NAM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mGlu&lt;sub&gt;8&lt;/sub&gt;</td>
<td>mGlu&lt;sub&gt;8a-c&lt;/sub&gt;</td>
<td>(S)-3,4-DCPG (orthosteric agonist) AZ12216052 (PAM)</td>
</tr>
</tbody>
</table>

Table 1.1. Classification, G protein coupling, splice variants and selective ligands for mGlu subtypes.
mGlu\textsubscript{7} is abundantly expressed in striatum, globus pallidus, and substantia nigra pars reticulata (Kosinski et al., 1999), whereas mGlu\textsubscript{8} is expressed primarily in hippocampus and piriform cortex (Ferraguti et al., 2005; Kinoshita et al., 1996). mGlu receptors also expressed in peripheral tissues and play important roles in pathological conditions including congenital stationary night blindness (Mathiesen et al., 2005; Zeitz et al., 2007) and malignant transformation, such as melanoma (Choi et al., 2011; Pollock et al., 2003).

Different mGlu subtypes have been suggested to colocalize at various synapses. Previous immunohistochemistry and in situ hybridization studies suggest that mGlu\textsubscript{2} and mGlu\textsubscript{4} are co-localized in several brain regions (Bradley et al., 1999; Neki et al., 1996; Ohishi et al., 1995; Ohishi et al., 1993) and mGlu\textsubscript{2} is also functionally expressed at corticostriatal synapses (Johnson et al., 2005).

All mGlu subtypes have been discovered to undergo alternative splicing, primarily at the C-terminus, and some of the better characterized splice variants are described below. In humans, 8 different splice variants of mGlu1 exist, named mGlu\textsubscript{1a}, 1b, 1c, 1d, 1e, 1g, 1g\textsubscript{-393}, 1g\textsubscript{-620} and 1h. Two newly identified exons in human \textit{GRM1} express a novel splice variant of metabotropic glutamate 1 receptor. mGlu\textsubscript{1a} is the longest variant and the others result from differential splice site usage, generating distinct isoforms with differing C-termini (reviewed in (Hermans and Challiss, 2001)). The splice variant containing only the VFD has been shown to act as a dominant negative, preventing full length mGlu\textsubscript{1} isoforms from signaling (Beqollari and Kammermeier, 2010). Also within the group I mGlus, mGlu\textsubscript{5a} and mGlu\textsubscript{5b} are two splice variants for mGlu\textsubscript{5} with similar pharmacological profiles (Joly et al., 1995; Minakami et al., 1994). Three splice variants of mGlu\textsubscript{3} exist in human brain due to exon skipping events: GRM3\Delta2 (lacking exon 2), GRM3\Delta4 (lacking exon 4), and GRM3\Delta2\Delta3 (lacking exons 2 and 3). Among the three variants, GRM3\Delta4 is most abundantly expressed and represents an mGlu\textsubscript{3} receptor without a seven-transmembrane domain, which may have unique functions and relate to non-coding single nucleotide polymorphisms (SNPs) in patients with cognitive dysfunction (Sartorius et al., 2006). As to the group III mGlus, the mGlu\textsubscript{4} gene was described as undergoing
alternative splicing to generate mGlu₄ₐ and mGlu₄ₐ (Thomsen et al., 1997); however, this result has not been replicated by other groups (Corti et al., 2002). Three mGlu₆ splice variants exist in human retina, with mGlu₆ₐ lacking 97 nucleotides from exon 6 and mGlu₆ₐ including 5 nucleotides from intron 5 (Valerio et al., 2001). Both mGlu₇ and mGlu₈ can undergo alternative splicing at the C-terminus, resulting in at least 5 splice variants for mGlu₇ and 2 variants for mGlu₈ (Corti et al., 1998; Schulz et al., 2002). In addition, another splice variant, mGlu₈ₐ, contains a 74 nucleotide insertion, resulting in a frame shift and termination of the polypeptide before the seven transmembrane domains (Malherbe et al., 1999). As the C-terminal intracellular domain plays important roles in protein-protein interactions and signal transduction, different splice variants may possess distinct profiles with regards to receptor activation, receptor modification and receptor internalization (Enz, 2012). With the advances in sequencing technology, the diversity of mGlu splice variants is increasing rapidly. For example, 7 splice variants have been predicted for mGlu₄ according to Ensembl genome database, although their existence still needs to be validated experimentally.

Several isoforms of mGlus also exist due to alterations at the N-terminus; for example, Taste mGlu₁ and Taste mGlu₄ (Chaudhari et al., 2000), which play roles in detecting the taste of umami. These receptor variants, with approximately 50% of the N-terminus truncated, are expressed in taste buds. Compared to full-length receptors, these N-truncated variants lack much of the glutamate binding domain and thus exhibit lower potency when activated by glutamate (Chaudhari et al., 2000).

The structure of mGlus

General structural features of mGlus

As members of the Family C 7TMRs, the mGlus are characterized by a large N-terminal domain, commonly referred to as the Venus Flytrap Domain (VFD). Studies of the crystal structures of the mGlu₁, ₃ and ₇ VFDs reveal that each VFD contains two lobes, which together form a clam shell-like structure, with the
glutamate binding site found between the two lobes (Acher and Bertrand, 2005; Kunishima et al., 2000; Muto et al., 2007; Tsuchiya et al., 2002). Besides glutamate, the VFDs of some mGlus also bind other endogenous agonists of mGlus, such as cinnabarinic acid (Fazio et al., 2012) and L-serine-O-phosphate (Klunk et al., 1991) (Hampson et al., 1999), as well as magnesium and calcium, which can modulate receptor activity (Francesconi and Duvoisin, 2004; Kubo et al., 1998; Kunishima et al., 2000). Both structural (Kunishima et al., 2000; Muto et al., 2007; Tsuchiya et al., 2002) and biochemical data (Romano et al., 1996) suggest that the VFDs from two distinct mGlu receptors sit back to back and dimerize together. Upon ligand binding, large conformational changes lead to closure of the two lobes. Closure of one or both VFDs within each mGlu dimer initiates receptor activation (Kniazeff et al., 2004).

Connecting the VFD and the seven transmembrane spanning domain (7TMD) is the cysteine-rich domain (CRD). Based on structural studies with mGlu3, the CRD contains 9 cysteine residues; 8 of them form internal disulfide bonds to stabilize the structure of this domain. In addition, the ninth cysteine forms a disulfide bond linked to the VFD (Muto et al., 2007), such that the CRD senses the conformational changes induced by ligand binding and transmits it to the 7TMD. The 7TMD and intracellular loops play important roles in receptor-G protein coupling and receptor modulation. It has been shown that a single mutation in the 7TMD that disrupts the hydrogen-bonding network in TM6 and TM7 induces high constitutive activity of mGlu8 (Yanagawa et al., 2013), suggesting that TM6 and TM7 constrain the receptor in an inactive conformation and that rearrangement between these two helixes is critical for receptor activation. The 7TMD also provides an opportunity to modulate receptor activity at a site other than the traditional agonist-binding VFD. All mGlu small molecule allosteric modulators discovered to date are believed to bind to receptor 7TMDs. Classification, action and therapeutic implications of such allosteric modulators will be discussed in the following sections. The intracellular loops of mGlus are involved in G protein coupling and receptor phosphorylation. Specifically, the second intracellular loop is implicated in G protein coupling
specificity of the mGlus (Gomeza et al., 1996; Havlickova et al., 2003; Pin et al., 1994), which is usually the function of the third intracellular loop for Class A, rhodopsin-like receptors.

The C-terminal domain of the mGlus is within close proximity to the inner leaflet of the lipid bilayer. Although recent structural studies of the purified intracellular C-terminal domains from mGlu₆, 7 and 8 suggest that the C-termini of unliganded mGlus are mediated by linear motifs rather than secondary/tertiary structures (Seebahn et al., 2011), these intracellular tails of mGlus interact with various intracellular proteins, and are subject to alternative splicing (see section 3.1 and 3.2.2), phosphorylation, and SUMOylation (reviewed in (Enz, 2012)).

**The dimeric complex of mGlus**

As mentioned above, mGlus form stable, covalently linked dimers. Data suggesting constitutive formation of mGlu homodimers emerged as early as 1996 (Romano et al., 1996), and was further supported by data obtained from mGlu VFD/CRD crystal structures (Kunishima et al., 2000). Evidence from biochemical studies reveals that that one or more cysteine residues on the N-terminal extracellular domain mediate the covalent and non-covalent interactions between two mGlu protomers (Ray and Hauschild, 2000; Romano et al., 2001). In addition, several studies have shown that mGlus do not appear to form higher-order oligomers (Brock et al., 2007; Doumazane et al., 2011b), although this is the case for some other Class C 7TMRs, such as the GABA_B receptor (Comps-Agrar et al., 2012).

The activation machinery of mGlu homodimers has been studied in depth, particularly by Jean Philippe Pin’s group, using a quality control system adapted from the GABA_B receptor to generate mGlu dimers bearing specific mutations within one of the protomers. These data suggest that closure of one VFD per dimer is sufficient to activate the receptor, although closure of both VFDs is required to achieve full activity (Kniazeff et al., 2004). When one or both VFDs are occupied,
the 7TM domain of either protomer can be activated through intersubunit rearrangement (Brock et al., 2007). These findings are consistent with the hypothesis that only a single 7TM domain is turned on upon activation of each homodimeric receptor (Goudet et al., 2005; Hlavackova et al., 2005; Hlavackova et al., 2012).

Besides forming homodimers, the VFD of mGlu₁ can heterodimerize with full length mGlu₅ and vice versa (Beqollari and Kammermeier, 2010). In addition, a splice variant of mGlu₁ that contains only the VFD functions as a dominant negative to potently block the signaling of full length mGlu₁ or mGlu₅ (Beqollari and Kammermeier, 2010). Unfortunately, there is a paucity of evidence demonstrating the extent to which heterodimers exist in vivo. The discovery of heteromers in native systems would dramatically shift our understanding of the functional roles of this important family of 7TMRs and would suggest that mGlus exhibit greater diversity in signaling and function than has previously been appreciated. In addition, mGlu receptor heteromerization could also explain discordant pharmacological findings observed in native brain tissue (Ayala et al., 2008; Niswender et al., 2010).

Evidence for full length mGlu heterodimers initially emerged in in vitro expression systems (Doumazane et al., 2011a; Kammermeier, 2012). By using a time-resolved FRET assay, Doumazane et al. demonstrated that group I mGlus can interact with each other, but do not associate with group II and group III mGlu subtypes; in contrast, group II and III mGlu receptors can co-assemble within and outside of the two groups. In addition, Kammermeier’s study utilizing injected superior cervical ganglion cells suggests that heterodimerization may alter the pharmacology of mGlus and their modulators. Together, these findings indicate that the functions and signaling of mGlus could be much more diverse and complex than previously estimated, although the existence and pharmacology of mGlu heterodimers still needs to be established using native tissue. However, examples already exist for heterodimers of mGlus and Class A 7TMRs in the CNS. Gonzalez-Maeso et al. reported that mGlu₂ receptors interact with 5-HT₂A receptors through transmembrane helix domains and form functional complexes in brain cortex (Gonzalez-Maeso et al.,
2008). Subsequent mutagenesis studies revealed that three residues within transmembrane domain 4 of mGlu2 are necessary to form the 5-HT2A-mGlu2 receptor heterocomplex (Moreno et al., 2012). Furthermore, hallucinogenic 5-HT2A agonists elicit unique responses at 5-HT2A/mGlu2 complexes, which is implicated in the pathogenesis of psychosis (Gonzalez-Maeso et al., 2008). However, it should be noted that, although the formation of mGlu2/5HT2A heterocomplexes has been validated by other groups, (Delille et al., 2012) the unique signal transduction pathways mediated by the heterodimeric complex was not replicated. In addition, it was also shown that mGlu2 can interact with 5-HT2B, indicating that complex formation is not specific to the 5-HT2A-mGlu2 pair and challenging the biological relevance of the 5-HT2A-mGlu2 complex.

**Signaling of mGlus**

As shown in Figure 1.1, mGlu receptors signal to intracellular machinery through both G protein-dependent and independent pathways.

**G protein-dependent signaling**

Classically, group I mGlus are generally coupled to Gq/11 and activate phospholipase Cβ, which hydrolyses phosphotinositides into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol, a pathway leading to calcium mobilization and activation of protein kinase C (PKC). Other effectors downstream of Gq include phospholipase D, ion channels, c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase/extracellular receptor kinase (MAPK/ERK), and the mammalian target of rapamycin (mTOR) pathway (Li et al., 2007; Page et al., 2006; Sayer, 1998; Servitja et al., 1999) (Figure 1.1). In addition, evidence has emerged that group I mGlus can also activate Gs and Gi/o and their downstream pathways, and that distinct regions on the receptor are responsible for coupling of different G proteins (Francesconi and Duvoisin, 1998; McCool et al., 1998).
Figure 1.1. Signaling pathways of mGlu receptors. The group I, II and III mGlu receptors induce signal transduction through both G protein-dependent and independent pathways, while the signaling profile of heterodimeric mGlu receptor remains unexplored. GKAP, guanylate kinase-associated protein; PSD-95, postsynaptic density protein 95; NMDAR, N-methyl-D-aspartate receptor; PI3K, phosphatidylinositol 3-kinase; PIKE-L, phosphatidylinositol 3-kinase enhancer-long; mTOR, mammalian target of rapamycin; ERK, extracellular signal-regulated kinase; ASK, apoptosis signal-regulating kinase; JNK, c-Jun N-terminal kinases; PIP2, Phosphatidylinositol 4,5-bisphosphate; DAG, diacyl-glycerol; IP3, inositol 1,4,5-trisphosphate; ER, endoplasmic reticulum; PKC, protein kinase C; AC, adenylate cyclase; PKA, protein kinase A.
As stated previously, the group II and group III mGlu receptors are coupled to $G_{i/o}$ proteins and negatively regulate the activity of adenylyl cyclase. In addition, many ion channels have also been reported to be regulated by $G_{ai}$ and the liberated $G_{ip}$ subunit (Guo and Ikeda, 2005; Kammermeier, 2012; Niswender et al., 2008a). $G_{i/o}$-mediated activation of MAPK and phosphatidylinositol 3-kinase (PI3 kinase) pathways, as supported by the inhibitory effect of pertussis toxin (Iacovelli et al., 2002), add another level of complexity to the G protein-mediated signaling of group II and III mGlus.

**G protein-independent signaling**

While 7TMRs transduce signals through various cellular pathways, their responsiveness may also be regulated by receptor desensitization. When a receptor is stimulated, activated G protein-coupled receptor kinase (GRK) then initiates a combination of events including receptor phosphorylation, arrestin binding, and receptor internalization (reviewed in (Krupnick and Benovic, 1998)), providing a feedback mechanism that prevents receptor over-stimulation. For mGlus, such regulation is more thoroughly studied for group I mGlus than the other two groups. For example, internalization of mGlu$_1$ has been shown to depend on GRK4 and β-arrestin-1 (Dale et al., 2001; Iacovelli et al., 2003). Desensitization of mGlu$_5$, however, seems to be dependent on GRK2 activity, suggesting different mGlu subtypes are regulated by distinct mechanisms (Sorensen and Conn, 2003).

Besides regulating receptor desensitization, recruited β-arrestins are also well-known as scaffolding protein for signaling molecules. Reports have shown that active Src is recruited to activated 7TMRs by interaction with β-arrestin, which then results in phosphorylation of downstream molecules and consequently activation of the MAPK cascade (Luttrell et al., 1999). In addition, arrestins also directly facilitate the subcellular localization and activation of two MAPK cascades (the RAF→MEK→extracellular signal-regulated kinases (RAF-MEK-ERK) cascade and
the apoptosis signal-regulating kinase → MKK → c-Jun N-terminal kinases (ASK-MKK-JNK cascade) (Pierce and Lefkowitz, 2001), further expanding the dimension and complexity of signal transduction upon GPCR activation. This role of arrestins in mediating signal transduction events has been demonstrated for mGlus as well. For example, activation of mGlu$_7$ significantly reduces N-methyl D-aspartate receptor (NMDAR)-mediated currents in prefrontal cortex pyramidal neurons in a β-arrestin/ERK signaling pathway-dependent manner (Gu et al., 2012).

In addition, mGlu receptors also demonstrate the ability to activate signaling cascades through protein-protein interactions. For instance, the C-terminal domains of mGlu$_1$ and mGlu$_5$ interact with Homer proteins, a group of scaffolding proteins for multiprotein complexes. Besides interacting with the receptor, Homer proteins demonstrate binding ability with inositol-1,4,5-triphosphate (IP3) receptors, ryanodine receptors, transient receptor channel-1 and 4 (TRPC1, TRPC4), P/Q-type Ca$^{2+}$ channels, Shank, the phosphoinositide 3-kinase (PI3K) enhancer-long (PIKE-L) etc., coupling receptor activation to other signaling components within the cell (Bockaert et al., 2004; Fagni et al., 2004; Rong et al., 2003). mGlu$_5$ has been found to interact with the NMDA receptor via Homer and other scaffolding proteins and potentiate receptor activity (Attucci et al., 2001; Pisani et al., 2001; Tu et al., 1999). In addition, it has been shown that disruption of mGlu$_5$–Homer interactions selectively blocks mGlu activation of the PI3K-Akt-mTOR pathway (Ronesi and Huber, 2008) and contribute to phenotypes of Fmr1 knockout mice, an animal model for Fragile X syndrome (Ronesi et al., 2012). Interestingly, Homer proteins include long Homer isoforms and short isoforms (Homer1a and Ania), which act as endogenous dominant-negatives and disrupt protein complexes containing the long Homer variant. The ratio of Homer 1a/long Homer bound to mGlu$_5$ may associate with cognitive aging (Menard and Quirion, 2012) and has been shown to be altered in Fmr1 knockout mice (Ronesi et al., 2012). In addition, genetic deletion of Homer1a rescues several phenotypes in Fmr1 knockout mice, suggesting the importance of Homer proteins in the mechanism of Fragile X syndrome and potential therapeutic intervention for this disease (Ronesi et al., 2012). Besides the
PI3K-Akt-mTOR pathway, Homer also links mGlu₅ to PIKE-L, which prevents cell apoptosis upon mGlu₅ activation (Rong et al., 2003). Interestingly, it has been shown that the disruption of Homer-mGlu₅ interaction reduces astrocyte apoptosis (Paquet et al., 2013), suggesting opposite functions of Homer in regulation of cell apoptosis in neurons and astrocytes. Besides the CNS, a point mutation of mGlu₁ within the Homer binding region that has been discovered in the somatic cells of lung cancer patients (Esseltine et al., 2013), indicating important roles of Homer in the periphery.

Another well-studied protein-protein interaction is the mGlu₇-protein interacting with C kinase 1 (PICK1) interaction. PICK1 was discovered as a peripheral membrane protein that interacts with protein kinase Cα (PKCα) (Staudinger et al., 1995). Besides PICK1, the C-terminus of mGlu₇ also interacts with Ca²⁺-calmodulin, G protein βγ subunits to modulate the activity of voltage-gated Ca²⁺ channel and negatively regulate neurotransmitter release (Dev et al., 2001). Interestingly, phosphorylation of the receptor by PKC increases receptor binding to PICK1, which is required for stable surface expression of mGlu₇ (Suh et al., 2013; Suh et al., 2008), but, at the same time, inhibits the binding of Gβγ subunits and Ca²⁺-calmodulin, providing a delicate regulatory machinery. Disruption of the mGlu₇-PICK1 interaction has been performed by genetic knock-in of an mGlu₇ mutant that does not bind PICK1. The resulting animals exhibited significant defects in hippocampus-dependent spatial working memory and high susceptibility to convulsant drugs (Zhang et al., 2008). Additionally, injection of a competing peptide to rodents also resulted in behavioral symptoms and EEG discharges that are characteristic of absence epilepsy (Bertaso et al., 2008). These data indicate that the mGlu₇-PICK1 interaction is important for regulating mGlu₇ signaling and may underlie certain disease mechanisms, including cognitive disorders and epilepsy.
Allosteric modulation of mGlu

Therapeutic indications of mGlu$_4$

Pharmaceutical manipulation of mGlus represent ideal therapeutic interventions for a wide range of neurological and psychiatric, as well as peripheral disorders, but this section will primarily focus on the therapeutic indication of mGlu$_4$ for Parkinson’s disease (PD). PD is a debilitating neurodegenerative disorder characterized by movement symptoms including tremor, rigidity, bradykinesia and postural instability, as well as disturbances in sleep, depression and cognition (Jankovic, 2008; Johnson et al., 2009). The pathology of PD stems from severe degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNc), a brain structure that plays important roles in the basal ganglia to control motor function (Surmeier and Sulzer, 2013). Within the basal ganglia, dopamine released from SNc neurons delicately controls the balance of output between the “direct pathway” and the “indirect pathway”, which oppose each other in controlling motor output in the basal ganglia. In PD patients, however, the loss of dopaminergic neurons leads to an overall increase of activity in the indirect pathway, which ultimately inhibits motor function in PD patients (reviewed in (Johnson et al., 2009)). Thus, according to this model, a rebalancing of the basal ganglia circuitry is predicted to alleviate disease symptoms.

The current gold standard treatment for PD is dopamine replacement therapy using L-DOPA, the precursor of dopamine. However, long-term treatment with L-DOPA results in “wearing-off” of efficacy and development of side effects, such as dyskinesias and psychiatric complications (Chen and Swope, 2007). In addition, no treatment is available to delay the progression of the disease. The mGlu$_4$ receptor is highly expressed presynaptically at the first synapse in the indirect pathway (the GABAergic striatopallidal synapse) (Bradley et al., 1999) and receives glutamate input from subthalamic nucleus, providing an exciting alternative approach to rebalance the basal ganglia circuitry for PD treatment. Administration of the group III mGlu agonists L-AP4 or L-SOP, and recently
the more mGlu₄-selective agonists LSP1-2111 and LSP4-2022, has been shown to reduce GABAergic transmission at the striatopallidal synapse and demonstrate efficacy in several rodent PD models, including haloperidol-induced catalepsy and 6-OHDA-induced motor deficits (Beurrier et al., 2009; Goudet et al., 2012; Macinnes and Duty, 2008; MacInnes et al., 2004; Matsui and Kita, 2003; Valenti et al., 2003; Wittmann et al., 2001). Recently, numerous highly selective mGlu₄ PAMs have been developed from different chemical series and exhibit robust efficacy in preclinical rodent models. Administration of either PHCCC or VU0155041, two mGlu₄ PAMs that bind to distinct binding sites on the receptor, reversed parkinsonian behavior in PD animal models, such as reserpine-induced akinesia as well as haloperidol-induced catalepsy (Marino et al., 2003; Niswender et al., 2008b). In another example, VU0364770, a systemically active mGlu₄ PAM, produced a reversal of forelimb asymmetry induced by unilateral 6-hydroxydopamine (6-OHDA) lesion of the median forebrain bundle either alone or in combination with L-DOPA (Jones et al., 2012). In contrast, Lu AF21934 alone exhibited no effect in the akinesia induced by unilateral 6-OHDA lesion of the SNc unless a sub-threshold dose of L-DOPA was co-administered. Similarly, ADX88178 alone had no impact on forelimb akinesia induced by a bilateral 6-OHDA lesion. However, coadministration of ADX88178 with a low dose of L-DOPA enabled a robust reversal of the forelimb akinesia deficit. The difference between PAMs in 6-OHDA lesion models could result from distinctions in the lesion protocols used by the different research groups. It is also possible that the discrepancy is mediated by different pharmacological profiles of mGlu₄ PAMs. Detailed studies of PAMs using the same lesion procedure will be required to elucidate the mechanism for these differences.

