# SITES OF ACTION AND PHYSIOLOGICAL IMPACT OF MGLUR5 POSITIVE ALLOSTERIC MODULATORS

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# CHAPTER I

# PHARMACOLOGICAL AND ELECTROPHYSIOLOGICAL PROPERTIES OF METABOTROPIC GLUTAMATE RECEPTORS

## Metabotropic Glutamate Receptors

### Molecular identity, structure, activation mechanism and signaling.

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) and is responsible for the majority of fast excitatory synaptic responses at CNS synapses (Dingledine et al., 1999). Fast synaptic responses at glutamatergic synapses are mediated by activation of glutamate gated cation channels termed ionotropic glutamate receptors (iGluRs). In addition, glutamate activates metabotropic glutamate receptors (mGluRs), which are coupled to GTP-bindinG-proteins (G-protein) (Conn and Pin, 1997). Unlike iGluRs, mGluRs modulate synaptic transmission and cell excitability through second messenger systems. Because of the ubiquitous distribution of glutamatergic synapses, mGluRs participate in regulating a wide variety of CNS functions (Conn and Pin, 1997; Anwyl, 1999; Coutinho and Knopfel, 2002).

mGluRs belong to family C of G-protein-coupled receptors (GPCRs). Eight family members of mGluRs have been cloned from rat and human genomes to date. Based on their sequence homology, pharmacological selectivity and primary G-protein coupling, mGluRs are further divided into three groups (Figure 1-1). Group 1 mGluRs include mGluR1 and mGluR5, both of which are coupled to $G_{q/11}$ to activate

phospholipase C (PLC). Group 2 mGluRs (mGluR2 and mGluR3) and Group 3 mGluRs (mGluR4, mGluR6, mGluR7 and mGluR8) are coupled to $G_{i/o}$ to inhibit adenylyl cyclase activity, which results in a decrease of cyclic AMP (cAMP) accumulation.

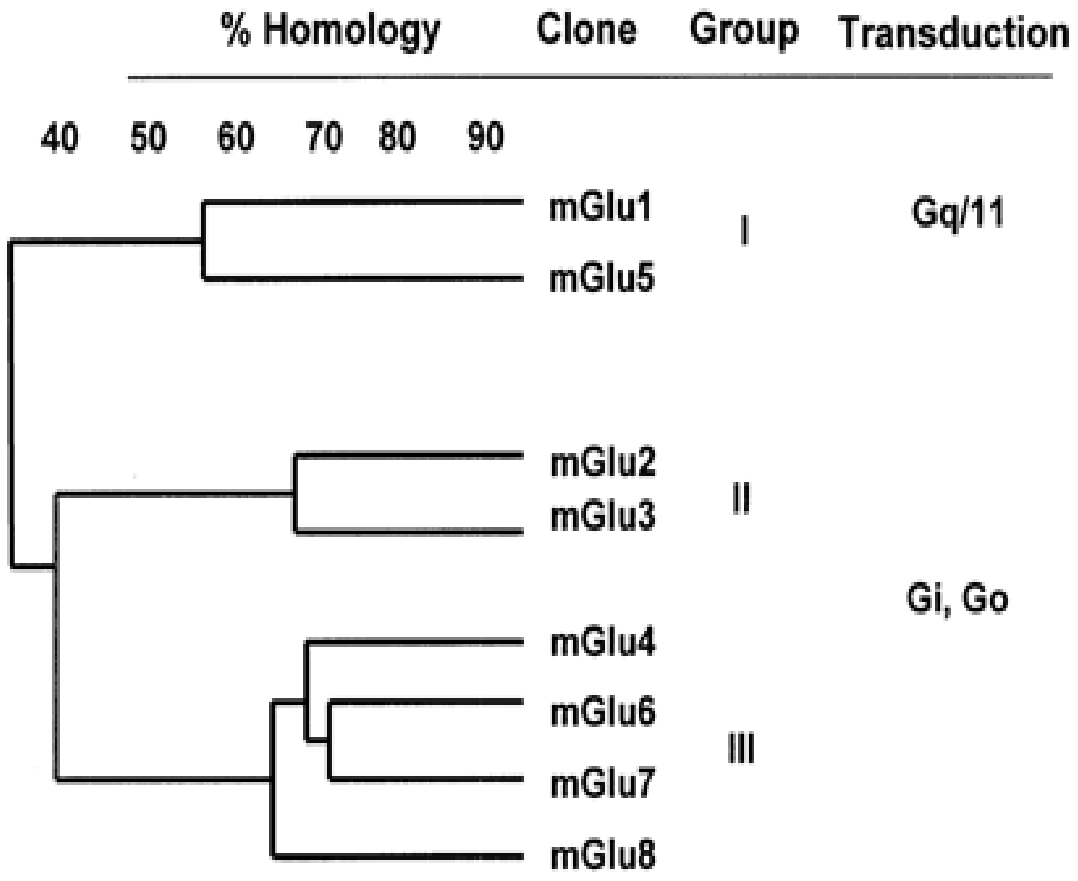


Figure 1-1: Classification of mGluRs. Eight mGluRs are divided into three groups based on their sequence homolog and primary G-protein coupling. (Figure modified from Conn 2003)

All eight mGluR subtypes contain three major domains, a large extracellular N-terminal domain, a heptahelical domain containing seven transmembrane regions linked by short loops, and an intracellular C-terminal domain (Figure 1-2). Unlike family A and B GPCRs, glutamate binds to the N-terminal extracellular domains of mGluRs. This orthosteric binding site is highly conserved through out all the mGluR subtypes. It has been proposed that the mGluR extracellular domain has a sequence similarity with bacterial periplasmic bindinG-proteins (PBP) (O'Hara et al. 1993). This homology has been used to construct a model based on the structure of PBPs, which predicted the mGluRs N-terminal domains to be made up of two globular domains with a hinge region. This "Venus FlyTrap" structure was confirmed by a crystallography study of the mGluR1 extracellular domain at 2.2Å resolution later (Kunishima et al., 2000). In addition, this model predicted glutamate binding to the N-terminal domain, which was supported by mutagenesis studies ((Takahashi et al., 1993; Tones et al., 1995 and Parmentier et al., 1998; Pin et al., 2003). Glutamate induced closure of the "Venus FlyTrap" domain leads to the activation of the receptor (Kunishima et al., 2000; Tsuchiya et al., 2002; Pin et al., 2004). mGluRs possess the typical seven transmembrane (TM) domains linked with loops as other families of GPCRs (Conn and Pin 1997; Bhave et al., 2003). It has been proposed that the TM domains interact with the N-terminal domain allosterically to transduce the extracellular stimulus into an intracellular response. The intercellular loops and C-terminal tail are both crucial for the selective coupling to different G-proteins (Pin et al., 1994; Gomeza et al., 1996 and De Blasi et al., 2001). The major splice isoforms of mGluRs' are only different in the intracellular C-terminal tails. For instance, at least 5 isoforms of mGluR1 have been shown to exist, named as mGluR1a/1b/1c/1d/1e

individually (Pin et al., 1992; Tanabe et al., 1992; Laurie et al., 1996; Mary et al., 1997). All mGluR1 sequences are identical up to the forty-sixth residue of the intracellular tail, followed by 313, 20, 11, and 26 residues for mGluR1a, mGluR1b, mGluR1c, and mGluR1d respectively (Mary et al., 1998). The different variants are all coupled to $G_{q/11}$ but show different activation kinetics and constitutive activities (Mary et al., 1998). This indicates that the intracellular tails of mGluRs may control their activation kinetics.

mGluRs can couple to multiple signaling pathways besides the primary G-proteins. mGluRs are classified as GPCRs because they activate intracellular signaling pathways that begin with G-proteins. However, evidence suggests that mGluRs also trigger signaling pathways independent of G-protein activation. For instance, in hippocampus, mGluR1 activation simultaneously triggers both G-protein-dependent and -independent signaling induced distinct currents (Heuss et al., 1999; Gee et al., 2004). Consistent with this, inward currents mediated by mGluRs in hippocampus still persisted in G-protein knockout mice (Krause et al., 2004). Additionally, in CA3 pyramidal neurons, N-methyl-D-aspartate (NMDA) receptor current is potentiated by mGluR5 by a G-protein-dependent manner, whereas NMDAR current potentiation by mGluR1 is independent of G-protein activation (Benquet et al., 2002). It is possible that different signaling pathways are responsible for different responses mediated by a single subtype of mGluR. Interestingly, increasing evidence suggests that different orthosteric agonists can differentially activate distinct signaling pathways of a single GPCR, a phenomenon termed as agonist receptor trafficking (Berg et al., 1998; Brink et al., 2000; Gazi et al., 2003). Thus, compounds with high selectivity to one certain signaling pathway of one receptor may have better therapeutic properties compared with the compounds only

selective to the receptor. This has become important for development of novel pharmacological reagents to regulate distinct signaling pathways differentially according to certain therapeutic purposes.

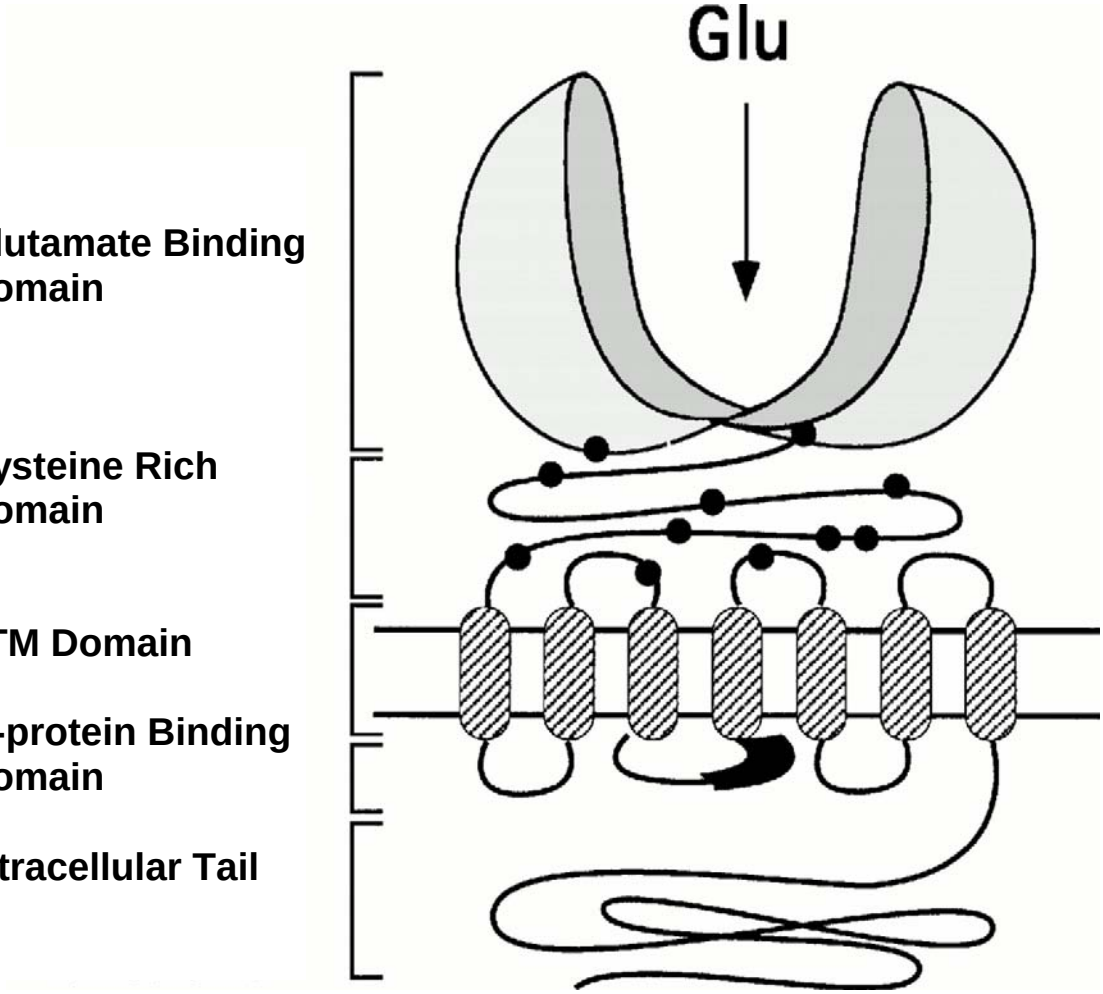


Figure 1-2: Schematic structure of an mGluR. The cysteine residues in the cysteins rich domain are indicated with black circles. The segment within the second intracellular loop that is important for G-protein coupling specificity is indicated in black. (Figure modified from Conn and Pin 1997)

**Physiological effects and diseases relevance.**

mGluRs exist predominantly in many regions of the mammalian nervous system, where they modulate neuronal excitability and synaptic transmission through activation of second messenger systems. Activation of mGluRs results in a diverse range of electrophysiological effects, such as inhibition of potassium and calcium currents, activation of potassium, calcium and non-specific cation currents, induction of slow excitatory postsynaptic potentials, inhibition of presynaptic neurotransmitter release, and potentiation of synaptic amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and N-methyl-D-aspartate (NMDA) receptor currents (Anwyl 1999). mGluRs are potential drug targets for many neurological and psychiatric disorders, including Parkinson's disease (Marino and Conn, 2002b), epilepsy (Doherty and Dingledine, 2002), Alzheimer's disease (Wisniewski and Carr, 2002), pain (Varney and Gereau, 2002), schizophrenia (Marino and Conn, 2002a), depression (Palucha and Pilc, 2002), and anxiety disorders (Chojnacka-Wojcik et al., 2001; Pilc, 2003). Thus, it has become critical to develop subtype selective pharmacological reagents to study the potential involvement of mGluRs in different human disorders that may be relevant to the development of new therapeutic agents.

**Pharmacology of mGluRs.**

Much effort has been focused on the development of selective compounds to either activate (agonist) or inhibit (antagonist) mGluR responses. The first generation of compounds includes primarily analogs or derivatives of glutamate, the endogenous agonist of mGluRs. As a result, these compounds also act at the same binding pocket as

glutamate, the N-terminal Venus Flytrap domain of mGluRs. Targeting the orthosteric glutamate binding site has been successful for generating many groups of non-selective or selective compounds for mGluRs, which have been useful in demonstrating the pharmacological properties and physiological roles of mGluRs in both recombinant and native systems (Schoepp et al., 1999).

**1. Orthosteric ligands of mGluRs.**

Quisqualic acid (quisqualate) is the first identified agonist for phosphoinositide linked mGluRs. It has sub-micromolar potencies at group 1 mGluRs in both native and recombinant systems (Palmer et al., 1988). However, its use as a group I mGluR-selective agonist is limited by its activity at mGluR3 and AMPA receptors (Watkins et al 1990; Scheopp et al., 1999). The first selective agonist for mGluRs was aminocyclopentane-trans-1,3-dicarboxylic acid (trans-ACPD), which is a conformationally restricted glutamate analog that activates multiple mGluRs but does not activate iGluRs (Desai and Conn, 1991; Palmer et al., 1989). Thus it was used widely to study mGluR functions both *in vitro* and *in vivo*. However, because it activates almost all mGluR subtypes (except mGluR7) with a similar potency, its use is also limited. 3,5-Dihydroxyphenylglycine (DHPG) is the first group 1-selective mGluR agonist. It is active to both recombinant and native group 1 mGluRs but displays no agonist or antagonist activity to other groups of mGluRs (Schoepp et al., 1994). DHPG has been used widely to investigate the pharmacological properties and physiological roles of group 1 mGluRs in many studies. For example, bath application of DHPG has been shown to have variety of physiological effects in hippocampal CA1 pyramidal neurons,

including direct depolarization, increasing of cell firing, decreasing of γ-aminobutyric acid (GABA)-mediated inhibition, potentiation of NMDAR current and intracellular calcium increase (Mannaioni et al., 2001). In addition, DHPG activates group 1 mGluRs to induce a chemical form of long term depression (LTD) at the Schaffer collateral CA1 synapse (Palmer et al., 1997). Other group selective agonists have also been identified but none of them achieves subtype selectivity among all the mGluRs (Schoepp et al., 1999). (RS)-2-chloro-5-hydroxyphenylglycine (CHPG) is a mGluR5 selective agonist but has very weak potency and efficacy (Doherty et al., 1997).

**2. Importance of developing novel mGluR ligands.**

Initial efforts to develop inhibitors of mGluRs, which also focused on the glutamate binding site, have identified a variety of competitive antagonists for mGluRs. However, similar to agonists acting at the glutamate site, these competitive antagonists are not able to achieve good selectivity for specific mGluR subtypes (Schoepp et al., 1999). Thus it has been proposed that because the glutamate binding pockets are relatively highly conserved among all the eight family members of mGluRs due to the evolutionary pressure of glutamate binding, the chance is low to develop subtype-selective compounds acting at the glutamate binding pockets of mGluRs. It is clear that different subtypes of mGluRs exist in the same neurons through out the CNS. For example, immunocytochemistry studies reveal that mGluR5 is localized in CA1 pyramidal cells, but not mGluR1a (Baude et al., 1993; Romano et al., 1995). Therefore, mGluR5 was thought to be the group 1 mGluR that regulates CA1 pyramidal neurons. However, although mGluR5 is the most abundant group I mGluR in CA1 pyramidal cells,

these cells also express mGluR1 mRNA (Shigemoto et al., 1992; Berthele et al., 1998), and a follow-up immunohistochemical study with antibodies that react with all splice variants of mGluR1 indicated some mGluR1 immunoreactivity in this region (Ferraguti et al., 1998). Although the short forms of mGluR1 and mGluR5 are both primarily coupled to the same G α q/11 second messenger system, it is still possible that they may elicit distinct physiological effects in the same cells because of different activation kinetics and coupling to different effector systems as well as different expression levels (Pin et al., 1992; Kawabata et al., 1996; Conn and Pin, 1997). Hence, it is important to develop subtype selective mGluR agonists and antagonists to demonstrate the distinct roles of different subtypes of mGluRs.

**3. Novel non-amino acid mGluR antagonists.**

In the late 1990s, the first generation of the non-amino acid class of mGluR antagonists was developed, among which, N-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC) and cyclopropan[b]chromen-1a-carboxylic acid ethylester (CPCCOEt) were characterized as mGluR1 selective antagonists compared with mGluR5, other groups of mGluRs and iGluRs (Annoura et al., 1996). CPCCOEt has no effect on mGluR2 or iGluRs. At a concentration much higher than its potency on mGluR1, CPCCOEt also blocks mGluR5 mediated response. However, at certain concentrations, it is possible for these compounds to achieve selective blockade of mGluR1 without acting on mGluR5.

CPCCOEt is a non-competitive mGluR1 antagonist. The non-amino acid origin of CPCCOEt led to a hypothesis that it may bind to a different site on mGluR1 instead of

the orthosteric glutamate binding pocket. This hypothesis has been supported by a series of studies. Classic amino-acid-like mGluR antagonists block mGluR responses competitively. In the absence of receptor reserve, competitive antagonists induce a parallel shift of the concentration response curve (CRC) of agonist to the right without reducing the maximal response. This kind of effect is obtained by the study of mGluR1 competitve antagonist (S)-*α*-methyl-4-carboxyphenylglycine ((S)-MCPG) (Hermans et al., 1998). Interestingly, CPCCOEt decreases the maximal response of the quisqualate CRC to a saturated plateau, which could not be reversed by increased concentration of quisqualate (Hermans et al., 1998). This indicates that CPCCOEt is a non-competitive antagonist of mGluR1, which might interact at a site completely distinct from the glutamate binding pocket. This is additionally supported by a study showing that CPCCOEt does not displace specific [$^3$H]quisqualate binding to cell membranes containing mGluR1 proteins (Okamoto et al., 1998).

CPCCOEt acts at the seven TM domains of mGluR1. In order to identify the site of action of CPCCOEt, a series of mutagenensis and chimeric receptor studies were performed. These studies were guided by the selectivity of CPCCOEt for mGluR1 relative to mGluR5. Thus, the switching of crucial residues in mGluR1 to their corresponding residues of mGluR5 should reduce the antagonism by CPCCOEt. By this strategy, two amino acids (Thr815 and Ala818) were identified to be responsible for the selective action of CPCCEOt (Litschig et al., 1999). These two residues are located at the extracellular surface of TM domain VII. In conclusion, these non-competitive antagonists act at a distinct site from glutamate binding site. This opens up a novel way to develop subtype selective compounds for mGluRs (Schoepp et al., 1999).

Mutel and colleagues developed the first mGluR positive allosteric modulators (PAM) for rat mGluR1 (rmGluR1) (Mutel et al., 2001; Knoflach et al., 2001), which are non-amino acid compounds (Ro67-7476, Ro 01-6128 and Ro 67-4853). These compounds have no effect on their own but potentiate the action of agonists. It has been shown that these compounds are devoid of any enhancing effect at recombinant mGluR2, mGluR4, and mGluR8 by various functional models including GTPγ[$^{35}$S] binding, $[Ca^{2+}]_{int}$ imaging, and activation of G-protein-regulated inwardly rectifying $K^+$ channels (GIRKs). Importantly, Ro 67-7476 and Ro 01-6128 do not enhance the glutamate-induced GIRK current in mGluR5 receptor-expressing cells. Mutagenesis experiments revealed that mutations within the seven TM domains of mGluR1 could abolish the potentiation of these compounds (Knoflach et al., 2001). There is no significant effect of these compounds, up to 10 μM, on the binding of [$^3$H]quisqualate to rat mGluR5 (rmGluR5), whereas they increase the binding of this ligand to the rmGluR1a (Knoflach et al., 2001). However, the increased affinity in competition binding assay is smaller than the increase of quisqualate potencies caused by the same concentrations of these mGluR1 PAMs (Knoflach et al., 2001). These results indicate that this novel family of mGluR1 PAMs act through binding to the 7 TM domain of the receptor to potentiate the mGluR1 response via a mechanism that enhances the agonist binding affinity, at least partially, to the receptor. The discovery of non-competitive mGluR antagonists and PAMs leads to the conclusion that there could be both negative and positive allosteric modulators acting on the 7 TM domains of mGluRs. The non-competitive antagonists of mGluRs are also called negative allosteric modulators (NAM) in contrast to PAMs.

**4. Advantages of mGluR allosteric compounds.**

Compared to classic orthosteric compounds, allosteric modulators could display better subtype selectivity among mGluR subtypes. This result provides a solution for the difficulty in developing potent subtype selective compounds targeting the orthosteric glutamate binding site. Additionally, classic orthosteric compounds have difficulty crossing the blood brain barrier (BBB) because their general hydrophilicity due to their amino acid origins. Thus, their usage is limited to *in vitro* studies. In contrast, the mGluR allosteric modulators are usually hydrophobic and theoretically should exhibit better penetration of BBB. Moreover, derivatives of amino acids are often easily metabolized; hence the non-amino acid nature of mGluR allosteric modulators could have more favorable pharmacokinetics. In conclusion, mGluR allosteric modulators have improved subtype selectivity, BBB penetration and pharmacokinetics compared with classic mGluR orthosteric compounds. These properties will allow these novel compounds to be used as better pharmacological tools for basic studies of mGluRs as well as potentially useful therapeutic reagents. Thus a lot of effort has been focused on the development and characterization of novel mGluR allosteric modulators. My graduate research has been focused on studies of the pharmacological properties and the physiological effects of mGluR5 PAMs.

## Metabotropic Glutamate Receptor Subtype 5

### Molecular identity, structure and signaling.

mGluR5 belongs to the group 1 mGluRs which primarily couple to $G_{\alpha\ q/11}$ signaling pathway. It is the most abundant mGluR and is expressed in many regions of mammalian CNS. There are two splice forms of mGluR5, mGluR5a and mGluR5b. The N-terminal domain and the heptahelical domain of the two splice isoforms are identical, but mGluR5b has an additional insertion of a 32 amino acids fragment after the fiftieth residue of the C-terminal tail (Joly et al., 1995). Both isoforms contain a long intercellular C-terminal tail of more than 300 residues, which is the target of many interactinG-proteins. For instance, the Homer proteins have been shown to interact with a Pro-Pro-X-X-Phe-Arg (PPXXFR) motif close to the C-terminus (Xiao et al., 2000). These proteins can therefore link mGluRs to large protein complexes and control many aspects of receptor function, such as membrane insertion, localization, kinetics of the intracellular calcium signal, and constitutive activity (Roche et al., 1999; Ango et al., 2000; Tu et al., 1998; Ango et al., 2001). In addition to coupling to phospholipase C through $G_{q/11,}$ mGluR5 also activates other signaling pathways. For example, mGluR5 activation increases extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation in cultured rat cortical astrocytes. This activation requires transactivation of the epidermal growth factor receptor but not phospholipase C activation (Peavy et al., 2001, 2002). This epidermal growth factor receptor-dependent ERK phosphorylation and phospholipase C-dependent responses are differentially regulated by protein kinase C, although they are both initiated by activation of mGluR5 (Peavy et al., 2002).