Besides a symptom-alleviating effect, mGlu₄ PAMs also possess other potential benefits, such as potential disease-modifying efficacy and a lack of L-DOPA-mediated side effects, such as dyskinesia. PHCCC and VU0155041 has been shown to reduce dopaminergic cell death (Battaglia et al., 2006; Betts et al., 2012), possibly due to reduced excessive excitatory drive onto dopamine neurons, and provide the potential to slow disease progression. In addition, LuAF21934, a selective mGlu₄ PAM related to the VU0155041
series, has been shown to decrease the incidence of L-DOPA-induced dyskinesia (Bennouar et al., 2013), further making mGlu₄ an attractive target for PD treatment.

**Mechanism of allosteric modulation**

Through years of research, orthosteric ligands useful in determining the physiological roles of mGlus have been developed which display group-selectivity. However, as all orthosteric ligands bind to the N-terminal VFD of mGlus, which is evolutionarily designed to bind glutamate, it is difficult to achieve subtype selectivity since the glutamate binding site is highly conserved across all eight subtypes. In addition, brain penetration and pharmacokinetics of these orthosteric ligands can be limited by their amino acid-like properties. Many of these hurdles have been overcome by targeting allosteric binding sites on the receptors. As indicated by the name, allosteric modulators bind to a site other than the endogenous agonist binding site, and provide modulatory effects on the affinity/efficacy of the orthosteric agonist, termed cooperativity. Indeed, the complex structure of mGlus offers a number of possibilities to develop novel allosteric modulators. Because of the greater sequence divergence demonstrated at allosteric sites, a series of subtype-selective allosteric agonists and positive, negative or silent allosteric modulators (PAM, NAM or SAMs) of mGlus have been identified through high through-put screening campaigns ((Kinney et al., 2005; Mitsukawa et al., 2005; Niswender et al., 2008b; Varney et al., 1999) and many others), which have greatly advanced studies on mGlu functions and accelerated the development of mGlu reagents as disease therapeutics.

The mechanism of action of allosteric modulators is exemplified by the dataset shown in Figure 1.2, PAMs potentiate the response to orthosteric agonists by shifting the concentration-response curve of an orthosteric agonist to the left (with or without increasing the maximum response), indicating that the agonist response is being pharmacologically amplified. NAMs are molecules that antagonize the activity of agonists in a noncompetitive fashion through negative cooperativity. SAMs are neutral allosteric modulators that exhibit no apparent effect on agonist responses on their own,
Figure 1.2. Mechanism of action of allosteric modulators. Instead of binding to the glutamate binding site on the N-terminal VFD, mGlu PAMs and NAMs bind to the 7TMD on the receptor and induce selective modulating effects, which can be blocked by SAMs. PAMs potentiate the response to orthosteric agonists by shifting the dose response curve of an orthosteric agonist to the left, whereas NAMs progressively antagonize the activity of agonists through negative cooperativity in a noncompetitive fashion.
however, such compounds can block the binding and subsequent receptor modulation induced by PAMs or NAMs.

Besides improved selectivity, PAMs and NAMs also provide some other advantages when compared to their orthosteric counterparts. First, the biological effects of PAMs and NAMs are dependent on the presence of the endogenous agonist; thus, they have the potential to preserve the spatial and temporal aspects of endogenous signaling. As PAMs which lack allosteric agonist activity will not constantly activate the receptor, they may also reduce the liability of receptor desensitization compared to the direct activation by orthosteric, or even allosteric, agonists. Allosteric modulators also bring a further advantage in that their modulating effect is saturable, thus providing a larger therapeutic window and potentially decreasing the risk of overdose. In addition, these allosteric molecules often possess drug-like properties and better pharmacokinetics and brain penetration compared to orthosteric ligands, an important feature considering the therapeutic indication of mGlus in CNS disorders.

Thusfar, the majority of identified mGlu allosteric modulators bind to the 7TMD region of the receptor. Results from mutagenesis studies indicate that the mGlu allosteric binding site likely corresponds to the orthosteric binding site in Class A 7TMRs (Chen et al., 2008; Gregory et al., 2013; Litschig et al., 1999; Malherbe et al., 2003; Pagano et al., 2000; Schaffhauser et al., 2003). Interestingly, PAMs can directly activate an N-terminal truncated mGlu (El Moustaine et al., 2012; Goudet et al., 2004), suggesting that VFD-CRD region prevents PAMs from activating the receptor until glutamate is bound. The modulating effects induced by allosteric modulators can be quantified using operational models of allosterism (Gregory et al., 2012; Leach et al., 2007):

\[
y = \text{basal} + \frac{(E_m - \text{basal})(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n}{(\tau_A[A](K_B + \alpha\beta[B]) + \tau_B[B]K_A)^n + ([A]K_B + K_AK_B + K_A[B] + \alpha[A][B])^n}
\]
where \( A \) and \( B \) are the molar concentration of the orthosteric agonist and the allosteric modulator, respectively; \( K_A \) and \( K_B \) are the equilibrium dissociation constant of the orthosteric agonist and the allosteric modulator, respectively; \( \tau_A \) and \( \tau_B \) quantify the efficacy of the orthosteric agonist and the allosteric modulator, respectively. \( \text{Basal}, E_m \) and \( n \) represent the basal system response, maximal possible system response and the transducer function that links occupancy to response. Importantly, these models have introduced two parameters, \( \alpha \) and \( \beta \), to describe cooperativity of an allosteric ligand on the affinity and efficacy of orthosteric agonist. The operational models of allosterism not only allow quantitative estimation of modulator affinity and cooperativity values, which can be used to guide compound optimization processes, but can be used to derive reliable estimates of modulator affinities when radioligand is not available (Gregory et al., 2012).

Based on molecular pharmacology data, it has been hypothesized that binding of one PAM per mGlu dimer is sufficient to potentiate receptor activity (Goudet et al., 2005). In contrast, binding of NAMs to both protomers appears to be necessary to inhibit receptor activation (Hlavackova et al., 2005).

**Allosteric modulators for mGlu_2 and mGlu_4**

As allosteric modulators have been discovered for each individual mGlu subtype, selective modulators for mGlu_2 and mGlu_4 will be discussed here in detail.

BINA, LY487379 (along with CBiPES) and THIIC represent three chemical scaffolds of highly selective mGlu_2 PAMs that lacks activity at mGlu_3 (Fell et al., 2011; Galici et al., 2006; Johnson et al., 2005). Several NAMs from the dihydrobenzo[1,4]diazepin-2-one series, as exemplified by MNI-137, have been reported to antagonized both mGlu_2 and mGlu_3 in a non-competitive fashion (Hemstapat et al., 2007; Woltering et al., 2008a; Woltering et al., 2008b). Additionally, a group of mGlu_2 SAMs have been identified through FRET-based binding assays and slight modification of these SAMs yields three mGlu_2 NAMs with mGlu_3 PAM activity, indicating that identification of SAMs is a useful approach to discover novel mGlu allosteric modulators (Schann et al., 2010).
Mutagenesis studies have revealed that amino acids that are essential for mGlu2 PAM activity are dispensable for NAMs, and the converse has also been reported (Lundstrom et al., 2011; Rowe et al., 2008; Schaffhauser et al., 2003). These data suggest that mGlu2 PAMs and NAMs may bind to different allosteric pockets, although binding studies using appropriate allosteric radioligands are required to validate this hypothesis.

Allosteric modulation has also been demonstrated to be a successful approach to selectively modulate mGlu4, 7 and 8. An mGlu1 partial antagonist, PHCCC, was the first identified PAM for mGlu4 with no activity on 6 of the other mGlu subtypes except for mGlu6 (Beqollari and Kammermeier, 2008; Maj et al., 2003; Marino et al., 2003). In the in vitro mGlu4 assays in which is has been examined, PHCCC exhibits no agonist activity by itself but increases the potency of glutamate at mGlu4 and acts as a proof-of-concept compound for targeting mGlu4 as a potential therapeutic strategy for disorders such as Parkinson’s disease (Marino et al., 2003). Further optimization of the PHCCC scaffold has been challenging (Niswender et al., 2008, Williams et al 2010); however, a high-throughput screening campaign led to the identification of VU0155041 and VU0080421 as examples of non-PHCCC scaffold mGlu4 PAMs (Niswender et al., 2008b; Niswender et al., 2008c). Recently, many other mGlu4 PAMs, including 4PAM-2, VU0364770, ADX88178, LuAF21934 and others (Bennouar et al., 2013; Drolet et al., 2011b; Jones et al., 2012; Le Poul et al., 2012), have emerged with significant improvements in potency, selectivity and pharmacokinetic profile compared to PHCCC. Particularly, VU0364770, ADX88178 and LuAF21934 exhibit high potency with good pharmacokinetic profiles for use as tool compounds; each of these ligands shows in vivo efficacy in animal models of PD and other disorders. Interestingly, VU0155041 also exhibits allosteric agonist activity at mGlu4 when tested in vitro (Niswender et al., 2008b), suggesting an alternative mechanism of action compared to PHCCC. Indeed, data obtained from radioligand binding assays has demonstrated that VU0155041 binds to a unique allosteric site on mGlu4 which is different from the binding site of PHCCC and 4PAM-2 (Drolet et al., 2011b).
Advantages and complications of allosteric modulation

In addition to the improved selectivity, PAMs and NAMs also provide some other advantages comparing to orthosteric counterparts. First, the biological effects of PAMs and NAMs are dependent on the presence of the endogenous agonist, thus they have the potential to preserve the spatial and temporal aspects of endogenous signaling. As PAMs themselves will not continuously activate the receptor, they may also reduce the liability of receptor desensitization comparing to the direct activation by orthosteric, or even allosteric, agonists. Allosteric modulators also bring a further advantage in that their modulating effect is saturable, thus providing a larger therapeutic window and potentially decreasing the risk of overdose. In addition, these allosteric molecules often possess drug-like properties and better permeability at the blood-brain barrier, an important feature considering the therapeutic indication of mGlus in CNS disorders.

Despite all the advantages mentioned above, many allosteric modulators are lipophilic, which diminishes their solubility, can affect their pharmacokinetic profile, and potentially increases off-target binding. The shallow structure-activity relationship among classes of allosteric ligands also represents a significant hurdle in the development of allosteric modulators. Additionally, because of the substantial activity alteration generated by minor structural changes, a number of mGlu allosteric modulator classes are susceptible to subtle “molecular switches” (Wood et al., 2011), by which compounds within a series can switch from NAM to PAM, PAM to NAM, or exhibit altered selectivity (Lamb et al., 2011; Sharma et al., 2009; Sheffler et al., 2012; Zhou et al., 2010).

Allosteric compounds have also complicated our understanding of receptor pharmacology. As allosteric modulators potentiate/inhibit the receptor through cooperativity with the orthosteric ligand being used, it is not surprising that “probe dependence” has been reported in some cases, in that modulators have differential effects depending upon the orthosteric ligand that is present (Suratman et al., 2011; Valant et al., 2012). Although examples still have yet to be discovered for mGlu receptors, caution
should be taken to choose the orthosteric ligand for *in vitro* studies. In addition, similar to what has been described with some orthosteric ligands (Urban et al., 2007), many allosteric ligands have been found to differentially stimulate multiple signaling cascades downstream of a receptor (Kenakin, 2005), a phenomenon often termed “functional selectivity” “biased signaling”, or “ligand directed trafficking” (Marlo et al., 2009; Zhang et al., 2005). Indeed, 7TMRs may adopt multiple structural conformations, and allosteric modulators may stabilize any of them, which can translate into the regulation of some signaling pathways but not others.

The first aim of this dissertation is to study the signaling bias of mGlu4 PAMs induced by concommittant activation of a Gq-coupled receptor. The second part of this dissertation is to study the heterodimerization of mGlu2/4 and the differentially regulated pharmacology of allosteric modulators by the formation of mGlu heterodimers. Although these findings highly complicate the application of allosteric modulators as disease therapeutics, it is conceivable that it could be utilized to enhance the therapeutic outcome or avoid adverse effects.
CHAPTER II

FUNCTIONAL SELECTIVITY INDUCED BY MGLU4 RECEPTOR ALLOSTERIC MODULATORS DURING CONCOMITANT ACTIVATION OF Gq COUPLED RECEPTORS

Introduction

Seven Transmembrane Spanning/G-Protein-Coupled Receptors (7TMRs/GPCRs) represent the majority of drug targets currently used in clinical practice. Much interest has recently been placed on the discovery and characterization of allosteric modulators for these receptors due to several potential advantages over traditional orthosteric ligands in terms of drug development (reviewed in (Keov et al., 2011)). For example, many natural endogenous ligands are peptides or small amino acids which possess limitations in pharmacokinetic properties, preventing their development as drug candidates. Additionally, the orthosteric agonist binding sites of many 7TMRs are highly conserved across family members, making selectivity for a particular receptor within one group difficult to achieve. Due to their interaction with the receptor at a site distinct from the orthosteric site, allosteric ligands often possess very high receptor selectivity. Allosteric modulators also have the ability to provide a more subtle and physiologically-relevant approach to increasing or decreasing target activity because receptor regulation will occur only in the presence of the endogenous ligand (Bridges and Lindsley, 2008; Conn et al., 2009). Furthermore, allosteric potentiators, or positive allosteric modulators (PAMs), may, in some cases, avoid receptor desensitization and/or down-regulation that can occur after chronic administration of an orthosteric agonist (Bridges and Lindsley, 2008; Conn et al., 2009). As allosteric modulators function by exerting either positive (PAMs) or negative (NAMs) cooperativity with the orthosteric ligand, mechanistically they will
exhibit a “ceiling” effect (i.e., maximal receptor occupancy may not translate to maximal effect on receptor activation), which may avoid target/mechanism-mediated side effects that could arise from accidental overdose.

While allosteric modulators of 7TMRs provide potential advantages/distinctions over orthosteric ligands, these compounds also greatly complicate our understanding of receptor pharmacology. In recent years, there has been a growing appreciation of the ability of a single 7TMR to simultaneously regulate multiple signaling cascades (Kenakin, 2005), some of which are G protein-independent, such as β arrestin-regulated pathways. This phenomenon, now well established for orthosteric ligands, has been termed “functional selectivity”, “biased signaling”, or “ligand directed trafficking” (Keov et al., 2011; Urban et al., 2007); we will refer to this phenomenon as functional selectivity. There are now also clear examples of 7TMR allosteric modulator-mediated functional selectivity (Marlo et al., 2009; Mathiesen et al., 2005). While this behavior introduces complexity into ligand development, it is anticipated that capitalizing on functionally selective effects will provide exciting opportunities to tailor new drug therapy to specifically regulate coupling of 7TMRs to beneficial signaling pathways but not others, potentially reducing adverse effects.

There are numerous mechanisms by which functionally selective pharmacology can be induced by allosteric ligands. For example, 7TMRs have the ability to adopt multiple structural conformations, any of which might be stabilized by an allosteric modulator. This can translate into the ability of a modulator to preferentially regulate some pathways and not others based on the particular conformation they stabilize. Receptor activity is also regulated by other cellular proteins, such as G-proteins, arrestins, or scaffolding proteins, which also act in an allosteric fashion to affect receptor conformations. In this case, compound pharmacology can be altered depending on the context in which a receptor is expressed (e.g., (Niswender et al., 2010)), presumably due to the different proteins or cellular components interacting with the receptor in various cell types.
An alternate possibility that may affect the outcome of functional selectivity would be convergent signaling pathways that are activated (or inhibited, or simply absent) in a certain temporal or spatial context. It has previously been demonstrated that activation of the G_{i/o}-coupled GABA_{B} receptor, in conjunction with the G_{q}-coupled metabotropic glutamate 1 (mGlu_{1}) receptor, produces a signaling convergence at the level of phospholipase C β3 (PLCβ3) to induce potentiated calcium mobilization (Pin et al., 2009; Rives et al., 2009). In these studies, this phenomenon was not due to heterodimerization/oligomerization of the receptors, was generalizable to other receptor pairs, and was demonstrated to exhibit functional relevance in cerebellar Purkinje cells and cultured cortical neurons where these two receptors are co-expressed (Rives et al., 2009). In the current study, we extend these findings to explore potentially functionally selective effects induced by this type of signaling convergence. We describe that, as for the GABA_{B} and mGlu_{1} receptor combination, activation of a G_{q} coupled histamine receptor, the H_{1} receptor, dramatically potentiates the ability of the G_{i/o}-coupled metabotropic glutamate 4 (mGlu_{4}) receptor to induce intracellular calcium mobilization. However, histamine does not potentiate the ability of mGlu_{4} activation to modulate other “common” G_{i/o}-regulated signaling cascades, such as cAMP inhibition. These results suggest that H_{1} co-activation biases mGlu_{4}-mediated signaling events toward certain signaling pathways. Furthermore, when small molecule PAMs of mGlu_{4}, are included in these assays, the potentiated signaling of mGlu_{4} is further biased by histamine toward calcium-dependent pathways. Our results suggest that convergence of these signaling pathways may result in substantial, and potentially unexpected, increases in calcium responses downstream of mGlu_{4} activation, particularly when receptor activity is potentiated using positive allosteric modulators.
Methods

Cell line establishment and cell culture

Establishment and culture of the human mGlu₄ (hmGlu₄)/Gₛ₅/CHO-DHFR(-) has been described in (Niswender et al., 2008b). All cell culture reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise noted.

Guinea pig H₁ (gp H₁)/CHO-K1 cells were obtained by stable transfection of CHO-K1 cells with guinea pig H₁ receptor in pcDNA3.1 vector (a generous gift of Mike Zhu, Ohio State University). Single G418-resistant clones were isolated and screened for H₁-mediated calcium mobilization as described below. Monoclonal gpH₁/CHO-K1 cells were cultured in 90% Dulbecco's modified Eagle's medium (DMEM), 10% dialyzed fetal bovine serum (FBS), 100 U/ml penicillin/streptomycin, 20 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 µg/ml proline (Sigma-Aldrich, Inc., St. Louis, MO) and 400 µg/ml G418 sulfate (Mediatech, Inc., Herndon, VA).

Rat mGlu₄/CHO-K1 cells, rat mGlu₂/CHO-K1 cells, rat mGlu₄/H₁/CHO-K1 cells, and rat mGlu₂/H₁/CHO-K1 cells were obtained by stable transfection of either CHO-K1 cells or gpH₁/CHO-K1 cells with rat mGlu₄ or mGlu₂ receptor in a pIRESpuro3 vector (Invitrogen). Polyclonal cells were cultured in 90% DMEM, 10% dialyzed FBS, 100 U/ml penicillin/streptomycin, 20 mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 µg/ml proline (Sigma-Aldrich, Inc., St. Louis, MO), 20 µg/ml puromycin (Sigma-Aldrich, Inc., St. Louis, MO) without or with 400 µg/ml G418 sulfate (for H₁ expressing cell lines, Mediatech, Inc., Herndon, VA).

Rat mGlu₄/M₁/CHO-K1 cells were generated by stable transfection of rat mGlu₄/CHO-K1 cells with rat M₁ muscarinic receptor DNA in pcDNA3.1 vector. Polyclonal cells were cultured in 90% DMEM, 10% dialyzed FBS, 100 U/ml penicillin/streptomycin, 20
mM HEPES, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 µg/ml proline (Sigma-Aldrich, Inc., St. Louis, MO), 400 µg/ml G418 sulfate (Mediatech, Inc., Herndon, VA) and 20 µg/ml puromycin (Sigma-Aldrich, Inc., St. Louis, MO).

**Calcium mobilization assays**

For assays performed using the Flexstation (Molecular Devices, Sunnyvale, CA), cells were seeded at a density of 60,000 cells/100 µl/well in Costar 96-well tissue culture-treated plates. For assays performed using the Hamamatsu FDSS 6000 or 7000 (Hamamatsu, Japan), cells were seeded at 30,000 cells/20 µl/well in Greiner 384-well clear-bottomed, tissue culture–treated plates. Cells were incubated in assay medium (90% DMEM, 10% dialyzed FBS, 20 mM HEPES and 1 mM sodium pyruvate) overnight at 37°C/5% CO₂ and assayed the following day.

Fluo-4/acetoxyethyl ester (Invitrogen) was dissolved as a 2.3 mM stock in DMSO and mixed in a 1:1 ratio with 10% (w/v) Pluronic acid F-127 and diluted in assay buffer (Hanks' balanced salt solution, 20 mM HEPES, and 2.5 mM probenecid; Sigma-Aldrich) to a final concentration of 2 µM. Cells were dye-loaded for 45 min at 37°C; dye was then removed and replaced by appropriate volume of assay buffer. For single-add experiments, a series of different concentrations of glutamate or histamine were diluted into assay buffer as 2× stock. For histamine fold-shift and potency experiments, histamine was diluted as 2× stock and added at the first add. After 150 sec, the appropriate volume of a 5× glutamate stock was added in a second addition. For experiments using antagonists or PAMs, compounds were added at a 2× final concentration in the first addition followed by the desired concentration of agonist in the second addition.
Total RNA isolation, reverse transcription and polymerase chain reaction (RT–PCR)

CHO-K1 cells, mGlu₄/CHO-DHFR(-) cells, mGlu₄/G₉₅/CHO-DHFR(-) cells, and mGlu₄/HEK/GIRK cells were seeded in 10 cm cell culture dishes one day before the experiment. On the second day, cells were harvested and total mRNA from each cell line was extracted using an RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA was quantified by Nanodrop and 0.5 ug was reversely transcribed into cDNA by iScript cDNA Synthesis Kit (Bio-Rad, Philadelphia PA) according to the manufacturer’s protocol. Reactions were carried out both in the presence and in the absence of reverse transcriptase (as negative controls). One tenth of each yielded cDNA sample was used to perform polymerase chain reaction (PCR) using primers for histamine H₂, H₃ and H₄ receptors. The primers were designed to match the conserved sequence for human, rat and mouse histamine receptors:

H₂ Forward: AGCTTTGGCCAGGTCTTCTGCA
H₂ Reverse: GGCTGCCAGTGTCACGGTGG
H₃ Forward: GCTGTGGCTGGTGGTAGACT
H₃ Reverse: AGGAGCTTGGTGAAGGCTCTGCGG
H₄ Forward: TGATAGGCAATGCTGTGGTC
H₄ Reverse: GCCAGTGACCTGGCTAGCTTC

Water was used as a negative control for PCR reactions for H₂-H₄ receptors. 1 ng of rat brain cDNA was used as the positive control for H₂ and H₃ receptor and pcDNA3.1-H₄ plasmid was used as positive controls for H₄ receptor. The amplification protocol for H₂ and H₃ was 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1.5 min. For H₄, the annealing temperature was set at 52°C. The final extension step was at 72 °C for 5 min. The PCR products were then electrophoresed on a 1% agarose gel containing ethidium bromide in parallel with 1 Kb Plus DNA Ladder (Invitrogen).
Phosphoinositide hydrolysis assays

mGlu₂/H₁/CHO-K1 cells were plated in 24-well plates at a density of 100,000 cells/well/0.5 mL in growth medium two days before the assay. On the following day, growth media was removed and replaced with 0.5 ml/well assay media containing 0.5 µCi/ml [³H]-inositol. Cell plates were incubated at 37°C/5% CO₂ overnight and assayed on the third day. For stimulation of phosphoinositide hydrolysis, the [³H]-inositol-containing assay medium was first aspirated from wells and replaced with 200 µl of assay buffer (HBSS supplemented with 20 mM HEPES and 30 mM LiCl). Cells were then treated with 250 µl of assay buffer or histamine (2×, 1 µM final concentration, diluted in assay buffer) and 50 µl of serial dilutions of glutamate (10×, diluted in assay buffer). After drug addition, the assay plates were incubated at 37°C/5% CO₂ for 1 h, and then 1 ml of stop solution (10 mM formic acid) was added into each well to terminate the reaction. Cells were incubated in stop solution for 1 h at room temperature then the cell extracts were transferred to anion exchange columns (AG 1-X8 Resin, 100-200 mesh, formate form; Bio-Rad Laboratories, Hercules, CA) for separation of [³H]inositol-containing compounds. After loading of cell extracts, each column was washed sequentially with 9 ml of water, 9 ml of 5 mM inositol, and 9 ml of water. Finally, the [³H]inositol-containing compounds that bound to columns were eluted with 9 ml of PI Eluent (200mM ammonium formate and 100 mM formic acid) into scintillation vials and measured by liquid scintillation counting. Baseline response was removed from both histamine-treated and control group respectively and data were fit with GraphPad Prism (La Jolla, CA) to a 4-parameter logistic equation.

Adenylate cyclase assays

Adenylate cyclase assays were performed according to the methods described in (Sheffler and Conn, 2008; Watts and Neve, 1996). Cells were plated at 60,000 cells/well in Assay Media in 96 well plates 24 h prior to assay. The next day, media was removed from the cells and replaced with 100 µL of serum free DMEM containing 20 mM HEPES. After 1
h incubation at 37 °C, the media was replaced with 50 µL 37 °C stimulation buffer
(DMEM, 15 mM HEPES, pH 7.4, 0.025% ascorbic acid). After a 10 min incubation at
room temperature, the stimulation buffer was removed and the cells were placed on ice.
For dose response curves of glutamate in rat mGlu4/CHO-K1 cells and rat
mGlu4/H1/CHO-K1 cells, 20 µL of the phosphodiesterase inhibitor 3-isobutyl-1-
methylxanthine (IBMX) (4×, 500 µM final concentration, diluted in stimulation buffer)
was first added to all wells to prevent cAMP breakdown. 20 µL of stimulation buffer was
then added, followed by 20 µL of forskolin (4×, 10 µM final concentration, diluted in
stimulation buffer) or DMSO-matched vehicle control. Finally, serial dilutions of
 glutamate (20 µL, 4×, diluted in stimulation buffer) were added to the wells. For
histamine fold-shift experiments in mGlu4/Gqi5/CHO-DHFR(-) cell line, cells were
treated with 20 µL IBMX (4×, 500 µM final concentration, diluted in stimulation Buffer),
20 µL stimulation buffer or histamine (4×, 10 µM final concentration, diluted in
stimulation buffer), 20 µL of forskolin (4×, 20 µM final concentration) or vehicle and 20
µL of serial dilutions of glutamate. For histamine fold-shift experiments in
mGlu4/H1/CHO-K1 cells, serial dilutions of mGlu4 PAMs were diluted as 4× stock in
stimulation buffer containing 4 µM glutamate (1 µM final concentration). Cells were
treated with 20 µL IBMX (4×, 500 µM final concentration, diluted in timulation Buffer),
20 µL of forskolin (4×, 20 µM final concentration) or vehicle, 20 µL stimulation buffer or
histamine (4×, 300 nM final concentration, diluted in stimulation buffer) and 20 µL of
serial dilutions of mGlu4 PAMs.

After 20 min incubation in water bath at 37 °C, drugs were then removed from the wells
and the reaction was terminated by addition of 40 µL ice-cold 3% trichloroacetic acid
(TCA). Cell lysates were chilled at 4 °C for at least 2 h. Accumulated cAMP was
quantified using a competitive binding assay adapted from (Nordstedt and Fredholm,
1990) with minor modifications. Briefly, TCA extracts (15 µL) from assay plates were
added to a deep well 96 well plate (Axygen Scientific) in triplicates. [3H]-cAMP
(PerkinElmer) (1 nM final concentration) was diluted in cAMP Assay Buffer (100 mM
Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA) and added to each well (25 µL/well).
Lastly, 500 µL of cAMP-binding proteins (approximately 100 µg of crude extract from
bovine adrenal cortex) was added to each well. The deep-well plates were incubated on ice for 2 h and harvested with a Brandel cell harvester (Gaithersburg, MD) onto Whatman GF/B filters. Radioactivity bound to filters was quantified by liquid scintillation counting using a PerkinElmer Top Count. The concentration of cAMP in each well was calculated according to a cAMP standard curve ranging from 0.01 to 1000 pM.

**Positive allosteric modulators of mGlu₄**

Glutamate, histamine and N-Phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC) were purchased from Tocris Biosciences (Ellisville, Missouri). Acetylcholine was purchased from Sigma-Aldrich Incorporated (St. Louis, MO). *cis*-2-[[(3,5-Dichlorophenyl)amino]carbonyl]cyclohexanecarboxylic acid (VU0155041), *N*-(4-[(N-(2-chlorophenyl)sulfamoyl)phenyl]picolinamide (4PAM-2), and 5-methyl-*N*-(4-methylpyrimidin-2-yl)-4-(1*H*-pyrazol-4-yl)thiazol-2-amine (ADX88178) were synthesized in-house according to methods in (Celanire et al., 2011; Engers et al., 2010; Niswender et al., 2008b; Reynolds, 2008).

**Astrocyte culture**

Rat cortical astrocytes were prepared using neocortices from 2-4 day old Sprague Dawley rats. Neocortics from pups were dissected and dissociated in DMEM by trituration and the cells were centrifuged and resuspended in growth media (DMEM supplemented with 10 % FBS, 1 mM sodium pyruvate, 2 mM L-glutamine and antibiotics) in poly-D-lysine-coated tissue culture dishes. Cells were maintained at 37 °C in an atmosphere of 95 % air/5 % CO₂ until ready for assay.

**Phosphoinositide hydrolysis assays using hippocampal slices**

Hippocampal slices were prepared according to the procedures described in (Ayala et al., 2009). Following tissue recovery, the tissue was combined, washed with warm Krebs
buffer (108 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃ and 10 mM glucose), and 25 ml of gravity packed slices were incubated with 175 ml Krebs buffer containing 0.5 mCi [³H]myo-inositol in 37°C water bath for 45 min. 10 mM LiCl was added to slices and incubated for an additional 15 min. Histamine and/or L-AP4 were then added, followed by 15 min incubation. Carbachol was used as positive control and Krebs buffer was used as negative control. The reaction was terminated and phosphoinositide was extracted, purified and quantified using liquid scintillation counting as previously described in (Ayala et al., 2009).

Results

Activation of histamine H₁ receptor biases the signaling of mGlu₄ toward calcium mobilization

We have long been interested in the identification and characterization of small molecule positive allosteric modulators (PAMs) of mGlu₄ for the symptomatic and disease modifying treatment of Parkinson’s disease. To characterize compounds, we employ a cell line in which the normally Gᵢₒ-coupled mGlu₄ is co-expressed with the chimeric G protein G₉₅ to permit induction of a calcium response downstream of mGlu₄ activation, a technique that is commonly employed in high throughput screening campaigns for various 7TMRs as it is an easy and cost-effective method to measure compound activity. In addition to identifying small molecule allosteric modulators, we had designed parallel studies to explore the impact of endogenous neurotransmitters and ligands on the modulation of mGlu₄ function. In the course of these studies, we discovered that application of the autacoid histamine to mGlu₄-expressing cells, prior to application of a concentration of glutamate designed to induce a 20% maximal response, resulted in a strong potentiation of the calcium mobilization signal normally induced by glutamate in mGlu₄/G₉₅-expressing cells. In these studies, the potentiation effect of histamine highly resembled the effects of synthetic PAMs of mGlu₄, such as the prototype PAM N-Phenyl-
7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC), with one important difference. While both histamine and PHCCC were able to potentiate the effects of an EC\textsubscript{20} concentration of glutamate (Figure 2.1A and B, “Glutamate Add”), unlike PHCCC, histamine induced a weak response when added alone to mGlu\textsubscript{4}/Gqi5 cells (Figure 2.1B, “Compound Add”). In our experience using this mGlu\textsubscript{4}/Gqi5 assay, response of compound alone is not common. In Figures 2.1C and D, concentration-response curves are shown for the compound response in the absence and presence of an EC\textsubscript{20} concentration of glutamate. The control PAM PHCCC was inactive when added alone but exhibited a potency of 5.1±0.3 µM when potentiating the response to a low concentration of glutamate. In contrast, histamine generated a concentration-dependent response alone with a potency of 8.3±1.4 µM; in addition, histamine also potentiated the EC\textsubscript{20} glutamate response (EC\textsubscript{50}=1.2±0.1 µM).

Small molecule PAMs of mGlu\textsubscript{4} have been shown to potentiate multiple responses downstream of mGlu\textsubscript{4} activation (Jones et al., 2011; Niswender et al., 2008b). Therefore, if histamine directly binds to the mGlu\textsubscript{4} protein and acts as a prototypical mGlu\textsubscript{4} PAM, it would be expected to modulate additional, G\textsubscript{i/o}-dependent pathways downstream of mGlu\textsubscript{4} activation, such as cAMP inhibition. In contrast to our studies with calcium mobilization (Figure 2.1B, 2.1D and 2.2A), histamine did not potentiate the ability of mGlu\textsubscript{4} activation to inhibit cAMP levels (Figure 2.2B). These results suggested that histamine induced biased signaling downstream of mGlu\textsubscript{4} activation. It also indicated that the mechanism of histamine’s potentiation might differ from previously identified small molecule PAMs.

While our previous results did not rule out the possibility that histamine directly interacted with mGlu\textsubscript{4}, the distinct signaling profile induced by histamine suggested that the mechanism of histamine’s potentiation might be due to activity at a site distinct from mGlu\textsubscript{4}, such as a histamine receptor. To determine if low levels of endogenous histamine receptors were involved in mediating the potentiation response, we performed RT-PCR experiments from our mGlu\textsubscript{4}-expressing cells and determined that they expressed low
Figure 2.1. Histamine differs from the small molecule mGlu₄ PAM, PHCCC, in its potentiation effect. A and B, fluorescence traces of PHCCC and histamine in calcium mobilization assays measured in CHO-DHFR(-) cells co-expressing mGlu₄ and the chimeric G protein Gqi5 in cells. PHCCC (A, ranging from 100 nM to 30 µM) or histamine (B, ranging from 100 nM to 100 µM) was added in the “Compound Add”, while an EC₂₀ concentration of glutamate (2.5 µM final) was added after 150 sec in the “Glutamate Add”. C and D, Compound activity alone (Agonist Response) and PAM activity (PAM Response) in the presence of an EC₂₀ concentration of glutamate (2.5 µM final) from traces represented in A and B are shown for PHCCC and histamine, respectively. For both responses, the increase in fluorescence units is normalized to the maximum response elicited by 1 mM glutamate in this cell line. PHCCC elicited no agonist response and possessed a potency of 5.1±0.3 µM for the PAM response. The potency of histamine for the PAM response was 1.2±0.4 µM and for the agonist response was and 8.3±1.4 µM. Data shown were performed in triplicate; Mean ± SEM.
Figure 2.2. Histamine potentiates calcium responses downstream of mGlu4 without impacting glutamate-dependent cAMP inhibition. A, Glutamate-induced calcium mobilization was measured in the presence of vehicle control (■) or 100 µM histamine (□). The responses are normalized to maximum effect of glutamate in the same cell line. Potencies in the absence or presence of 100 µM histamine were 5.1±0.9 µM vs. 2.0±0.3 µM (*p=0.029; unpaired t-test). Maximal responses in the absence or presence of 100 µM histamine were: 100.0±1.0% vs. 179.1±20.0% (*p=0.017; unpaired t-test). B, Glutamate-dependent intracellular cAMP concentration was quantified in the absence (■) or presence (□) of 100 µM histamine using a competitive binding assay as described in 2.5. Adenylate cyclase assays. Data were normalized to the 20 µM forskolin-induced response. Potencies in the absence or presence of 100 µM histamine were 4.1±0.3 µM vs. 4.3±0.8 µM (p=0.76; unpaired t-test). Maximal inhibition values in the absence or presence of 100 µM histamine were: 88.1±8.3% vs. 83.9±2.3% (p=0.65; unpaired t-test). Data shown were performed in triplicate; Mean ± SEM. Statistical analysis was performed using GraphPad Prism (La Jolla, CA).
levels of H1 histamine receptor mRNA (Figure 2.3A). PCR with primers against H2, H3 and H4 receptor did not yield any specific bands to support the existence of other histamine receptors (Figure 2.4). To address the potential contribution of functional activity of Gq-coupled H1 receptors, we co-applied the H1 receptor antagonist mepyramine and 100 µM histamine to mGlu4-expressing cells in the first addition and then added an EC20 concentration of glutamate in the second addition. These studies revealed that mepyramine blocked the response induced by histamine application alone to a baseline level (Figure 2.3B), consistent with full blockade of H1. While mepyramine was also able to block the response induced by the glutamate EC20 addition, the blockade saturated at the level of the EC20 glutamate response (Figure 2.3B).

These findings suggested that the potentiation effect was likely mediated by low levels of endogenous H1 protein present in our mGlu4 cell line. However, they did not completely rule out the possibility of direct histamine binding to mGlu4, since mepyramine might have exerted its effect by displacing binding of histamine from the mGlu4 receptor. Additionally, our original cell line contained the chimeric G protein Gqi5 to induce mGlu4-mediated calcium mobilization, which would not be reflective of mGlu4 signaling in native tissues. In order to test the hypothesis that the potentiation effect was definitively mediated by H1 activity versus direct histamine interaction with mGlu4 and occurred in presence of native cellular G proteins, we screened a panel of cell lines to identify a line that did not express H1 mRNA. We found that CHO-K1 cells, in contrast to the CHO-DHFR(-) cell background used for the initial screening, did not express H1 mRNA (Figure 2.3A). By utilizing the CHO-K1 cells as the parental cell line, we generated two new cell lines that contained either the mGlu4 receptor alone, or mGlu4 co-expressed with the H1 receptor; neither of these cell lines contained Gqi5. As shown in Figure 2.5A, mGlu4 was functional and coupled to glutamate-induced cAMP inhibition in cells expressing either mGlu4 alone or the mGlu4+H1 combination. We then performed studies in which we attempted to potentiate glutamate-induced calcium mobilization using increasing concentrations of histamine in cells without (Figure 2.5B) and with (Figure
Figure 2.3. The histamine H₁ receptor may be involved in the potentiation effect of histamine. A, mRNA expression of histamine H₁ receptor in different cell lines. mRNA was extracted from CHO-K1 cell line and mGlu₄/Gqi₅/CHO-DHFR(-) cell line and RT-PCR was performed as described under Materials and Methods. “+RT” indicates presence of reverse transcriptase during the reaction of reverse transcription, whereas “–RT” represents absence of reverse transcriptase as negative controls. Predicted size of the PCR product for the H₁ receptor was 423 bp, as shown with arrow. B, Mepyramine abolishes the agonist response and potentiation effect of histamine. Increasing concentrations of mepyramine were added to mGlu₄/Gqi₅/CHO-DHFR(-) cells with 100 µM histamine prior to addition of an EC₂₀ concentration of glutamate (2.5 µM glutamate final). Calcium mobilization induced by the histamine addition and the subsequent glutamate addition was measured as described. Potencies of mepyramine in the histamine add or the glutamate add were: 1.55±0.5 µM vs. 393±154 nM (p=0.10; unpaired t-test). Data shown were performed in triplicate; Mean ± SEM. Statistical analysis was performed using GraphPad Prism (La Jolla, CA).
Figure 2.4. mRNA expression of histamine H$_2$, H$_3$ and H$_4$ receptor (A-C respectively) in different cell lines. mRNA was extracted from different cell lines as labeled and RT-PCR was performed as described under Supplemental Methods. “+RT” indicates presence of reverse transcriptase during the reaction of reverse transcription, whereas “–RT” represents absence of reverse transcriptase as negative controls. Predicted size of PCR products for H$_2$, H$_3$ and H$_4$ were 462 bp, 957 bp and 834 bp, respectively, as shown with arrows.
Figure 2.5. The histamine H\textsubscript{1} receptor is required for the potentiation effect of histamine. A, Glutamate exhibits potencies of 1.0±0.03 µM and 2.3±0.1 µM, respectively, in adenylate cyclase assays in mGlu\textsubscript{4}/CHO-K1 cells and mGlu\textsubscript{4}/H\textsubscript{1}/CHO-K1 cells (*p=0.0048, unpaired t-test). Intracellular cAMP concentration was measured as described in 2.5. Adenylate cyclase assays and responses were normalized to the 10 µM forskolin response in each cell line, respectively. B, The effect of 1 µM (▲), 10 µM (▼) and 100 µM (◆) histamine on glutamate-induced calcium mobilization in mGlu\textsubscript{4}/CHO-K1 cells is shown. Maximal responses of vehicle, 1 µM, 10 µM or 100 µM histamine-treated cells were 3391±1033, 3254± 841, 3067± 527 and 3214± 643 relative fluorescence units, respectively (p=0.99; One-way ANOVA). C, The effect of 30 nM (▲), 100 nM (▼) and 300 nM (◆) histamine in potentiating calcium responses mediated by glutamate in mGluR\textsubscript{4}/H\textsubscript{1}/CHO-K1 cells is shown. Maximal responses in vehicle, 30 nM, 100 nM or 300 nM histamine-treated cells were 1548±230, 3390±636, 10099±819, 21261±1356 relative fluorescence units, respectively (*p<0.0001; One-way ANOVA). Data shown were performed in triplicate; Mean ± SEM. Statistical analysis was performed using GraphPad Prism (La Jolla, CA).
2.5C) H₁. These results clearly showed that the potentiation required the presence of H₁ receptors.

**Concomitant activation of Gq coupled receptors pathway-selectively potentiate the calcium signaling of Gi-coupled receptors**

According to our findings, the potentiated calcium response that we observed was mediated by concomitant activation of the G_q-coupled H₁ receptor and G_/i/o-coupled mGlu₄ receptor. We speculated that, if this potentiation was due to a signaling convergence, the phenomenon would extend to other G_q and G_/i/o coupled receptor pairs. To test this hypothesis, we co-expressed mGlu₄ with the muscarinic acetylcholine M₁ receptor, another G_q-coupled receptor which is also extensively expressed in the CNS. We observed that activation of the M₁ receptor via acetylcholine in this mGlu₄-co-expressing cell line induced similar glutamate-dependent calcium mobilization compared to cells co-expressing H₁ and mGlu₄ (Figure 2.6A). We also hypothesized that such signaling crosstalk might be generalizable to other G_/i/o-coupled mGlu receptors. As carried out for mGlu₄, we constructed two mGlu₂ cell lines in a CHO-K1 background, one of which expressed mGlu₂ alone and the other in combination with H₁ receptor. As shown in Figure 2.5B, cells expressing mGlu₂ alone did not respond to histamine; in contrast, cells co-expressing H₁ and mGlu₂ exhibited robust potentiation of calcium responses after co-application of histamine and glutamate (Figure 2.6C). As shown previously (Rives et al., 2009), signaling of G_/i/o and G_q receptors converges on the PLC_β pathway. To determine if this was also the mechanism of potentiated calcium responses for the receptors examined here, phosphoinositide hydrolysis assays were performed in cells co-expressing mGlu₂ and H₁ receptors. Consistent with our observations in calcium mobilization assays, histamine dramatically potentiated mGlu₂-induced phosphoinositide hydrolysis (Figure 2.6D).
Functionally selective effects of mGlu$_4$ positive allosteric modulators induced by co-activation of H$_1$ and mGlu$_4$

We are very interested in the development of small molecule positive allosteric modulators of mGlu$_4$ and were particularly intrigued with our initial studies (Figure 2.2) that suggested that histamine may induce functionally selective activation of signaling pathways downstream of mGlu$_4$. Furthermore, we hypothesized that co-application of histamine with mGlu$_4$ PAMs could further selectively potentiate calcium responses compared to signaling induced by other G$_i$/o-dependent pathways. To evaluate this hypothesis, we used our generated mGlu$_4$/H$_1$/CHO-K1 cell line which does not express G$_{qi5}$. For these studies, we chose PAMs from four distinct chemical scaffolds (Celanire et al., 2011; Niswender et al., 2008b; Reynolds, 2008) (Figure 2.7). These compounds were chosen based on differential in vitro potency and efficacy at mGlu$_4$; additionally, VU0155041 displays allosteric agonist activity in some assays (Niswender et al., 2008b) and has been proposed to bind to a different site on the mGlu$_4$ receptor compared to PHCCC and 4PAM-2 (Drolet et al., 2011a). In these experiments, we added increasing concentrations of each PAM either alone or in combination with histamine in the first addition. As shown in Figure 2.8, addition of each PAM alone (white traces, “Compound/Histamine Add”) resulted in no calcium mobilization, even after glutamate addition (“Glutamate Add”). Addition of 300 nM histamine alone induced a relatively strong calcium response (dark gray traces); no potentiation of glutamate (second addition) was observed in this case due to the low concentration of glutamate added in these experiments. In contrast, addition of histamine + PHCCC, 4PAM-2, or ADX88178 (Figure 2.8A, B, and C) resulted in a prolonged calcium transient after the first addition and a very strong potentiation of the glutamate addition. Consistent with its potential to display allosteric agonist activity in some assays, VU0155041 behaved differently from the other PAMs, and substantial potentiation was observed in the first addition when this compound was added with histamine (Figure 2.8D); in contrast, no further potentiation was observed during the glutamate addition. In Figure 2.9, the concentration-response curves for these compounds, plus and minus 100 nM and 300 nM histamine, are plotted.
Figure 2.6. Phospholipase C pathway potentiation extends to additional Gq and Gi/o pairs. A, Acetylcholine (Ach) potentiates calcium responses induced by mGlu4 activation in mGlu4/M1/CHO-K1 cells. 3 nM Ach (□) or vehicle (■) control was added to cells in the first add, while increasing concentrations of glutamate were applied 150 sec later in the second add and calcium mobilization was measured. Maximal responses in the absence or presence of 3 nM Ach were: 2889±878 vs. 6175±280 relative fluorescence units (*p=0.024; unpaired t-test). Data shown were performed in triplicate; Mean ± SEM.

B and C, histamine potentiates the calcium response of mGlu2 in the presence of the histamine H1 receptor in mGlu2/H1/CHO-K1 cells. In mGlu2/CHO-K1 cells, maximal responses in the absence or presence of 100 µM histamine were: 2072±23.7 vs. 2122±272 relative fluorescence units (p=0.86; unpaired t-test). In mGlu2/H1/CHO-K1 cells, maximal responses in the absence or presence of 1 µM histamine were: 2796±285 vs. 8223±1128 relative fluorescence units (*p=0.010; unpaired t-test). Data shown were performed in triplicate; Mean ± SEM.