**mGluR1 and mGluR5 mediate different physiological responses when co-existing in the same cell population.**

Both mGluR1 and mGluR5 are abundantly expressed in many neuronal populations, including those in the hippocampus, subthalamic nucleus (STN), substantia nigra, globus pallidus and other forebrain and midbrain regions (Awad et al., 2000; Poisik et al., 2003; Wittmann et al., 2001). Our group has examined the physiological roles of mGluR1 and mGluR5 in multiple neuronal populations including hippocampal CA1 pyramidal cells, STN neurons, substantia nigra neurons and globus pallidus neurons. Interestingly, in each cell population from which we have recorded, we find that mGluR1 and mGluR5 have distinct effects (Valenti et al., 2002). In addition, the precise role of each group I mGluR subtype is cell-type specific.

While mGluR1 and mGluR5 are closely related and couple to the same G-proteins and effector systems in cell lines, it is clear that these receptor subtypes do not usually have redundant effects in native preparations, but often couple to distinct electrophysiological responses (Valenti et al., 2002). For instance, selective group 1 mGluR agonist DHPG induces a robust increase in intracellular calcium as measured by an increase in fluorescence in cells loaded with the calcium-sensitive fluorescent dye Fluo3 and an inward current that leads to cell depolarization when recording in current clamp mode in CA1 pyramidal cells (Mannaioni et al., 2001). Interestingly, both of these responses were completely blocked LY367385, a competitive antagonist that is selective for mGluR1. In contrast, neither response is blocked by 2-methyl-6-(phenylethynyl) pyridine (MPEP), a noncompetitive antagonist that is selective for mGluR5 (Mannaioni et al., 2001). This result is surprising and suggests that although mGluR5 is heavily expressed in these cells, only mGluR1 is involved in eliciting the somatic calcium

transient and inward current. However, DHPG also elicits a number of other responses in these cells, including inhibition of multiple potassium currents and changes in synaptic transmission. For instance, DHPG induces a complete inhibition of a slow calcium-activated potassium current termed $I_{AHP.}$ In contrast to the effect on calcium transients and the inward current, DHPG-induced suppression of $I_{AHP}$ is not blocked by LY367385 but is completely blocked by the mGluR5-selective antagonist MPEP, suggesting that this response is exclusively mediated by mGluR5 (Mannaioni et al., 2001). Similarly, MPEP, but not LY367385 blocks DHPG-induced potentiation of the NMDA receptor current (Mannaioni et al., 2001). Thus, while mGluR1 and mGluR5 can both couple to similar signal transduction mechanisms (i.e. $G_q$ and activation of PLC), these receptors can have clearly distinct effects when present in the same hippocampal CA1 pyramidal neuron population.

Similar results have been shown in other neuron populations. Electrophysiology studies have revealed that the group I mGluR agonist DHPG induces a depolarization of STN neurons and potentiates NMDA receptor currents (Awad et al., 2000). Both the depolarization and the increase in NMDA receptor currents are completely blocked by the mGluR5 antagonist MPEP but not by the selective mGluR1 antagonists LY367385 or CPCCOEt (Awad et al., 2000). Thus, while both mGluR1 and mGluR5 are abundantly expressed, DHPG–induced depolarization and potentiation of NMDA receptor currents are mediated exclusively by mGluR5. Interestingly, either the mGluR1-selective antagonist LY367385 or the mGluR5 antagonist MPEP completely blocks the DHPG-induced calcium response (Marino et al., 2002). These intriguing results suggest that while mGluR5 activation alone is necessary and sufficient to elicit depolarization and

potentiation of NMDA receptor currents, neither mGluR1 nor mGluR5 alone can elicit a detectable somatic calcium transient response; only co-activation of both receptors with DHPG elicits a robust response. In GABAergic projection neurons of the substantia nigra pars reticulata (SNr), DHPG-induced depolarization is mediated exclusively by mGluR1. This is in clear contrast to the role of mGluR5 in depolarization of STN neurons. However, in common with STN neurons, either LY367385 or MPEP blocks the somatic calcium transient, suggesting that both receptor subtypes are functionally active (Marino et al., 2002).

Group 1 mGluRs are also activated via synaptic transmission. In most cases, these receptors are involved in slow mGluR-mediated synaptic responses consistent with responses to exogenous agonists, such as the slow synaptic responses of GABAergic projection neurons in the SNr and a dopamine neuron in the substantia nigra pars compacta (SNc) to stimulation of glutamatergic afferents. In SNr neurons, stimulation of excitatory afferents elicits a depolarization with increased action potential firing (Marino et al., 2001). In contrast, for dopamine neurons, stimulation of glutamatergic afferents induces a slow hyperpolarization (Fiorillo and Williams, 1998; Marino et al., 2001). In both cases, the response is mediated exclusively by mGluR1 and completely blocked by the mGluR1 antagonists LY367385 or CPCCOEt, but not by MPEP (Marino et al., 2001; Ciombor et al., 2002).

**mGluR5 is a potential target for novel therapeutic agents for the treatment of schizophrenia.**

Recent studies revealed mGluR5 as a potential drug target for agents that may provide better treatment of schizophrenia. Schizophrenia is a prevalent chronic

psychiatric disorder, affecting 1% of the population worldwide. This disorder is characterized by a combination of negative and positive symptoms as well as cognitive impairments. However, the etiology remains unknown. Traditional dopamine D2 receptor blocker therapeutics have several obvious shortcomings. They normally do not have any effect on about 25% of the treated patients and are often unable to relieve the negative symptoms and cognitive deficits and can elicit a variety of severe side effects. Atypical antipsychotic drugs have shown some improvement in the relief of all the symptoms. However, their efficacy in the treatment of negative symptoms and cognitive impairment is low and severe side effects still remain a problem. The large affected population, lack of effective therapeutics and relatively early onset lead to a tremendous amount of money ($20 to 35 billion annually) spent on schizophrenia in the United States alone (Marino et al., 2002; Chavez-Noriega et al., 2002).

Recent clinical and basic studies suggest that changes in signaling through the NMDA subtype of glutamate receptor may play an important role in some of the pathological changes associated with schizophrenia (Coyle et. al., 2002; Marino and Conn, 2002a; Tsai and Coyle 2002). For instance, NMDA receptor antagonists, phencyclidine (PCP) and ketamine, produce cognitive deficits and positive and negative symptoms in normal human volunteers that are reminiscent of those observed in schizophrenic patients. In addition, these agents exacerbate existing symptomatology in schizophrenic patients. Furthermore, administration of agents that enhance NMDAR function, such as agonists at the glycine binding site on the receptor, results in a symptomatic improvement in schizophrenic patients. Also, NMDA co-agonists are able to relieve all the positive and negative symptoms as well as cognitive deficits in

schizophrenic patients (Tsai et al., 1998). Recently, the broad benefits of clozapine, an atypical antipsychotic drug, have been correlated with its indirect capability to enhance NMDA receptor activity via one of its main metabolites (Sur et al., 2003; Weiner et al., 2004). An NMDA hypofunction model of schizophrenia has been developed based on these observations (Marino et al., 2002). Therefore, it is predicted that any drug that enhances NMDA receptor activity should have the potential to treat schizophrenia (Marino et al., 2002).

Several approaches have been taken to potentiate NMDA receptor function by activation of either NMDA receptor itself or NMDA receptor regulatory proteins, such as mGluR5. mGluR5 is primarily localized postsynaptically, where it potentiates NMDA receptor currents in a wide range of neuronal populations. One possible mechanism for mGluR5 to enhance NMDA receptor activity is that G-protein activation of PLC in turn activates PKC (protein kinase C) and SFK (src family kinase) via multiple $2^{nd}$ messenger system components. SFK in turn up-regulates NMDA receptor function (Salter and Kalia, 2004; Chavez-Noriega et al., 2002; Benquet et al., 2002). A number of recent studies suggest that mGluR5 is a closely associated signaling partner with the NMDA receptor and may play an integral role in regulating and setting the tone of NMDA receptor function in a variety of forebrain regions (Marino and Conn, 2002a). Based on this and a large number of cellular studies suggesting that activation of mGluR5 could have robust effects in forebrain circuits thought to be disrupted in schizophrenia, we and others postulated that activators of mGluR5 could provide novel therapeutic agents that may be useful for treatment of this disorder (Marino and Conn, 2002a; Mohgaddam et al., 2004). Consistent with this, other evidence has emerged that suggests a possible involvement of

mGluR5 in schizophrenia. A genetic study of a large Scottish family successfully links an mGluR5 variation with high risk of schizophrenia (Devon et al., 2001). Behavioral studies also demonstrate that MPEP is able to potentiate hyperactivity, disruption in pre-pulse inhibition (PPI) and cognitive deficits in the PCP-induced schizophrenia rat model. In addition, mGluR5 knock-out mice display consistent deficits in PPI relative to their wildtype controls (Kinney et al., 2003b; Henrey et al. 2002; Campbell et al., 2004; Brody et al., 2004). Moreover, CHPG, an mGluR5 selective agonist, reverses amphetamine-induced disruption of PPI in rat (Kinney et al., 2003). Taken together, mGluR5 selective activators may have promise as novel potential therapeutic agents for schizophrenia.

**mGluR5 is a potential target for multiple other neuronal and psychiatric disorders.**

Hyperactivity of the STN has long been associated with some of the hallmark symptoms of Parkinson's disease (PD) (DeLong, 1990). In the STN, mGluR5 mediates excitatory effects (Awad et al., 2000). Thus, it is possible that blockade of mGluR5 activity in the STN could be beneficial in treating PD pathophysiology. Additionally, globus pallidus (GP) neurons send inhibitory projections to the STN and MPEP potentiates the mGluR1-mediated depolarization of GP neurons. MPEP could exert an anti-parkinsonian effect by facilitating the mGluR1-mediated increased activity of the pallidosubthalamic pathway (Poisik et al., 2003). Consistent with this, there are reports demonstrating that systemic administration of MPEP ameliorates parkinsonian-like symptoms in rodent models of the disease (Ossowska et al., 2001; Spooren et al., 2001). Moreover, studies with mGluR5 selective antagonists, MPEP and MTEP, suggest that mGluR5 may be involved in the physiological and pathological responses related to

neurodegeneration, depression, anxiety, epilepsy, pain, and drug addiction (Chapman et al., 2000; Palucha A and Pilc A, 2002; Chojnacka-Wojcik et al., 2001; Lea et al., 2006). Further studies are necessary to refine our understanding of the roles of mGluR5 in these disorders and to evaluate the possibility that selective blockade of mGluR5 could be used as potential strategy for their treatment.

**mGluR5 is a potential target for learning and memory enhancing reagents.**

Plasticity of synaptic transmission has been proposed to be the molecular corelates of learning and memory. Long term potentiation (LTP) and long term depression (LTD) are two basic forms of synaptic plasticity. Activation of mGluR5 has been suggested to be essential for the induction of synaptic plasticity including both LTP and LTD in the hippocampal CA1 region. mGluR5 knockout mice have impaired LTP in the hippocampal CA1 region and show impairments in memory tasks, such as the Morris water maze and contextual information in the fear-conditioning test (Lu et al., 1997). Also, the mGluR5 selective antagonist MPEP blocks theta burst stimulation (TBS)-induced LTP in the rat CA1 region (Francesconi et al., 2004; Shalin et al., 2006). Meanwhile, the group 1 mGluR selective agonist DHPG has been reported to prime LTP induction at relatively low concentrations (Cohen et al., 1998; Raymond et al., 2000) and induce LTD at higher concentrations in the CA1 region (Gasparini et al. 1999; Huber et al., 2001). DHPG-induced LTD is absent in mGluR5 null mice and can be blocked by MPEP (Faas et al. 2002; Gasparini et al. 1999; Hou and Klann 2004; Huang and Hsu 2006; Huang et al. 2004 and Huber et al., 2001). Moreover, low frequency stimulation (LFS) also induces an mGluR-dependent LTD in addition to NMDAR dependent LTD

(Oliet et al., 1997). Thus it has been postulated that certain manipulations of mGluR5 could affect cognition performance of mammals.

**Allosteric modulators of mGluR5.**

**1. Non-competitive antagonist - MPEP.**

MPEP is one of the earliest allosteric modulators of mGluR5, which is a highly selective full antagonist with nanomolar potency (Gasparini et al., 1999). Schild analysis indicates that MPEP acts on mGluR5 in a non-competitive manner (Pagano et al., 2000). MPEP also inhibits the constitutive receptor activity in cells transiently overexpressing mGluR5, suggesting that MPEP is an inverse agonist. A mutagenesis study using both chimeras and single amino acid substitutions of human mGluR1 and human mGluR5 has been successful to show the molecular determinants of MPEP action and binding. The studies with chimeric receptors show that TM3 and TM7 are two critical domains for the selective inhibitory effect on mGluR5 compared with mGluR1 (Pagano et al., 2000). Replacement of Ala810 in TM7 or Pro655 and Ser658 in TM3 with the homologous residues of mGluR1 abolishes radiolabeled ligand binding to the MPEP site (Pagano et al., 2000). In addition, a reciprocal mGluR1 mutant bearing these three residues of mGluR5 shows high affinity for radio-labeled MPEP analog (Pagano et al., 2000). These results were confirmed by a different group in rat mGluR5 (Malherbe et al., 2003). MPEP has been widely used to study the physiological and behavioral roles of mGluR5 compared with mGluR1 (Lea et al., 2006).

## 2. DFB, DCB and DMeoB.

The success of MPEP stimulated the search for positive allosteric modulators of mGluR5. The first mGluR5 PAM is difluorobenzaldazine (DFB) (O'Brien et al., 2003). DFB has no effect on mGluR5 mediated response alone, but shifts the concentration response curve of glutamate and other orthosteric agonists to the left (Figure 1-3A). DFB is highly selective for mGluR5 and has no activity at other mGluR subtypes (O'Brien et al., 2003). DFB does not alter binding of [$^3$H]quisqualate to the orthosteric glutamate binding site but displaces radioligand binding to the binding site of the allosteric antagonist MPEP, suggesting that this allosteric potentiator might share the same site with the previously identified NAMs of mGluR5 (O'Brien et al., 2003). A series of analogs of DFB have been developed to have a range of activities. While DFB is a PAM, a closely related analog, 3,3'-dimethoxybenzaldazine (DMeOB), is a NAM of mGluR5. Another DFB analog 3,3'-dichlorobenzaldazine (DCB) acts as an allosteric ligand with neutral cooperativity, preventing the positive allosteric modulation of mGluR5 by DFB as well as the negative modulatory effect of DMeOB. Similar to DFB, neither DMeOB nor DCB alters binding of [$^3$H]quisqualate to the orthosteric glutamate site, but they reduce [$^3$H]3-methoxy-5-(2-pyridinylethynyl)pyridine ([$^3$H]MethoxyPEPy) binding to the allosteric MPEP site (Figure 1-3B). These interesting results suggest that structurally related compounds can bind to a single allosteric site to exert effects ranging from negative to positive as well as neutral allosteric activities (O'Brien et al., 2003).

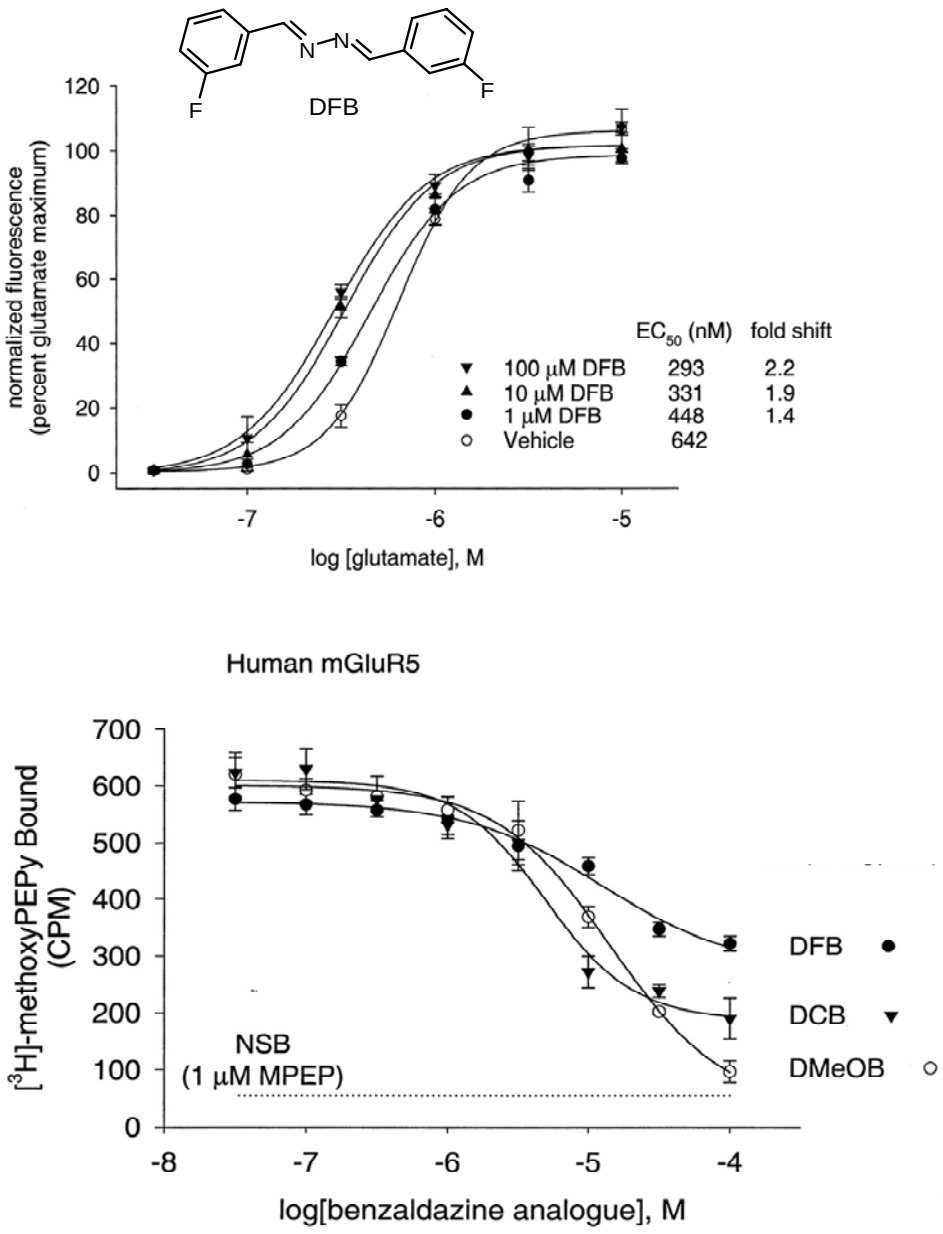


Figure 1-3: DFB family of mGluR5 allosteric modulators. A. Structure of DFB and its role to dose dependently potentiated glutamate concentration dose response curve using calcium mobilization assay of mGluR5 expressing cells. B. DFB, DCB and DMeOB reduced [$^3$H]MethoxyPEPy binding to mGluR5 expressing membrane. (Modified from O'Brien et al., 2003)

### 3. CPPHA

Recently, a second class of mGluR5 PAMs represented by *N*-{4-chloro-2-[(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)methyl] phenyl}-2-hydroxybenzamide (CPPHA) has been discovered (O'Brien et al., 2004). Similar to DFB, CPPHA has no effect on mGluR5 alone and does not alter [$^3$H]quisqualate binding to the orthosteric gulatmate site, but shifts the glutamate concentration response curve to the left (O'Brien et al., 2004). CPPHA is more potent that DFB (EC50 = 100 nM) and induces more robust shift in the glutamate concentration response curve. Interestingly, CPPHA does not compete with ligand binding to the MPEP binding site (Figure 1-4). These data suggest that mGluR5 PAMs may act at multiple sites and have multiple mechanisms of the action. It also suggests that CPPHA and DFB act at different sites on the receptor to potentiate mGluR5 mediated responses. Alternatively, this data suggests that DFB might bind to multiple sites on mGluR5: the MPEP site is one of these sites DFB acts at, but it is not the site that is required for DFB to potentiate mGluR5 responses.

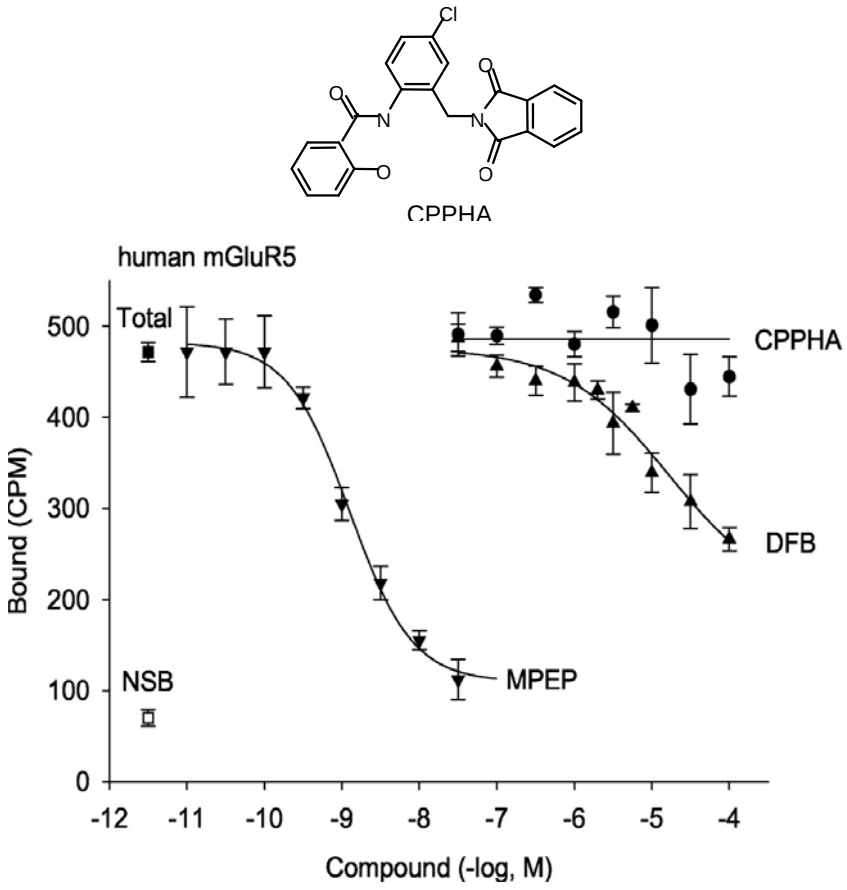


Figure 1-4: CPPHA does not bind to MPEP site. Membranes prepared from human mGluR5 CHO cells were incubated with the radiolabeled MPEP analog [$^3$H]MethoxyPEPy. (Modified from O'Brien et al., 2004)

**4. CPPHA and DFB differentially regulate mGluR5-mediated ERK phosphorylation.**

Recently, increasing evidence suggests that different agonists could differentially activate different signaling pathways of a single GPCR, a phenomenon termed agonist receptor trafficking (Brink et al., 2000; Gazi et al., 2003; Berg et al., 1998). Based on this, it is possible that PAMs of mGluRs could differentially regulate different signaling pathways coupled to a single mGluR subtype. mGluR5 has been shown to couple to multiple signaling pathways and physiological responses. For example, in secondary cultured rat cortical astrocytes, mGluR5 activates PI hydrolysis and extracellular signal regulated kinase (ERK2) phosphorylation by completely independent mechanisms (Peavy et al., 2001; 2002). Both DFB and CPPHA induce parallel leftward shifts of the concentration-response curves of DHPG- and glutamate-induced calcium transients in secondary cultured rat cortical astrocytes. DFB induced a similar shift of concentration-response curve of DHPG-induced ERK1/2 phosphorylation (Zhang et al., 2005). However, CPPHA induces an increase in basal mGluR5-mediated ERK1/2 phosphorylation and potentiates the effect of low concentrations of agonists. In contrast, CPPHA significantly decreases ERK1/2 phosphorylation induced by high concentrations of DHPG. Thus, CPPHA has qualitatively different effects on mGluR5-mediated calcium responses and ERK1/2 phosphorylation (Zhang et al., 2005). Together, these data suggest that different PAMs could differentially modulate different signaling pathways coupled to a single receptor.