D, Histamine potentiates mGlu2 responses in
phosphoinositide hydrolysis assays in mGlu_{2}/H_1/CHO-K1 cells. Cells were treated with increasing concentrations of glutamate in the presence of 1 μM histamine (□) or vehicle control (■). After 1h incubation at 37°C, accumulated inositol phosphates were measured according to description in 2.4. *Phosphoinositide hydrolysis assays*. Maximal responses in the absence or presence of 1 μM histamine were: 6.9±1.8 vs. 68.7±5.8 cpm (*p=0.0005; unpaired t-test). Data from triplicate experiments are shown (Mean ± SEM) with relative baseline responses subtracted from each group. All Statistical analysis was performed using GraphPad Prism (La Jolla, CA).

Figure 2.7. Chemical structures of mGlu_4 PAMs used in these studies: PHCCC, 4PAM-2, ADX88178 and VU0155041.
Figure 2.8. Histamine dramatically potentiates the effect of PAMs on mGlu₄-mediated calcium mobilization in cells co-expressing mGlu₄ and H₁ receptors. Traces of calcium transients showing the effects of mGlu₄ PAMs, histamine or the combination of both in potentiating the response of an EC₂₀ concentration of glutamate (1 µM glutamate final) are shown. In these traces, a 10 µM concentration of each mGlu₄ PAM (PHCC, 4PAM-2, ADX88178 or VU0155041, in A-D respectively; (◊)), 300nM histamine (♦) or combination of both (♦) were applied in the first add (“Compound/Histamine Add”). After 150 sec, 1 µM glutamate (concentration determined based on cAMP experiments shown in Figure 5A) was applied in the second add (“Glutamate Add”). Calcium responses were measured as the fluorescence ratio, which involves dividing all fluorescence data for each point in the kinetic trace by the fluorescence value obtained in the first baseline sample read, which corrects for differences in dye loading and cell plating.
Figure 2.9. Histamine dose-dependently potentiates the efficacy of PAMs on mGlu4-mediated calcium mobilization in cells co-expressing mGlu4 and H1 receptors. Results of traces in Figure 7 were plotted in concentration-response curve format. Increasing concentrations of the mGlu4 PAMs PHCCC, 4PAM-2, ADX88178 or VU0155041 (A-D, respectively) were applied either alone (■) or together with 100 nM (▲) or 300 nM (●) histamine in the first add. After 150 sec, a 1 µM glutamate concentration was applied in the second add. Calcium responses were measured as the fluorescence ratio, which involves dividing all fluorescence data for each point in the kinetic trace by the fluorescence value obtained in the first baseline sample read, which corrects for differences in dye loading and cell plating. Data were further normalized by taking the maximum calcium response minus the minimum response measured 3 seconds prior to either the first or second addition. For PHCCC (A), 4PAM-2 (B), and ADX88178 (C) responses, the effect on the second addition (“Glutamate Add”) window is shown. For VU0155041 (D), the effect on the first addition (“Compound Add”) is shown.
Potencies for the different conditions were: PHCCC alone, no fit, PHCCC+100 nM histamine, 8.2±5.1 µM, PHCCC+300 nM histamine, 7.6±1.4 µM; 4PAM-2 alone, no fit, 4PAM-2+100 nM histamine, 54.2±29.0 nM, 4PAM-2+300 nM histamine, 40.8±10.0 nM; ADX88178 alone, no fit, ADX88178+100 nM histamine, 37.2±15.1 nM, ADX88178+300 nM histamine, 30.0±5.2 nM; VU0155041 alone, no fit, VU0155041+100 nM histamine, 9.5±3.9 µM, VU0155041+300 nM histamine, 6.5±1.6 µM. Maximal responses in the absence or presence of 100 nM or 300 nM histamine were: for PHCCC, 0.016±0.003, 0.089±0.010, 0.259±0.018 (*p<0.0001; One-way ANOVA); for 4PAM-2, 0.017±0.002, 0.212±0.013, 0.460±0.022 (*p<0.0001; One-way ANOVA); for ADX88178, 0.016±0.002, 0.197±0.003, 0.438±0.020 (*p<0.0001; One-way ANOVA); and for VU0155041, 0.018±0.003, 0.221±0.032, 0.864±0.043 (*p<0.0001; One-way ANOVA). Data shown were performed in triplicate; Mean ± SEM. Statistical analysis was performed using GraphPad Prism (La Jolla, CA).
Figure 2.10. In contrast to effects on calcium mobilization, histamine has no effect on the activity of mGlu4 PAMs in adenylate cyclase assays in cells expressing both mGlu4 and H1 receptors. Increasing concentration of mGlu4 PAMs (PHCCC, 4PAM-2, ADX88178 or VU0155041, A-D, respectively) were co-diluted with 1 μM glutamate and incubated with mGlu4/H1/CHO-K1 cells either alone or together with 300 nM histamine. Intracellular cAMP concentration was measured as described and then normalized to either 20 μM forskolin response or 20 μM forskolin+300nM histamine, respectively. Potencies in the absence or presence of 300 nM histamine were: PHCCC, 2.5±0.6 μM vs. 1.8±0.4 μM (p=0.40; unpaired t-test); 4PAM-2, 55.5±12.6 nM vs 66.1±8.6 nM (p=0.53; unpaired t-test); ADX88178, 11.7±2.0 nM vs. 16.0±4.3 nM (p=0.42; unpaired t-test); and VU0155041, 287.3±23.1 nM vs. 360.0±38.8 nM (p=0.18; unpaired t-test). Maximal inhibition values in the absence or presence of 300 nM histamine were: PHCCC, 85.3±3.4% vs. 82.0±2.7% (p=0.49; unpaired t-test); 4PAM-2, 89.0±1.2 nM vs. 89.4±0.6% (p=0.77; unpaired t-test); ADX88178, 90.4±1.5% vs. 91.1±0.6% (p=0.70;
unpaired t-test); and VU0155041, 89.5±0.4% vs. 90.0±0.1% (p=0.85; unpaired t-test). Data shown were performed in triplicate; Mean ± SEM. Statistical analysis was performed using GraphPad Prism (La Jolla, CA).
for the PAM (Figure 2.9A, B, and C) and agonist (Figure 2.9D) windows of the experiments shown in Figure 2.8; the responses to histamine in this assay are clearly concentration-dependent and the potencies of the PAMs obtained are consistent with the potencies observed in other assays (Celanire et al., 2011; Engers et al., 2010; Niswender et al., 2008b).

In parallel experiments designed to test the potential for functional selectivity, we assessed the ability of histamine to affect cAMP inhibition responses induced by these PAMs in these same cells. For these studies, we performed concentration-response curves of these compounds, in the presence of an EC$_{20}$ concentration of glutamate, with or without 300 nM histamine. Again, in contrast to calcium mobilization assays and as can be seen in Figure 2.10, histamine did not affect the cAMP responses to any of the PAMs tested. These results further suggest that co-activation/potentiation of the histamine H$_1$ receptor and mGlu$_4$ results in functionally selective effects, resulting in unexpected calcium mobilization induced by mGlu$_4$ PAMs.

**Exploration of H$_1$-mGlu$_4$ signaling crosstalk in native tissues**

Based on these interesting findings, we sought to explore the signaling crosstalk between H$_1$ and mGlu$_4$ using native tissues that coexpress the two receptors. It has been reported that the histamine H$_1$ receptor is functionally expressed in astrocytes (Lipnik-Stangelj and Carman-Krzan, 2004a, b). mGlu$_4$ levels have been reported to be very low in astrocytic cultures relative to mGlu$_5$ (Peavy and Conn, 1998, *J Neurochem*, 71(2):603-612 and Besong et al., 2002, *J Neurosci*, 2213, 5403-5411). However, a low level of mGlu$_4$ was detected in astrocytes by Besong et al, which was shown to be mediated astrocytic cytokine release (Besong et al., 2002). Therefore, primary rat cortical astrocyte cultures were prepared according to the method described in Besong et al (see Method for details) and evaluated in calcium assays. Consistent with previous studies, we performed immunoblot studies which revealed expression of mGlu$_4$ monomers in astrocytes but not mGlu$_4$ dimers, the functional format of the receptor (Figure 2.11A). When tested in
calcium assays, we did not observe effects of mGlu_4 activation on calcium mobilization or crosstalk between H_1 and mGlu_4 receptors, although histamine was able to induce profound calcium responses in these astrocytes (Figure 2.11B). We further explored the possibility of signaling crosstalk in hippocampal slices, where both receptors are expressed. 100 µM carbachol (Cch), a muscarinic agonist as a positive control, induced significant hydrolysis of phosphoinositide (Figure 2.11C). While 100 µM histamine triggered modest responses when added alone, this effect was not potentiated by co-addition of 1 mM L-AP4 (Figure 2.11C), supporting an absence of signaling crosstalk in this brain region.

**Discussion**

In this chapter, we have explored the ability of convergent signaling to induce functionally selective effects downstream of allosteric modulation. These studies capitalized on initial reports, such as those of Rives et al. (Rives et al., 2009), showing that convergent signaling downstream of the G_i/o-coupled GABA_B and G_q-coupled mGlu_1 receptors could result in potentiated calcium signaling. The effects reported in Rives et al. were apparent in transfected cells as well as in neurons, indicating that this potentiation can be observed in native tissues. We observed a similar interaction between mGlu_4 and H_1 histamine receptors in terms of calcium mobilization. In previous studies (Rives et al., 2009), the mechanism for potentiation was a convergence of signaling via G_q G proteins and the G_{βγ} subunits of the G_i/o G protein at the level of PLC_β3. To explore the generalizability of this phenomenon for different mGlus, in addition to our mGlu_4 and H_1 cells lines, we generated cells expressing mGlu_4 and the G_q coupled M_1 muscarinic receptor as well as cells co-expressing mGlu_2 and H_1. As shown in Figure 2.6, activation of the M_1 receptor via acetylcholine in mGlu_4-co-expressing cells induced similar glutamate-dependent calcium mobilization compared to cells co-expressing H_1 and mGlu_4. As shown for mGlu_4, CHO-K1 cells expressing mGlu_2 alone did not respond to
Figure 2.11. Interaction of H₁ receptor and mGlu₄ was not detected in rat cortical astrocytes or hippocampal slices. A. expression of mGlu₄ in rat cortical astrocytes were determined using Western blotting. Lanes from left to right: mGlu₄/HEK/GIRK (positive control); HEK/GIRK (negative control); astrocytes from batch 1, day 6 after in vitro culture; astrocytes from batch 2, day 5 after in vitro culture. B. Agonist-induced responses were measured using calcium mobilization assay as described above. Vehicle or 100 nM, 1 µM, 3 µM and 10 µM histamine was added in the first add and serial dilutions of L-AP4 were added in the second add. Serial dilutions of histamine were used as positive control. C. Agonist-induced phosphoinositide hydrolysis was measured as described and the measured scintillation values were normalized to vehicle response (as 0%), with 100 µM carbachol response as 100%. 
histamine; in contrast, cells co-expressing H₁ and mGlu₂ exhibited robust potentiation of calcium responses when histamine and glutamate were co-applied. Finally, consistent with signaling that converges on the PLCβ pathway by co-activated Gᵢ/o and Gq receptors (Rives et al., 2009), histamine dramatically potentiated mGlu₂-induced phosphoinositide hydrolysis, suggesting that the potentiation mechanism we are observing here is similar. Since PLCβ can be activated by both Gαq and Gβγ subunit from the Gᵢ G-protein, we propose that occupation of both might have a synergistic effect on IP₃ production, inducing potentiated calcium mobilization. Indeed, mutagenesis studies of the PLCβ₂ protein have shown that distinct binding sites may exist for Gαq and Gβγ on the enzyme (Lee et al., 1993).

The present studies extend previous observations by showing that the potentiation effect induced by Gq and Gᵢ/o convergent activation is signaling pathway specific and does not extend to other Gᵢ/o-mediated signaling events, such as cAMP inhibition. These findings suggest that this cascade convergence effectively results in functional selectivity at a signaling level. During the course of our development of mGlu₄ PAMs, we have been interested in potential signaling bias or functionally selective effects induced by these compounds, particularly ligands belonging to different chemical scaffolds. In the assays we have examined, the majority of mGlu₄ PAMs will potentiate multiple signaling pathways downstream of mGlu₄ activation, including calcium mobilization induced using the chimeric G protein Gqi5 and GIRK channel activation (Jones et al., 2011; Niswender et al., 2008b), in addition to potentiation of cAMP inhibition as shown in this chapter. Our studies here show that the signal bias induced by histamine can be greatly potentiated in the presence of small molecule PAMs, with PAMs and histamine inducing dramatic potentiation of calcium responses versus other Gᵢ/o-dependent responses. More importantly, our studies now show that PAMs with no ability to potentiate glutamate-dependent calcium mobilization alone in the absence of chimeric G proteins (Figure 2.8, white traces) can induce substantial calcium signaling when the H₁ receptor, and presumably other Gq coupled receptors, are co-activated (Figure 2.8, black traces).
This unmasking of a substantial calcium response could be highly important in the physiological effects induced by PAMs in vivo. While the H\textsubscript{1} receptor and mGlu\textsubscript{4} do exhibit different expression patterns in neurons (for example, mGlu\textsubscript{4} is predominantly presynaptic and H\textsubscript{1} predominantly postsynaptic), which may account for the absence of crosstalk in hippocampal slices (Figure 2.11C), there are locations where their expression may overlap, such as dendritic cells of the immune system (Fallarino et al., 2010; Vanbervliet et al., 2011). Additionally, the observations that signaling convergence can extend to other receptor pairs suggests that it is highly likely that there are locations in which G\textsubscript{q} and G\textsubscript{i/o} receptors may co-localize. In particular, mGlu\textsubscript{2} is expressed in many postsynaptic neurons (Neki et al., 1996; Petralia et al., 1996) and mGlu\textsubscript{2} PAMs are currently being developed for schizophrenia treatment, suggesting that a similar phenomenon may also impact mGlu\textsubscript{2} PAM signaling in postsynaptic neurons.

Another interesting point of speculation is that the strategy outlined here might provide a viable mechanism to potentiate the signaling of an intractable target. For example, there is substantial evidence indicating that activation of H\textsubscript{1} in neurons may have beneficial effects in terms of attention and wakefulness (reviewed in (Thakkar, 2011)). Due to the substantial expression of H\textsubscript{1} in various immune system cells, however, it would be difficult to pursue direct H\textsubscript{1} agonists or PAMs as drugs for attention without inducing substantial adverse effects. Exploiting the activity of a convergent signaling partner, however, might be an alternate mechanism by which to increase signaling of a G\textsubscript{q} coupled 7TMR that is difficult to modulate directly. While mGlu\textsubscript{4} expression in dendritic cells of the immune system may preclude it as a strategy to potentiate H\textsubscript{1} signaling, restricted postsynaptic neuronal expression of other G\textsubscript{i/o}-coupled receptors that are not expressed in immune cells could be an interesting strategy to explore.

In conclusion, we have shown that co-activation of mGlu\textsubscript{4} and the H\textsubscript{1} histamine receptor induces strong potentiation of calcium mobilization but not traditional G\textsubscript{i/o} signaling pathways, indicating functional selectivity in signal transduction. These functionally selective effects are observed in the absence of chimeric or promiscuous G proteins and are synergistically potentiated in the presence of small molecule PAMs. Finally, these
studies reveal that signaling events induced by PAMs may be “unmasked” in the presence of convergent signaling by $G_q$ coupled receptors, which may lead to complex and unexpected pharmacology. The concept of functional selectivity may be the next frontier in the translation of novel therapeutics into patient populations, and it is anticipated that further exploration of compound pharmacology will certainly aid in the understanding of therapeutic efficacy and adverse effects.
CHAPTER III

HETERODIMERISATION OF MGLU2/4 DIFFERENTIALLY REGULATE EFFECT OF ALLOSTERIC MODULATORS

Introduction

The metabotropic glutamate (mGlu) receptors are members of the Family C Seven Transmembrane Spanning/G Protein Coupled Receptors (7TMRs/GPCRs) and are activated by the major excitatory neurotransmitter, glutamate. In their simplest context, Group I mGlus (mGlu1 and mGlu5) primarily modulate postsynaptic neuronal activity, whereas the Group II mGlus (mGlu2 and mGlu3) are found in both presynaptic and postsynaptic locations, and the Group III receptors (mGlu4, 6, 7, and 8) are predominantly expressed presynaptically, where they act as auto- and heteroreceptors to regulate neurotransmitter release (reviewed in (Niswender and Conn, 2010)). The eight mGlu receptor subtypes have been historically thought to function as homodimers (Kunishima et al., 2000; Romano et al., 1996). However, recent in vitro studies suggest that mGlu receptors can also form heterodimers (Doumazane et al., 2011a; Kammermeier, 2012) with group I mGlus interacting with each other but not associating with other subtypes, and members of group II and III receptors co-assembling in vitro.

Among the group III mGlu receptors, mGlu4 plays an important role in the basal ganglia, a primary site of pathology in movement disorders such as Parkinson’s disease (PD).
Activation of mGlu4 reduces transmission at synapses that project from the striatum to the globus pallidus (striatopallidal synapses) as well as synapses between the subthalamic nucleus and the substantia nigra pars compacta (STN-SNc synapses (Marino et al., 2003; Valenti et al., 2005; Valenti et al., 2003)), two synapses that are overactive in PD. At each of these synapses, the response to the general group III mGlu agonist L-AP4 is potentiated by PHCCC (Marino et al., 2003; Valenti et al., 2005), a positive allosteric modulator (PAM) that is selective for mGlu4. In contrast to findings at the striatopallidal and STN-SNc synapses, we now report the surprising observation that PHCCC is without effect in regulating mGlu4-modulated transmission at corticostriatal synapses.

Previous immunohistochemistry and in situ hybridization studies suggest that mGlu2 and mGlu4 are co-localized in several brain regions (Bradley et al., 1999; Neki et al., 1996; Ohishi et al., 1995; Ohishi et al., 1993) and mGlu2 is also functionally expressed at corticostriatal synapses (Johnson et al., 2005). We tested the hypothesis that heterodimers of mGlu2/4 may display a unique profile in response to selective mGlu4 PAMs and that these mGlu subtypes would form hetero-complexes in the striatum. Through evaluation of mGlu4 PAMs from different chemical scaffolds, we show here that hetero-interactions between mGlu2 and mGlu4 differentially impact responses to individual mGlu receptor PAMs and an mGlu2 negative allosteric modulator (NAM). Furthermore, co-immunoprecipitation studies suggest that mGlu2 and mGlu4 receptors form hetero-complexes in the striatum and the unique pharmacological profile of effects of selected mGlu4 receptor PAMs, as well as an mGlu2 NAM, is recapitulated at the corticostriatal synapse. These studies directly impact our understanding of mGlu
receptors and regulation by allosteric modulators in the basal ganglia and provide critical insights into potential functions and pharmacological properties of mGlu receptors that are co-expressed in multiple other regions and cell populations.

**Methods**

**Cell line establishment and cell culture**

Cell culture reagents were purchased from Life Technologies (Carlsbad, CA) unless otherwise noted. Rat mGlu\textsubscript{2} or rat mGlu\textsubscript{4} were cloned into the pIRESpuro3 vector, transfected into HEK/GIRK cells and selected with puromycin. Polyclonal rat mGlu\textsubscript{2}/HEK/GIRK and rat mGlu\textsubscript{4}/HEK/GIRK cells were cultured in growth media as previously described in (Niswender et al., 2008a), supplemented with Non-Essential Amino Acids. Rat mGlu\textsubscript{4} was also subcloned into the pIREShyg3 vector and the resulting plasmid was transfected into rat mGlu\textsubscript{2}/HEK/GIRK cells; cells were then selected with 200 µg/mL hygromycin B. Polyclonal cells were cultured in growth media supplemented with 100 µg/ml hygromycin B.

**Western blot analysis**

Cells were scraped into lysis buffer (50 mM Tris·HCl, pH 7.5, 150mM NaCl, 0.5% Nonidet P40 and 0.5% Deoxycholate) containing protease inhibitor cocktail (Roche, Indianapolis, IN) and incubated on ice for 20-30 min. The supernatant was separated from cell debris by centrifugation at 16,000×g for 10 min at 4°C. Protein concentrations
in cell lysates were quantified by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) or Bradford protein assay (Bio-Rad) and aliquots of lysate were heated in SDS sample buffer (containing 10% SDS and 9.3% DTT) at 65°C for 5 min. Samples were loaded to SDS–polyacrylamide gel for electrophoresis and transferred to nitrocellulose membranes (Bio-Rad). After transfer, membranes were blocked in TBST (25mM Tris, 150mM NaCl and 0.05% Tween-20) containing 5% non-fat milk at room temperature for 1 h. mGlu$_2$ antibodies (Advanced Targeting Systems, San Diego, CA, cat # AB-N32) and mGlu$_4$a antibodies (Upstate, Billerica, MA, cat # 06-765) were diluted in blocking solution and incubated with the membranes at 4°C overnight. Membranes were then washed with TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Santa Cruz, Santa Cruz, CA, cat # sc-2060, 1:7,500 diluted in blocking buffer (Jackson ImmunoResearch, West Grove, PA, cat # 115-035-166, 1:10,000 diluted in blocking buffer) for mGlu$_2$ or horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Santa Cruz, Santa Cruz, CA, cat # sc-2004, 1:7,500 diluted in blocking buffer or Jackson ImmunoResearch, West Grove, PA, cat # 111-035-144, 1:10,000 diluted in blocking buffer) for mGlu$_4$ at room temperature for 1 h. Membranes were washed again with TBST and an enhanced chemiluminescent assay (Thermo Scientific, Waltham, MA, cat # 32106 or 34075) was performed to detect immunoreactive proteins.

**Co-immunoprecipitation**

In cell line experiments, mGlu$_2$/HEK/GIRK, mGlu$_4$/HEK/GIRK and mGlu$_{2/4}$/HEK/GIRK cells were lysed with IP lysis buffer (50 mM Tris·HCl, pH 7.5, 150mM NaCl, 2mM
EDTA, 1% Nonidet P40 with Complete Mini protease inhibitor cocktail (Roche, Basel, Switzerland, cat # 04693159001)) for IP experiments using the mGlu4 antibody; buffer was supplemented with 0.5% sodium deoxycholate for experiments using the mGlu2 antibody. Cell lysates were passed through G27 needles and incubated on ice for 30 min. The supernatant was centrifuged and pre-cleared with protein A/G beads (Santa Cruz, sc-2003) at 4°C for 2-3 h. mGlu2- (Advanced Targeting Systems, San Diego, CA, cat # AB-N32) or mGlu4 (Upstate, Billerica, MA, cat # 06-765) antibodies were bound to protein A/G beads by rotating at 4°C for 2-3 h. Pre-cleared cell lysates were then added to antibody-bound protein A/G beads or to beads without antibody as a negative control. After overnight rotation at 4°C, the beads were washed 3 times with washing buffer (IP lysis buffer without EDTA or protease inhibitors) and pelleted by low-speed centrifugation. SDS sample buffer was added to elute bound proteins. Samples were heated at 65°C for 5 min and subjected to SDS-PAGE and western blot analysis.

For co-immunoprecipitation assays in rat or mouse brain samples, Sprague Dawley rats of mixed gender (Charles River, Wilmington, MA) and ICR(CD-1) or male mice (Harlan, Indianapolis, IN) 22-30 days old were anesthetized under isoflurane anesthesia, decapitated, and brains were rapidly removed and cut into 0.5-1 mm coronal slices using a brain matrix or a vibratome (Leica, Buffalo Grove, IL). Slices were transferred to a chilled metal surface and dorsal striatum and medial prefrontal cortex (prelimbic and infralimbic regions) were dissected using a scalpel blade and immediately frozen on dry ice. Samples were homogenized in buffer containing (in mM): 50 Tris HCl, pH 7.4, 50 NaCl, 10 EGTA, 5 EDTA, 2 NaF, 1 Na3VO4, 1 PMSF supplemented with 1× Complete
Mini protease inhibitor cocktail, phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich, St. Louis, MO). Homogenized samples were centrifuged at 16,100×g for 15 min at 4 °C and pelleted membranes were stored at -80°C. Membrane pellets were resuspended in IP lysis buffer (same as IP lysis buffer for IP mGlu₄ in cell lines, supplemented with 1mM PMSF) and nutated at 4°C for 1 h. Supernatant was prepared by centrifugation at 16,100×g for 15 min and pre-cleared by protein A/G beads at 4°C for 2-3 h. mGlu₄ antibodies or normal rabbit IgG (Millipore, Billerica, MA, cat# 12-370) were bound to protein A/G beads by rotating at 4°C for 2-3 h. Pre-cleared cell lysates were then added to mGlu₄ antibody bound protein A/G beads or rabbit IgG-coupled beads as a negative control. After overnight rotation at 4°C, the beads were washed and samples were eluted and analyzed as described above.

**Thallium flux assays**

Thallium flux assays were performed according to methods described in (Niswender et al., 2008b) with minor modifications. For dye loading, media was exchanged with Assay Buffer (Hanks Balanced Salt Solution (HBSS) containing 20mM HEPES, pH 7.4) using an ELX405 microplate washer (BioTek), leaving 20 µL/well, followed by addition of 20 µL/well 2× FluoZin-2 AM (330 nM final) indicator dye (Life Technologies, prepared as a DMSO stock and mixed in a 1:1 ratio with pluronic acid F-127) in Assay Buffer. After a 1 h incubation at room temperature, dye was exchanged with Assay Buffer, leaving 20 µL/well. Thallium flux was measured at room temperature using a Functional Drug Screening System 7000 (FDSS 7000, Hamamatsu). Baseline readings were taken (2 images at 1 Hz; excitation, 470 ± 20 nm; emission, 540 ± 30 nm), and test compounds
(2×) were added in a 20 µL volume and incubated for 140s before the addition of 10 µL of Thallium Buffer with or without agonist (5×). Data were collected for an additional 2.5 min and analyzed using Excel (Microsoft Corp, Redmond, WA) as previously described (Niswender et al., 2008b), and the concentration-response curves were fitted to a four-parameter logistic equation to determine potency estimates using GraphPad Prism:

\[ y = \text{bottom} + \frac{\text{top} - \text{bottom}}{1 + 10^{(\text{Log EC}_{50} - A) \text{HillSlope}}} \]

where \( A \) is the molar concentration of the compound; \( \text{bottom} \) and \( \text{top} \) denote the lower and upper plateaus of the concentration-response curve; HillSlope is the Hill coefficient that describes the steepness of the curve; and \( \text{EC}_{50} \) is the molar concentration of compound required to generate a response halfway between the \( \text{top} \) and \( \text{bottom} \).

**Operational modeling of allosterism**

Shifts of agonist concentration-response curves by allosteric modulators were globally fitted to an operational model of allosterism (Leach et al., 2007):

\[ y = \text{basal} + \frac{(E_m - \text{basal}) (\tau_A[A](K_B + \alpha B) + \tau_B[B]K_A)^n}{(\tau_A[A](K_B + \alpha B) + \tau_B[B]K_A)^n + ([A]K_B + K_AK_B + K_A[B] + \alpha[A][B])^n} \]

where \( A \) is the molar concentration of the orthosteric agonist; \( B \) is the molar concentration of the allosteric modulator; \( K_A \) is the equilibrium dissociation constant of the orthosteric agonist, and \( K_B \) is the equilibrium dissociation constant of allosteric modulator. Affinity modulation is governed by the cooperativity factor \( \alpha \), and efficacy modulation is governed by \( \beta \). The parameters \( \tau_A \) and \( \tau_B \) relate to the ability of orthosteric agonist and allosteric ligands, respectively, to directly activate the receptor. \( \text{Basal}, E_m, \text{and } n \) represent
the basal system response, maximal possible system response and the transducer function that links occupancy to response.

Alternatively, a simplified version of this model was applied to estimate a composite cooperativity parameter ($\alpha\beta$) for PAMs (Leach et al., 2007):

$$y = \text{basal} + \frac{(E_m-basal)(\tau_A[A](K_B+a\beta[B])+\tau_B[B]K_A)^n}{(\tau_A[A](K_B+a\beta[B])+\tau_B[B]K_A)^n+(K_A(K_B+B))^n}$$

where all parameters are as described above.

For the simulation of mGlu$_4$ PAM effects, the log$K_A$ of L-AP4 for mGlu$_4$ was set to -6.759 according to literature values (Monastyrskaia et al., 1999), and was assumed to be unaltered at mGlu$_2$/4 heterocomplexes. For PHCCC and 4PAM-2, log$\tau_B$ was set to -100 due to the lack of allosteric agonist activity but was allowed to float for compounds exhibiting allosteric agonism (VU0155041, Lu-AF29134). For the simulation of MNI-137, the log$K_A$ of DCG-IV for mGlu$_2$ was set to -6.959 according to literature values (Schweitzer et al., 2000), and was assumed to be unaltered at mGlu$_2$/4 heterocomplexes; log$\tau_B$ was set to -100.

**Transient transfections**

Two days before the assay, combinations of pIRES-hyg3-rat mGlu$_2$, pIRES-hyg3-rat mGlu$_4$, and pIRES-hyg3-rat mGlu$_7$ were co-transfected with ratios of 0 µg:1 µg, 0.1 µg:1 µg, 0.2 µg:1 µg, 0.5 µg:1 µg or 1 µg:1 µg of DNA into HEK/GIRK cells using Fugene 6 (Promega, Fitchburg, WI) according to the manufacturer’s protocol. After 24 hours, cells
were trypsinized and plated in 384-well poly D-lysine-coated plates using assay media. Plates were then tested the next day using the thallium flux assay as described above.

**Whole-cell patch-clamp recordings**

Whole-cell patch-clamp recordings were performed using coronal slices prepared from 15- to 19-day-old Sprague-Dawley rats of mixed gender (Charles River, Wilmington, MA). Animals were anesthetized with isoflurane and brains were removed and submerged into ice-cold cutting solution (in mM: 220 sucrose, 2.5 KCl, 1.25 NaH₂PO₄, 26.2 NaHCO₃, 10 D-glucose, 0.5 CaCl₂, 8 MgCl₂). Coronal slices containing the striatum were cut at 300 µm using a vibratome (Leica VT 1200S) or a compressstome (Precisionary Instruments, Greenville, NC). Slices were transferred to a holding chamber containing ACSF (in mM: 126 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 25 NaHCO₃, 11 D-glucose, 2.4 CaCl₂, 1.2 MgCl₂) supplemented with 5 µM glutathione, for slice viability, for 25 min at 32°C. All buffers were continuously bubbled with 95% O₂/5% CO₂. Subsequently, slices were maintained at room temperature for at least 30 minutes in ACSF, then transferred to a submersion recording chamber where they were perfused with room temperature ACSF at a rate of 2 ml/min. Neurons were visualized with a 40× water immersion lens with Hoffman modulation contrast optics coupled with an Olympus BX50WI upright microscope (Olympus, Lake Success, NY). Borosilicate glass patch electrodes were pulled using a Flaming/Brown micropipette puller (Sutter Instruments, CA) and had a resistance of 4-6 MΩ when filled with an intracellular solution containing (in mM: 123 potassium gluconate, 7 KCl, 0.025 CaCl₂, 1 MgCl₂, 10 HEPES, 0.1 EGTA, 2 magnesium-ATP, 0.2 sodium-GTP; pH adjusted to 7.3 with 1 N KOH; 295 mOsm).
Whole-cell recordings were made from medium spiny neurons which were visually identified and then confirmed by determining the current-voltage relationship of positive or negative current injections; a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA) was used for current-clamp recordings. Data were digitized with a DigiData 1331 system (Molecular Devices, Sunnyvale, CA) and acquired using pClamp10.2 (Molecular Devices, Sunnyvale, CA). EPSPs were evoked in medium spiny neurons by placing a bipolar electrode in the white matter between the cortex and striatum. After formation of a whole-cell configuration, membrane potential was recorded and current was injected to maintain resting membrane potential at -75 mV and changes in membrane potential were recorded. Compounds were diluted in ACSF and bath applied as noted. Data were analyzed using Clampfit 10.2 (Molecular Devices, Sunnyvale, CA).

**Drugs**

Glutamate, DCG-IV, CBiPES and LY487379 was purchased from Tocris Biosciences (Ellisville, Missouri). L-AP4, LY379268 and N-Phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC) were purchased from Abcam Biochemicals (Cambridge, UK). cis-2-[(3,5-Dichlorophenyl)amino]carbonyl]cyclohexanecarboxylic acid (VU0155041), N-(4-(N-(2-chlorophenyl)sulfamoyl)phenyl)picolinamide (4PAM-2), (1S,2R)-N1-(3,4-dichlorophenyl)cyclohexane-1,2-dicarboxamide (Lu AF21934) and biphenylindanone A
(BINA) were synthesized in-house. Synthesis of VU0155041, BINA, and MNI-137 was performed according to methods in the following references: (Galici et al., 2006; Hemstapat et al., 2007; Niswender et al., 2008b).

Synthesis of 4PAM-2 and Lu AF21934 was performed according to methods described below:

**General.** All NMR spectra were recorded on a 400 MHz AMX Bruker NMR spectrometer. $^1$H chemical shifts are reported in δ values in ppm downfield with the deuterated solvent as the internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), integration, coupling constant (Hz). Low resolution mass spectra were obtained on an Agilent 1200 series 6130 mass spectrometer with electrospray ionization. High resolution mass spectra were recorded on a Waters Q-TOF API-US plus Acquity system with electrospray ionization. Analytical thin layer chromatography was performed on EM Reagent 0.25 mm silica gel 60-F plates. Analytical HPLC was performed on an Agilent 1200 series with UV detection at 214 nm and 254 nm along with ELSD detection. LC/MS: Restek-C18, 3.2×30mm, 2 min gradient,

10%[0.05%TFA/CH$_3$CN]:90%[0.05%TFA/H$_2$O] to 100%[0.1%TFA/CH$_3$CN] or Phenomenex-C18, 2.1 X 30 mm, 1 min gradient,

7%[0.1%TFA/CH$_3$CN]:93%[0.1%TFA/H$_2$O] to 95%[0.1%TFA/CH$_3$CN]. Preparative purification was performed on a custom HP1100 purification system with collection triggered by mass detection. Solvents for extraction, washing and chromatography were
HPLC grade. All reagents were purchased from Aldrich Chemical Co. and were used without purification.

**4PAM-2 (N-(4-(N-(2-chlorophenyl)sulfamoyl)phenyl)picolinamide):**

![Chemical Structure]

To a solution of 2-chloroaniline (0.95 mL, 9.02 mmol, 1.0 eq) in pyridine (5 mL) and DCM (5 mL) at 0°C was added 4-nitrobenzenesulfonyl chloride (2.0 g, 9.02 mmol, 1.0 eq). After 15 min, the cold bath was removed. After an additional 24 h at rt, the rxn was added to 1N HCl (aq) (50 mL) and DCM (50 mL). The organic layer was separated, washed with 1N HCl (aq) (20 mL), H₂O (2 x 20 mL), brine (20 mL) and dried (MgSO₄). The mixture was filtered and concentrated to afford **1** (2.73 g, 97%). The residue was carried through without further purification.

R_f 0.85 (50% EtOAc/hexanes);

LCMS: R_f=1.403 min, M+H=313.0; >98% @ 215 and 254 nm

^1^H NMR (400 MHz, CDCl₃): δ 8.27 (d, J = 9.0 Hz, 2H), 7.92 (d, J = 9.0 Hz, 2H), 7.70 (dd, J = 8.1 Hz, 1H), 7.32-7.26 (m, 2H), 7.13 (dd, J = 8.8, 8.8, 1.5 Hz, 1H), 7.01 (br s, 1H)
To a solution of 1 (2.73g, 8.73 mmol, 1.0eq) in EtOAc (40 mL) was added 5% Pd/C (~150 mg). The rxn atmosphere was evacuated and purged with H₂ balloon (1 atm). The rxn was followed by TLC. After 4h, the rxn mixture was filtered through Celite and concentrated to provide a white solid (2.45g, 99%).

R_f 0.50 (50% EtOAc/hexanes);

^1^H NMR (400 MHz, CDCl₃): δ 7.64 (dd, J = 8.2, 1.4 Hz, 1H), 7.54 (d, J = 8.7 Hz, 2H), 7.24-7.19 (m, 2H), 7.01 (ddd, J = 7.8, 7.8, 1.5 Hz, 1H), 6.91 (br s, 1H), 6.58 (d, J = 8.8 Hz, 2H), 4.11 (br s, 2H)

To a solution of 2 (2.45g, 8.66 mmol, 1.0eq) in DMF (16 mL) and Hunig’s Base (3.64 mL, 25.98 mmol, 3.0eq) at 0°C was added picolinoyl chloride hydrochloride (1.70g, 9.53 mmol, 1.1eq). After 15 min, the ice bath was removed. After an additional 24h at rt, the rxn was added to EtOAc:H₂O (1:1, 100 mL). The organic layer was separated and washed with H₂O (2 x 50 mL), brine (50 mL), dried (MgSO₄) and concentrated. The residue was purified by reverse phase liquid column chromatography (40-80%
acetonitrile: H₂O with 0.1% trifluoroacetic acid). The collected fractions were added to EtOAc:NaHCO₃(aq) (1:1, 100 mL). The organic layer was separated and washed with H₂O (50 mL), brine (50 mL), dried (MgSO₄), filtered and concentrated to afford a white solid. The white solid was dissolved in DCM (25 mL) and 4N HCl in dioxane (5 mL) was added. After 5 min, the solvent was removed to yield 4PAM-2 as an HCl salt (1.06 g, 32% yield).

LCMS: Rₜ=1.455 min, M+H=387.8; >98% @ 215 and 254 nm

¹H NMR (400 MHz, d-DMSO): δ 11.01 (s, 1H), 9.92 (s, 1H), 8.77 (d, J = 4.2 Hz, 1H), 8.18 (d, J = 7.6 Hz, 1H), 8.10 (d, J = 8.7 Hz, 2H), 8.09-8.08 (m, 1H), 7.73-7.71 (m, 1H), 7.71 (d, J = 8.8 Hz, 2H), 7.41 (dd, J = 7.8, 1.2 Hz, 1H), 7.32-7.26 (m, 2H), 7.23-7.19 (m, 1H);

HRMS, calc’d for C₁₈H₁₄N₃O₃NaSCl (M+Na⁺), 410.0342; found 410.0339.

Lu AF21934 (N¹-(3,4-dichlorophenyl)cyclohexane-1,2-dicarboxamide):

Into a 50 mL round bottom flask, containing a magnetic stir bar, was weighed 1.3751 mmol (212 mg) of cyclohexyldicarboxylic anhydride (predominantly cis) followed by 6 mL chloroform. To this solution was added 0.9167 mmol (148.5 mg) of 3,4-dichloroaniline. After being fitted with a reflux column the reaction mixture was heated in an oil bath to reflux at 70°C for 2 h, with magnetic stirring. Over this time a white
solid crashed out of the reaction mixture. After cooling the reaction to ambient
temperature, the solid was isolated via vacuum filtration and washed with cold
chloroform to obtain 434.8 mg of the desired product, 3 (78.4% yield) as a crystalline
white powder.

LCMS: R_t = 0.741 min, M+H=315.7; >98% @ 215 and 254 nm;

HRMS calcd for C_{14}H_{15}Cl_{2}NO_{3}[M+H]: 315.0667 found 315.0668;

^1^H NMR (400 MHz, methyl sulfoxide-d_6 calibrated to 2.54) δ 10.02 (s, 1H), 7.99 (d, J =
2.39 Hz, 1H), 7.53 (d, J = 8.79 Hz, 1H), 7.46 (dd, J = 8.87, 2.43 Hz, 1H), 2.92 (q, J =
4.78 Hz, 1H), 2.64-2.60 (m, 1H), 2.12-2.03 (m, 1H), 1.99-1.94 (m, 1H), 1.76-1.61 (m,
3H), 1.41-1.29 (m, 3H).

To a 4 mL vial were weighed 0.3115 mmol (98.5 mg) compound 3, 0.9345 mmol (98.5
mg) ammonium chloride, 0.3738 mmol (71.5 mg) 1-ethyl-3-(3-
dimethylaminopropyl)carbodiimide, and 0.3115 mmol (42.1 mg) hydroxybenzotriazole,
followed by 3 mL of 9:1 dimethylformamide:diisopropylethylamine. The reaction vial
was rotated at room temperature overnight. The reaction was diluted with ethyl acetate
(~5 mL) and washed with brine. The organic phase was separated and dried over sodium
sulfate. Solvent was removed under reduced pressure and the crude product was purified
via flash column chromatography. Product-containing fractions were combined and the
solvents removed under reduced pressure to obtain 31 mg of the desired product, **Lu AF21934** (31.5% yield) as an off-white powder.

LCMS: 0.690 min, M+H=314.7; >98% @ 215 and 254 nm;

HRMS calcld for C_{14}H_{16}Cl_{2}N_{2}O_{2}[M+H]: 316.0507 found 316.0507;

^{1}H NMR (400 MHz, methyl sulfoxide-\textit{d}\textsubscript{6}, calibrated to 2.54): \delta 9.92 (s, 1H), 8.01 (d, J = 2.27 Hz, 1H), 7.51 (d, J = 8.91 Hz, 1H), 7.44 (dd, J = 8.88, 2.42, 1H), 7.06 (s, 1H), 6.72 (s, 1H), 2.80 (q, J = 4.83 Hz, 1H), 2.50-2.46 (m, 1H), 2.15-1.98 (m, 1H), 1.70-1.48 (m, 1H), 1.39-1.23 (m, 1H).

**Animal studies**

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 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.

**Statistical analysis**
All data shown represent Mean±SEM value for at least three replicates. Statistical significance between groups was determined using unpaired Student’s t tests or ANOVA (with Dunnett’s or Bonferroni’s post-test) as specified in each Figure Legend.

Results

**mGlu₄ interacts with mGlu₂ to form heterocomplexes both in vitro and in brain tissue**

PHCCC is the first described mGlu₄ positive allosteric modulator (PAM) (Maj et al., 2003; Marino et al., 2003) and has been used to probe the activity of mGlu₄ at various synapses in the basal ganglia and other brain regions (Jones et al., 2008; Marino et al., 2003; Valenti et al., 2005). The efficacy of PHCCC at striatopallidal and STN-SNc synapses is consistent with its symptomatic and disease modifying effects in PD animal models (Battaglia et al., 2006; Marino et al., 2003). In addition to striatopallidal and STN-SNc synapses, immunohistochemistry studies reveal that mGlu₄ is expressed at corticostriatal synapses, which represent the primary input to the basal ganglia from the motor cortex (Corti et al., 2002). Consistent with expression studies, Bennouar et al. recently reported that the mGlu₄ PAM Lu AF21934 reduces corticostriatal transmission (Bennouar et al., 2012). To further understand the role of mGlu₄ in regulation of basal ganglia function, we evaluated the ability of PHCCC to reduce corticostriatal transmission. In agreement with previous results (Pisani et al., 1997), 100 μM L-AP4, a group III selective mGlu receptor agonist, reduced evoked excitatory postsynaptic potentials (eEPSPs) measured in striatal medium spiny neurons following stimulation in
the corpus callosum (42.8 ± 5.8% of baseline; Figure 3.1A). To determine if PHCCC could potentiate the response to L-AP4, a concentration of L-AP4 that resulted in a small reduction in the eEPSP amplitude was identified. 500 nM L-AP4 resulted in reduction in the eEPSP amplitude that was at the threshold for detection (90.5 ± 6.2% of baseline; Figure 3.1B). Surprisingly, PHCCC did not potentiate the response to L-AP4 at this synapse; 30 µM PHCCC, followed by the co-addition of 30 µM PHCCC + 500 nM L-AP4, resulted in no change in eEPSP amplitude (90.1 ± 7.1% of baseline; Figure 3.1C) relative to 500 nM L-AP4 alone.

The lack of effect of PHCCC was surprising and contrasts with the ability of this compound to potentiate mGlu4 activity at other synapses (Jones et al., 2008; Marino et al., 2003; Valenti et al., 2005). In addition, this finding does not align with the ability of the structurally distinct mGlu4–selective PAM Lu AF21934 to potentiate mGlu4 agonist effects at corticostriatal synapses (Bennouar et al., 2012). Interestingly, both the group II mGlu agonist LY379268 (Picconi et al., 2002) and the mGlu2 PAM cyPPTS (Johnson et al., 2005) inhibit excitatory transmission at corticostriatal synapses via a presynaptic mechanism of action, suggesting that mGlu2 receptors are also expressed on corticostriatal terminals. Recent studies including time-resolved FRET and co-expression studies have shown that mGlu2 and mGlu4 form heterodimers in vitro (Doumazane et al., 2011a; Kammermeier, 2012) and we hypothesized that mGlu4-containing heteromers may be expressed on corticostriatal terminals and may not display the same response to PHCCC as that observed with mGlu4 homomers.
To test this hypothesis, an mGlu$_{2/4}$ cell line was constructed by transfecting rat mGlu$_4$ into rat mGlu$_2$/HEK cells stably expressing G Protein Inwardly Rectifying Potassium (GIRK) channels, which allows assessment of receptor activity using a GIRK-mediated thallium flux assay (Niswender et al., 2008a). We established that the resulting mGlu$_{2/4}$ cell line expressed similar amounts of both mGlu$_2$ and mGlu$_4$ protein compared to the parental cell lines expressing either receptor alone (Figure 3.2). We then assessed the physical interaction between mGlu$_2$ and mGlu$_4$ using co-immunoprecipitation techniques. mGlu$_4$ antibodies immunoprecipitated mGlu$_4$ protein (∼240 kDa in dimeric form) from the cell lysate of the mGlu$_4$ and mGlu$_{2/4}$ cell lines (Figure 3.3B). A band of approximately 100 kDa was present in all of the immunoprecipitation (IP) samples and obscured specific identification of monomeric receptor protein; this band was present in IP samples without any cell lysate (data not shown), suggesting that it resulted from the antibody itself. In cells co-expressing mGlu$_2$ and mGlu$_4$, mGlu$_2$ proteins were co-precipitated along with mGlu$_4$ by mGlu$_4$ antibody-coupled beads (Figure 3.3D; ∼100 kDa and ∼240 kDa for monomeric and dimeric forms, respectively). In contrast, precipitation using the protein A/G beads alone did not yield any specific bands. Additionally, precipitated mGlu$_2$ was not detected in IPs from the control mGlu$_2$ or mGlu$_4$ cell line. To eliminate the possibility that mGlu$_2$ and mGlu$_4$ proteins nonspecifically aggregated after the cells were lysed, we mixed the cell lysates from the mGlu$_2$-expressing cell line and the mGlu$_4$-expressing cell line and subjected the mixed sample to co-IP. The absence of precipitated mGlu$_2$ in this mixed sample indicated that mGlu$_{2/4}$ complexes were formed before, but not during or after, the lysis process. We further confirmed the physical interaction between mGlu$_2$ and mGlu$_4$ by swapping the bait and prey in additional co-IP
Figure 3.1. The mGlu₄ PAM PHCCC fails to potentiate L-AP4-induced decreases in evoked EPSPs at corticostriatal synapses. EPSPs were recorded in medium spiny neurons following stimulation of the white matter between the cortex and striatum with a bipolar electrode. All compounds were bath applied. Data are normalized to the average baseline EPSP amplitude. Insets are sample traces from an individual, representative experiment (black – averaged traces from minute prior to L-AP4 application; gray – averaged traces from last minute of L-AP4 application). Slices were treated with 100 µM L-AP4 (A), 500 nM L-AP4 (B) or 30 µM PHCCC followed by co-application of 30 µM PHCCC and 500 nM L-AP4 (C). Solid and dashed lines represent time of compound additions. Values represent mean ± SEM (n=5).
Figure 3.2. Similar expression levels of mGlu$_2$ and mGlu$_4$ in various cell lines. 15 µg of cell lysates from mGlu$_2$, mGlu$_{2+4}$ and mGlu$_4$ cell lines were prepared as described. Receptor expression was analyzed by western blots using anti-mGlu$_2$ (panel A; 1:1000 dilution) and anti-mGlu$_4$ (panel B; 1:1000 dilution) antibodies.
experiments. When mGlu2 was used as the bait, mGlu4 proteins were also co-immunoprecipitated by mGlu2 antibodies only in the cell line that expressed both receptors (Figure 3.3H). These data support the hypothesis that mGlu2 specifically interacts in some manner with mGlu4 in vitro and can be co-immunoprecipitated with antibodies recognizing the native receptor proteins.