It has been found that activation of mGluR5 can have a wide variety of effects on different neuronal populations, including cell depolarization, modulation of different potassium currents, potentiation of NMDA receptor currents, and a variety of other

responses (Valenti et al., 2002). It is possible that these responses are mediated by different signaling mechanisms and could be differentially regulated. In addition, in many neuronal populations, both mGluR1 and mGluR5 are co-expressed, but they elicit different responses (Valenti et al., 2002; Marino et al., 2002; Awad et al., 2000; Poisik et al., 2003; Mannaioni et al., 2001). Since mGluR1 and mGluR5 are closely related in terms of G-protein coupling, this raises the possibility that PAMs could induce a qualitative change in the physiological impact of activation of these receptors in some neuronal populations. Thus it becomes critical to determine how different PAMs could differentially regulate the different signaling responses coupled to a single receptor. One possibility is that DFB and CPPHA could elicit their distinct effects on ERK1/2 phosphorylation by acting at the different sites of mGluR5. This question remains largely unaddressed.

**5. CDPPB.**

The discovery of DFB and CPPHA represents a major advance in establishing the utility of developing selective PAMs at a cellular and molecular level. However, these compounds have relatively low potency and inadequate pharmacokinetic properties needed for *in vivo* studies. More recently, a third series of PAMs of mGluR5 has been identified (Lindsley et al., 2004). These compounds are suitable for *in vitro* studies in rat brain slices and *in vivo* studies to test the hypothesis that mGluR5 PAMs will have an antipsychotic-like and cognition-enhancing activity in animal models (Kinney et al., 2005). These compounds are represented by 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl)benzamide (CDPPB). CDPPB induces a robust potentiation of mGluR5 mediated

responses with an $EC_{50}$ about 25 nM. At 1 μM, CDPPB shifts mGluR5 agonist concentration response curves 9-fold to the left. As with DFB and CPPHA, CDPPB has no effect on agonist binding to the orthosteric agonist binding site of mGluR5 and is highly selective for mGluR5 and has no effect on any other mGluR subtype. Furthermore, the activity of CDPPB was tested against a panel of 175 receptors, transporters, ion channels and enzymes and had no sub-micromolar activities at any of these known receptors (Kinney et al., 2005). The increased potency and solubility of CDPPB relative to DFB and CPPHA make this compound highly suitable for electrophysiology studies in rat brain slices. Furthermore pharmacokinetic studies in Sprague-Dawley rats reveal that CDPPB (2 mg/kg in DMSO) has a plasma half-life of 4.4 hours and readily crosses the blood brain barrier (Kinney et al., 2005). Thus, while CDPPB behaves in a manner similar to DFB and CPPHA at a cellular level, this compound represents a major advance relative to the previous compounds in that its properties make it highly useful for electrophysiology studies in brain slices and for determination of the behavioral effects of mGluR5 potentiators *in vivo*. As discussed above, previous anatomy, electrophysiology, and behavioral studies with mGluR5 antagonists have led to the hypothesis that activation of mGluR5 could have behavioral effects in animal models that are used to predict antipsychotic and cognition-enhancing activity. Interestingly, CDPPB is found to be brain penetrant and to reverse amphetamine-induced locomotor activity and amphetamine-induced deficits in prepulse inhibition in rats, two models sensitive to antipsychotic drug treatment (Kinney et al., 2005). These results demonstrate that positive allosteric modulation of mGluR5 produces significant behavioral effects, suggesting that such modulation serves as a viable

approach to increasing mGluR5 activity *in vivo*. These effects are consistent with the hypothesis that allosteric potentiation of mGluR5 might be a novel approach for development of antipsychotic agents.

**6. 5MPEP and partial antagonists.**

It has been reported that three novel MPEP analogs bind to the allosteric MPEP site on mGluR5 but have only partial inhibition or no functional effects on the mGluR5 response. Two of these compounds, 2-(2-(3-methoxyphenyl)ethynyl)-5-methylpyridine (M-5MPEP) and 2-(2-(5-bromopyridin-3-yl)ethynyl)-5-methylpyridine (Br-5MethoxyPEPy), act as partial antagonists of mGluR5 because they only partially inhibit the response of this receptor to glutamate. The third compound, 5-methyl-6-(phenylethynyl)-pyridine (5MPEP), has no effect on mGluR5 mediated responses alone but still fully displaces MPEP site binding. Interestingly, 5MPEP blocks the effects of both the allosteric antagonist MPEP and allosteric potentiators CDPPB, similar to DCB's effects on DFB and DMeoB. Importantly, 5MPEP has better potency and solubility than DCB. Furthermore, electrophysiological studies show that 5MPEP is active in brain slices preparations. Schild analysis using 5MPEP shows that 5MPEP inhibits MPEP antagonism via a competitive manner. It has been concluded that 5MPEP is a neutral mGluR5 allosteric modulator at the MPEP site, which provides a unique tool to study the pharmacological properties and physiological roles of mGluR5 allosteric modulators in both recombinant and native systems.

**N-terminal truncated mutant of mGluR5.**

Interestingly, it has been shown that mGluR5 retains its constitutive activity on phosphoinositide hydrolysis (PI hydrolysis) with truncation of its N-terminal domain, including the orthosteric glutamate binding site, in recombinant systems. Interestingly, this constitutive activity is inhibited by the mGluR5 NAM, MPEP. Furthermore, this N-terminal truncated mutant is able to be activated by the mGluR5 PAM, DFB, when it is expressed in the recombinant cell line systems (Goudet et al., 2004). This finding illustrates that, like Class 1 GPCRs, the heptahelical domain of mGluR5 can constitutively couple to G-proteins and be negatively and positively regulated by ligands. Furthermore, it provides insights into the unique activation mechanism of family 3 GPCRs. The fact that PAMs do not directly activate wildtype mGluR5 but can activate the N-terminal truncated mutant suggests that there is an allosteric interaction between the Venus Flytrap glutamate binding domain and the heptahelical effector domain that controls the activation of the receptor. A conserved disulfide bond between these two domains has been shown to be necessary for this allosteric interaction which is consistent with this hypothesis (Ronard et al., 2006). Moreover, this discovery provides a unique tool to better understand the mechanism and pharmacological properties of PAMs.

## Objective of This Study

The studies outlined above suggest the mGluR5 PAMs are useful tools to understand the mGluR5 physiological responses and can be used as potential reagents for the treatment of schizophrenia and other CNS disorders. However, the sites of action as well as other pharmacological properties of these compounds remain unclear.

According to the background studies, we hypothesized that CDPPB and CPPHA families of mGluR5 PAMs act through distinct sites in the receptor. CDPPB act at the same site as MPEP, while CPPHA acts at a different site. In chapter II of this thesis, a series of CDPPB analogs were synthesized and we found that they bound to the MPEP site with affinities that are closely related to their potencies as mGluR5 potentiators. Furthermore, allosteric potentiation was blocked by 5MPEP, the neutral ligand at the MPEP site and reduced by a mutation in mGluR5 that eliminates MPEP binding. Together, these data suggest that interaction with the MPEP site is important for allosteric potentiation of mGluR5 by CDPPB and related compounds. In addition, whole-cell patch-clamp studies in midbrain slices reveal that a highly potent analog of CDPPB, 4-nitro-N-(1,3-diphenyl-1H-pyrazol-5-yl)benzamide (VU-29), selectively potentiates mGluR5 but not mGluR1-mediated responses in midbrain neurons, whereas a previously identified allosteric potentiator of mGluR1 had the opposite effect.

In chapter III of this thesis, we found that VU-29- and CPPHA-induced potentiation of mGluR5 responses were both blocked by 5MPEP. However, increasing concentrations of 5MPEP induced parallel rightward shifts in the VU-29 concentration response curve (CRC) whereas 5MPEP inhibited CPPHA potentiation in a non-ompetitive manner. Consistent with this, one mutation that reduced binding of ligands to the MPEP site also eliminated the effect of VU-29 but had no effect on the response to CPPHA. Conversely, a mutation (F585I/mGluR5) that eliminated the effect of CPPHA did not alter the response to VU-29. CPPHA was also a PAM at mGluR1. Interestingly, the corresponding mutation of F585I/mGluR5 in mGluR1 (F599I/mGluR1) eliminated CPPHA's effect without altering the potentiation of a known PAM of mGluR1 (Ro 67-

7476). Likewise, another mutation (V757L/mGluR1) abolished potentiation of Ro 67-7476 had no effect on CPPHA. Finally, CPPHA did not displace binding of a radioligand for the previously characterized mGluR1 allosteric antagonist. Together, these data suggest that CPPHA acts at a novel allosteric site on group 1 mGluRs to potentiate their responses.

There is evidence suggesting that mGluR5 may play a role in learning and memory, but its precise role is still unknown. Taking the advantage of the novel mGluR5 PAMs, we hypothesize that mGluR5 PAMs could affect the plasticity of the rat hippocampal slices by enhancing mGluR5 activation. In chapter IV of this thesis, we test the effect of mGluR5 PAms on rat hippocampal LTP induction. Specifically, we found 500 nM VU-29 potentiated DHPG-induced PI hydrolysis in hippocampal slices, which could be completely blocked by 5MPEP. 500 nM VU-29 did not affect basic synaptic transmission in the CA1 region. Interestingly, pre-incubation of VU-29 significantly enhanced threshold theta burst stimulation (TBS)-induced long term potentiation (LTP). This LTP induction was eliminated by NMDA receptor antagonist, D-AP5. The LTP enhancement was completely blocked by 5MPEP, which had no effect on 10 HZ TBS-induced LTP. Additionally, saturated TBS-induced LTP occluded VU-29 facilitated LTP. VU-29 was not able to further enhance saturated TBS-induced LTP. These results indicate that VU-29 facilitated LTP shares the same mechanism with TBS-induced LTP. Meanwhile, ADX-47273, a novel mGluR5 selective PAM from a distinct structural family, also significantly facilitated threshold TBS-induced LTP. Thus, we conclude mGluR5 PAMs are able to facilitate threshold TBS-induced LTP in the rat hippocampal CA1 region, which could be used as potential cognition enhancing reagents.

In summary, we have investigated two distinct crucial sites action for two different families of mGluR5 PAMs respectively and their differential pharmacological properties. Additionally, we have reported the first evidence that mGluR5 PAMs have the potential to be cognition enhancing reagents by facilitating rat hippocampal CA1 LTP induction.

Table 1-1: Summary of mGluR ligands pharmacology.

| Compound | Activity | Selectivity | Site of Action |
|---|---|---|---|
| ADX-47273 | PAM | mGluR5 | Allosteric |
| CDPPB | PAM | mGluR5 | Allosteric |
| CPPHA | PAM | mGluR1/5 | Allosteric |
| CPCCOEt | Antagonist | mGluR1 | Allosteric |
| DCB | Neutral Modulator | mGluR5 | Allosteric |
| DFB | PAM | mGluR5 | Allosteric |
| DHPG | Agonist | mGluR1/5 | Orthosteric |
| DMeOB | Antagonist | mGluR5 | Allosteric |
| L-AP4 | Agonist | mGluR4/6/7/8 | Orthosteric |
| LY367385 | Antagonist | mGluR1 | Orthosteric |
| MPEP | Antagonist | mGluR5 | Allosteric |
| PHCCC | Antagonist | mGluR1 | Allosteric |
| Quisqualate | Agonist | iGluRs/mGluRs | Orthosteric |
| Ro 01-6128 | PAM | mGluR1 | Allosteric |
| Ro 67-4853 | PAM | mGluR1 | Allosteric |
| Ro 67-7476 | PAM | mGluR1 | Allosteric |
| R214127 | Antagonist | mGluR1 | Allosteric |
| VU-29 | PAM | mGluR5 | Allosteric |
| 5MPEP | Neutral Modulator | mGluR5 | Allosteric |

# CHAPER II

## INTERACTION OF NOVEL POSITIVE ALLOSTERIC MODULATORS OF MGLUR5 WITH THE NEGATIVE ALLOSTERIC ANTAGONIST SITE IS REQUIRED FOR POTENTIATION OF RECEPTOR RESPONSES

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### Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian CNS. In addition to eliciting fast excitatory synaptic responses, glutamate has important neuromodulatory effects by the activation of G-protein-coupled receptors (GPCRs) termed metabotropic glutamate receptors (mGluRs). The mGluRs play important roles in a broad range of central nervous system functions and have potential as novel targets for the development of new therapeutic agents for a number of neurological and psychiatric disorders, including Parkinson's disease (Marino and Conn, 2002b), epilepsy (Doherty and Dingledine, 2002), Alzheimer's disease (Wisniewski and Carr, 2002), pain (Varney and Gereau, 2002), schizophrenia (Marino and Conn, 2002a), depression (Palucha and Pilc, 2002), anxiety disorders (Chojnacka-Wojcik et al., 2001; Pilc, 2003), and others. Since the initial discovery of the mGluRs, there has been an increasing focus on developing subtype-selective modulators of these receptors for use as potential clinical agents and as pharmacological tools that could aid in developing a better understanding of mGluR function.

Although mGluRs have a seven transmembrane (7TM)-spanning domain similar to other GPCRs (Conn and Pin, 1997; Bhave et al., 2003), glutamate binds these receptors

on a large N-terminal extracellular glutamate binding domain that is composed of two globular domains and a hinge region (O'Hara et al., 1993; Jingami et al., 2003). As expected for a region involved in binding a common endogenous agonist, the glutamate binding sites share high homology across the mGluR subtypes relative to other regions of the receptor (Conn and Pin, 1997). Based on this, we and others have begun to take a novel approach and develop compounds that interact with potentially less evolutionary conserved allosteric sites of mGluRs (Knoflach et al., 2001; Gasparini et al., 2002; Marino et al., 2003; May and Christopoulos, 2003; O'Brien et al., 2003, 2004; Schaffhauser et al., 2003). For instance, we have developed DFB, CPPHA, and CDPPB as three distinct structural classes of allosteric potentiators of mGluR5 (O'Brien et al., 2003, 2004; Kinney et al., 2005). These compounds do not activate mGluR5 directly but potentiate the response of mGluR5 to glutamate, inducing a leftward shift of the glutamate concentration-response curve. It is noteworthy that these allosteric modulators do not affect binding of ligands to the orthosteric glutamate binding site. Thus, in contrast to known allosteric modulators of family A GPCRs, they do not act by altering agonist affinity. However, competition binding with [$^3$H]methoxyPEPy, an analog of the allosteric mGluR5 antagonist MPEP, reveals that two potentiators, DFB and CDPPB, displace binding to this site. This led to the suggestion that allosteric potentiators and allosteric antagonists act at overlapping sites in the transmembrane domain. However, whereas CDPPB fully displaces [$^3$H]methoxyPEPy binding, it is not clear whether this compound interacts competitively with [$^3$H]methoxyPEPy at this site. Furthermore, the potency of CDPPB as an allosteric potentiator of mGluR5 is more than one magnitude higher than the apparent affinity of this compound at the [$^3$H]methoxyPEPy site. Finally,

at least one mGluR5 allosteric potentiator, CPPHA, has been identified that does not displace [$^3$H]methoxyPEPy binding (O'Brien et al., 2003, 2004; Kinney et al., 2005). Based on this, it is unclear whether the allosteric potentiator activity of CDPPB requires interaction with the site occupied by [$^3$H]methoxyPEPy. In addition, the majority of studies that have been focused on characterizing mGluR5 potentiators have relied on cultured cell lines rather than native neuronal populations. Thus, it is unclear whether mGluR5 potentiators will selectively potentiate the regulation of mGluR5 neuronal excitability by native neurons.

We report studies in which we use synthetic chemistry, along with molecular pharmacology approaches, to systematically examine the relationship between interaction of CDPPB and related compounds to the allosteric MPEP site and allosteric potentiator activity. Our studies suggest that activities of CDPPB and its analogs as allosteric potentiators are closely related to their affinities for the MPEP site. Furthermore, the discovery of an analog of CDPPB (VU-29) with low nanomolar potency provides an excellent tool for determining the effects of allosteric potentiators on excitation of neurons by mGluR5 and its closest relative, mGluR1. These compounds selectively potentiate mGluR5-mediated responses in midbrain slices without altering responses that are mediated by mGluR1.

## Materials and Methods

### Mutagenesis and transient transfection.

HEK 293A cells (Invitrogen, Carlsbad, CA) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen), 1mM L-glutamine (Invitrogen) and 1× Antibiotic-Antimycotic (Invitrogen). Cells were collected and plated in clear-bottom-96-well plates (Costar, Corning Life Sciences) pretreated with poly-D-lysine (Sigma) in normal growth medium with a density of 40,000 cells per well overnight before transfection. Cells were transiently transfected with wild type and mutant forms of rat mGluR5a cDNA using the pRK5 vector (BD Biosciences Clontech, Palo Alto, CA). Point mutations were generated using the Quick Change II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). All mutations were verified by sequencing. The transfection plasmid was prepared using Sigma Maxi Prep kit (Sigma-Aldrich, St Louis, MO). Cells were transfected with lipofectamine (Invitrogen) for 6 h according to the manufacturer's instructions (80ng DNA and 0.2 l lipofectamine per well) before switching to normal growth medium. Rat GLAST pCDNA3.1 (20 ng per well) was co-expressed with mGluR5 pRK5 to reduce extracellular glutamate concentration. Glutamate/glutamine free medium (glutamine free DMEM plus 10% dialyzed fetal bovine serum, Invitrogen) was applied to substitute growth medium at least 4 hours before performing functional assays. Cell culture, transfection and starving were performed at 37°C in an atmosphere of 95% air plus 5% carbon dioxide. Transfected cells were tested about 48 h after transfection. Rat mGluR2

and human mGluR4 were co-expressed with $G_{qi5}$ which enables coupling to the calcium mobilization as reported by Galici et al., (2006).

**Secondary rat cortical astrocytes culture.**

Secondary rat cortical astrocytes were prepared as described (Peavy et al., 2001; Zhang et al., 2005). Astrocytes were plated into poly-D-lysine coated 96-well plates with a density of 30,000 cells per well on day zero in DMEM containing 10% FBS, 1 mM L-glutamine (Invitrogen) and 1× Antibiotic-Antimycotic (Invitrogen) for overnight. Then G-5 supplement (Invitrogen), which contains epidermal growth factor (10 ng/ml), basic fibroblast growth factor (5 ng/ml), insulin (5 μg/ml) and other factors, was added to the growth medium on day 1 and switched to glutamine-free DMEM with 10% dialyzed FBS on the day 3. Calcium mobilization assay was performed on the day 4. Cell culture and starving are were performed at 37°C with 5% carbon dioxide.

**Calcium fluorescence measurement.**

Cells were loaded with calcium-sensitive dye according to the manufacturer's instructions (Calcium 3 kit; Molecular Devices Corp., Sunnyvale, CA) after incubated in glutamate/glutamine free medium (DMEM and 10% dialyzed fetal bovine serum) for five hours. 1 ml compound A from Calcium 3 kit was dissolved in 20 ml of 1× Hanks' balanced salt solution (HBSS, Invitrogen/Gibco) containing 2.5 mM probenicid (Sigma), adjusted to pH 7.4. Cells were loaded for 50 min at 37°C with 5% carbon dioxide. Dye was then carefully removed and cells were washed with HBSS containing probenicid. Cells were maintained in the same buffer at room temperature for the following assay.

For calcium fluorescence measurement of rat cortical astrocytes, allosteric modulators were added 5 min before the addition of agonist manually. For transient transfected cells, allosteric modulators were added 1 min before the addition of agonist using Flexstation II (Molecular Devices Corp.). Agonist was added at a speed of 52 μl/s and Calcium flux was measured using Flexstation II at 25°C. All the peaks of the calcium response were normalized to the maximum response to a saturated dose of glutamate (10 μM). The submaximal concentration ($EC_{20}$) of glutamate was determined for every separate experiment, allowing for a response varying from 10% to 30% of the maximum peak.

**Radioligand Binding Assays.**

The MPEP analog [$^3$H]methoxyPEPy was used to test the binding of MPEP site on mGluR5 (Cosford et al., 2003). Membranes were prepared from stable rat mGluR5-HEK293A cells (Rodriguez et al., 2005). [$^3$H]methoxyPEPy were incubated with membrane (10 μg/well) in the binding buffer (50 mM Tris/0.9% NaCl, pH 7.4) with the presence or absence of CDPPB analogs at room temperature for 1 h with shaking. Then, the membrane-bound ligand was separated from free ligand by filtration through glass-fiber 96 well filter plates (Unifilter-96, GF/B, PerkinElmer Life and Analytical Sciences, Boston, MA) and washed 3 times with binding buffer (Brandel Cell Harvester, Brandel Inc., Gaithersburg, MD). 30 μL scintillation fluid was added to each well and the membrane-bound radioactivity determined by scintillation counting (TopCount, PerkinElmer Life and Analytical Sciences). Non-specific binding was estimated using 5 μM MPEP. For Scatchard analysis, [$^3$H]methoxyPEPy concentrations of 2.5, 5, 10, 20

and 40 nM were used, whereas 2 nM of [$^3$H]methoxyPEPy was used for competition binding assay. The $K_D$ of [$^3$H]methoxyPEPy by saturation binding was 3.4 nM.

**Compound preparation and application.**

5MPEP, CDPPB, VU-20 to VU-24, VU-28, VU-29, VU-35, and VU-36 were synthesized as described (Rodriguez et al., 2005; Lindsley et al., 2005; de Paulis et al., 2006). Compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma) and stored at -80ºC. Stock solutions were dissolved in 1× HBSS containing 0.1% BSA (Albumin Bovine Serum, Sigma) on the day of experiment. Final DMSO concentration was 0.12% to 0.15% for all the assays.

**N-terminal truncated mGluR5 and Inositol Phosphate determination.**

Construction of the N-terminal truncated mutant of mGluR5 and inositol phosphate (IP) accumulation measurement were performed as reported by Goudet et al. (2004). Briefly, the mGluR5 mutant possesses the signal peptide of the wild-type mGluR5 followed by the HA epitope and the coding sequence of the 7TM region starting at P568, and terminating at L864. IP measurements were performed after transient transfection by electroporation of HEK 293a cells with the plasmid expressing the truncated mGluR5. The cells were incubated overnight with $^3$H-myoinositol (23.4 Ci/mol; NEN; France). Afterwashing, cells were stimulated with the indicated compounds for 30 min in the presence of 10mM LiCl. Inositol phosphate accumulated was recovered by ion exchange chromatography using a Dowex resin (Biorad) in 96 well microfilter plates.

Results are expressed as the ratio between IP and the total radioactivity (IP fraction plus the radioactivity in the membranes).

**Electrophysiology in Subthalamic Nucleus and Substantia Nigra Neurons.**

Whole cell recordings were performed using midbrain brain slices prepared from 12 to 18 day old male Sprague–Dawley rats, as described (Awad et al., 2000; Marino et al., 2001). After decapitation, brains were rapidly removed and submerged in an ice-cold choline replacement solution containing 126 mM choline chloride, 2.5 mM KCl, 1.2 mM $NaH_2PO_4$, 1.3 mM $MgCl_2$, 8 mM $MgSO_4$, 10 mM glucose, and 26 mM $NaHCO_3$, equilibrated with 95% $O_2$, 5% $CO_2$. Sagittal brain slices (350 μm) containing subthalamic nucleus and substantia nigra were cut using a microtome (Leica Microsystems, Nussloch, Germany) and transferred to a holding chamber containing artificial cerebrospinal fluid (ACSF) with 124 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, 1.0 mM $NaH_2PO_4$, 2 mM $CaCl_2$, 20 mM glucose, and 26 mM $NaHCO_3$, equilibrated with 95% $O_2$/5% $CO_2$ and maintained at room temperature. For all experiments, both choline replacement buffer and holding chamber ACSF buffer were supplemented with 5 μM glutathione, 500 μM pyruvate, and 250 μM kynurenic acid to increase slice viability.