After optimizing conditions for co-immunoprecipitation of mGlu2 and mGlu4 in cell lines, we tested the hypothesis that these receptors interact in brain tissue. Both mGlu2 and mGlu4 are expressed in dorsal striatum and medial prefrontal cortex of Sprague-Dawley rats, as indicated by the immuno-reactive monomeric and dimeric bands in tissue lysates (Figure 3.4, input). While both mGlu4 antibodies and a rabbit IgG control generated antibody bands at around 100 kDa, mGlu4 antibodies were able to precipitate dimeric mGlu4 from the dorsal striatum and medial prefrontal cortex (~240 kDa in dimeric form; Figure 3.4A and B). Conversely, immunoprecipitation using rabbit IgG did not yield any mGlu4-specific bands. In addition, when detected using mGlu2-specific antibodies, we found that mGlu2 proteins were co-immunoprecipitated by mGlu4 antibodies in both monomeric and dimeric forms (~100 kDa and 240 kDa respectively), but not by rabbit IgG (Figure 3.4C and D). Similar results were also obtained in mouse dorsal striatum and medial prefrontal cortex (Figure 4E-H). Taken together, these data present the first evidence consistent with the existence of mGlu heteromers in vivo and suggest that mGlu2/4 heteromers may participate in the regulation of CNS function.
mGlu\textsubscript{2/4} heteromer differentially regulate the effect of mGlu\textsubscript{4} allosteric modulators

To further test the hypothesis that the lack of efficacy of PHCCC at the corticostriatal synapse was due to expression of mGlu\textsubscript{4}-containing heteromers, the pharmacology of mGlu\textsubscript{2/4} receptors was extensively characterized using the thallium flux assay (Niswender et al., 2008a). In these studies, the initial slopes (starting 5 seconds after thallium addition and then measured over a 10 second time span) of inward flux of thallium are calculated and plotted as agonist-induced concentration-response curves (samples traces of thallium flux assays are shown in Figure 3.5A-C and represent the data used to generate the L-AP4 curves in panel 3.5F). Glutamate, the orthosteric agonist for both mGlu\textsubscript{2} and mGlu\textsubscript{4}, exhibited a pEC\textsubscript{50} value of 6.22±0.03 in the mGlu\textsubscript{2/4} cell line. This potency was similar to the pEC\textsubscript{50} of glutamate at mGlu\textsubscript{2} (6.08±0.03) compared to that of mGlu\textsubscript{4} (4.79±0.03) (Figure 3.5D, Table 3.1). To further characterize each of the subtypes in the heterocomplex, more selective agonists were utilized for mGlu\textsubscript{2} (LY379268) or mGlu\textsubscript{4} (L-AP4) (Figure 5E and F). In the mGlu\textsubscript{2/4} cell line, the mGlu\textsubscript{2} agonist LY379268 elicited a full agonist response with a slightly reduced potency comparing to cells expressing mGlu\textsubscript{2} alone (Figure 3.5E, Table 3.1). Likewise, L-AP4, the mGlu\textsubscript{4} selective agonist, induced an agonist response with similar potency in cells expressing mGlu\textsubscript{2/4} and mGlu\textsubscript{4} alone. Unlike LY379268, L-AP4 was only able to elicit about 70% of the maximum response generated by glutamate in mGlu\textsubscript{2/4} cells. Additionally, we observed a significant decrease in the Hill slope of the curve fits for both LY379268 and L-AP4 in mGlu\textsubscript{2/4}-co-expressing cells (Table 3.1), indicating an interaction between the two proteins and supporting the hypothesis that the mGlu\textsubscript{2/4} complex possesses distinct pharmacological properties.
We then investigated whether mGlu\textsubscript{2} and mGlu\textsubscript{4} agonist sites interact with each other in mGlu\textsubscript{2/4}-expressing cells. A serial dilution of L-AP4 was applied either alone or in the presence of 0.5 nM or 1 nM LY379268. In the mGlu\textsubscript{4} cell line, neither concentration of LY379268 produced any effect on L-AP4 responses compared to buffer control (Figure 3.6A). When assessed in the mGlu\textsubscript{2/4} cell line, LY379268 dose-dependently shifted the L-AP4 dose response curve to the left (Figure 3.6B), suggesting a potentiating effect by co-activation of the mGlu\textsubscript{2} subunit. In support of an interaction between subunits, 0.1, 10 and 100 nM L-AP4 dramatically potentiate the responses induced by LY379268 as well (Figure 3.6C and D).

After assessing the activity of orthosteric agonists in cells expressing either receptor alone or expressing the combination, we moved to an analysis of potential effects on the pharmacology of allosteric modulators for mGlu\textsubscript{4} and mGlu\textsubscript{2} (structures shown in Figure 3.7). Advantages if focusing our studies on mGlu\textsubscript{2} and mGlu\textsubscript{4} are that 1) these receptors are co-expressed in many brain regions, and 2) there are a number of orthosteric and allosteric ligands that differentiate between mGlu\textsubscript{2} and mGlu\textsubscript{4}, allowing us to generate a tool set of ligands appropriate for native tissue studies. 10 µM PHCCC induced a 4.7±0.02 fold leftward shift of the L-AP4 response in cells expressing mGlu\textsubscript{4} alone.
Figure 3.3. mGlu\textsubscript{2} and mGlu\textsubscript{4} are co-immunoprecipitated from Human Embryonic Kidney (HEK) cells. mGlu\textsubscript{4} antibodies (A-D) or mGlu\textsubscript{2} antibodies (E-H) were used for co-immunoprecipitation. Cell lysates from mGlu\textsubscript{2/4}, mGlu\textsubscript{2}, and mGlu\textsubscript{4} cell lines, together with lysates from mGlu\textsubscript{2} cells and mGlu\textsubscript{4} cells that were mixed after lysis, were subjected to co-IP experiments. Cell lysates from mGlu\textsubscript{2/4} cells were also precipitated by protein A/G beads without antibody as a negative control (A/G control). Precipitated proteins from different samples were analyzed by western blots. Cell lysates before co-IP experiments were loaded as indication of IP input (left side panels). Molecular sizes of mGlu\textsubscript{2} or mGlu\textsubscript{4} (~100 kDa and ~240 kDa for monomeric and dimeric forms, respectively) are indicated with arrows.
IB: mGlu4 (1:1000)

RAT DORSAL STRIATUM

A

IB: mGlu2 (1:500)

B

MOUSE DORSAL STRIATUM

E

MOUSE PREFRONTAL CORTEX

F

IB: mGlu4 (1:1000)

G

IB: mGlu2 (1:500)

H
**Figure 3.4. mGlu₄ antibodies co-immunoprecipitate mGlu₂ protein from rodent dorsal striatum and medial prefrontal cortex.** Dorsal striatum and medial prefrontal cortex extracts from rat (A-D) or mouse (E-H) samples were prepared as described and tissue lysates were precipitated using anti-mGlu₄ antibody (IP-mGlu₄) or rabbit IgG (IgG Control). The precipitated proteins were analyzed via western blots and tissue lysates before IP experiments were loaded as IP input. Molecular sizes of mGlu₂ or mGlu₄ (~100 kDa and ~240 kDa for monomeric and dimeric forms, respectively) are indicated with arrows.
Figure 3.5. Orthosteric agonist responses are distinct in mGlu<sub>2</sub>, mGlu<sub>4</sub> or mGlu<sub>2/4</sub>-expressing cell lines. A-C, sample traces of L-AP4 responses in mGlu<sub>2</sub>, mGlu<sub>4</sub> and mGlu<sub>2/4</sub> cells with concentrations ranging from 0.1 nM to 10 µM. The initial slopes of the raw traces were used to generate concentration response curves shown in F. D-F, serial dilutions of glutamate (D), the group II agonist LY379268 (E) and the group III agonist L-AP4 (F) were applied to HEK/GIRK/mGlu<sub>2</sub> (■), HEK/GIRK/mGlu<sub>4</sub> (▲) and HEK/GIRK/mGlu<sub>2/4</sub> (●) cell lines and GIRK-mediated thallium flux was measured according to protocols described above. Responses were normalized to the maximal response induced by 1 mM glutamate in each individual cell line, and pEC<sub>50</sub> values for concentration-response curves are shown in Table 1. All values represent mean ± SEM (n≥3).
Table 3.1. Potencies and efficacies of orthosteric agonists in various cell lines. Data represent the Mean±SEM of at least three experiments performed in duplicate.

<table>
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<th>Hill Slope</th>
<th>pEC_{50}</th>
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<td>1.10±0.10</td>
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<td>N/A</td>
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<td>6.64±0.05</td>
<td>72.8±2.0</td>
<td>0.91±0.02</td>
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</tbody>
</table>

\( ^a p=0.0034 \) for mGlu\(_2\) versus mGlu\(_2/4\) lines; \(^b p=0.0161 \) for mGlu\(_2\) versus mGlu\(_2/4\) lines; 
\(^c p=0.0049 \) for mGlu\(_2\) versus mGlu\(_2/4\) lines; \(^d p<0.0001 \) for mGlu\(_2\) versus mGlu\(_2/4\) lines; 
\(^e p<0.0001 \) for mGlu\(_4\) versus mGlu\(_2/4\) lines; \(^f p<0.0001 \) for mGlu\(_4\) versus mGlu\(_2/4\) lines.

Unpaired Student’s T test (n≥3, two-tailed).
Figure 3.6. Orthosteric agonist of mGlu₂ and mGlu₄ potentiates effect of each other in mGlu₂₄-expressing cell line. Buffer control ( ■), 0.5 nM ( ●) or 1 nM LY379268 ( □) were added 140 s before addition of serial dilutions of L-AP4. GIRK channel-mediated thallium flux was measured as described in HEK/GIRK/mGlu₂, HEK/GIRK/mGlu₄ or HEK/GIRK/mGlu₂₄ cell line and was normalized to the maximal response induced by 1 mM glutamate. A and B, L-AP4 dose response curves were plotted in the absence or presence of LY379268 in mGlu₄ or mGlu₂₄ cells. C and D, the responses induced by 0, 0.5 or 1 nM LY379268 were plotted in the absence or presence of L-AP4 in mGlu₂ or mGlu₂₄ cells. Basal responses induced by 0.1, 10 or 100 nM L-AP4 alone were deducted.
(Figure 3.8A). In contrast, PHCCC only induced a negligible shift of the L-AP4 response in the mGlu2/4 cell line (1.7±0.04 fold, Figure 3.8B). When assessed using glutamate, 10 µM PHCCC shifted the concentration-response curve to the left by 3.5±0.53 fold in mGlu4 cells (Figure 3.8C) but did not potentiate the glutamate response in cells expressing both mGlu2 and mGlu4 (1.0±0.04 fold; Figure 3.8D). This loss of efficacy in the mGlu2/4 cell line is consistent with a previous report (Kammermeier, 2012) and aligns with the lack of significant potentiation of the L-AP4 response we observed with PHCCC at corticostriatal synapses (Figure 3.1C). The inability to potentiate mGlu2/4 heteromers was not limited to PHCCC alone. 4PAM-2 is a selective and efficacious PAM of mGlu4 which binds to the same allosteric site as PHCCC (Drolet et al., 2011c). In cells expressing mGlu4 alone, 10 µM 4PAM-2 shifted concentration-response curves of L-AP4 and glutamate by 18.8±2.6 and 15.2±3.1 fold, respectively (Table 3.2). When mGlu2 was co-expressed, however, 4-PAM2 only weakly potentiated the L-AP4 response (2.7±0.2 fold) and was completely ineffective at shifting the glutamate concentration-response curve (1.0±0.02 fold; Table 3.2).

VU0155041 is another mGlu4 PAM derived from a different chemical scaffold compared to PHCCC or 4PAM-2. Consistent with our previous report (Niswender et al., 2008b), VU0155041 (10 µM) shifted the L-AP4 and glutamate concentration-response curves to the left by 3.9±0.3 and 4.0±0.3 fold, respectively, in cells expressing mGlu4 alone (Figures 3.8E and G). Interestingly, VU0155041 also induced leftward shifts in the agonist concentration response curves in the mGlu2/4 cell line, shifting the L-AP4 response substantially (9.7±1.0 fold shift), retaining its efficacy in shifting the glutamate response (3.5±0.3 fold shift) (Figure 3.7F and H), and, surprisingly, even showing
significant potentiation of the LY379268 response (4.2±0.12 fold, Table 3.2). As VU0155041 is predicted to bind only to the mGlu4 protein and LY379268 should only activate mGlu2, this finding suggests that there is transactivation between the subunits within the heteromer. We noted a slight decrease in the maximal response induced by the VU0155041/glutamate combination (Figure 3.8H); this was not present when L-AP4 was used as the agonist (Figure 3.8) or when mGlu4 was expressed alone (Figure 3.8G). There are several possibilities that may explain this phenomenon. For example, there could be differences in receptor desensitization induced by the allosteric agonist activity of VU0155041 (Niswender et al., 2008b). When the endogenous agonist activity of VU0155041 was assessed in the absence of orthosteric agonist, the potency of VU0155041 was similar in cells expressing mGlu4 versus those containing mGlu2/4 as was the maximal level of potentiation (pEC50 value, mGlu4, -5.38±0.17, and mGlu2/4 cells, 5.25±0.10 (p=0.5719); maximal response, mGlu4, 40.5±4.8% of glutamate maximal response, and mGlu2/4 cells, 38.9±6.3% of glutamate maximal response (p=0.8459)), suggesting differential desensitization is not the cause of this discrepancy. It is possible that the use of glutamate in these experiments may contribute to this change in maximal response as glutamate will activate both mGlu2 and mGlu4. The decrease in the maximal response occurs at glutamate concentrations that would activate both mGlu2 and mGlu4, suggesting that there could be unique responses elicited when both the mGlu2 and mGlu4 orthosteric sites are occupied. The use of L-AP4, however, would not carry such a caveat, and the maximal responses in Figure 3.7F are similar with or without VU0155041. The enhanced ability of VU0155041 to potentiate L-AP4 responses was not due to non-
Figure 3.7. Structures of allosteric ligands used in these studies.
Figure 3.8. The efficacies of PHCCC and VU0155041 are differentially regulated by mGlu$_{2/4}$ co-expression. A-H, 10 µM compound (■) or DMSO (●) were added 140 s before addition of serial dilutions of L-AP4 or glutamate. GIRK channel-mediated thallium flux was measured as described in HEK/GIRK/mGlu$_4$ (left panels) and HEK/GIRK/mGlu$_{2/4}$ (right panels) cell lines. Responses were normalized to the maximal response induced by 1 mM glutamate in each individual cell line. pEC$_{50}$ values for dose response curves in panels A-D without or with PHCCC were: A, 6.61±0.11 vs. 7.28±0.11 (p=0.0115); B, 6.63±0.06 vs. 6.85±0.05 (p=0.0557); C, 4.77±0.05 vs. 5.27±0.10 (p=0.0017); D, 6.11±0.05 vs. 6.09±0.06 (p=0.8383). pEC$_{50}$ values for dose response curves in panel E-H without or with VU0155041 were: E, 6.61±0.11 vs. 7.21±0.08 (p=0.0113); F, 6.63±0.06 vs. 7.61±0.07 (p=0.0004); G, 4.77±0.05 vs. 5.36±0.04 (p<0.0001); H, 6.11±0.05 vs. 6.64±0.02 (p<0.0001). All values represent mean ± SEM (n≥3).
Table 3.2. The ability of mGlu4 PAMs to left-shift agonist concentration-response curves is distinct for different groups of PAMs. Data represent the Mean ± SEM of at least three experiments performed in duplicate. a p=0.0009 for mGlu4 versus mGlu2/4 lines; b p<0.0001 for mGlu4 versus mGlu2/4 lines; c p=0.0003 for mGlu4 versus mGlu2/4 lines; d p=0.0034 for mGlu4 versus mGlu2/4 lines; e p<0.0001 for mGlu2 versus mGlu2/4 lines; f p=0.0054 for mGlu4 versus mGlu2/4 lines; g p=0.0175 for mGlu4 versus mGlu2/4 lines; h p<0.0001 for mGlu2 versus mGlu2/4 lines; i p=0.0021 for mGlu4 versus mGlu2/4 lines. Unpaired student’s t-test (n≥3, two-tailed). Note the ability of VU0155041 to shift responses of the mGlu2 agonist, LY379268.
selective activity of VU0155041 on mGlu2 receptors (Table 3.2, (Niswender et al., 2008b)). These results reveal that VU0155041 can be used as a chemical probe to potentiate responses to activation of mGlu2/4 heterodimers.

The marked distinction between VU0155041 and PHCCC led us to speculate that the divergence in effect between the two PAMs may arise from their different chemical structures or binding sites; PHCCC and 4-PAM2 have been reported to bind to the same site on mGlu4 while VU0155041 appears to bind to a distinct site on the mGlu4 protein ((Drolet et al., 2011c), assessed using a racemic mixture of VU0155041 regioisomers). Consistent with this hypothesis, the VU0155041-related compound, Lu AF21934, also exhibited an enhanced ability to potentiate L-AP4 responses (3.9±0.1 fold for mGlu4 cells alone and 8.2±1.0 fold for mGlu2/4 cells) and retained the ability to potentiate glutamate responses (3.4±0.1 fold, mGlu4 cells; 2.7±0.2 fold, mGlu2/4 cells, Table 3.2) in cells co-expressing both receptors.

To gain further insight into the mechanism of the pharmacological changes, the ability of increasing amounts of compound to induce progressive leftward shifts in agonist concentration-response curves was measured using each individual mGlu4 PAM and the operational model of allosterism was applied to compare the affinity (log K_B) and cooperativity (log αβ—a combined parameter that represents the effects of a modulator on affinity (α) as well as effects on efficacy (β)) of PAMs in cells expressing mGlu4 alone vs. mGlu2/4. As shown in Table 3.3, the estimated affinity of PHCCC was similar in mGlu2/4 cells compared to cells expressing mGlu4 alone. However, the positive cooperativity of PHCCC decreased significantly, from 0.57 ± 0.03 in the mGlu4 cell line
to 0.12 ± 0.02 in mGlu2/4 cell line (p=0.0017). Similar to PHCCC, 4PAM-2 also
demonstrated a significant decrease in positive cooperativity in mGlu2/4 cells (log αβ:
1.29±0.04 in mGlu4 vs. 0.82±0.02 in mGlu2/4 cells, p=0.0006); again, the affinity of the
compound was not significantly different in the cell line expressing both receptors (Table
3.3). In contrast, VU0155041 and Lu AF21934 exhibited significant changes in affinity
(Log K_B -5.27±0.01 vs. -4.78±0.13 for VU0155041, p=0.0188 and -5.88±0.04 vs. -
5.26±0.09 for Lu AF21934, p=0.0042) as well as increases in positive cooperativity (log
αβ 0.95 ± 0.05 vs. 1.83 ± 0.27 for VU0155041, p=0.0334 and 0.66 ± 0.03 versus 1.7 ±
0.11 for Lu AF21934, p=0.0008) in mGlu2/4 expressing cells compared to cells
expressing mGlu4 alone. These data suggest the altered pharmacology of mGlu4 PAMs is
due to differential changes in their positive cooperativity.

The lack of efficacy of PHCCC and 4PAM-2 suggests that the mGlu2/4 cell line in which
we performed our studies contains few or no mGlu4 homomers, indicating that mGlu2/4
interactions may be dominant and actually preferred. To further probe the interactions
between the receptors, we transiently transfected either increasing amounts of mGlu2
alone, increasing amounts of mGlu2 in the presence of a constant amount of mGlu4, or
increasing amounts of mGlu2 in the presence of another group III mGlu, mGlu7. mGlu7
was chosen as Kammermeir previously reported that mGlu2 and mGlu7 do not appear to
interact in the same fashion as mGlu2 and mGlu4, suggesting that there is some specificity
to the interaction (Kammermeier, 2012). In these studies, we observed gradual increases
in the maximal LY379268 response when mGlu2 was expressed alone in increasing
amounts (data not shown); at the concentrations used here, we saw no significant
differences in the potency (Figure 3.9A) or Hill slope (Figure 3.9B) of the LY379268 response when mGlu₂ was assessed in the absence of other mGlus. In the presence of mGlu₇, responses appeared similar to those in which mGlu₂ alone was expressed, with no differences in LY379268 potency or Hill slope (Figure 3.9A and B). In the presence of mGlu₄, however, the potency of LY379268 was progressively shifted to the left when mGlu₂ levels were increased; additionally, the Hill slope of the curve fit also progressively increased, indicating alterations in cooperativity between the subunits. These differences suggest that the mGlu₂/₄ combination appears to be distinct from that of mGlu₂/₇, indicating some specificity in this interaction and confirming previous work by Kammermeier.