After one hour of recovery in the holding chamber, brain slices were then transferred to the slice recording chamber and maintained fully submerged with continuous perfusion of ACSF (2-3 ml/min). Neurons in the subthalamic nucleus (STN) or substantia nigra pars reticulata (SNr) were visualized with a 40x water immersion lens with Hoffman modulation contrast optics. Patch electrodes were pulled from borosilicate glass on the Narishige (East Meadow, NY) vertical patch pipette puller and filled with

internal solution: 125 mM potassium gluconate, 4 mM NaCl, 6 mM $NaH_2PO_4$, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 10 mM BAPTA-tetrapotassium salt, 10 mM HEPES, 2 mM Mg-ATP, and 0.3 mM $Na_2$-GTP; pH adjusted to 7.3 with 1 N KOH. Electrode resistance was 3 to 7 MΩ. All whole-cell patch-clamp recordings were performed using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA). Data were digitized with DigiData 1322A (Molecular Devices), filtered (2 kHz), and acquired by the pClamp 9.2 program (Molecular Devices). After formation of a whole-cell configuration, the recorded neurons were current-clamped to -60 mV. Membrane potentials of STN or SNr neurons were recorded. All compounds were applied by adding into perfusion solution. Data were analyzed using Clampfit 9.2 (Molecular Devices). All results are expressed as mean±SEM, statistical significance was determined using Student's t test.

## Results

### CDPPB displaces [$^3$H]methoxyPEPy binding on mGluR5 competitively.

We previously reported that CDPPB completely displaces binding of the allosteric site ligand [$^3$H]methoxyPEPy to membranes from cells stably expressing mGluR5 (Kinney et al., 2005). We now performed saturation binding experiments with increasing concentrations of [$^3$H]methoxyPEPy in the presence or absence of two concentrations of CDPPB and data transformed using a Scatchard analysis to determine whether this is consistent with competitive interaction of CDPPB with the [$^3$H]methoxyPEPy binding site (Limbird, 1996) (Figure 2-1). Non-specific binding was defined as binding in the presence of 5 μM MPEP and subtracted from total binding. In the absence of CDPPB, Scatchard analysis of [$^3$H]methoxyPEPy binding reveals a straight line ($r^2$ = 0.77), demonstrating interaction at a single binding site. The X-intercept indicates a binding density ($B_{max}$) of approximately 2300 fmol/mG-protein in this membrane preparation and the slope reveals an apparent $K_D$ value of 6.2 nM, consistent with previous results. Addition of CDPPB induced a shift in the slope of the regression line but no effect on the X-intercept, suggesting that CDPPB has no effect on the receptor density. However, the apparent affinity of [$^3$H]methoxyPEPy was reduced by CDPPB, with $K_D$ values of 8.3 nM and 12 nM for 1 μM CDPPB and 2.5 μM CDPPB respectively. The maintenance of a linear Scatchard regression ($r^2$ = 0.74 and $r^2$ = 0.69 for 1 μM and 2.5 μM CDPPB, respectively) with change in apparent $K_D$ and no change in $B_{max}$ is consistent with the hypothesis that CDPPB competitively displaces [$^3$H]methoxyPEPy binding at the MPEP binding site.

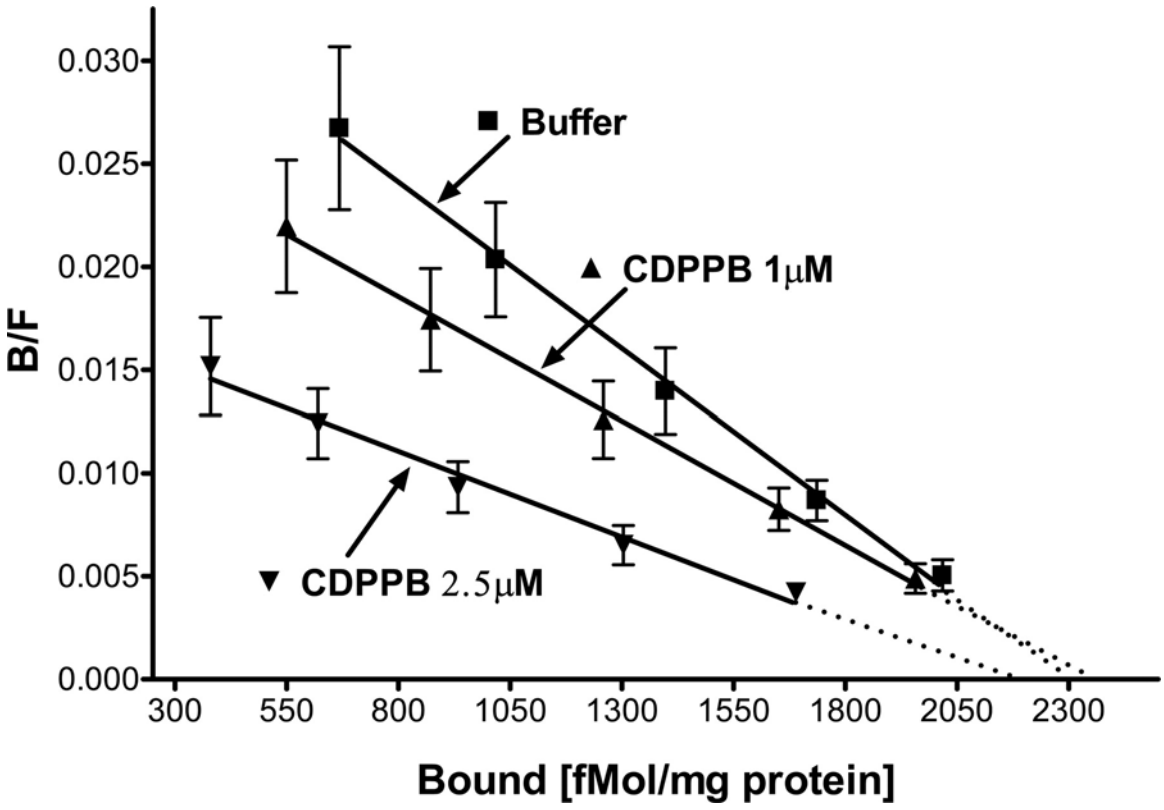
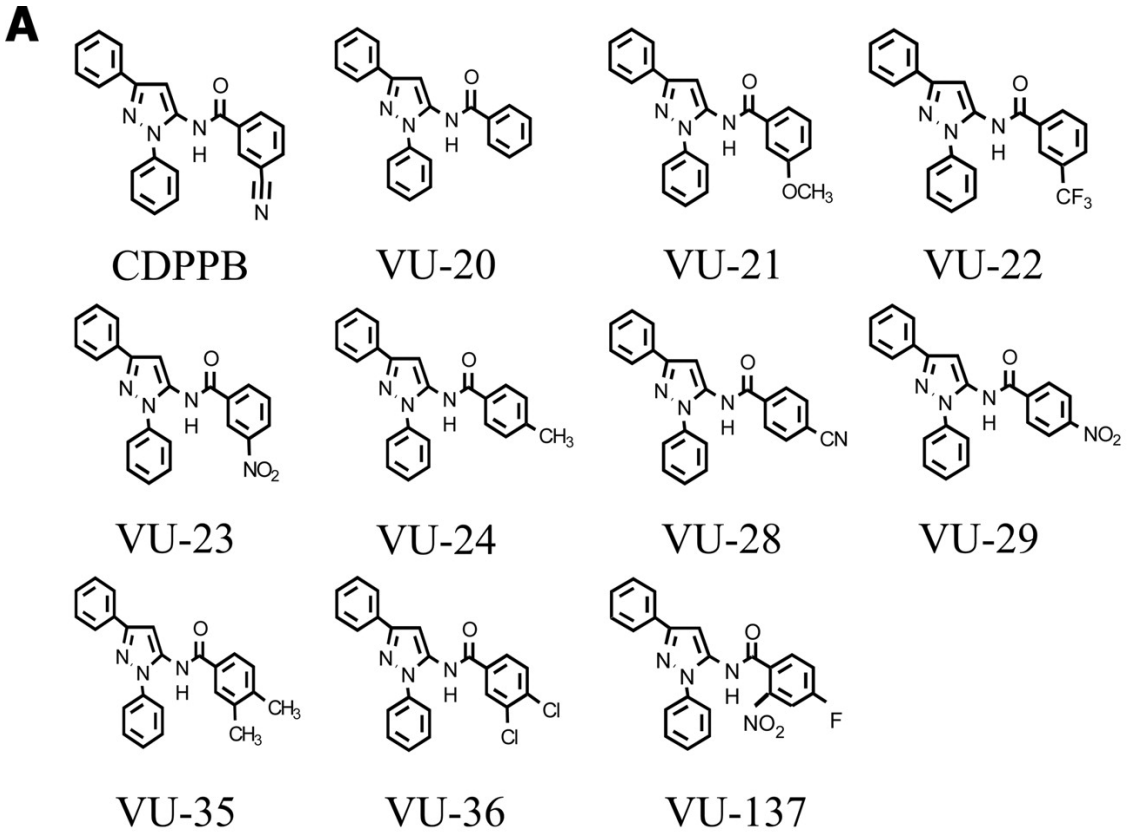



Figure 2-1: CDPPB reduces [$^3$H]methoxyPEPy binding to mGluR5 in a competitive manner. Scatchard analysis showed that CDPPB dose-dependently decreases [$^3$H]methoxyPEPy binding affinity but does not alter maximum binding. Saturation binding on membranes from mGluR5 stable HEK cell line was performed in the absence or presence of CDPPB. X-intercepts showed maximum binding under different binding conditions. In the absence of CDPPB, $B_{max}$ was 2312 ± 355 fmol/mg of protein, with 1 or 2.5 μM CDPPB, $B_{max}$ was 2350 ± 366 or 2135 ± 251 fmol/mg of protein, respectively, showing no significant differences in $B_{max}$ [$^3$H]methoxyPEPy (Student's *t* test). Linear regression lines were generated from four independent experiments in duplicate. Error bars represent S.E.M.

**Potencies of CDPPB analogs at potentiating mGluR5 responses correlate significantly with their affinities at the [$^3$H]methoxyPEPy binding site.**

The finding that CDPPB displaces [$^3$H]methoxyPEPy binding in a manner that is consistent with competitively interaction with this allosteric MPEP binding site raises the possibility that binding to this site is necessary for allosteric potentiator activity. However, another allosteric potentiator of mGluR5, CPPHA, does not bind to this site (O'Brien et al., 2004). Also, the potency of CDPPB as the mGluR5 allosteric potentiator is more than one order of magnitude higher than its apparent affinity at the MPEP binding site (Kinney et al., 2004, de Paulis et al., 2006). Thus, it is possible that CDPPB-induced potentiation of mGluR5 responses is unrelated to its interaction with the allosteric MPEP site. To address this, we synthesized a series of structural analogs of CDPPB to determine whether affinities of these compounds at the MPEP site are closely related to their potencies at potentiating mGluR5 (de Paulis et al., 2006). We selected ten of these compounds based on their close structural similarity to CDPPB, and with no changes to the diphenylpyrazole portion of the molecule (de Paulis et al., 2006). Concentration-response analysis revealed that these compounds potentiate calcium mobilization responses to the mGluR5 agonist glutamate with potencies that range from 9 nM to 228 nM as mGluR5 allosteric potentiators (Figure 2-2; Table 2-1). One compound, VU-137, was inactive as an mGluR5 potentiator. VU-29 was the most potent allosteric potentiator in this group with potency of 9 nM. None of these ten compounds showed significant activities as allosteric potentiators of mGluR1 concentrations up to 10 μM (Hemstapat et al., 2006). Radioligand binding studies revealed that 9 of the 10 CDPPB analogs displace [$^3$H]methoxyPEPy in a concentration-dependent manner (data not shown). Interestingly, the one compound that was inactive at displacing [$^3$H]methoxyPEPy binding, VU-137,

was also inactive at potentiating mGluR5-mediated calcium mobilization responses (Figure 2-2). Similar to CDPPB, the potencies of multiple compounds in the CDPPB series at potentiating glutamate-mediated functional responses were higher than their affinities at displacing [$^3$H]methoxyPEPy from its binding site (Table 1). However, regression analysis of the affinities at the MPEP site versus allosteric potentiator potencies revealed that there is a close correlation between binding affinities to this site and potentiator activity (Figure 2-3) ($r = 0.89$; $p \leq 0.001$). This, together with the finding that VU-137 is inactive in either binding to the MPEP site or as an allosteric potentiator is consistent with the hypothesis that binding to this site is required for allosteric potentiator activity. Having identified VU-29 as a highly potent allosteric potentiator of mGluR5, we used this compound in further studies aimed at characterizing this response.


A
CDPPB
VU-20
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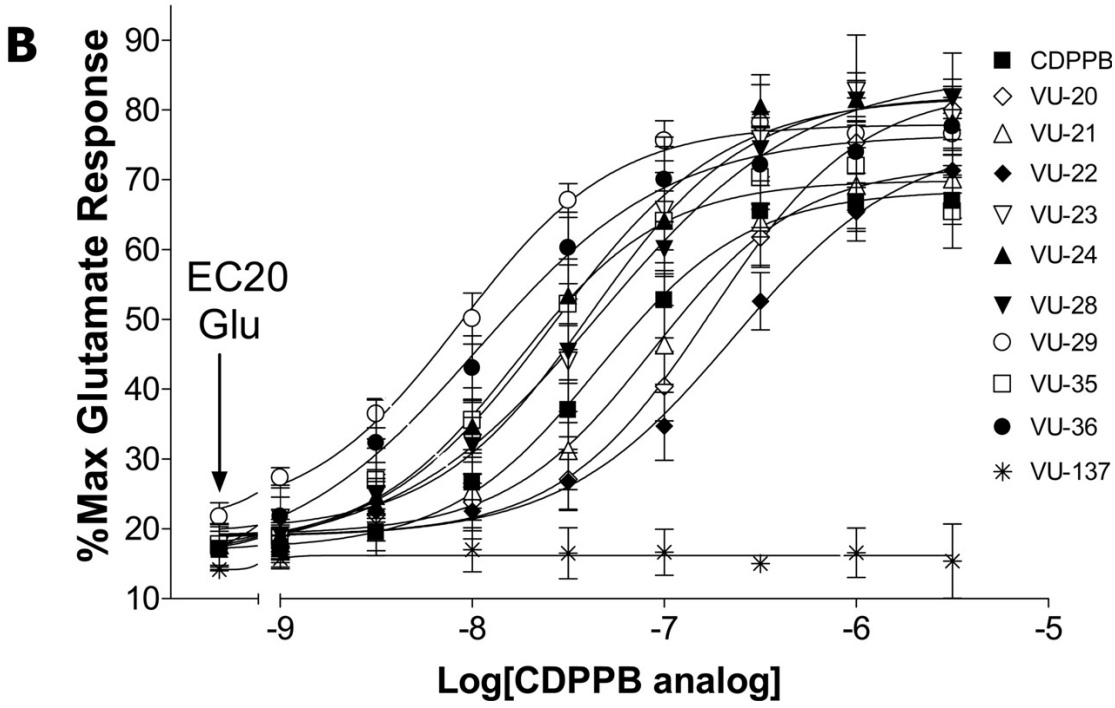
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VU-35
VU-36
VU-137

Figure 2-2: CDPPB analogs have a range of potencies on secondary cultured rat astrocytes as mGluR5 allosteric potentiators. A, chemical structures of selected CDPPB analogs. B, intracellular calcium mobilization responses were measured in response to an $EC_{20}$ concentration of glutamate in the absence or presence of different concentrations of CDPPB analogs. The $EC_{20}$ concentration for glutamate was determined each day and ranged from approximately 270 to 320 nM. CDPPB and its analogs induced a concentration-dependent increase in the response to this $EC_{20}$ glutamate concentration. Data were normalized to the maximum response of each reaction determined by 10 μM glutamate. Concentration-response curves were generated from three independent experiments each performed in duplicate. Error bars represent S.E.M.

Table 2-1: Affinities of CDPPB analogs at the MPEP binding site and their potencies as mGluR5 PAMs. Affinities (Ki) were obtained from [$^3$H]methoxyPEPy competition binding using membrane prep from the rmGluR5 stable cell line and potencies from glutamate-induced calcium release in cultured rat cortical astrocytes. Data are average ± S.E.M. from three or four independent experiments in duplicate.

| | Ki (nM) | $EC_{50}$ (nM) |
|---|---|---|
| **CDPPB** | 1,340 ± 233 | 44.8 ± 20.8 |
| **VU-20** | 3,800 ± 854 | 175 ± 46.4 |
| **VU-21** | 1,400 ± 428 | 87.1 ± 22.2 |
| **VU-22** | 1,310 ± 162 | 228 ± 39 |
| **VU-23** | 604 ± 180 | 41.4 ± 17.8 |
| **VU-24** | 606 ± 263 | 26.2 ± 9.4 |
| **VU-28** | 920 ± 362 | 46.5 ± 18.6 |
| **VU-29** | 244 ± 90 | 9.0 ± 1.5 |
| **VU-35** | 287 ± 64 | 16.9 ± 6.2 |
| **VU-36** | 94.9 ± 41 | 10.6 ± 3 |

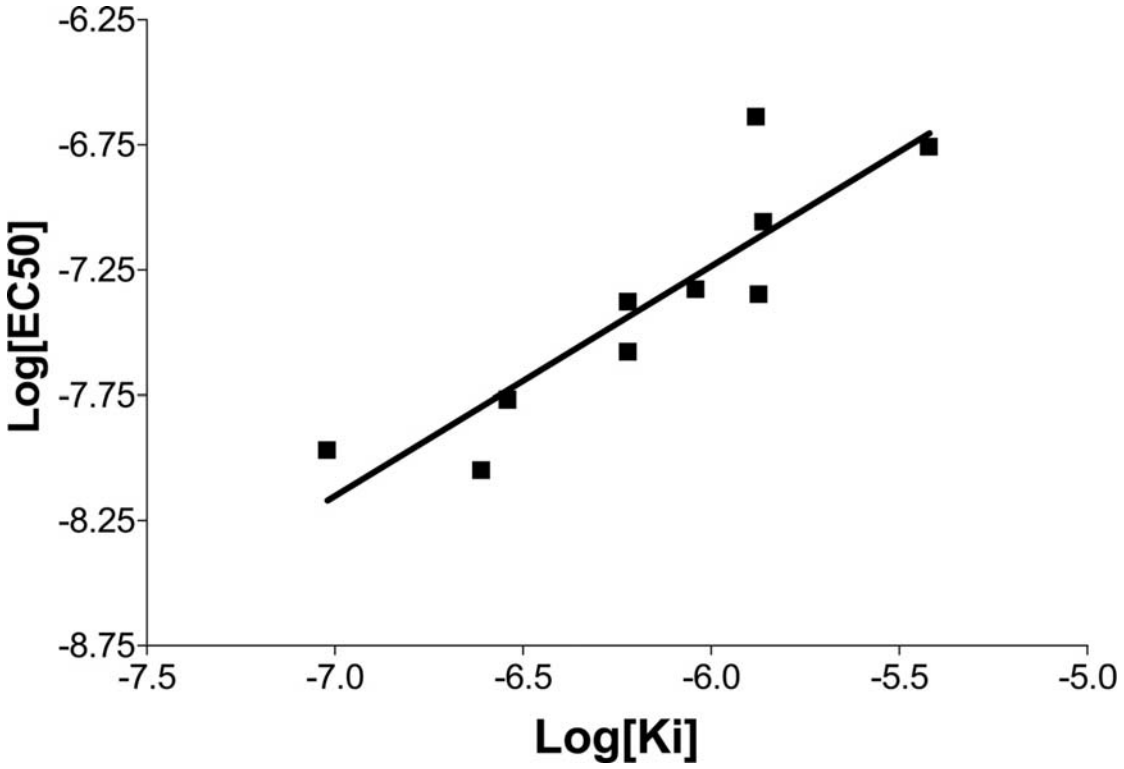
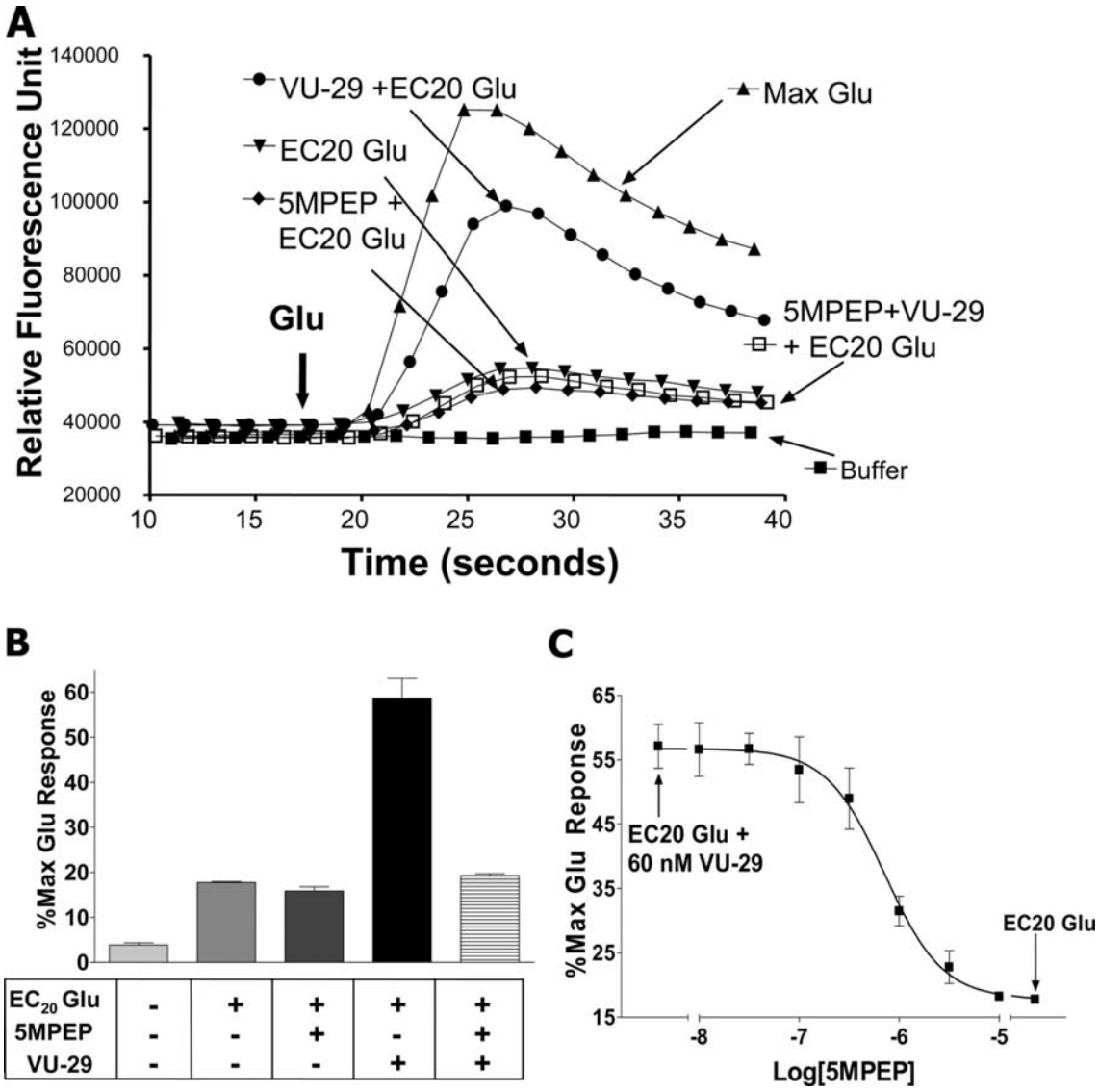



Figure 2-3: The potencies of CDPPB analogs as mGluR5 PAMs significantly correlate with their affinities at the [$^3$H]methoxyPEPy binding site ($r$ = 0.89, $p$ < 0.001). Membranes were prepared from rmGluR5 stable Chinese hamster ovary cell line; 2 nM [$^3$H]methoxyPEPy was used for the competition binding assay, and 5 μM MPEP was used to determine nonspecific binding. Data were obtained from three or four separate experiments each performed in duplicate.

**5MPEP antagonizes VU-29 mediated potentiation of mGluR5 response.**

We recently reported the discovery and characterization of a novel compound that is a positional isomer of MPEP, 5MPEP, that acts as a neutral ligand at the allosteric MPEP site and blocks responses of both allosteric antagonists and potentiators (Rodriguez et al., 2005). Consistent with previous results, 10 μM 5MPEP completely blocked the potentiation of the calcium mobilization response to glutamate by 60 nM VU-29 ($p<0.001$; Figure 2-4A, 2-4B). Concentration response analysis revealed that blockade of the response to VU-29 by 5MPEP is concentration-dependent with an $IC_{50}$ value of 5MPEP of 710 ± 170 nM (Figure 2-4C), which is consistent with the $IC_{50}$ of 5MPEP at blocking the antagonist effect of MPEP (Rodriguez et al., 2005).


A
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Time (seconds)
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Glu
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5MPEP+VU-29
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Buffer
B
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60
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EC20 Glu +
60 nM VU-29
EC20 Glu
-8
-7
-6
-5
Log[5MPEP]

Figure 2-4: 5MPEP is a neutral antagonist of VU-29. A, calcium fluorescence responses of cortical astrocytes to an $EC_{20}$ concentration of glutamate added in the absence and presence of VU-29 and, in some cases, the neutral allosteric site ligand 5-MPEP. Glutamate was added (arrow) 5 min after the addition of 5MPEP and/or VU-29. B, mean (± S.E.M.) calcium fluorescence responses for each condition. 5MPEP (10 μM) did not alter the $EC_{20}$ glutamate response. VU-29 enhanced the $EC_{20}$ glutamate response approximately 3-fold (Student's *t* test, one tail, $p < 0.001$). 10 μM 5MPEP completely blocked the enhancement by 60 nM VU-29 to the baseline (Student's *t* test with VU-29 potentiated response, one tail, $p < 0.001$). C, concentration-response relationship for inhibition of the response to VU-29 by 5MPEP. The response to 60 nM VU-29 plus and $EC_{20}$ concentration of glutamate in the absence of 5MPEP is shown on the left. 5MPEP dose-dependently antagonized VU-29-induced potentiation to a level similar to that of an $EC_{20}$ concentration of glutamate in the absence of VU-29 (shown at right). 5MPEP, 10 μM; VU-29, 60 nM; $EC_{20}$ glutamate, approximately 300 nM. Data were obtained from three or four separate experiments each performed in duplicate.

**VU-29 is an agonist of N-terminal truncated mGluR5.**

If the allosteric potentiator of VU-29 and related compounds is due to actions in the 7TM spanning domain, where the allosteric MPEP binding site resides, it is possible that this compound could retain activity at a truncated mutant of mGluR5 in which the N-terminal extracellular domain, including the glutamate binding domain, is missing. We have recently reported that MPEP and other allosteric modulators of mGluR5 can retain their activity in cells expressing this truncated form of the receptor (Goudet et al., 2004). However, with the glutamate binding site absent, this activity does not depend on the presence of glutamate and these ligands behave in a manner similar to orthosteric ligands at family A GPCRs. Consistent with our previous studies, in HEK cells transiently expressed N-terminal truncated mutant of mGluR5 MPEP behaved as an inverse agonist and inhibited baseline accumulation of inositol phosphates, a measure of coupling of mGluR5 to PI hydrolysis (Goudet et al., 2004) (Figure 2-5A). Consistent with its activity as a neutral ligand at the MPEP site, 5MPEP did not behave as an inverse agonist of the truncated receptor and also did not activate the truncated mutant (Figure 2-5A). Interestingly, VU-29, behaved as an agonist in this system and directly activated PI hydrolysis in a concentration-dependent manner in cells expressing the N-terminal truncated form of mGluR5 (Figure 2-5A). The agonist effect of VU-29 was inhibited by 5MPEP (Figure 2-5B), which is consistent with the hypothesis that activation of PI hydrolysis in the cells is mediated by actions of VU-29 on the 7TM binding domain of mGluR5.

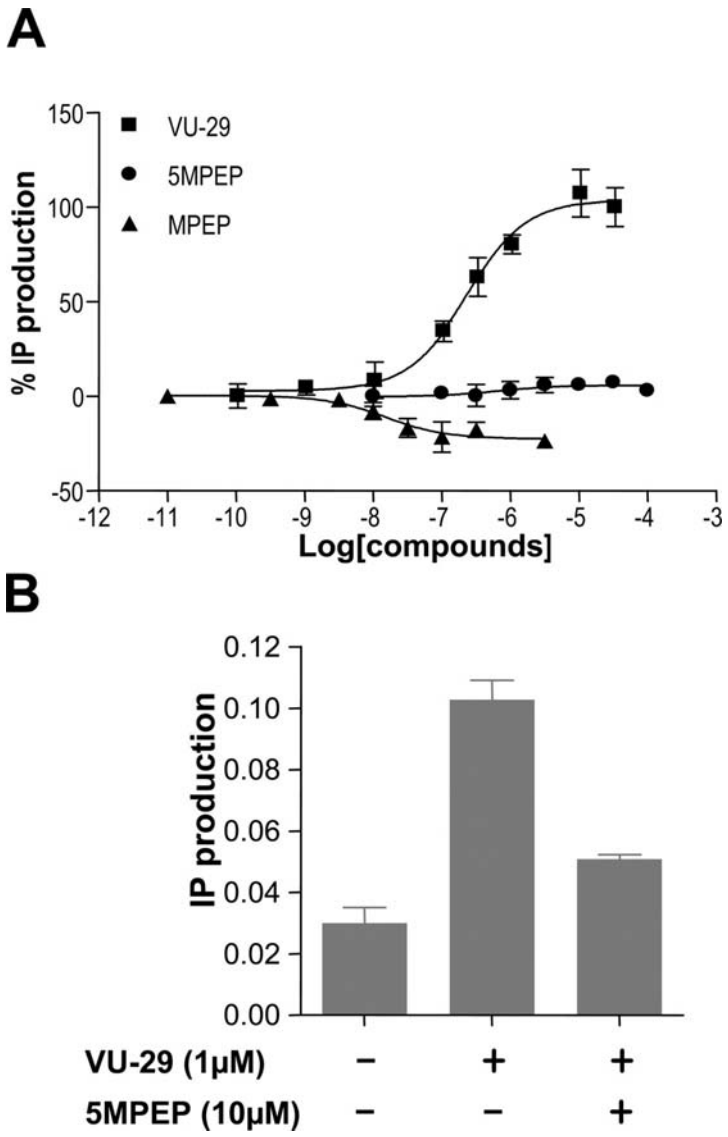



Figure 2-5: VU-29 is an agonist of N-terminal truncated mGluR5. A, VU-29 directly activated N-terminal truncated mGluR5. MPEP was an inverse agonist and 5MPEP did not activate or antagonize the truncated mutant. B, 5MPEP antagonized VU-29-induced IP accumulation through truncated mGluR5. Error bars represent S.E.M. (This figure was contributed by Dr. Cyril Goudet)

**Mutation that eliminates binding of allosteric antagonists to the MPEP binding also reduces the potentiation of mGluR5 by VU-29.**

Mutation of alanine at the 809 position of mGluR5 to valine mGluR5 (A809V) reduces binding of MPEP to mGluR5 and severely reduces the potency of MPEP as an mGluR5 allosteric antagonist (Pagano et al., 2000; Malherbe et al., 2003). In contrast, mutation of a neighboring amino acid, M801T, which is also in the 7TM domain, has little effect on the affinity and function of MPEP (Pagano et al., 2000; Malherbe et al., 2003). If the allosteric potentiator activity of CDPPB and VU-29 require interaction with the allosteric MPEP site, a mutation that reduces binding of ligands to the MPEP site should also reduce activity of these compounds as allosteric potentiators, whereas mutation of this neighboring amino acid should not. Consistent with the previous report, the potency of MPEP on mGluR5 (A809V) was about 10 fold lower than its potency on the wild type receptor, whereas the potency of MPEP on mGluR5 (M801T) remained intact (Pagano et al., 2000 and data not show). Consistent with the hypothesis that allosteric potentiator activity of these compounds requires binding to the MPEP site, both CDPPB and VU-29 were inactive as allosteric potentiators at mGluR5 (A809V), whereas both compounds retained activity at mGluR5 (M801T). Thus, as demonstrated in stable mGluR5-expressing cell lines and astrocytes, CDPPB (1 μM) and VU-29 (200 nM), induced parallel leftward shifts of the glutamate concentration response curves in cells transiently transfected with wildtype mGluR5 (Figure 2-6A) or with mGluR5 (M801T) (Figure 2-6B). In contrast, neither compound induced a significant shift in the glutamate concentration response curve of mGluR5 (A809V) (Figure 2-6C).

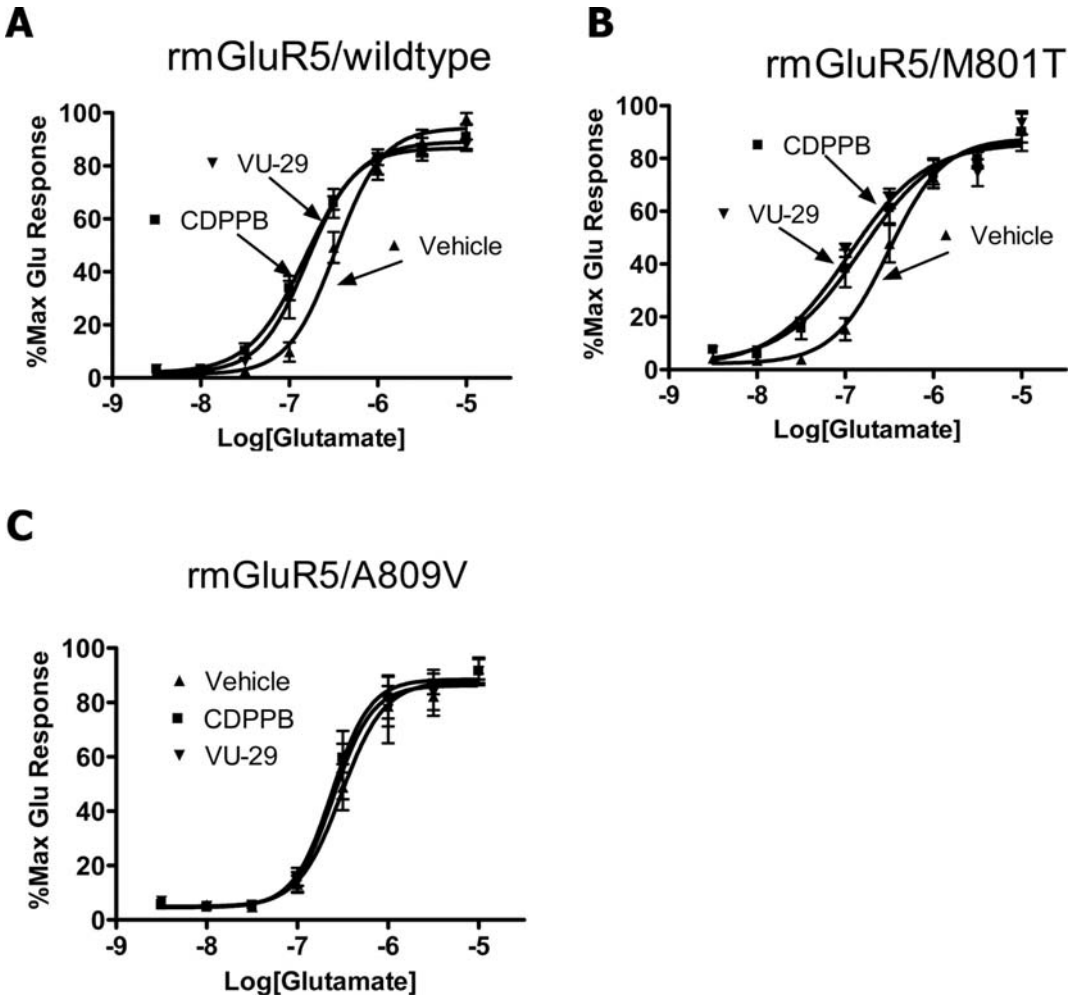



Figure 2-6: Single point mutation that eliminates radiolabeled MPEP binding also eliminates CDPPB- or VU-29-induced potentiation of mGluR5-mediated calcium mobilization in transiently transfected HEK293A cells. A, CDPPB analogs shifted glutamate concentration-response curves of wild-type rmGluR5 2.8 ± 0.6-fold (CDPPB, 1 μM) and 2.5 ± 0.5-fold (VU-29, 200 nM) to the left, respectively. B, CDPPB analogs shifted glutamate concentration-response curves of rmGluR5 (M801T) 3.16 ± 0.91-fold (CDPPB, 1 μM) and 3.55 ± 0.54-fold (VU-29, 200 nM) to the left. C, CDPPB (1 μM) and VU-29 (200 nM) did not shift the glutamate concentration-response curves of rmGluR5 (A809V). Concentration-response curves were generated from three or four independent experiments performed in duplicate. Error bars represent S.E.M.

**VU-29 is selective for mGluR5 relative to other mGluR subtypes.**

Discovery of VU-29 as an allosteric potentiator of mGluR5 with low nanomolar potency provides one of the most potent allosteric potentiators of mGluR5 to date. The potency and solubility properties of this compound make VU-29 well suited to use in functional studies aimed at determining the physiological effects of allosteric potentiators of mGluR5. However, before using this compound to probe mGluR5 function, it is important to determine the selectivity of VU-29 for mGluR5 relative to other mGluR subtypes. We previously reported that this compound is without allosteric potentiator activity on mGluR1 (Hemstapat et al., 2006). We now determined the effects of this compound on mGluR2 and mGluR4 as representative members of the other major subgroups of mGluRs (group II and group III respectively). For measurement of responses to activation of mGluR2 and mGluR4, these receptors were co-transfected with chimeric G-protein-$G_{qi5}$ which allows coupling of these receptors to activation of phospholipase C (PLC) and calcium mobilization. A concentration of VU-29 (1 μM) that is capable of inducing maximal potentiation of mGluR5-mediated responses did not potentiate responses to activation of mGluR2 (Figure 2-7A) or mGluR4 (Figure 2-7B).

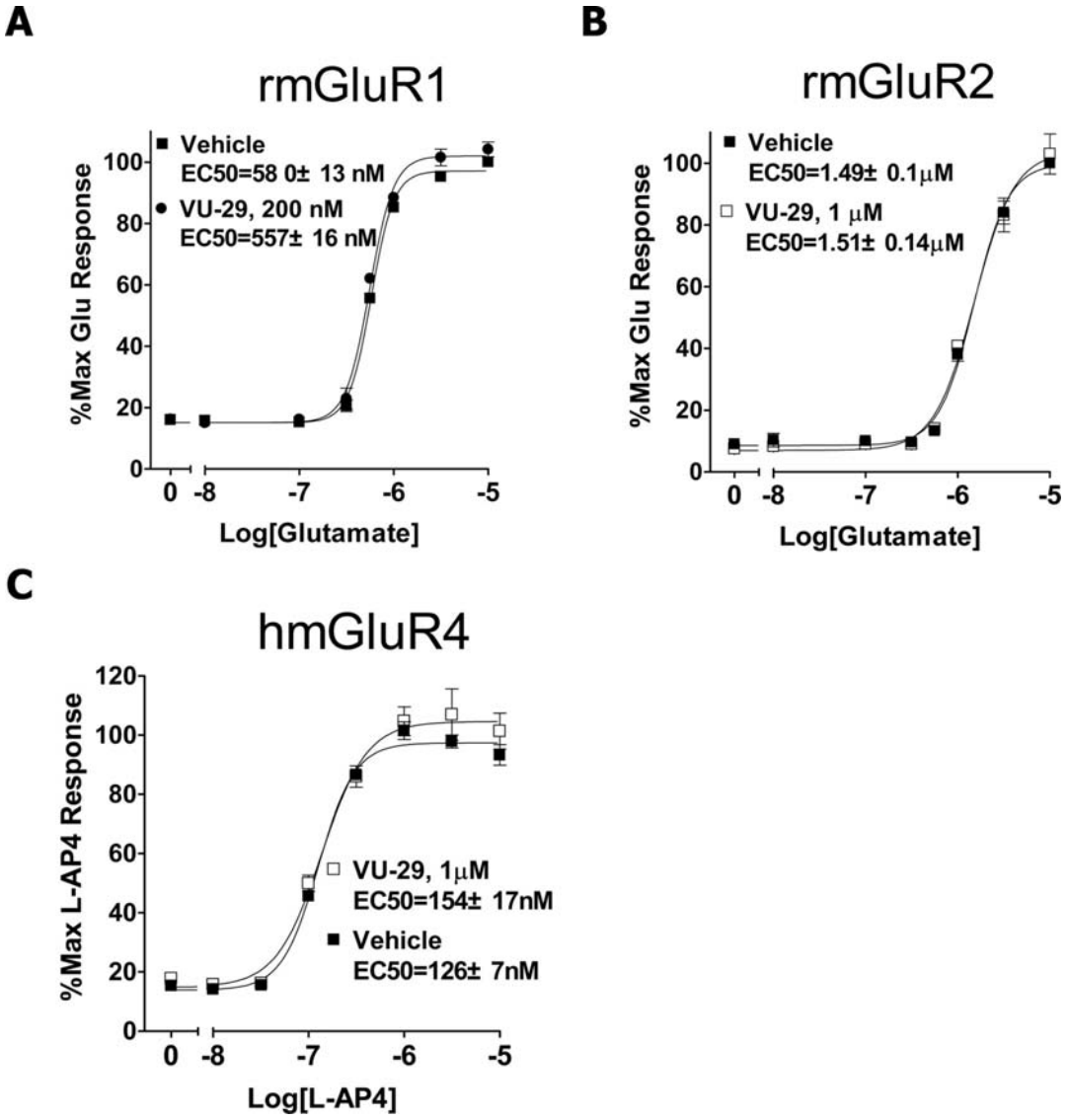



Figure 2-7: VU-29 does not potentiate mGluR1-, -2-, or -4-mediated responses. A concentration of VU-29 that maximally potentiates mGluR5 responses (0.5 μM) neither potentiates nor antagonizes mGluR1- (A), mGluR2- (B), and mGluR4-mediated (C) responses in transiently transfected HEK 293A cells as measured using the calcium mobilization assay. Concentration-response curves were generated from three to four independent experiments performed in duplicate. Error bars represent S.E.M. ((This figure was contributed by Dr. Kamondanai Hemstapat)

**CDPPB and its analogs potentiate excitatory effects of DHPG on neurons in the STN neurons but not the SNr.**

Previous studies reveal that the metabotropic receptor subtypes mGluR5 and mGluR1 are postsynaptically localized on neurons in the STN and SNr. Activation of mGluR5 by DHPG elicits depolarization in STN neurons, which can be blocked by the mGluR5-selective antagonist MPEP (Awad et al., 2000), whereas, mGluR1 plays a predominant role in regulating activity of neurons in the SNr (Marino et al., 2001). However, both of these neuronal populations express both mGluR1 and mGluR5 and previous studies suggest that each of these receptor subtypes can contribute to depolarization of either neuronal population under certain pathological conditions such as dopamine depletion (Marino et al., 2002a). Thus, while it is possible that selective mGluR5 potentiators will selectively potentiate responses that are normally mediated by the respective mGluR subtype, it is also possible that these compounds could recruit mGluR5 activity in neurons in which this receptor is present but does not normally contribute to neuronal depolarization. Whole-cell recordings were performed in STN and SNr neurons in rat midbrain slices to determine whether CDPPB and its analogs selectively potentiate mGluR5-mediated responses in STN neurons while exerting no effect on DHPG-induced depolarization of neurons in the SNr. Consistent with previous reports, bath application of 10 μM DHPG induced a depolarization in STN neurons (6.9 ± 1.2 mV, n=6 cells, Figure 2-8), and CDPPB (10 μM) enhanced the DHPG-induced responses (13.4 ± 1.5 mV, n=8 cells, Figure 2-8). The new higher potency CDPPB analog VU-29 (1 μM) had no effect on the membrane potential of STN neurons during 10 to 15 min application period. However, in the presence of 1 μM VU-29, DHPG (10 μM) elicited stronger depolarization in STN neurons (13.2 ± 1.3 mV, n=8 cells, Figure 2-8)

when compared with the effect of DHPG (10 μM) alone. In addition, VU-23, the 3-nitro analog of CDPPB, potentiated DHPG-induced responses in STN neurons at a concentration of 10 μM (12.4 ± 2.1 mV, n=7 cells). In contrast to CDPPB and its analogs, the mGluR1-selective allosteric potentiator Ro 67-7476 (Knoflach et al, 2001) (3 μM) did not potentiate DHPG-induced (10 μM) depolarization in STN neurons (7.64 ± 1.23 mV, n=9 cells, Figure 2-8), suggesting that the mGluR1 potentiator does not recruit mGluR1-mediated depolarization of these cells and the mGluR5 mediated response in STN neurons is specifically enhanced by the mGluR5 allosteric modulators.

We next determined the effect of CDPPB analogs on DHPG-induced depolarization of SNr neurons, a response that is normally mediated exclusively by mGluR1 (Marino et al., 2001). Consistent with previous reports (Marino et al, 2001), DHPG (3μM) induced a depolarization in SNr neurons (5.45 ± 0.93 mV, n=6, Figure 2-9) and the DHPG-induced response was blocked by the mGluR1 selective antagonist CPCCOEt (7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester) (data not show). In contrast to its effects in STN neurons, VU-29 (1 μM) did not potentiate DHPG-induced (3 μM) depolarization in SNr neurons (7.7 ± 1.9 mV, n=11 cells, Figure 2-9). However, the mGluR1 potentiator Ro 67-7476 (3 μM) enhanced DHPG-induced (3 μM) depolarization in SNr neurons (13.3 ± 2.7 mV, n=7 cells, Figure 2-9). Taken together, these data indicate that mGluR5 allosteric modulator CDPPB and its analogs act as potent and selective positive allosteric modulators of native mGluR5 in STN neurons, whereas mGluR1 allosetric modulator Ro 67-7476 selectively potentiates mGluR1-mediated responses in SNr neurons.