To further demonstrate that the altered pharmacology of mGlu₄ PAMs was due to mGlu₂/₄ interaction, we tested the activity of mGlu₄ PAMs after transient transfection of increasing amounts of mGlu₂ in the presence of a constant amount of mGlu₄. In these experiments, a 30 µM concentration of each PAM was used. In cells transfected with just mGlu₄, 30 µM of PHCCC induced 7.2±1.5 and 8.5±0.3 fold leftward shifts of the glutamate or L-AP4 concentration-response curves, respectively. However, in cells co-transfected with 0.1, 0.2 and 0.5 µg mGlu₂ DNA, the shift of the L-AP4 response progressively decreased to 5.8±1.2, 3.5±0.6 and 3.4±0.2 fold, and in cells transfected with equal amounts of mGlu₂ and mGlu₄, the shift was only 1.9±0.5 fold (Figure 3.9C and Table 3.4). In addition, the shift of the glutamate response, even with only 0.1 µg of mGlu₂ DNA present (10% of the amount of mGlu₄) drastically decreased to only 1.3±0.1 fold (Figure 3.8D), suggesting a quite dramatic and dominant effect induced by the
presence of mGlu2. As with the responses observed with PHCCC, 4PAM-2 demonstrated similar efficacy changes in transiently transfected cells, which is consistent with our findings in the stable cell lines and the observation that these two PAMs bind to the same allosteric pocket. Interestingly, the potentiation induced by VU0155041 and Lu AF21934 remained similar as the amount of mGlu2 increased, further supporting the observation that distinct classes of mGlu4 PAMs are differentially regulated by mGlu2/4 interactions. It should be noted that, in these experiments, the similarity in potencies and Hill slopes of LY379268 in mGlu2,4-expressing cells when the mGlu2:4 ratio is 1:1, compared to cells expressing mGlu2 alone, suggest that mGlu2 homomers may exist along with mGlu2/4 heteromers in these experiments. In contrast, our data also suggest that mGlu2/4 heteromers are the dominant entity for mGlu4 in these experiments. This interpretation is supported by the lack of potentiation induced by PHCCC and 4PAM-2 when glutamate is used as the agonist and the ratio of mGlu2 to mGlu4 is 1:10, suggesting that co-expression of even small amounts of mGlu2 dramatically regulates activity of mGlu4.

**Co-addition of mGlu2 PAM and mGlu4 PAM does not result in further potentiation**

We were also intrigued to investigate the cooperativity of mGlu2 PAM and mGlu4 PAM and examined if co-addition of these compounds led to further potentiation in mGlu2/4 cells compared to the effect induced by single PAM. 1 μM BINA, an mGlu2 PAM, was applied either alone or in combination with mGlu4 PAM VU0155041 (Figure 3.10A). 1 μM BINA, when added alone, induced allosteric agonist activity as can be seen by the elevated baseline of the dose response curve, and produced a 2.9±0.8 fold shift of the glutamate response. Consistent with previous data, 10 μM VU0155041 was able to shift
Table 3.3. Analysis of mGlu4 PAMs using the operational model of allosterism reveals differential alterations in affinity or cooperativity for distinct groups of PAMs. Data were generated by progressive fold shift experiments using increasing concentrations of four mGlu4 PAMs (ranging from 0 to 30 µM) prior to application of a full concentration-response range of L-AP4 (ranging from 0.1 nM to 10 µM). The log $K_A$ of L-AP4 for mGlu4 was set to -6.759 according to literature values (Monastyrskaia et al., 1999). For PHCCC and 4PAM-2, log $\tau_B$ was set to -100 due to the lack of allosteric agonist activity but was allowed to float for compounds exhibiting allosteric agonism (VU0155041, Lu AF29134). Data represent the Mean±SEM of at least three experiments performed in duplicate. $^a$ p=0.0017 between mGlu4 cells and mGlu2/4 cells; $^b$ p= 0.0006 between mGlu4 cells and mGlu2/4 cells; $^c$ p=0.0188 between mGlu4 cells and mGlu2/4 cells; $^d$ p=0.0334 between mGlu4 cells and mGlu2/4 cells; $^e$ p=0.0042 between mGlu4 cells and mGlu2/4 cells; $^f$ p=0.0008 between mGlu4 cells and mGlu2/4 cells. Unpaired student’s t-test (n≥3, two-tailed).
Table 3.4. Differential leftward fold shifts of glutamate or L-AP4 concentration-response curves are induced by different PAMs after transient expression of increasing amounts of mGlu2 (0, 0.1, 0.2, 0.5 or 1 µg) with a constant amount (1 µg) of mGlu4. Data represent the Mean ± SEM of three independent experiments performed in duplicate. Data were analyzed using one way ANOVA and Dunnett’s Multiple Comparison Test comparing to cells transfected with mGlu4 alone: *p<0.05, **p<0.01, ***p<0.001.

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<td>Lu AF21934</td>
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Figure 3.9. Co-expression of varying amounts of mGlu$_2$ and mGlu$_4$ regulates responses to both orthosteric and allosteric ligands. HEK/GIRK cells were transfected with 0, 0.1, 0.2, 0.5 or 1 µg mGlu$_2$ DNA in the absence or presence of co-transfection of 1 µg of vector control, 1 µg mGlu$_7$, or 1 µg mGlu$_4$. A and B. Potencies and Hill slope of the LY379268 response were determined; responses were unaffected by empty vector or mGlu$_7$ but dramatically altered in the presence of mGlu$_4$. C and D. The fold shifts induced by 30 µM of PHCCC, 4PAM-2, VU0155041 or Lu AF21934 are summarized for L-AP4 (C) and glutamate (D) using bar graphs. Values represent mean ± SEM (n=3).

Statistics were performed using 1way ANOVA, Bonferroni’s Multiple Comparison Test was used for A and B and Dunnett’s Multiple Comparison Test was used for C and D. * denotes p < 0.05; ** denotes p<0.01 and *** denotes p<0.001.
100

Figure 3.10. Co-addition of mGlu$_2$ PAM and mGlu$_4$ PAM does not result in further potentiation. A-C, DMSO control (■), 1 µM BINA (▼), 10 µM mGlu$_4$ PAM (●, VU0155041, PHCCC or 4PAM-2) or BINA plus mGlu$_4$ PAM (□) were added 140 s before addition of serial dilutions of glutamate. GIRK channel-mediated thallium flux was measured as described in HEK/GIRK/mGlu$_2$/4 cell line and was normalized to the maximal response induced by 1 mM glutamate. pEC$_{50}$ values for dose response curves of DMSO and 1 µM BINA were 6.10±0.05 and 6.48±0.16, respectively. pEC$_{50}$ values for dose response curves of mGlu$_4$ PAM and BINA plus mGlu$_4$ PAM in panels A-C were: A, 6.64±0.02 vs. 6.79±0.05; B, 6.09±0.06 vs. 6.44±0.15; C, 6.12±0.05 vs. 6.60±0.14. All values represent mean ± SEM (n≥3).
glutamate dose response curve to the left by 3.5±0.3 fold. When added together, the induced fold shift was 5.0±0.3 fold, considerably lower than the expected value if the mGlu$_2$ PAM and mGlu$_4$ PAM exhibited an additive or cooperative effect. As shown in Table 3.2, PHCCC or 4PAM-2 was not able to shift glutamate responses when added alone in mGlu$_{2/4}$ cells (Figure 3.10 B and C). The effect of these two mGlu$_4$ PAMs were then assessed to determine if their efficacies could be modified in the presence of BINA. Co-addition of BINA resulted in a fold shift value that is not significantly higher than BINA alone (2.61±0.81 for PHCCC and 3.37±0.96 for 4PAM-2), suggesting no cooperative effect between mGlu$_2$ and mGlu$_4$ PAM sites.

**Allosteric modulators of mGlu$_2$ are also differentially regulated upon formation of mGlu$_{2/4}$ heteromer**

We also sought to investigate the influence of mGlu$_{2/4}$ interaction on mGlu$_2$ allosteric modulators. CBiPES and LY487379 are two PAMs from the pyridylmethylsulfonamide series that selectively potentiate mGlu$_2$ (Johnson et al., 2003; Johnson et al., 2005), whereas BINA was identified from a different chemical scaffold (Galici et al., 2006). We took advantage of these structurally distinct compounds and compared their efficacy in cells expressing mGlu$_2$ alone versus cells co-expressing mGlu$_2$ and mGlu$_4$. As shown in Table 3.5, none of the mGlu$_2$ PAMs potentiated responses to the mGlu$_4$ agonist L-AP4 in either mGlu$_4$ or mGlu$_{2/4}$-expressing cells. 1 µM BINA induced a 6.7 ±0.6 fold leftward shift of the glutamate concentration-response curve in mGlu$_2$ cells (Figure 3.11A). However, this number significantly decreased, to 3.0 ± 0.5 fold, in cells expressing both
mGlu$_2$ and mGlu$_4$ (Figure 3.11B). Likewise, the fold shift of the LY379628 response significantly decreased from 4.0 ± 1.1 fold in mGlu$_2$ cells to 1.7 ± 0.2 fold in mGlu$_2$/4 cells (Figure 3.11C, D). In contrast, the ability of the mGlu$_2$ PAMs LY487379 and CBiPES to potentiate either glutamate or LY379268 responses was not significantly altered (Figure 3.11E-L), suggesting that distinct mGlu$_2$ PAMs also possess different pharmacological profiles when mGlu$_2$ is expressed alone relative to when mGlu$_2$ and mGlu$_4$ are co-expressed.

In contrast to what was observed with VU0155041/Lu AF21934 and their ability to potentiate LY379268 responses, we did not see potentiation of L-AP4 responses with any of the mGlu$_2$ PAMs. Each of the mGlu$_2$ PAMs used here exhibits similar potentiation of mGlu$_2$ responses compared to the responses of VU0155041 and Lu AF21934 at mGlu$_4$ (3-6 fold), suggesting that our assay system should be sensitive enough to detect potentiation of an mGlu$_4$ agonist response. However, in contrast to responses to mGlu$_4$ agonists, we would note that there is possibly some masking of potentiation due to expression of mGlu$_2$ homodimers in our cells. Each of the mGlu$_2$ PAMs used here shows some degree of allosteric agonist activity, which may complicate measurement of the signal when L-AP4 is used as the orthosteric agonist. In contrast to mGlu$_4$, where we hypothesize most of the receptors are in heteromeric form, this may result in a loss of sensitivity. Additionally, these compounds could be engaging distinct sites on each receptor that translate to distinct abilities to induce potentiation. Our data actually are most consistent with the hypothesis that the two halves of the heteromer may not function symmetrically or may differentially interact with signaling components, such as G-
proteins. If correct, this might suggest that one half of the dimer may be more sensitive to potentiation (or antagonism). While this remains to be determined experimentally for mGlu$_{2/4}$ heteromers and will require an assay system in which absolutely no mGlu$_2$ homomers are present, it could eventually contribute to signaling differences induced downstream of heteromic receptors when specific modulators are used.

Unlike PHCCC, all mGlu$_2$ PAMs evaluated had some activity at both mGlu$_2$ and mGlu$_{2/4}$, suggesting that either these cells express some level of homomeric mGlu$_2$ or that the effect on potentiation is not as dramatic as that observed with the mGlu$_4$ PAMs examined thusfar; regardless, the ability of these PAMs to each retain some degree of potentiation indicates that these compounds may not be useful as selective probes for differentiating homomeric versus heteromeric receptors in native systems.

In addition to the evaluation of the efficacy of mGlu$_2$ PAMs, we also assessed the efficacy of MNI-137, a selective group II mGlu NAM, on mGlu$_2$ versus mGlu$_{2/4}$ responses (Figure 3.12). For these studies, we chose to use the group II agonist DCG-IV, as LY379268 will weakly activate mGlu$_4$ at the higher concentrations needed to assess whether any potential interaction was competitive or noncompetitive in nature. In cells expressing mGlu$_2$ alone, increasing concentrations of MNI-137 non-competitively antagonized activation of mGlu$_2$ by DCG-IV, completely abolishing the response (Figure 3.10A). Consistent with its previously reported selectivity profile (Hemstapat et al., 2007), MNI-137 showed no significant effect in blocking L-AP4 responses in cells expressing mGlu$_4$ alone (Figure 3.10B). In mGlu$_{2/4}$ cells, MNI-137 was still able to
Table 3.5. The efficacy of mGlu₂ PAMs to left-shift agonist concentration-response curves differs between classes of PAMs. Data represent the Mean ± SEM of three independent experiments performed in duplicate. \( ^a \)\( p=0.0003 \) for mGlu₂ versus mGlu₂/4 lines; \( ^b \)\( p=0.0466 \) for mGlu₂ versus mGlu₂/4 lines. Unpaired student’s t-test (\( n\geq3 \), two-tailed).
mGlu₂ cells

BINA

Log Glutamate, [M] Percent Max Glutamate Response

-10 -9 -8 -7 -6 -5 -4 -3 -2 0 20 40 60 80 100 120 140

1 uM BINA

BINA

Log LY379268, [M] Percent Max Glutamate Response

-12 -11 -10 -9 -8 -7 -6 -5 -4 0 20 40 60 80 100 120 140

LY487379

CBiPES

Log Glutamate, [M] Percent Max Glutamate Response

-12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 0 20 40 60 80 100 120 140

1 uM CBiPES
Figure 3.11. The efficacies of mGlu2 PAMs are also differentially regulated by mGlu2/4 co-expression. 1 µM compound (■) or DMSO (●) were added 140 s before addition of serial dilutions of glutamate or LY379268. GIRK channel-mediated thallium flux was measured as described in HEK/GIRK/mGlu2 (left panels) and HEK/GIRK/mGlu2/4 (right panels) cell lines. Responses were normalized to the maximal response induced by 1 mM glutamate in each individual cell line. pEC50 values for concentration response curves in panels A-D without or with BINA were: A, 6.01±0.03 vs. 6.81±0.05 (p<0.0001); B, 6.18±0.04 vs. 6.59±0.10 (p<0.0001); C, 8.25±0.02 vs. 8.79±0.10 (p<0.0001); D, 8.02±0.09 vs. 8.22±0.13 (p=0.0058). pEC50 values for dose response curves in panel E-H without or with LY487379 were: E, 5.96±0.01 vs. 6.47±0.03 (p<0.0001); F, 6.27±0.01 vs. 6.69±0.05 (p<0.0001); G, 8.20±0.02 vs. 8.52±0.05 (p=0.0003); H, 8.15±0.03 vs. 8.45±0.05 (p=0.0009). pEC50 values for dose response curves in panel I-L without or with CBiPES were: I, 5.97±0.01 vs. 6.76±0.02 (p<0.0001); J, 6.25±0.01 vs. 6.96±0.03 (p<0.0001); K, 8.20±0.01 vs. 8.68±0.01 (p<0.0001); L, 8.13±0.05 vs. 8.54±0.08 (p=0.0132). All values represent mean ± SEM (n≥3) and statistics were performed using unpaired t test.
Figure 3.12. MNI-137 exhibits reduced efficacy when mGlu4 and mGlu2 are co-expressed and non-competitively antagonizes mGlu4-mediated responses in mGlu2/4-expressing cells. A and B, the effect of MNI-137 on DCG-IV responses were tested in the mGlu2 or mGlu2/4 cell line. C and D, the effect of MNI-137 on L-AP4 responses was tested in mGlu4 or mGlu2/4 cell line. DMSO (●) or 100 nM, 300 nM, 1 µM or 10 µM MNI-137 were added 140 s before addition of serial dilutions of LY379268 or L-AP4. GIRK channel-mediated thallium flux was measured as described and responses were normalized to the maximal response induced by 1 mM glutamate in each individual cell line. All values represent mean ± SEM (n=3).
antagonize DCG-IV responses in a non-competitive manner (Figure 3.12C). Analysis using the operational model of allosterism suggested that the affinity of MNI-137 was slightly higher in mGlu2/4 cells. In addition, MNI-137 demonstrates increased cooperativity for affinity modulation but decreased cooperativity for efficacy modulation at mGlu2/4 (Table 3.6), evidenced by an inability to completely abolish the response to DCG-IV. Surprisingly, MNI-137 was also able to noncompetitively block activation of the mGlu4 subunit induced by stimulation with L-AP4, further supporting a structural and functional inter-subunit interaction within the mGlu2/4 complex and suggesting that MNI-137 binding to mGlu2 can negatively modulate the function of mGlu4. Since MNI-137 does not block responses to L-AP4 unless mGlu2 is co-expressed with mGlu4, this NAM provides an excellent tool to evaluate responses to L-AP4 that may be mediated by mGlu2/4 in native systems.

**mGlu2 and 4 allosteric modulators exhibit unique pharmacological effect at the corticostriatal synapse**

The unique pharmacology of VU0155041 and MNI-137 on mGlu2/4-elicited responses suggests that these compounds provide a pair of tool compounds that can be utilized to provide evidence for the existence of mGlu2/4 heteromers in native systems. Therefore, the effect of these two compounds were tested at the corticostriatal synapses by Meredith Noetzel and Kari Johnson in our laboratory. Treatment of slices with 10 µM VU0155041, followed by the co-addition of 10 µM VU0155041 and 500 nM L-AP4, resulted in a robust decrease in the eEPSP amplitude (51.3 ± 4.0% of baseline; Figure 3.13) relative to that observed with 500 nM L-AP4 alone (90.5 ± 6.2 % of baseline; Figure 3.1). These results, together with the lack of efficacy of PHCCC at the corticostriatal synapse, suggest that, in a native system, the potentiation of the L-AP4 responses by mGlu4 PAMs mimics the differential responses observed in cells co-expressing mGlu2 and mGlu4.
Table 3.6. MNI-137 exhibits enhanced affinity but decreased efficacy in modulating DCV-IV responses in mGlu<sub>2/4</sub> cells compared to cells expressing mGlu<sub>2</sub> alone. Data were analyzed using the operational model of allosterism as described in the Materials and Methods. The log<sub>K_A</sub> of DCG-IV for mGlu<sub>2</sub> was set to -6.959 according to literature values; log<sub>τ_B</sub> was set to -100. Data represent the Mean ± SEM of three independent experiments performed in duplicate. <sup>a</sup><i>p</i>=0.0028 between mGlu<sub>2</sub> cells and mGlu<sub>2/4</sub> cells; <sup>b</sup><i>p</i>=0.0206 between mGlu<sub>2</sub> cells and mGlu<sub>2/4</sub> cells. Unpaired student’s t-test (n≥3, two-tailed).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>mGlu&lt;sub&gt;2&lt;/sub&gt;</th>
<th>mGlu&lt;sub&gt;2/4&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log &lt;i&gt;K_A&lt;/i&gt;</td>
<td>-6.82±0.04</td>
<td>-7.18±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Log &lt;i&gt;α&lt;/i&gt;</td>
<td>-0.47±0.05</td>
<td>-0.74±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Log &lt;i&gt;β&lt;/i&gt;</td>
<td>-100</td>
<td>-0.69±0.04</td>
</tr>
</tbody>
</table>
Furthermore, these results suggest that homomeric mGlu4 receptors, which would be predicted to respond to PHCCC, are expressed at extremely low abundance, if at all, in these synaptic terminals.

If mGlu2/4 heteromeric receptors play a dominant role in regulating transmission at corticostriatal synapses, we would also predict that the mGlu2 and mGlu2/4 NAM MNI-137 would inhibit the effect of L-AP4 at this synapse. Treatment of slices with L-AP4 (100 µM) robustly inhibited the amplitude of electrically evoked EPSPs in striatal MSNs (62.3 ± 4.8% of baseline, Figure 3.14A). Bath application of MNI-137 (10 µM) for 10 min produced a small increase in EPSP amplitude (112.7 ± 3.5% of baseline, Figure 3.14B). Following pretreatment with MNI-137, 10 min bath application of L-AP4, in combination with MNI-137, returned EPSP amplitudes to baseline values (100.1 ± 3.4% of baseline, Figure 3.14B) and produced significantly less inhibition of EPSP amplitude compared with L-AP4 alone. The average inhibition of EPSP amplitude for L-AP4 alone was 37.1 ± 5.8%, whereas the L-AP4-induced inhibition of EPSP amplitude following MNI-137 treatment was only 12.5 ± 2.7% (P<0.05, unpaired t test, Figure 3.14C). These results indicate that an mGlu2 NAM can regulate the responses of an mGlu4 agonist at corticostriatal synapses, providing additional evidence for mGlu2/4 heteromer expression in vivo.
Figure 3.13. In contrast to PHCCC, VU0155041 potentiates the L-AP4-induced decrease in evoked EPSPs at the corticostriatal synapse. EPSPs were recorded in medium spiny neurons following stimulation of the white matter between the cortex and striatum with a bipolar electrode. All compounds were bath applied. Data are normalized to the average baseline EPSP amplitude. Insets are sample traces from an individual experiment (black – averaged traces from minute prior to L-AP4 application; gray – averaged traces from last minute of L-AP4 application). Slices were treated with 10 µM VU0155041 followed by co-application of 10 µM VU0155041 and 500 nM L-AP4. Two slices exhibited responses when VU0155041 was applied alone. Solid and dashed lines represent time of compound additions (A). Bar graphs summarizing the normalized peak EPSP response measured during the last two minutes of compound addition (B). Values represent mean ± SEM (n=5). * denotes p < 0.05 when compared to 500 nM L-AP4 using Dunnett’s Multiple Comparison Test.
Figure 3.14. MNI-137 blocks L-AP4-induced inhibition of corticostriatal transmission. EPSPs were recorded in medium spiny neurons following stimulation of the white matter between the cortex and striatum with a bipolar electrode. Data are normalized to the average baseline EPSP amplitude. Insets represent sample traces from a representative experiment (black – averaged traces from minute prior to L-AP4 application; gray – averaged traces from last minute of L-AP4 application). Slices were treated with 100 µM L-AP4 alone (A) or following a 10 minute pretreatment with 10 µM MNI-137 (B). (C) Bar graph representation of the inhibition of corticostriatal transmission by L-AP4 in the absence (control) and presence of MNI-137. The difference between EPSP amplitudes during the minute prior to L-AP4 application and the last minute of L-AP4 application were calculated for each cell, and the average difference for each treatment group is shown in the bar graph. Data represent mean ± SEM (n=5-7). * denotes P < 0.05, unpaired t test.
As recombinant cell lines are used to identify and characterize allosteric reagents, it is important to recognize potential discrepancies between \textit{in vitro} and \textit{in vivo} properties of these novel compounds. For example, MMPIP, an allosteric antagonist of mGlu\textsubscript{7}, inhibits mGlu\textsubscript{7} activity in recombinant cell lines (Mitsukawa et al., 2005; Niswender et al., 2010; Suzuki et al., 2007) ; however, when applied to brain slices, it fails to inhibit mGlu\textsubscript{7}-mediated L-AP4 responses at SC-CA1 synapses (Niswender et al., 2010). We show here that PHCCC fails to potentiate mGlu\textsubscript{4} at corticostriatal synapses, despite its well-established efficacy \textit{in vitro} and at other synapses. These data suggest that the activity of allosteric compounds may be context-specific and could be dramatically altered by the \textit{in vivo} environment, such as differential expression of signaling pathway components or variations in receptor assembly.

By performing co-IP experiments in dorsal striatum and medial prefrontal cortex from both rat and mouse, our data demonstrate mGlu\textsubscript{2/4} interaction in native tissue. The detection of mGlu\textsubscript{2/4} heteromers in dorsal striatum is consistent with electrophysiology data that both mGlu\textsubscript{2} and mGlu\textsubscript{4} act presynaptically to reduce excitatory transmission at corticostriatal synapses (Bennouar et al., 2012; Johnson et al., 2005), although mGlu\textsubscript{2} and mGlu\textsubscript{4} may colocalize on other axon terminals in the striatum as well. Similarly, our medial prefrontal cortex samples contain many axon terminals where mGlu\textsubscript{2} and mGlu\textsubscript{4} might colocalize. While we cannot conclude the precise localization of mGlu\textsubscript{2/4} heteromers in this region, physiological evidence suggests that presynaptic expression of
mGlu$_2$ and mGlu$_4$ at thalamocortical synapses (Benneyworth et al., 2007; Marek et al., 2000; Zhang and Marek, 2007) is also a potential source of the mGlu$_{2/4}$ heterocomplexes.