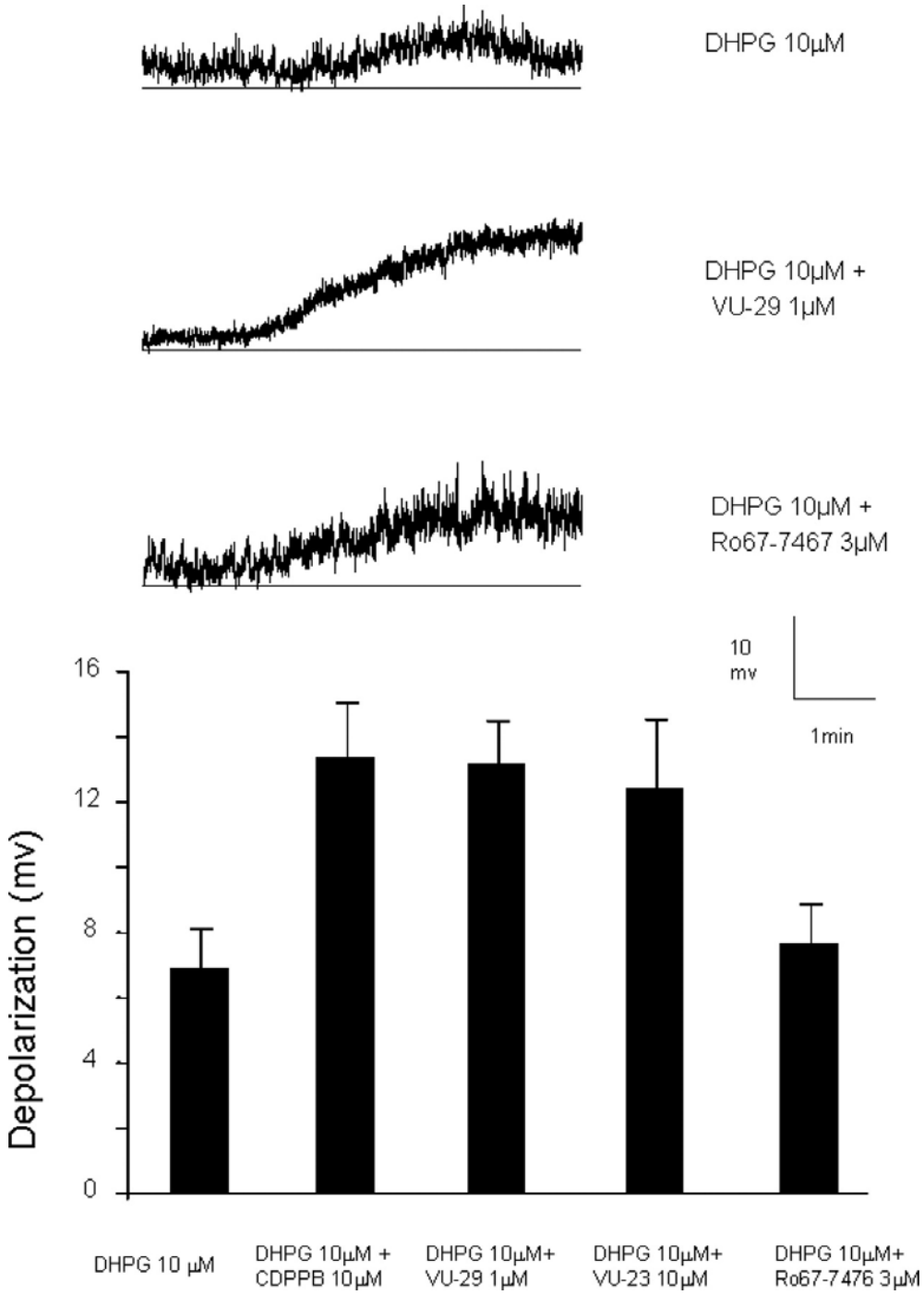



Figure 2-8: CDPPB and its analogs potentiate DHPG-induced depolarization in STN neurons. A, representative traces show depolarization elicited by application of DHPG (10 μM) alone, DHPG plus VU-29 (1 μM), and DHPG plus Ro 67-7476 (3 μM) in STN neurons. B, bar graph (mean ± S.E.M.) illustrates depolarization values elicited by DHPG ($n$ = 6 cells), DHPG plus CDPPB ($n$ = 8 cells), DHPG plus VU-29 ($n$ = 8 cells), DHPG plus VU-23 ($n$ = 7 cells), and DHPG in the presence of plus Ro 67-7476 ($n$ = 9 cells) in STN neurons. *, $p < 0.01$, Student's $t$ test. (This figure was contributed by Dr. Yi Nong)

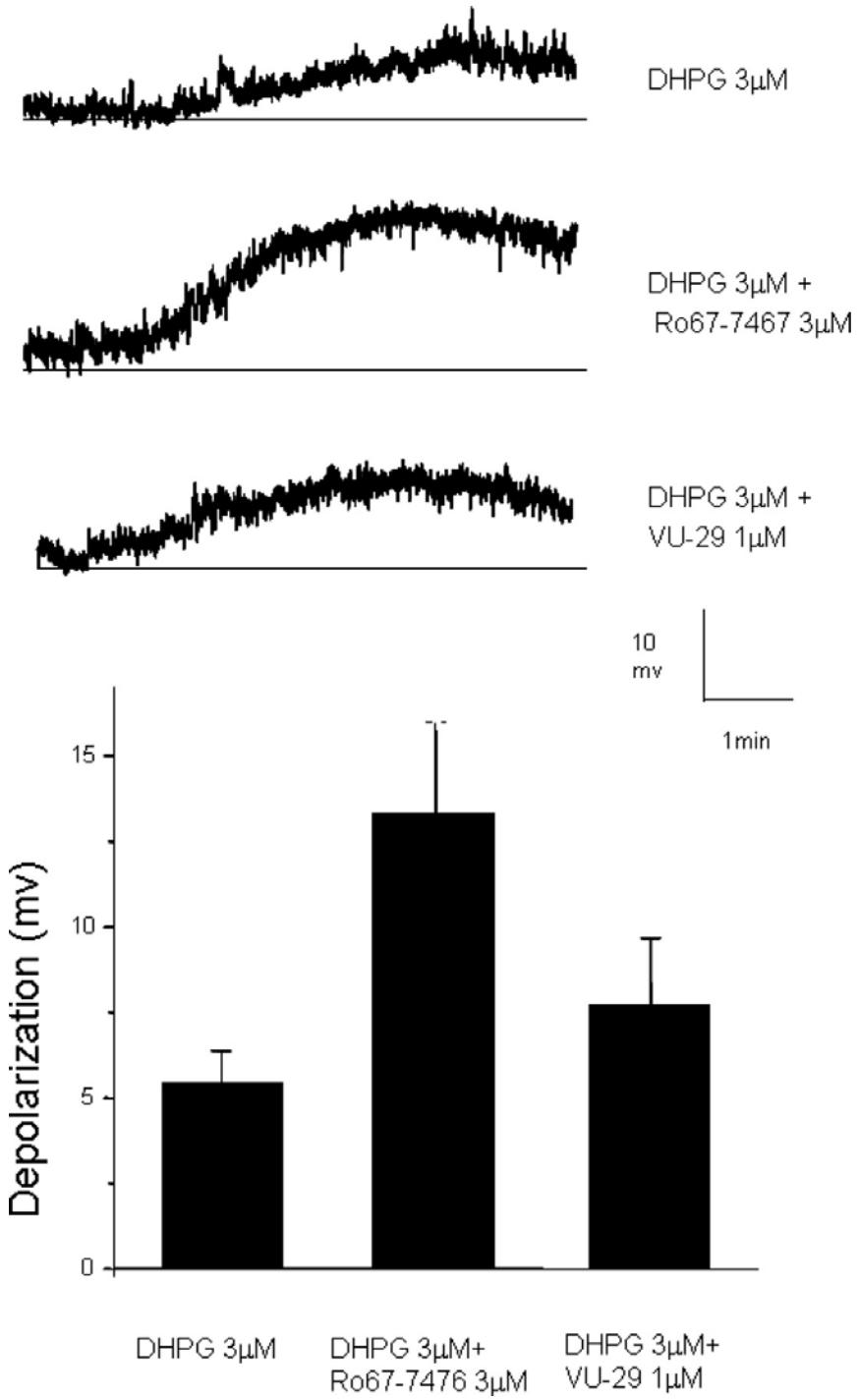



Figure 2-9: CDPPB analogs do not potentiate DHPG-induced depolarization in SNr neurons. A, representative traces showing depolarization of SNr neurons by application of DHPG (3 μM), DHPG (3 μM) plus Ro 67-7476 (3 μM), and DHPG plus VU-29 (1 μM) in SNr neurons. B, bar graph (mean ± S.E.M.) illustrates depolarization of SNr neurons by DHPG ($n$ = 6 cells), DHPG plus Ro 67-7476 ($n$ = 7 cells), and DHPG in the presence of VU-29 ($n$ = 11 cells). *, $p < 0.01$, Student's $t$ test. (This figure was contributed by Dr. Yi Nong)

## Discussion

Three families of mGluR5 selective allosteric potentiators have now been reported from distinct structural groups. These are represented by DFB, CPPHA and CDPPB (O'Brien, et al., 2003; O'Brien et al., 2004; Kinney et al., 2005). DFB and CDPPB inhibit binding of ligands to the site labeled by allosteric antagonists, such as [$^3$H]methoxyPEPy and [$^3$H]MPEP. However, CPPHA does not displace binding to this site, suggesting that allosteric potentiator activity does not always require interaction with the MPEP site. Furthermore, we recently reported that three structurally distinct families of allosteric potentiators of mGluR1 do not share a common binding site with allosteric antagonists at this receptor (Hemstapat et al., 2006). Finally, the potency of CDPPB at displacing antagonist binding to the MPEP site is considerably lower than the potency of this compound as an mGluR5 allosteric potentiator. Together, these data raise the question as to whether displacement of [$^3$H]methoxyPEPy binding is important for the allosteric potentiator activity of this compound. The present results provide strong evidence that CDPPB inhibits allosteric antagonist binding to the MPEP site in a manner consistent with a competitive interaction and that interaction with this site is important for allosteric potentiator activity. Scatchard analysis reveals the competitive nature of CDPPB interactions with this site. This is consistent with the hypothesis that CDPPB is not binding to a separate site to allosterically reduce [$^3$H]methoxyPEPy binding (Limbird, 1996). Further, our results show that there is a close correlation between the apparent affinities of several structural CDPPB analogs at the MPEP site and their activities as allosteric potentiators of mGluR5, suggesting that these two activities are interrelated. In addition, point mutations that reduce binding of MPEP and related compounds to the

allosteric mGluR5 antagonist site also reduce the ability of CDPPB and its analogs to potentiate mGluR5 responses. Taken together, these data suggest that CDPPB and its analogs share a common or partly overlapping binding site with mGluR5 allosteric antagonists and that binding to this site is important for both the activity of CDPPB and related allosteric potentiators and previously known allosteric mGluR5 antagonists.

Given the finding that interaction with the MPEP site is critical for allosteric potentiator activity, it is interesting that allosteric potentiators of mGluR5 consistently have higher potencies at potentiating responses to glutamate than their apparent affinities at the MPEP binding site. This is the case in multiple systems, including astroctyes studied here as well as cell lines stably expressing mGluR5 (Kinney et al., 2005) and cells transiently transfected with mGluR5 (unpublished findings). Thus, full allosteric potentiator activity can be achieved with concentrations of allosteric potentiators that displace a relatively small fraction of ligand binding to the MPEP sites. Importantly, CDPPB can also directly activate mGluR5 in some systems. For instance, unlike the situation in astrocytes, we previously reported that higher concentrations of this compound can directly activate mGluR5, as measured by calcium fluorescence, in the absence of glutamate in cell lines stably expressing mGluR5 (Kinney et al., 2005). Interestingly, the EC50 for direct activation of mGluR5 by CDPPB in the absence of glutamate is in the low μM range, which is closer to the CDPPB apparent Ki value at mGluR5. We have observed a similar activation of mGluR5-mediated calcium responses by CDPPB and its analogs in another stable cell line with consistently lower potencies relative to potentiation of responses to glutamate in the same cell line (unpublished findings). Similarly, we report here that VU-29 directly activates a truncated mutant form

of mGluR5 in which the extracellular glutamate binding domain has been deleted. The EC50 value of VU-29 at activating the truncated receptor is similar to its apparent Ki value at the allosteric MPEP site. The simplest explanation for the difference between modulator potency when tested against glutamate and modulator affinity for the free receptor is that the former parameter is influenced by the positively cooperative interaction between the modulator and glutamate when both occupy the receptor simultaneously. The greater the positive cooperativity, the greater the degree of left-shift in modulator potency. In contrast, when the modulator is tested on its own as an agonist, the potency derived from this latter type experiment will simply reflect the affinity of the modulator for the free receptor and the strength of stimulus-response coupling. Since CDPPB is a very weak agonist in its own right, coupling efficiency is very low and hence the potency of CDPPB as an agonist should be close to its binding affinity.

Current studies indicate that there are multiple allosteric potentiation sites on mGluR5. Among the current 3 families of mGluR5 allosteric potentiators, DFB has been shown to interact at the MPEP binding site as evidenced by competitive [$^3$H]methoxyPEPy binding and by point mutations that eliminate [$^3$H]MPEP binding and reduce DFB potentiation (O'Brien, et al., 2003; Chen et al., 2004). Here, we have confirmed that CDPPB series of allosteric potentiators also acts through interaction to the same site as MPEP. Giving the fact that CPPHA does not inhibit [$^3$H]methoxyPEPy binding to its site up to 100 μM (O'Brien et al., 2004), it is clear that CPPHA does not interact with MPEP binding site. Thus, we propose that there are multiple allosteric potentiatior sites on mGluR5. Allosteric potentiators that bind to different sites may regulate mGluR5 activity differentially. A recent study has shown that CPPHA and DFB

have different modulatory profiles on mGluR5-mediated ERK1/2 phosphorylation in secondary cultured rat cortical astrocytes (Zhang et al., 2005). Based on this, it is possible that allosteric modulators that potentiate activity of a single receptor by different mechanisms may have distinct physiological effects. The finding that CDPPB series of allosteric potentiators act through a shared binding site with MPEP does not necessarily imply that CDPPB analogs interact with identical amino acid residues as MPEP. It is more likely that they are interacting through binding to different amino acids in a largely overlapping pocket. Several residues in TM3, 6 and 7 have been mapped out as crucial interaction sites for MPEP (Pagano et al., 2000; Malherbe et al., 2003). However, the exact amino acid residues that constitute the binding pockets for MPEP or CDPPB are not currently known.

Interestingly, we have now identified ligands that interact with the MPEP site that have a range of activities from allosteric antagonists to allosteric potentiators, and include neutral ligands that interact with this allosteric site but have no intrinsic activity. This is in some ways analogous to the range of activities of ligands at orthosteric sites that includes agonists, inverse agonists, and neutral antagonists. However, it is interesting to note that compounds within a single chemical series that span this entire range of activity appear to be rare. For instance, analysis of the effects of almost 50 analogs of CDPPB has not revealed any compounds in this series that act as allosteric antagonists or neutral ligands (DePaulis et al., 2006; Hemstapat et al., 2006). Similarly, many members of the CDPPB series can also act as allosteric potentiators of mGluR1, but none of these compounds have neutral or allosteric antagonist activity at mGluR1 (Hemstapat et al., 2006). Furthermore, analysis of many analogs of MPEP has yielded many allosteric

antagonists and a small number of neutral ligands but no allosteric potentiators in the MPEP series. (Gasparini et al., 1999; Rodriguez et al., 2005; Alagille et al., 2005a; 2005b; Iso et al., 2006). This suggests clear differences in the structural requirements of different activities at allosteric sites on mGluRs. However, it is also important to note that the benzaldazine series of compounds, exemplified by 3,3'-Difluorobenzaldazine (DFB), also interacts with the MPEP site and includes closely related members that act as allosteric antagonists, allosteric potentiators, and neutral ligands (O'Brien et al., 2003).

Discovery of VU-29 as an allosteric potentiator of mGluR5 with nM potency provides a useful tool for studying the physiological impact of selective potentiation of this receptor subtype. Importantly, VU-29 was found to be selective for mGluR5 relative to mGluRs 1, 2, and 4. In recent years, it has become clear that multiple neuronal populations express both mGluR1 and mGluR5 but that these receptors have distinct physiological effects. For instance, neurons in the STN express both of these mGluR subtypes but under normal conditions only mGluR5 participates in depolarization of STN neurons by the mGluR1/5 agonist DHPG. However, under some conditions, activation of mGluR1 can substitute for mGluR5 and depolarize STN neurons in response to DHPG (Awad et al., 2000; Marino et al., 2001; 2002a). The opposite is true in SNr projections neurons. Only mGluR1 is involved in DHPG-induced depolarization under normal conditions but mGluR5 is also present and can induce calcium transients and can depolarize these cells under some conditions (Marino et al., 2001; 2002a). Theoretically, it is possible that VU-29 and other allosteric potentiators could selectively potentiate responses to DHPG that are normally mediated by mGluR5. However, it is also possible that by potentiating mGluR5 activity, these compounds could also lead to coupling of

mGluR5- to responses in which it does not normally participate in cells that express this receptor. The finding that VU-29 and related mGluR5 potentiators enhanced DHPG-induced depolarization of STN neurons but not of SNr neurons and that the mGluR1 potentiator Ro 67-7476 had the opposite effects suggests that, in these cells, these compounds potentiate the normal response to mGluR5 or mGluR1 activation but maintain the normal physiological roles of these receptor subtypes. This is of critical importance in considering the physiological impact of these compounds in intact systems. In future studies these compounds will provide excellent tools for understanding the impact of selective potentiation of mGluR1 and mGluR5-mediated responses in a range of neuronal populations and circuits.

# CHAPTER III

## CPPHA ACTS THROUGH A NOVEL SITE AS A POSITIVE ALLOSTERIC MODULATOR OF GROUP 1 METABOTROPIC GLUTAMATE RECEPTORS.

### Introduction

Group I mGluRs are distributed widely in multiple brain regions. Positive allosteric modulators (PAMs) of group 1 mGluRs have recently emerged as useful tools to study their physiological roles and provide a promising approach for developing novel therapeutic agents for treatment of psychiatric and cognitive disorders (Anwyl 1999; Marino et al 2002; O'Brien, et al., 2003; O'Brien et al., 2004; Campbell et al., 2004; Moghaddam, 2004; Kinney et al., 2005;).

Three distinct families of mGluR5 PAMs have been developed including DFB, CPPHA and CDPPB (O'Brien, et al., 2003; 2004; Kinney et al., 2005). The mGluR5 PAMs do not activate the receptor directly but potentiate its response to glutamate. Unlike allosteric modulators of other GPCRs, mGluR5 PAMs do not affect binding of ligands to the orthosteric glutamate binding site (Christopolous and Kenakin, 2002; O'Brien, et al., 2003; O'Brien et al., 2004; Kinney et al., 2005). DFB and CDPPB displace binding of a radioligand to a well characterized binding site for mGluR5 negative allosteric modulators (NAMs), such as MPEP. Furthermore, we have provided evidence that the CDPPB family of PAMs (including VU-29) exert their potentiation effects by actions at this site (Chen et al., 2007). Interestingly, a third PAM, CPPHA, does not bind to the MPEP site at concentrations several magnitudes higher than its PAM potency (O'Brien et al., 2004), suggesting that CPPHA acts by a different mechanism

than do CDPPB, VU-29, and DFB. However, PAMs and orthosteric agonists activate mGluR5 in a cooperative manner so that relatively low occupancy at the MPEP site can induce robust potentiation of mGluR5 responses (Chen et al., 2007; DePaulis et al., 2006). Thus, CPPHA could potentiate mGluR5 by actions at this site but have low affinity and high cooperativity so that low binding is not detectable at concentrations that have been used in radioligand binding studies. We now report a series of studies that suggest that CPPHA potentiates responses to activation of both mGluR5 and mGluR1 by actions at a site that is clearly distinct from the previously identified allosteric sites on either of these receptor subtypes. These data suggest that multiple distinct allosteric sites exist on mGluRs that can serve as targets for PAMs to induce qualitatively similar effects on receptor function.

## Materials and Methods

### Mutagenesis and transient transfection.

HEK 293A cells (Invitrogen, Carlsbad, CA) were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen), 1mM L-glutamine (Invitrogen) and 1× Antibiotic-Antimycotic (Invitrogen). Cells were collected and plated in clear-bottom-96-well plates (Costar, Corning Life Sciences) pretreated with poly-D-lysine (Sigma) in normal growth medium with a density of 40,000 cells per well overnight before transfection. Cells were transiently transfected with wild type and mutant forms of rat mGluR5a/mGluR1 cDNA using the pRK5 vector (BD Biosciences Clontech, Palo Alto, CA). Point mutations were generated using the Quick Change II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Construction of the N-terminal truncated mutant of mGluR5 was performed as reported by Goudet et al. (2004). All mutations were verified by sequencing. The transfection plasmid was prepared using Sigma Mini Prep kit (Sigma-Aldrich, St Louis, MO). Cells were transfected with lipofectamine (Invitrogen) for 6 h according to the manufacturer's instructions (80ng DNA and 0.2 µl lipofectamine per well) before switching to normal growth medium. Rat GLAST pCDNA3.1 (20 ng per well) was co-expressed with mGluR5 pRK5 to reduce extracellular glutamate concentration for the test of VU-29. Glutamate/glutamine free medium (glutamine free DMEM plus 10% dialyzed fetal bovine serum, Invitrogen) was applied to substitute growth medium at least 4 hours before performing functional assays. Cell culture, transfection and starving were performed at 37°C in an atmosphere of 95% air plus 5% carbon dioxide. Transfected cells

were tested about 48 h after transfection. Rat mGluR2 and human mGluR4 were co-expressed with $G_{qi5}$ which enables coupling to the calcium mobilization as reported by Gallici et al., (2006).

**Secondary rat astrocytes culture.**

Secondary rat cortical astrocytes were prepared as described (Peavy et al., 2001; Zhang et al., 2005; Chen et al., 2007). Detailed protocol has been described in Chapter II.

**Calcium fluorescence measurement.**

Cells were loaded with calcium-sensitive dye according to the manufacturer's instructions (Calcium 3 kit; Molecular Devices Corp., Sunnyvale, CA) after incubated in glutamate/glutamine free medium (DMEM and 10% dialyzed fetal bovine serum) for five hours. 1 ml compound A from Calcium 3 kit was dissolved in 20 ml of 1× Hanks' balanced salt solution (HBSS, Invitrogen/Gibco) containing 2.5 mM probenicid (Sigma), adjusted to pH 7.4. Cells were loaded for 50 min at 37°C with 5% carbon dioxide. Dye was then carefully removed and cells were washed with HBSS containing probenicid. Cells were maintained in the same buffer at room temperature for the following assay. For calcium fluorescence measurement of rat cortical astrocytes, allosteric modulators were added 5 min before the addition of agonist manually. For transient transfected cells, VU-29 was added 1 min before the addition of agonist using Flexstation II (Molecular Devices Corp.) at a speed of 52 µl/s. CPPHA was added 5 min before agonist. Calcium flux was measured at 25°C. All the peaks of the calcium response were normalized to the maximum response to a saturated dose of glutamate (10 µM). The submaximal

concentration ($EC_{20}$) of glutamate was determined for every separate experiment, allowing for a response varying from 10% to 30% of the maximum peak.

**Inositol Phosphate measurement.**

Inositol phosphate (IP) accumulation experiments were performed in 96-well microplates as already described by Goudet et al. (2004). Briefly, after transfection of the truncated mGluR5 receptor, HEK293 cells were incubated overnight with $^{3}$H-myoinositol (16 Ci/mmole, Amersham, Buckinghamshire, UK). The following day, cell medium were washed and ambient glutamate degraded by incubation in presence of GPT. Cells were stimulated by the indicated compounds for 30 minutes then the medium was removed and cells incubated with cold 0.1 M formic acid which induced cell lysis. Then $^{3}$H-IP produced following receptor stimulation were recovered by ion exchange chromatography using a Dowex resin (Biorad). IP were then eluted by a highly concentrated formate solution (4 M), collected in a 96-wells sample plate and mixed with liquid scintillator (Perkin Elmer). The radioactivity remaining in the membranes is used to normalize the IP produced. Membranes were solubilized with a solution of NaCl (0.1 M) containing 10% of Triton X100 (Sigma), collected and mixed with liquid scintillator in a 96-well sample plate. Radioactivity was counted using a Wallac 1450 Microbeta stintillation and luminescence counter (Perkin Elmer). Results are expressed as the ratio between IP and the total radioactivity corresponding to IP plus membrane. All points are realized in triplicate.

**[$^3$H]R214127 Radioligand Binding Assays.**

Membranes preparation from BHK cells stably expressing rat mGluR1a and [$^3$H]1-(3,4-dihydro-2*H*-pyrano[2,3-*b*]quinolin-7-yl)-2-phenyl-1-ethanone ([$^3$H]R214127) competition binding assay was performed following the protocol described in Hemstapat et al., (2006). Compounds were dissolved in DMSO as stocks kept in -20°C. The final DMSO concentration was 0.12% in the assay.

**Compound preparation.**

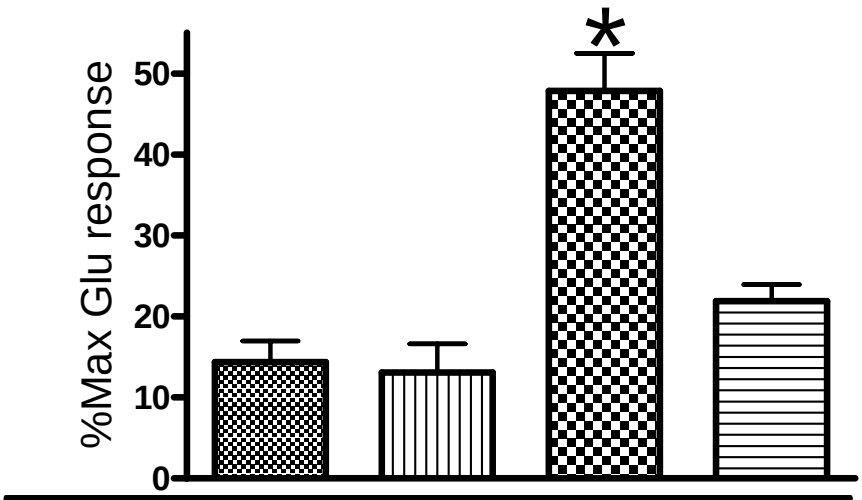
Compounds were dissolved in dimethyl sulfoxide (DMSO, Sigma) and stored at -80ºC. Stock solutions were dissolved in 1× HBSS containing 0.1% BSA (Albumin Bovine Serum, Sigma) on the day of experiment. Final DMSO concentration was 0.12% to 0.15% for all the assays.

## Results

### 5MPEP blocks responses to CPPHA and VU-29 with different potencies.

We used secondary cultured rat cortical astrocytes for the functional assay of mGluR5 (calcium mobilization assay). As previously described, the calcium fluorescence response to a threshold (EC20) concentration of glutamate (about 300 nM) was potentiated by 3 μM CPPHA (Figure 3-1A; student's t-test, $P<0.01$). The neutral MPEP site allosteric modulator, 5MPEP (10 μM) (Rodriguez et al., 2005) had no effect on the EC20 glutamate response, however it blocked potentiation of this response by 3 μM CPPHA (Figure 3-1A; student's t-test, $P<0.01$) in a manner similar to that previously shown for VU-29 (Chen et al., 2007). Comparable sub-maximal concentrations of CPPHA and VU-29 were chosen (3 μM CPPHA and 60 nM VU-29) to generate 5MPEP concentration response curves. The potency of 5MPEP at blocking the response to VU-29 was 0.75 ± 0.2 μM whereas its potency at blocking the response to CPPHA was 2.27 ± 0.7μM on (Figure 3-1B). These values were significantly different (student's t-test, $P<0.05$). Furthermore, the slope of the inhibition curve for blockade of the response to CPPHA appeared to be more shallow that for blockade of the response to VU-29.

**A**




| | | | | |
|---|---|---|---|---|
| EC20 Glu | + | + | + | + |
| 5MPEP | - | + | - | + |
| CPPHA | - | - | + | + |

**B**

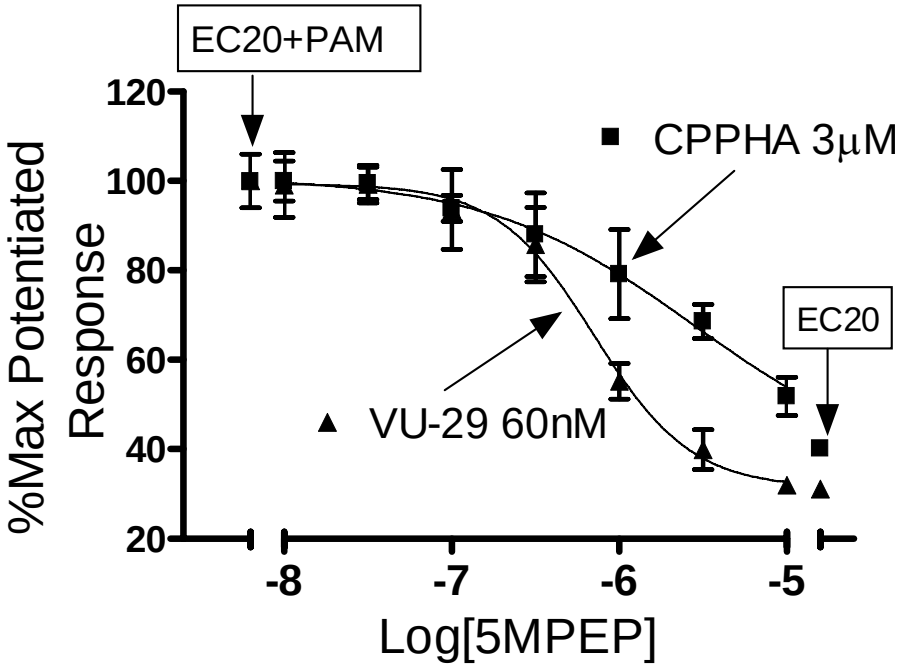


Figure 3-1: 5MPEP have different potencies to block CPPHA and VU-29-induced potentiation of mGluR5-mediated intracellular calcium flux. A, 5MPEP blocked CPPHA-induced potentiation of mGluR5-mediated intracellular calcium flux in the secondary cultured rat cortical astrocytes. Pretreatment with 10 μM 5MPEP did not affect the response to glutamate (EC20, about 300nM). 3 μM CPPHA enhanced the response. 5MPEP blocked the potentiation significantly. Asterisk means $P<0.01$ (Student's t test). B, 5MPEP blocks the effects of VU-29 and CPPHA in a dose dependent manner in the secondary cultured rat cortical astrocytes via calcium mobilization assay. Data was normalized to the maximum potentiation of each potentiator determined by 3μM of CPPHA or 60nM of VU-29 respectively showing as Control A. Control B shows the EC20 response of glutamate without any allosteric potentiator. The potency of 5MPEP on VU-29 was $0.745 \pm 0.2$ μM while it was $2.27 \pm 0.72$ μM for CPPHA (Student's t test, $P < 0.05$). Concentration-response curves were generated from three independent experiments, each performed in triplicate. Error bars represent S.E.M of 3 separate experiments.

**5MPEP and MPEP have actions consistent with non-competitive blockade of the response to CPPHA.**

To determine whether 5MPEP blocks responses to VU-29 and CPPHA by similar mechanisms, we determined the effects of increasing concentrations of 5MPEP on the concentration response relationships of VU-29 and CPPHA-induced potentiation of mGluR5 responses (Figure 3-2). 5MPEP induced progressive parallel rightward shifts of the VU-29 CRC when added at concentrations up to 10 μM. Thus, VU-29 was capable of overcoming inhibition by 5MPEP across this concentration range. Schild regression showed a linear line ($r^2$=0.9921) with a slope of 0.9704. The X-intercept was 100 nM, which fitted its Ki obtained from competition binding (Figure 3-2A; Rodriguez et al., 2005). This is consistent with a competitive mechanism of action of 5MPEP at blocking the VU-29 response. In contrast, increasing concentrations of 5MPEP induced progressive decreases in the maximum potentiation of CPPHA (Figure 3-2B) suggesting that 5MPEP blocks the response to CPPHA by a non-competitive mechanism of action.

We next performed similar experiments with MPEP. Since MPEP is an mGluR5 NAM instead of neutral modulator, this compound decreased the baseline response of the concentration response curves of both VU-29 and CPPHA. Consistent with 5MPEP, MPEP induced dose dependent parallel rightward shifts in the VU-29 CRC but downward shifts in CRCs for CPPHA. This is consistent with the effects of 5MPEP and the hypothesis that ligands acting at the MPEP site do not interact with CPPHA in a competitive manner (Figure 3-3A,B).

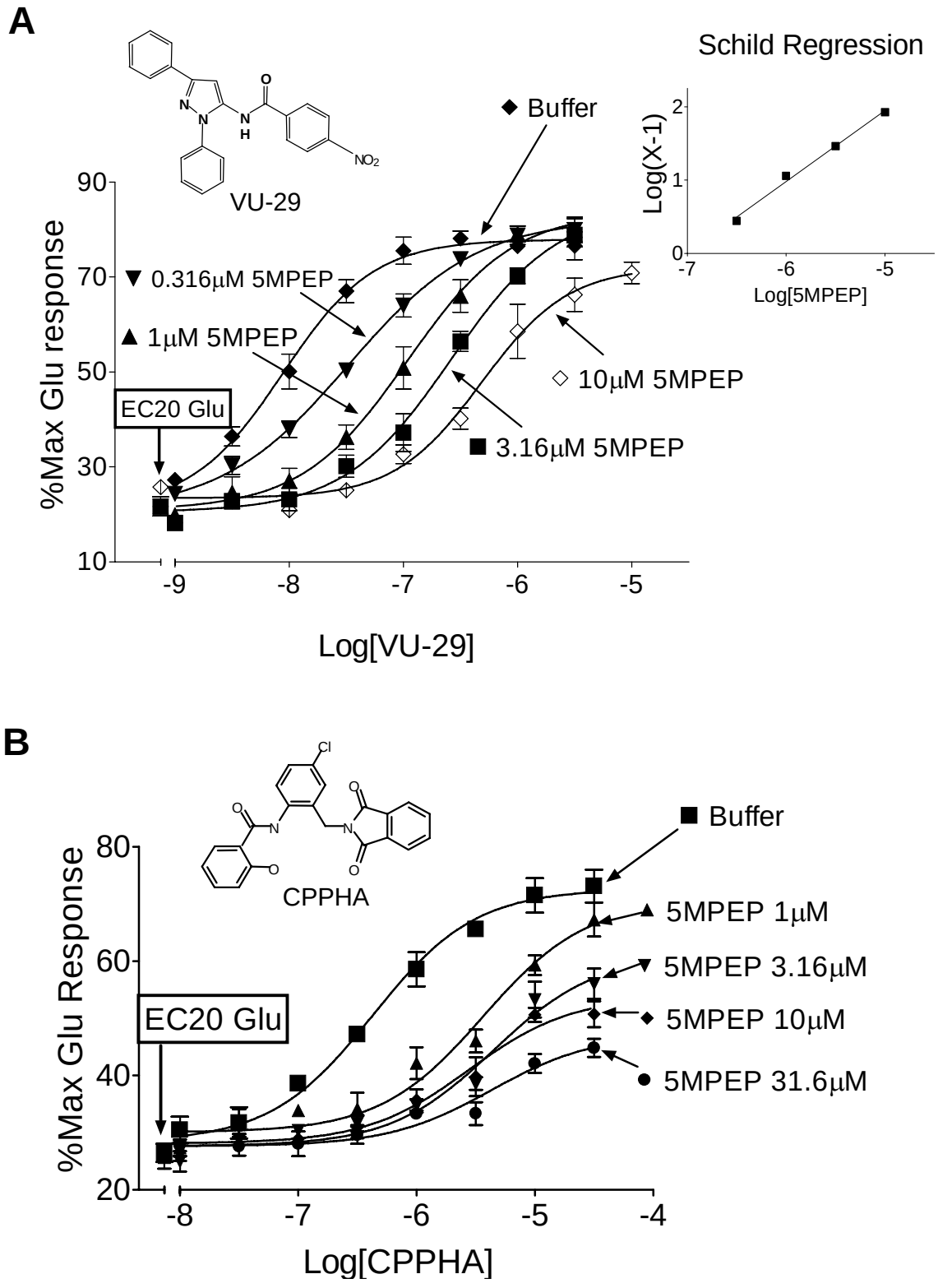

A
Schild Regression
Buffer
VU-29
0.316μM 5MPEP
1μM 5MPEP
10μM 5MPEP
3.16μM 5MPEP
EC20 Glu
%Max Glu response
Log[VU-29]
Log(X-1)
Log[5MPEP]
B
CPPHA
Buffer
5MPEP 1μM
5MPEP 3.16μM
5MPEP 10μM
5MPEP 31.6μM
EC20 Glu
%Max Glu Response
Log[CPPHA]

Figure 3-2: 5MPEP induces parallel rightward shifts in the VU-29 CRC but has non-competitive actions on the response to CPPHA using calcium mobilization assay of rat astrocytes. A, The concentration response curves of VU-29 were shifted to the right in a concentration-dependent manner by 5MPEP at concentrations up to 10 μM. Schild regression showed a slope of 0.97 and X-intercept of 100nM. B. 5MPEP blocks the CRC of CPPHA in a non-competitive manner. Increasing concentrations of 5MPEP induced progressive decreases in the maximum response to CPPHA. Data were obtained from 4 separate experiments each performed in triplicate. Error bars represent S.E.M.

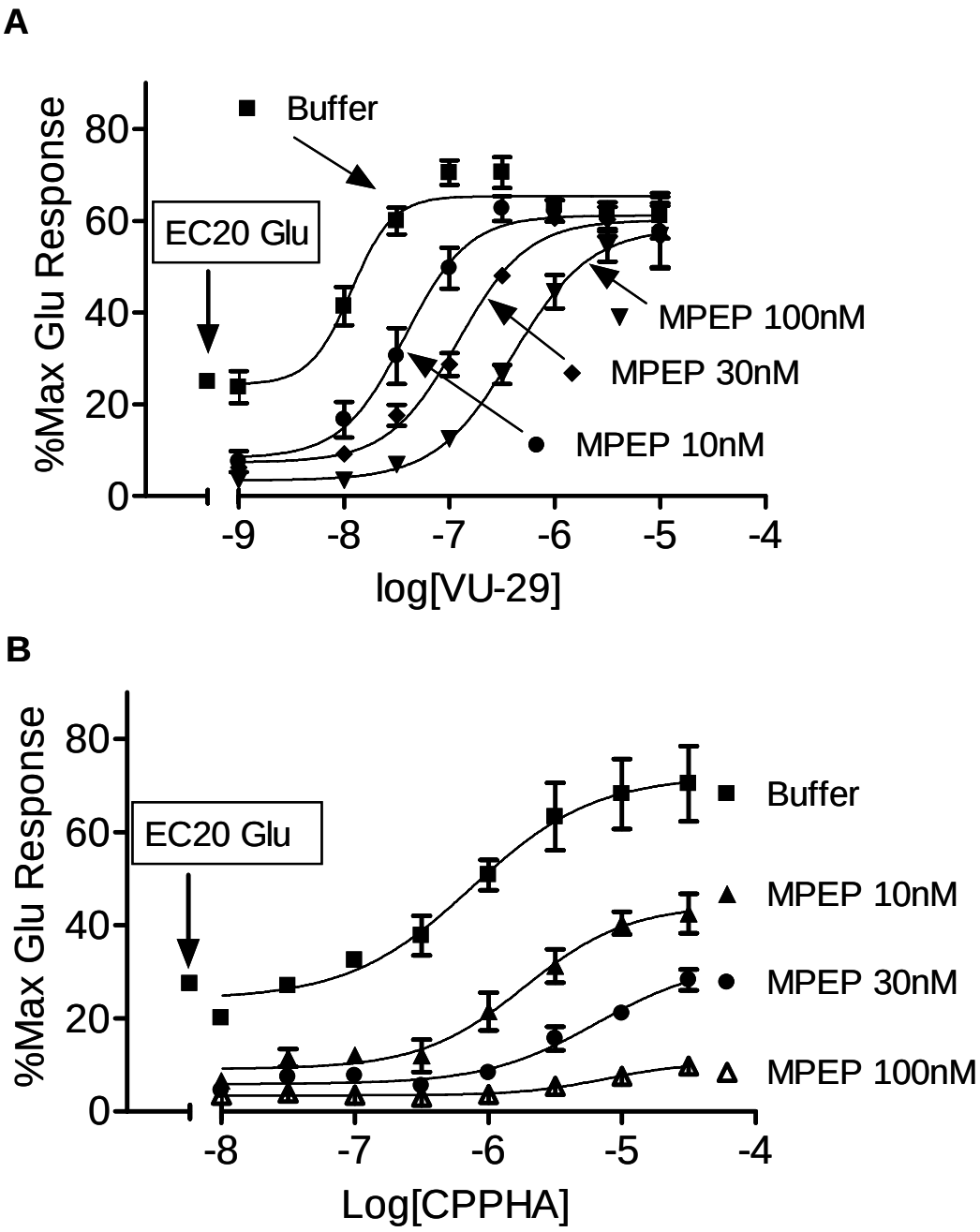



Figure 3-3: MPEP differentially shifts the concentration responses curves of VU-29 and CPPHA using the calcium mobilization assay of cultured rat cortical astrocytes. A, MPEP induced a parallel rightward shift in the CRC of VU-29 with no decrease of its maximum potentiation, suggesting competitive blockade. B, MPEP decreased the maximum effect of CPPHA, suggesting a non-competitive inhibitory action. Data were obtained from 3 separate experiments each performed in triplicate. Error bars represent S.E.M.

**CPPHA has PAM activity at both mGluR1 and mGluR5.**

We next evaluated the selectivity of CPPHA as a potentiator of mGluR5 relative to mGluR1 and representative mGluR subtypes belonging to group II (mGluR2) and group III (mGluR4). CPPHA (10 μM) potentiated responses of both mGluR5 and mGluR1d to glutamate but had no effect on responses to mGluR2 (Figure 3-4C). Consistent with the original report, CPPHA showed slight antagonism on mGluR4 mediated response (Figure 3-4D). CPPHA also potentiated rmGluR1a and 1b mediated calcium mobilization (data not shown).

CRC analysis revealed that CPPHA had a potency of 239 ± 27 nM on rmGluR5 and a potency of 3,460 ± 517 nM on rmGluR1d (Figure 3-5A,B). Thus, while somewhat selective for mGluR5, CPPHA has clear effects at both group I mGluR subtypes. This 10 fold selectivity likely accounts for the failure to see an effect of a single lower concentration of CPPHA on mGluR1 in previous studies (O'Brien et al., 2004).

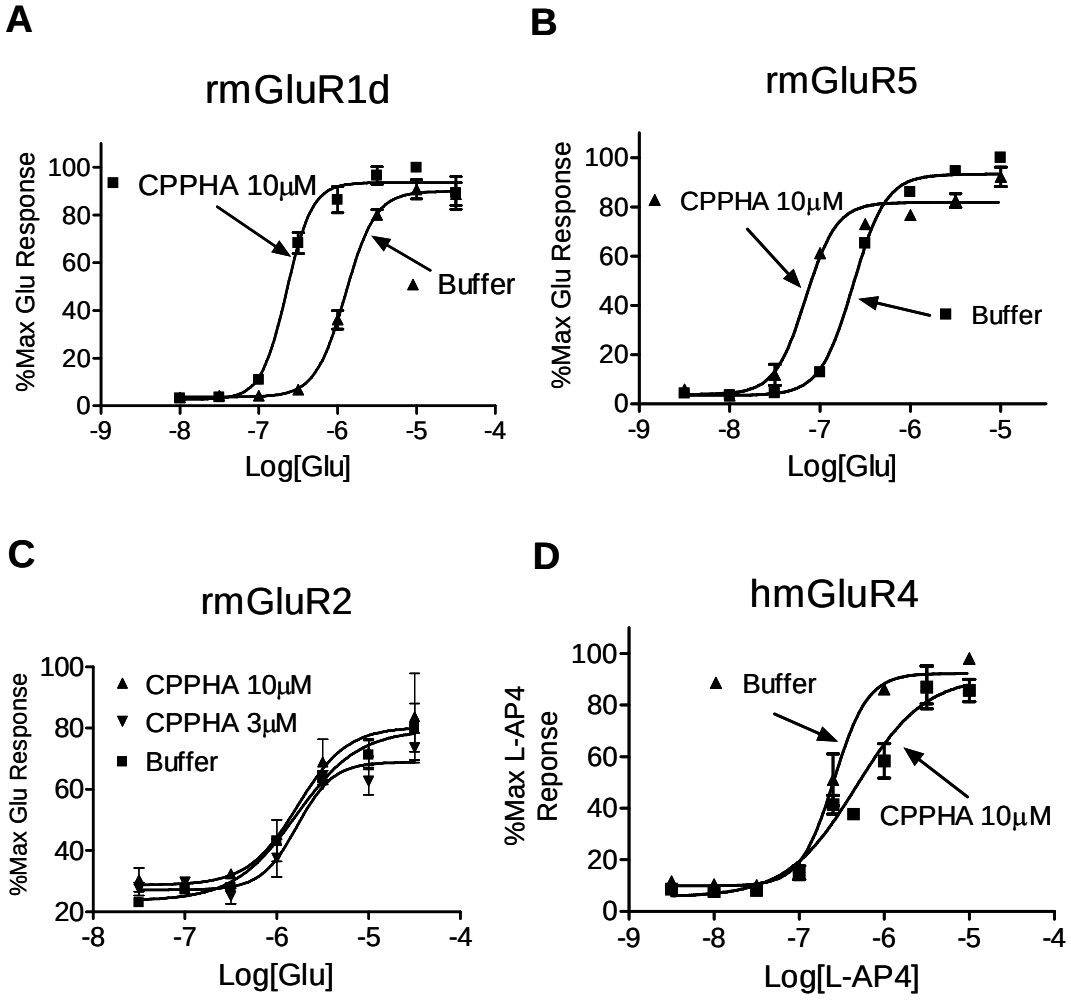



Figure 3-4: Selectivity of CPPHA for different mGluR subtypes in HEK 293 cells. A and B, 10 μM CPPHA shifted the glutamate CRC for mGluR5 and mGluR1. C, 3 or 10 μM CPPHA did not affect the CRC of mGluR2 significantly. D, 10 μM CPPHA slightly antagonized the response of mGluR4. Data were obtained from 3 separate experiments each performed in triplicate. Error bars represent S.E.M.

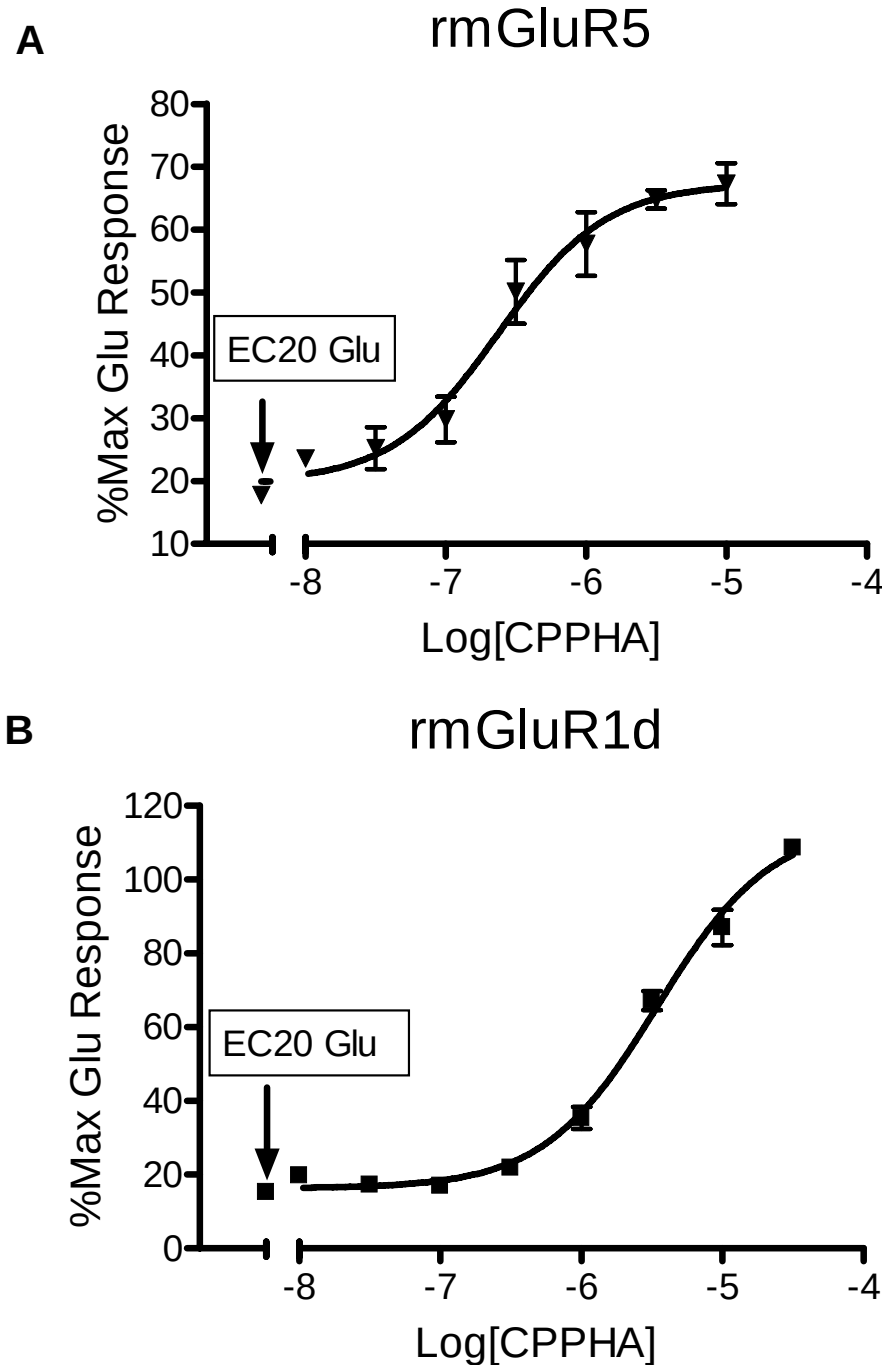



Figure 3-5: The potencies of CPPHA on mGluR1 and mGluR5 are slightly different. We pre-incubated transiently transfected HEK cells with multiple concentrations of CPPHA for 5 minutes, before adding EC20 concentrations of glutamate to elicit calcium mobilization. CPPHA had a potency of 239 ± 27 nM on rmGluR5 (A) and a potency of 3,455 ± 517 nM on rmGluR1d (B). Data were obtained from 3 separate experiments performed in triplicate. Error bars represent S.E.M.