We have observed altered efficacies of mGlu$_4$ PAMs in cells co-expressing mGlu$_2$ and mGlu$_4$ (Figure 3.8 and Table 3.2). These data are consistent with Kammermeier’s finding in *in vitro* cultured neurons co-injected with mGlu$_2$ and mGlu$_4$ cDNA (Kammermeier, 2012). We have also shown that effects of mGlu$_2$ allosteric modulators can also be significantly altered. Modeling of mGlu$_4$ PAM interactions using the operational model of allosterism suggests that binding to two distinct allosteric pockets results in differential pharmacological profile changes with regards to affinity and cooperativity. This suggests that these allosteric binding pockets may encounter differential conformation changes upon heterointeraction of the two receptor subunits, although more detailed structural studies are needed.

In our experiments, mGlu$_2$ and mGlu$_4$ were co-transfected without being forced to form an interaction. Advantages of this approach are that it avoids tagging of the receptors, which may affect pharmacology, and that it more closely mimics the receptor assembly in an *in vivo* environment where mGlu homomers and heteromers may co-exist. At the receptor levels expressed in our stable cell lines, the pharmacology of orthosteric agonists, such as LY379268 and L-AP4, was not dramatically altered in the mGlu$_{2/4}$ line (Figure 3.5). However, although both agonists elicited a response alone, the Hill slopes of the concentration-response curves were significantly decreased compared to cells expressing a single mGlu subtype, suggesting an interaction between the subunits. We did observe
differences in the ability of LY379268 and L-AP4 to achieve maximal responses (LY379268 was a full agonist while L-AP4 only achieved a 70% maximal response), which could result from the presence of mGlu2 homodimers under these experimental conditions. In contrast, our data appear to be consistent with little to no expression of mGlu4 homodimers in our mGlu2/4 cell line. For example, the fact that PHCCC and 4PAM-2 exhibit significantly decreased/no efficacy in potentiating L-AP4 responses suggests that most mGlu4 subunits in mGlu2/4 cell line appear to be in a complexed form. While we cannot definitively conclude that the receptors are forming strict heterodimers (as opposed to oligomers) in our system, the work of Doumazane et al. suggests that mGlu2 and mGlu4 appear to form heterodimers, rather than higher order oligomers, in vitro (Doumazane et al., 2011a).

To overcome the caveat of mGlu2 homodimers in the stable cell line, we performed transient transfection experiments with a constant amount of mGlu4 or mGlu7 and variable amounts of mGlu2. Results from these studies suggest that expression of mGlu4 specifically results in changes in the potency and cooperativity of an mGlu2 orthosteric agonist, and that alterations in mGlu4 PAM pharmacology are dependent on the amount of mGlu2 co-expression. Quite strikingly, 1/10 of the amount of mGlu2 compared to mGlu4 resulted in a nearly complete loss of potentiation of the glutamate response by PHCCC and 4PAM-2, suggesting that the heterointeractions may be dominant.

We would note that, when mGlu2 and mGlu4 were co-transfected in similar amounts, the potency and Hill slope of the LY379268 response was the same as when mGlu2 is
expressed alone. The lack of effect of PHCCC and 4-PAM2, along with the similarities in Hill slope and potency once the amount of mGlu$_2$ equals that of mGlu$_4$, suggests that the interpretation that there are homomeric pools of mGlu$_2$ and heteromeric mGlu$_{2/4}$ under these conditions are most consistent with the current data. These data also recapitulate Kammermeier’s finding that mGlu$_2$ homodimers existed when mGlu$_2$ and mGlu$_4$ were transfected with a 1:1 ratio but not a 1:3 ratio (Kammermeier, 2012).

It has previously been shown that heterodimerization/hetero-interactions of receptors can substantially alter the effect of pharmacological reagents. For example, Gonzalez-Maeso et al. reported that 5-HT$_{2A}$ receptors interact with mGlu$_2$ and form functional complexes in cerebral cortex (Gonzalez-Maeso et al., 2008). In the presence of this 5-HT$_{2A}$/mGlu$_2$ complex, hallucinogenic 5-HT$_{2A}$ agonists triggered unique cellular responses, which may contribute to the pathogenesis of psychosis. The combination of unique orthosteric and allosteric ligands for mGlu$_2$ and mGlu$_4$ now allows us to pharmacologically interrogate the functional expression of mGlu$_4$-containing heteromers at synapses in the CNS. For example, we found that VU0155041 potentiated L-AP4 responses at corticostriatal synapses, whereas PHCCC, which binds to a distinct allosteric site, showed no effect (Figure 3.1 and 3.13). In addition, the mGlu$_2$ NAM MNI-137 was able to block L-AP4 induced responses at the corticostriatal synapse, recapitulating the pharmacological profile of this compound in the mGlu$_{2/4}$ cell line.
Although the results reported here cannot be seen as definitive evidence of mGlu$_{2/4}$ heterodimer formation in the CNS and could be explained by other potential mechanisms, such as involvement of other partner proteins, multiple lines of evidence are consistent with functional existence of predominantly mGlu$_{2/4}$ heteromers at corticostriatal synapses: 1) time-resolved FRET studies by Doumazane et al. indicate that mGlu$_{2}$ and mGlu$_{4}$ form strict heterodimers when expressed in the same cells, 2) our co-immunoprecipitation data using rodent striatal tissue demonstrate some type of physical interaction between mGlu$_{2}$ and mGlu$_{4}$ in this brain region, and 3) pharmacological properties at corticostriatal synapses recapitulate the results seen in the mGlu$_{2/4}$ recombinant cell line. Regardless of the mechanism, these studies provide compelling evidence that the function of mGlu receptors can be context-dependent and that mGlu$_{4}$ may display fundamentally distinct responses to selective allosteric modulators at different synapses upon coexpression of other mGlu receptor subtypes.
CHAPTER IV

GENERAL DISCUSSION AND FUTURE DIRECTION

Functional selectivity and therapeutic implications of mGlu allosteric modulators

Seven transmembrane receptors (7TMRs) are intrinsic plasma membrane proteins that function as information transducers to pass stimuli from binding of natural ligands to activation of cytosolic signaling cascades. The fundamental mechanism of these actions is conformational changes of the receptors. Receptor proteins are dynamic molecules that are “breathing” and keep changing conformations, which are stabilized by natural ligands and synthetic orthosteric/allosteric reagents. Traditionally, these conformations are classified into “fully on”, “partially on” or “off” conformations using criteria of G protein activation.

In addition to G proteins, however, numerous cytosolic proteins interact with various domains on the 7TMRs and constitute a part in the whole “breathing system”. These proteins include arrestins and 7TMR interacting proteins, many of which are PDZ domain proteins. β-arrestin is able to recruit signaling molecules and induce long-lasting activation of ERK. Some examples of PDZ domain proteins include Na\(^+\)/H\(^+\) exchange regulator factor 1 and PICK1. As each receptor conformation possesses a specific energy map, the affinities to signaling molecules also varies. It has recently been shown by bioluminescence resonance energy transfer that G protein and non-G protein pathways
are directly correlated with unique receptor conformations (Galandrin et al., 2008). Therefore, a ligand may not elicit responses in all signaling pathways and theoretically, no two ligands would produce the same receptor behavior in terms of signaling and receptor regulation. And such phenomenon is termed “functional selectivity” (Gesty-Palmer and Luttrell, 2011).

Even naturally occurring ligands may exhibit functionally selective effect. CCL19 and CCL20 represent the first examples, which induce different receptor desensitization profile on CCR7 receptor (Kohout et al., 2004). Other examples of orthosteric ligands stem from serotonin (5-HT) receptors. Orthosteric ligands at the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors have revealed different rank orders of agonists to stimulate phospholipase C versus phospholipase A2 pathways (Berg et al., 1998). In addition, there are now examples of ligands with inverse agonist properties in certain pathways but clear agonist activity in others (De Deurwaerdere et al., 2004). It has been observed as well, that compounds classified as “antagonists” can induce desensitization, internalization, and downregulation of receptors, a property that might be considered “agonistic” in nature (discussed in (Urban et al., 2007)).

These observations deviate from “linear efficacy” and has breached the traditional view of signal propagation via the receptors. To better quantify such biased effects, useful measurements have been proposed to describe agonist effects. The term activity ratio, calculated using Max/EC$_{50}$, serves to characterize the ability of a certain agonist to activate a given signaling pathway (Ehlert, 2005). When two pathways are compared, the
relative activity ratios can thus be used to determine preference of one pathway over the other as a measurement of signaling bias.

As the search for more selective and “drug-like” 7TMR ligands has grown, the development of allosteric ligands has emerged. As these compounds bind to alternate sites on a 7TMR compared to the endogenous ligand, it might be expected that they could as well place the receptor in a unique structural conformation that might not be achieved, or at least favored, in their absence and result in preferential regulation of certain pathways in the presence of an allosteric ligand. For example, the mGlu5 PAM N-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isooindol-2-yl) methyl]phenyl}-2-hydroxybenzamide (CPPHA), which binds to an alternate site on the mGlu5 receptor versus other PAMs (O’Brien et al., 2004), exhibits differential effects on calcium mobilization and ERK1/2 phosphorylation downstream of mGlu5 activation in cultured cortical astrocytes (Zhang et al., 2005). This is in contrast to the PAM 3,3’-difluorobenzaldazine (DFB), which potentiates both responses similarly. Additionally, the study in Chapter II has introduced a second mechanism of functional selectivity where mGlu4 PAMs usually do not induce biased signaling yet can exhibit functional selective effects upon co-activation of a Gq coupled receptor. This indicates that the pharmacodynamics of compounds could be far more complicated in native environment and the substantial calcium response induced by mGlu4 PAMs could be of importance in the physiological and pathological conditions.
Functional selectivity could be used for rational design of drugs to maximize their therapeutic effects. In a case where functional selectivity may have therapeutic relevance, activation of the GPR109 receptor by nicotinic acid is effective in dyslipidemia by lowering triglycerides and elevating high-density lipoprotein. However, serious side effects were observed in patients including cutaneous flushing (Pike, 2005). It has been shown that activation of the receptor by nicotinic acid activates the $G_i$ signaling pathway, as well as phospholipase A2 activation followed by recruitment of β-arrestins. Studies using β-arrestin1 knockout mice revealed reduced dermal side effects while maintaining beneficial efficacies. In addition, MK-0354, a biased ligand that is devoid of β-arrestin recruitment, decreases serum levels of free fatty acids without inducing cutaneous flushing, and may represent an improved option for the treatment of dyslipidemia (Lai et al., 2008; Walters et al., 2009).

β-arrestin signaling, however, can also be associated with additional therapeutic effects. For example, the β-adrenergic receptor blocker carvedilol acts as an inverse agonist in cAMP pathways but as an agonist in stimulating β-arrestin-mediated ERK phosphorylation (Wisler et al., 2007); this compound clinically shows advantages over other β-blockers in the treatment of heart failure. Another example comes from parathyroid hormone (PTH) receptor. Intermittent Activation of PTH receptor increases bone mass by stimulating osteoblasts. However, bone resorption can also occur through the coupling of osteoblasts to osteoclasts, which is not desired for the treatment of osteoporosis. Studies using β-arrestin2 knockout mice suggest that β-arrestin pathway limits PTH-induced osteoclastogenesis and contributes to increased bone mass (Ferrari et
Indeed, a biased agonist, D-Trp(12),Tyr(34))-PTH(7-34) (also named PTH-betaarr), activates β-arrestin but not classic G protein signaling and induces bone formation without bone resorption, offering improved therapy for osteoporosis (Gesty-Palmer et al., 2009).

As to the biased signaling described in Chapter II, calcium is involved in various cellular functions including enzyme activation, signal-transduction, and electrophysiological responses. In the central nervous system, intracellular calcium mobilization is vital for neurotransmitter release and synaptic plasticity, including long-term potentiation and long-term depression. It is well studied that massive calcium mobilization mediated by 7TMR cross-talk can have significant physiological implications. For example, mGlu₁-mediated calcium mobilization in Purkinje neurons is enhanced by concomitant activation of GABAB receptor (Kamikubo et al., 2007). Induction of such a calcium signal is suggested to act as a co-incidence detector for parallel- and climbing-fiber inputs, and is required for long-term depression at parallel-fiber–Purkinje cell synapses (Hirono et al., 2001; Wang et al., 2000). Similarly, potentiated calcium release by cross-talk between group I and group II mGluRs is shown to be critical for LTD in the perirhinal cortex under resting-membrane potentials (Cho et al., 2000). Therefore, signaling bias induced by mGlu₄ PAMs could be utilized to treat disease conditions where calcium signaling of a G₄ coupled receptor is desired in mGlu₄-coexpressing cells but not in other tissues. On the other hand, however, an inappropriate intracellular calcium surge could induce apoptosis via activation of calpain (Camins et al., 2009). Therefore, caution may
need to be taken when mGlu4 PAMs are used in combination with other G\(_q\) receptor agonists.

As more allosteric modulators are pursued in the hopes of achieving highly selective drug leads with good pharmacokinetic properties, further complexity induced by signal bias will continue to develop. While functional selectivity clearly complicates the use of compounds as general tools for probing receptor function and the progression of drugs with these properties through clinical development, there is great promise in this approach to eventually tailor drug therapy to a particular pathway or subset of signaling cascades to enhance therapeutic efficacy and/or reduce side effects.

**Receptor assembly-selective modulators of mGlu**

As mentioned above, allosterism of the receptor by ligands/modulators and interacting cytosolic proteins is essential for the signal transduction from environment into the cells. In addition to the guest allosterism and cytosolic allostery induced by these factors, receptor dimerization/oligomerization, as well as the lipid environment, comprise important components for the lateral allostery of 7TMRs (Kenakin and Miller, 2010).

Although oligomerization of family A 7TMRs is a topic of extensive debate, family B 7TMRs have recently been shown to form stable homodimers with the interacting interface at TM4 (Harikumar et al., 2008; Harikumar et al., 2007). In addition, family C
7TMRs (including mGlus) have shown compelling evidence for
dimerization/oligomerization via both covalent and non-covalent bonds (Ray and
Hauschild, 2000; Romano et al., 2001).

As it is common to consider small molecules as receptor modulators, large receptor
protomers may also induce conformational change to other protomers. It is thus important
to realize two-way transmission by which receptor assembly can alter the behavior of
small molecule modulators as well. This effect has been well established in cell line
settings. Using the angiotensin receptor as an example, the agonist angiotensin II exhibits
significantly increased efficacy when the receptor form a heterocomplex with bradykinin-2
receptor (AbdAlla et al., 2000). Receptor dimerization may also affect intracellular
signaling pathways. The G protein coupling of dopamine D2 receptor switches from G_{i/o}
to G_{q/11} when dimerized with dopamine D1 receptor (Rashid et al., 2007). In addition,
SKF83959, a compound with no effect on either D1 or D2 monomer, activates the D1/D2
heterodimer toward G_{q/11} (Rashid et al., 2007).

As dimerization/oligomerization of receptors clearly complicated the use of
pharmaceutical reagents in in vivo settings. While selective compounds for receptor
heterodimers help to avoid off-target effects, reagents that target all receptor assemblies
may produce superior pharmaceutical efficacy. The results described in Chapter III also
shed light on targeting distinct mGlu receptor assembly for drug development purposes.
Taking PD as an example, highly selective mGlu4 PAMs have been developed from
different chemical series and exhibit robust efficacy in preclinical rodent models.
Interestingly, both PHCCC and VU0155041 have been shown to reverse reserpine-induced akinesia in rodents, suggesting that the anti-parkinsonian effects induced by mGlu$_4$ PAMs may not be dependent on their activity at corticostriatal synapses and the assembly of mGlu$_{2/4}$ heterodimer. However, corticostriatal synapses have been shown to be overactive in dopamine-depleted animals, which contributes to the loss of spines of striatal medium spiny neurons in PD (Garcia et al., 2010). The work of Picconi et al. demonstrates that dysregulated plasticity at these synapses, such as long-term depression and depotentiation, may underlie the mechanism of L-DOPA-induced dyskinesia (Picconi et al., 2011; Picconi et al., 2003). Therefore, mGlu$_4$ PAMs that potentiate mGlu$_{2/4}$ heteromers may potentially provide additional therapeutic benefits, such as restoring morphology of striatal neurons and reversing L-DOPA-induced dyskinesias. In contrast, PAMs with selectivity for homodimers over heteromers might be beneficial for targeting mGlu$_4$ activation in regions predominantly expressing mGlu$_4$ alone if activation of heteromers proves to engender side effects. Additionally, selective mGlu$_{2/4}$ modulators without mGlu$_2$ or mGlu$_4$ activity might also be of interest to achieve selective modulatory effect without affecting receptor homomers expressed in other regions.

Although the signaling pathway of mGlu$_{2/4}$ heterodimers does not deviate from the G$_{i/o}$ coupling of each individual subtype, whether hetero-assembly lead to biased signal is of interest to explore. The effect of heterodimerization on other signaling cascades, especially arrestin binding, receptor desensitization, and internalization is worth further examination. Additionally, binding of modulators to one protomer on the heterodimers may also affect the pharmacology of allosteric sites on the other protomer. Detailed
studies using binding assays and functional assays are required to answer this question. Beside receptor dimerization, the effects of other interacting proteins and lipid bilayer composition on mGlu allosteric modulators are also largely unknown.

In addition to mGlu$_{2/4}$, other combinations of mGlu subtypes are co-localized in the CNS as well. For instance, mGlu$_4$ and mGlu$_8$ are co-expressed at the lateral olfactory tract-piriform cortex synapse and suppress synaptic transmission (Jones et al., 2008). mGlu$_1$ and mGlu$_5$ are co-expressed in several neuronal populations including CA1 hippocampal pyramidal cells, striatal cholinergic interneurons, STN glutamatergic neurons and SNr GABAergic neurons (reviewed in (Valenti et al., 2002)). In addition, both mGlu$_7$ and mGlu$_8$ receptors modulate the Schaffer collateral-CA1 synapse in neonatal rats (Ayala et al., 2008). Although the assembly of other mGlu heteromers has yet to be determined in vivo, previous studies showing aberrant activity of mGlu-selective compounds may eventually be explained by heteromer-specific pharmacology (Ayala et al., 2008; Niswender et al., 2010). As characterization of other combinations of mGlu heteromers are underway, localizing mGlu homomers and heteromers will help elucidate the complexity of mGlu receptor signaling and function and eventually contribute to rational development of therapeuetic reagents that target specific tissues through selective modulation of individual receptor assemblies.
mGlu$_{2/4}$ heteromer as a biomarker for Parkinson’s Disease

As defined by the National Institutes of Health, a biomarker is a parameter that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Group, 2001). Biomarkers for PD are of critical importance for patient treatment, since neuroprotective therapy starting at an advanced stage (after motor symptoms develop) is usually unsuccessful in progressive neurodegenerative diseases when compared to early stages.

An ideal biomarker for PD should be able to facilitate PD treatment in the following aspects: 1) Aid in the early diagnosis of PD, preferably in a premotor period; 2) Accurately reflect disease progression; 3) Aid in therapeutic assignment of patients; and 4) Aid in accessing therapeutic efficacy of an intervention.

Since its first description, the diagnosis of PD has been made based on motor deficits and associated non-motor symptoms. Recently, however, a number of biomarkers have been developed to aid in the diagnosis of PD, which can be further divided into pathological biomarkers, biochemical biomarkers, genetic biomarkers, electrophysiological biomarkers and imaging biomarkers. A recent study discovered that $\alpha$-synuclein and Lewy bodies exist in the gut biopsies of PD patients that were obtained several years before the onset of motor symptoms (Shannon et al., 2012), serving as a potential pathological biomarker for early PD detection. Schmid et al. has shown that biochemical
profiling of the post-translational modification of α-synuclein in the cerebrospinal fluid or blood plasma might be a more relevant biomarker than total α-synuclein level (Schmid et al., 2013). Altered levels of brain-derived neurotrophic factor (BDNF) have been reported in the circulation (serum or plasma) of patients with PD. However, BDNF might not serve as a specific biomarker since decreased levels of this neurotrophic factor were also observed in other neurodegenerative disorders, including Alzheimer’s disease and Huntington’s disease, as well as mood disorders (major depression and bipolar disorder) (Teixeira et al., 2010). Mutations in five genes, such as α-synuclein, Parkin, PTEN-induced kinase 1 (PINK1), DJ-1 and Leucine-rich repeat kinase 2 (LRRK2) have been shown to lead to PD (Klein and Lohmann-Hedrich, 2007). However, these cases only account for 2–3% of all PD patients (Klein and Lohmann-Hedrich, 2007), and, even in these patients, age and environmental exposures greatly affect disease progression.

Electrophysiologically, Hohlefeld et al. reported long-range temporal correlation of oscillation activities in subthalamic nucleus of PD patients, which can be modulated by levodopa treatment (Hohlefeld et al., 2012). However, the surgical procedures needed to measure neuronal activity in patients limited the usage of this parameter as a biomarker. In contrast, non-invasive imaging approaches have demonstrated great potential as a biomarker for PD diagnosis and treatment. For example, imaging of the dopamine transporter (DAT) with Single Photon Emission Computer Tomography (SPECT) is a main diagnostic imaging procedure for the assessment of patients with parkinsonism (Varrone and Halldin, 2012).
Although with caveats, these existing biomarkers have brought valuable advances in PD diagnosis and the drug development process, including early proof-of-concept studies and dosing regimen determination. As PD is much more complicated than a single-cause disease, it is equally important to assign patients to suitable therapeutic interventions, and ideally, as quickly as possible to prevent further neurodegeneration. Unfortunately, no such biomarker has yet been developed. mGlu4 PAMs have been validated as potential therapeutic agents for PD with one possible mechanism being reduction of excessive GABA release at striatopallidal synapses (Marino et al., 2003; Valenti et al., 2003), thereby rebalancing basal ganglia circuitry. In addition, activation of mGlu4 inhibits glutamate release onto substantia nigra dopamine neurons (Valenti et al., 2005), which has been proposed to protect against excitotoxicity. However, our study described in Chapter III reveals that the efficacy of mGlu4 PAMs are differentially regulated by formation of mGlu2/4 heterodimers, with PHCCC losing efficacy and VU0155041 retaining efficacy at the corticostriatal synapse. In addition, transient transfection experiment using cell lines suggests that the regulatory effect is dependent on the expression level of mGlu2. Due to the heterogeneity within PD population, it is conceivable that mGlu2 expression levels in the basal ganglia circuitry could vary among PD patients and may suggest that mGlu4 PAMs that are ideal for some patients may not benefit others with high mGlu2 expression colocalized with mGlu4. Therefore, with help from future development of selective and powerful imaging reagents for mGlu2 and mGlu4, the expression pattern of mGlu2 and mGlu4 within the basal represent an attractive biomarker in PD clinical trials and future clinical practice.
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