**CPPHA acts in the 7TM domain of group I mGluRs at a site distinct from that of VU-29.**

The studies above suggest that CPPHA and VU-29 likely act at distinct sites. Since VU-29 and other previously identified allosteric modulators of mGluR5 act at a common site that is shared with MPEP, this raises the question of what domains of the receptor are most important for the action of CPPHA. The MPEP site and sites for other previously identified allosteric modulators of mGluRs are in the 7TM domain of the receptor. However, it is conceivable that an allosteric modulator could act in the extracellular or intracellular domains. The finding that CPPHA is an allosteric potentiator of mGluR1d suggests that CPPHA is unlikely to act in the intracellular C-terminal domain of the receptor. Thus, mGluR1d is a C-terminal truncated receptor that lacks the large intracellular C-terminal domain. We previously reported that other allosteric potentiators of mGluRs act as agonists of N-terminal truncation mutants that lack the large extracellular domain (Goudet et al., 2004; Chen et al., 2007). If CPPHA has a similar action, this compound is unlikely to act at a site in the N-terminal domain. Thus, we determined the effect of CPPHA on the previously described N-terminal truncation mutant of mGluR5. Interestingly, CPPHA induced a robust activation of the truncated mutant as assessed by measures of activation of PI hydrolysis. This was qualitatively similar to the effect of VU-29 (Figure 3-6). These data suggest that, like other allosteric modulators, CPPHA is likely to act in the heptahelical domain of mGluR5.

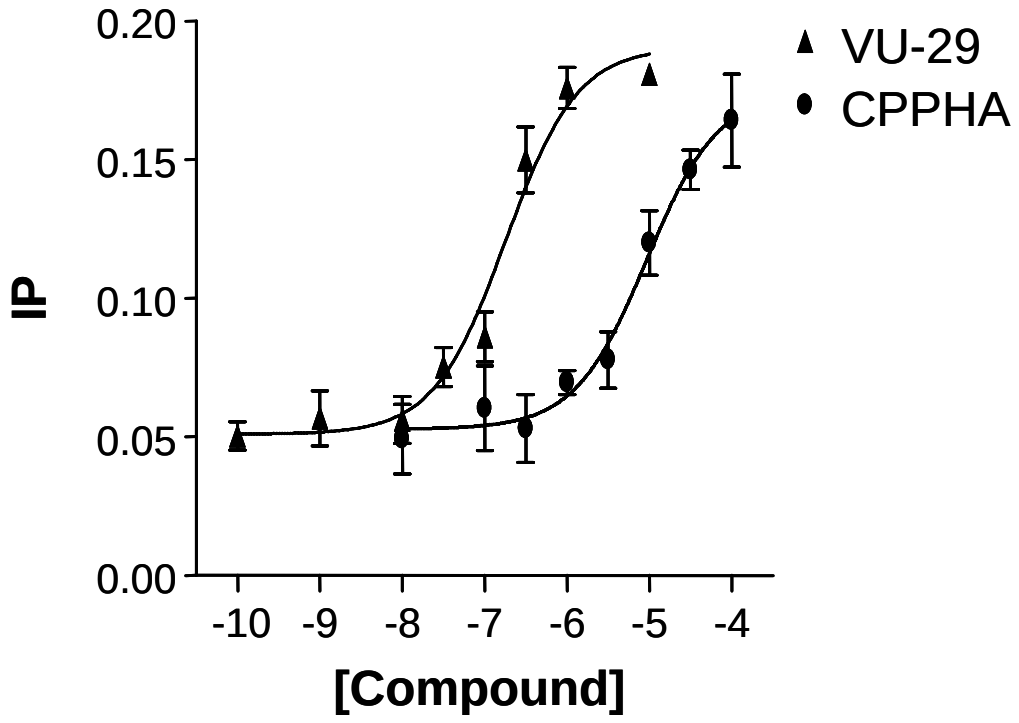



Figure 3-6: CPPHA and VU-29 directly activated N-terminal truncated mGluR5. IP productions were expressed as the ratio between IP and the total radioactivity. (This figure was contributed by Dr. Cyril Goudet)

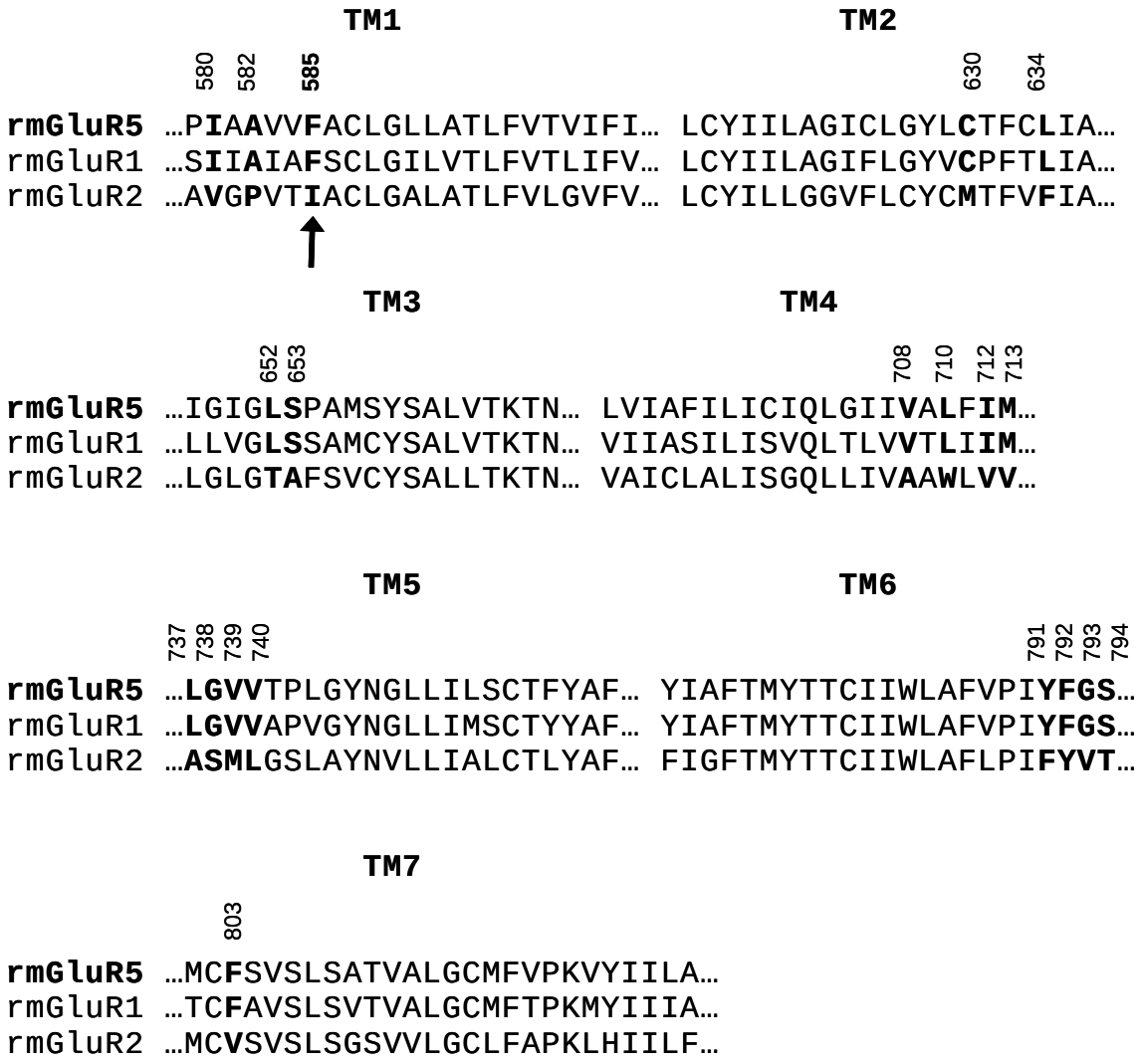



Figure 3-7: Sequence alignments of mGluR1, 5 and 2 transmembrane domains. Bold letters show the amino acids have been mutated.

We took advantage of the ability of CPPHA to potentiate responses at mGluR1 and mGluR5 but not mGluR2 to guide selection of mutations that may provide insights into specific amino acids that may be required for CPPHA action. Thus, we aligned the 7TM domain of these receptors and searched for residues that were identical between mGluR1 and mGluR5 but in which there was a different amino acid in mGluR2. We selected 20 amino acids in the transmembrane domain that were identical between mGluR1 and 5 but were divergent in mGluR2. Mutants of mGluR5 were constructed in which each of these 20 amino acids was singly mutated to the corresponding amino acids in mGluR2. Sequence homology and all the mutants studied are shown in Figure 3-7. All the mutated plasmids were tested for responses to 1 μM CPPHA in transiently transfected HEK cells using the calcium mobilization assay. Among all the mutants, only A582P/F585I/mGluR5 and F585I/mGluR5 in the first transmembrane domain eliminated the potentation of mGluR5 by 1 μM CPPHA (Figure 3-8B). CPPHA induced a significant potentiation of responses for all other mutants (data not shown) as well as wildtype receptor (Figure 3-8A). In contrast, the potentiation of 200 nM VU-29 remained for F585I/mGluR5 compared with wildtype (Figure 3-8A,B).

Previously we reported potentiation of VU-29 was abolished by A809V, which also eliminated MPEP site binding (Chen et al., 2007; Pagano et al., 2000 and Malherbe et al., 2003). As can be seen in figure 3-8C, A809V eliminates the effect of VU-29 without affecting the potentiation by CPPHA.

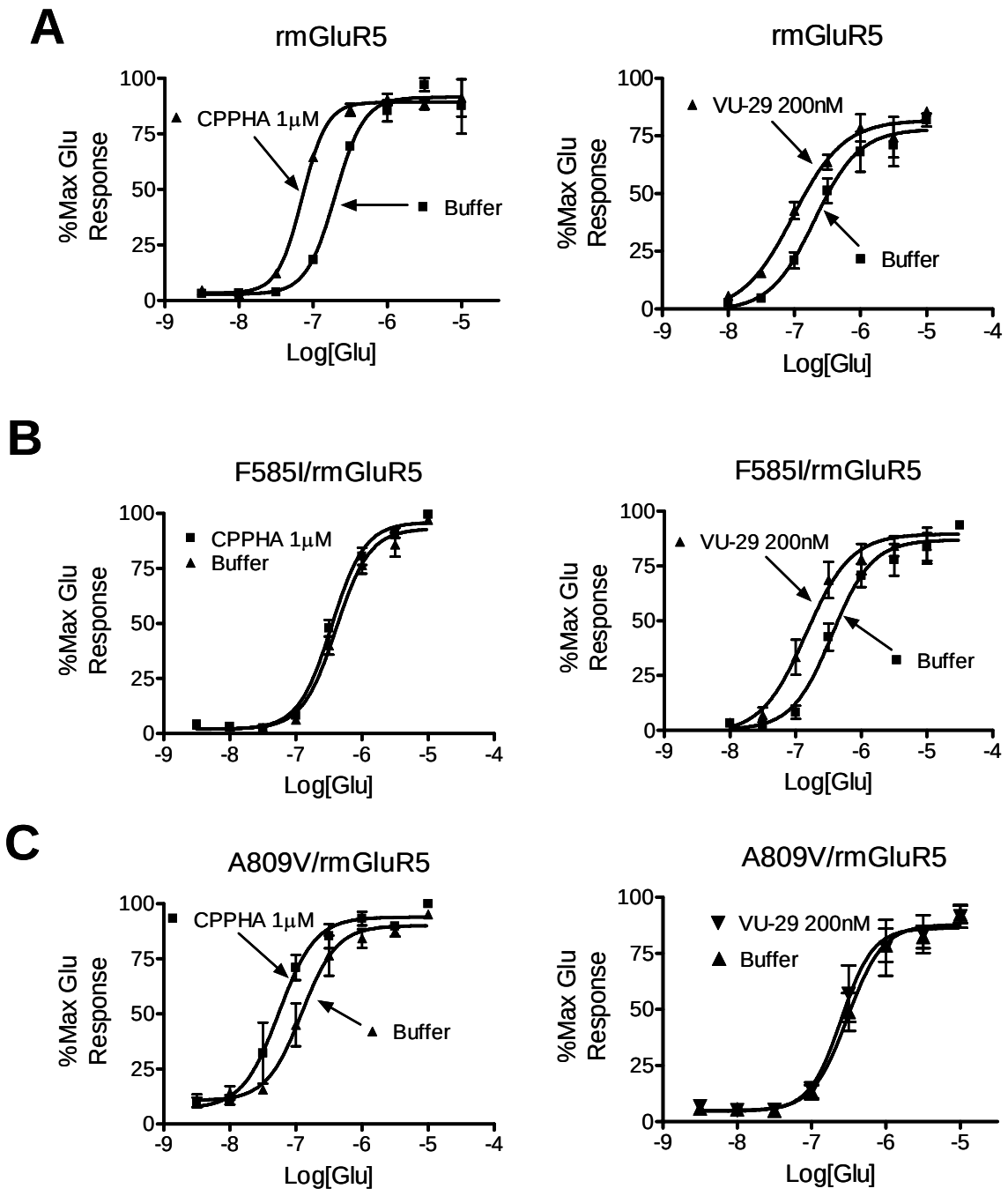



Figure 3-8: Two different point mutations in mGluR5 eliminate CPPHA or VU-29-induced potentiation respectively. A,1 μM CPPHA and 200 nM VU-29 induced leftward shifts of the glutamate CRC for wildtype rmGluR5 in transiently transfected HEK293 cells as measured by the calcium mobilization assay. B, single point mutation F585I completely abolished CPPHA potentiation without changing VU-29 potentiation. C, A809V eliminated the VU-29 potentiation but remained CPPHA potentiation intact. Data shown represent means ± S.E.M of 3 separate experiments, each performed in triplicate.

To further verify the impact of this mutation on CPPHA responses**,** we determined the effect of mutation of the homologous amino acid in mGluR1. In transiently transfected HEK cells, both 3 μM CPPHA and 200 nM Ro 67-7476 potentiate WT mGluR1-mediated calcium mobilization (Figure 3-9A). However, F599I/mGluR1, the homologous mutation of F585I/mGluR5, eliminated the potentiation of mGluR1 responses by CPPHA but not a previously characterized mGluR1 PAM, Ro 67-7476 (Knoflach et al., 2001; Hemstapat et al., 2006) (Figure 3-9B). Furthermore, another mutation, V757L/mGluR1 abolished the potentiation of mGluR1 by Ro 67-7476 as previously reported (Knoflach et al., 2001; Hemstapat et al., 2006) but did not abolish the potentiation of CPPHA (Figure 3-9C).

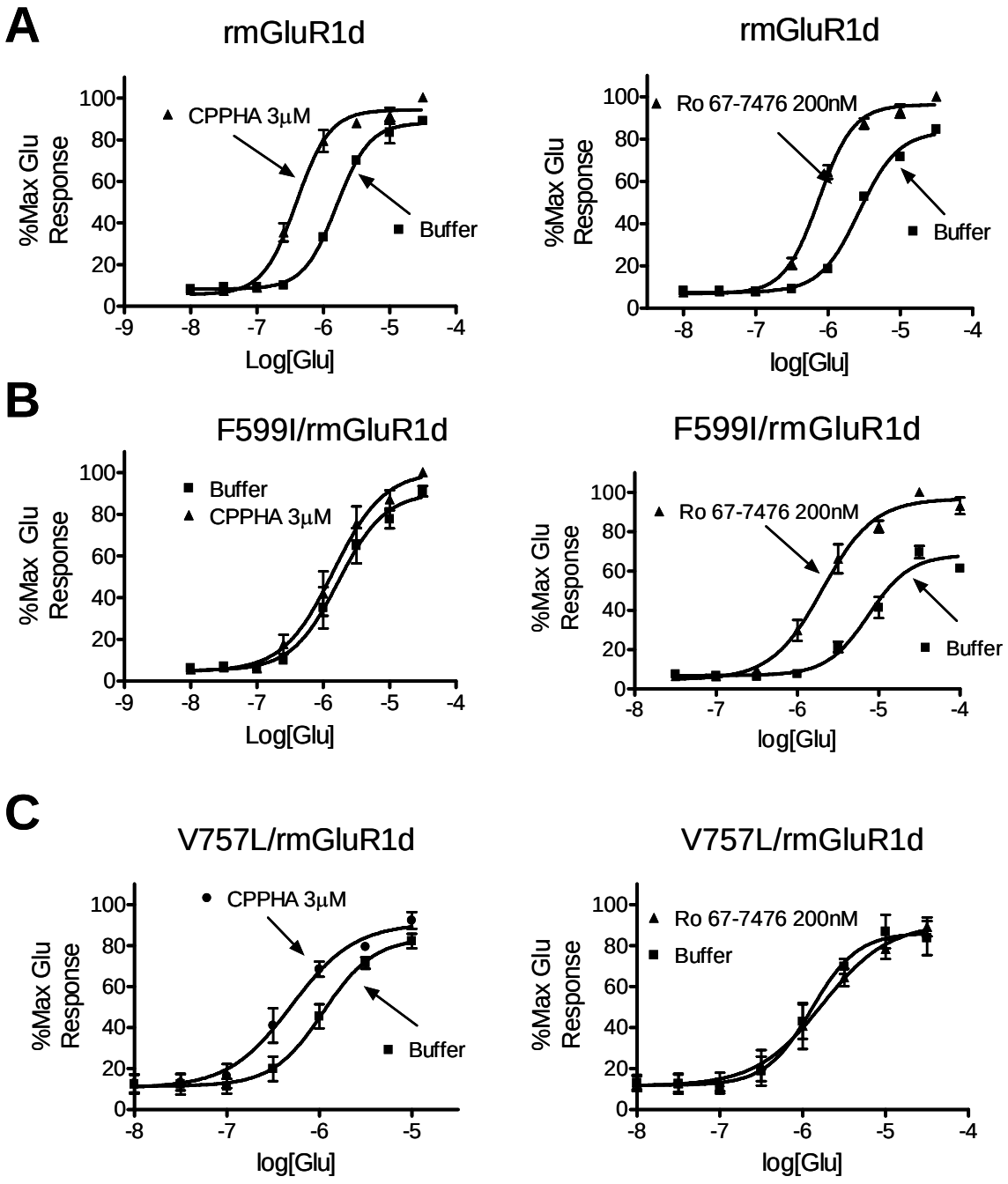



Figure 3-9: Two different point mutations in mGluR1 eliminate CPPHA or Ro 67-7476-induced potentiation respectively. A, 3 μM CPPHA and 200 nM Ro 67-7476 potentiated the CRC of glutamate to the left for wildtype rmGluR1d in transiently transfected HEK cells via calcium mobilization assay. B, Single point mutation F599I completely abolished CPPHA potentiation without changing Ro 67-7476 potentiation. C, V757L eliminated the Ro 67-7476 potentiation but remained CPPHA potentiation intact. Data is shown as means ± S.E.M of 3 separate experiments.

**CPPHA does not compete for binding of to the allosteric antagonist R214127 site on mGluR1.**

The allosteric mGluR1 antagonist radioligand [$^3$H]R214127 binds to mGluR1 at a site that is thought to be homologous with the MPEP site on mGluR5 (Pagano et al., 2000; Lavreysen et al., 2003). If CPPHA acts at a site that is conserved between mGluR1 and mGluR5 but is distinct from the MPEP and R214127 sites on these receptors, CPPHA should not compete for binding to the R214127 site. Thus, we determined the effect of CPPHA on specific binding of 2.5 nM [$^3$H]R214127 to mGluR1. Binding of [$^3$H]R214127 to membranes from cells expressing mGluR1 was reduced in a concentration dependent manner by cold R214127 with a IC50 of 3.4 nM (Figure 3-10). Under the same conditions, CPPHA did not affect the [$^3$H]R214127 binding up to 32 μM (Figure 3-10).

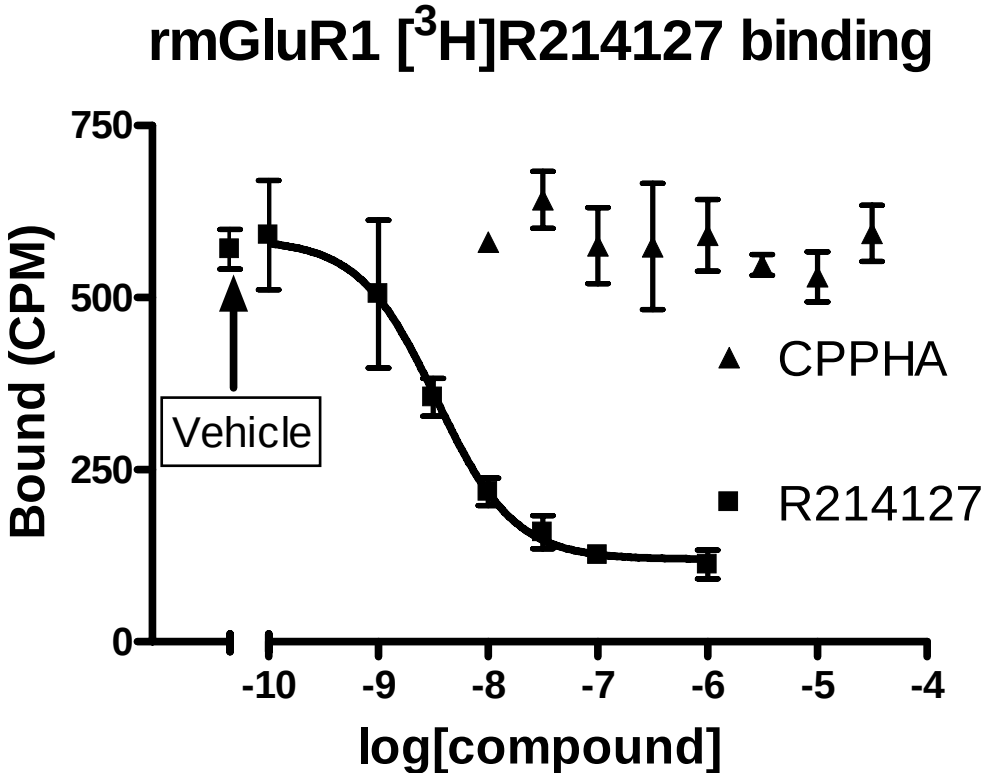



Figure 3-10: CPPHA does not bind to the R214127 NAM site on mGluR1. R214127 induced a concentration-dependent decrease in binding to of 2.5 nM [$^3$H]R214127 to a membrane preparation from an mGluR1 stable cell line with a IC50 of 3.4 nM. CPPHA did not affect the binding at concentrations up to 32 μM. Data are shown as means ± S.E.M of 3 separate experiments each performed in triplicate.

## Discussion

The data reported in the in this chapter provide strong support for the hypothesis that CPPHA acts at a novel allosteric site on mGluR5 and mGluR1 to potentiate activation of the receptor by orthosteric agonists. Our data suggest that the action of CPPHA is similar to actions of other known allosteric modulators in that it is due to actions in the 7TM domain but that the specific site differs from previously defined sites for allosteric potentiators or allosteric antagonists on these receptors. Most of the mGluR5 allosteric antagonists are derived from the same chemical scaffold and are believed to act on a shared site represented by the MPEP binding site (Varney et al., 1999; Gasparini et al., 1999). There are three families of PAMs for mGluR5, DFB, VU-29 and CPPHA (O'Brien, et al., 2003; O'Brien et al., 2004; Kinney et al., 2005). The recent finding that DFB and VU-29 act through binding to an overlapping site of MPEP (Mühlemann et al., 2006; Chen et al., 2007) suggests that the MPEP site is a common site shared by multiple allosteric modulators. Similarly, multiple allosteric ligands at mGluR1 appear to interact with a common site that is homologous with the MPEP site on mGluR5. Thus, all known mGluR1 NAMs that have been examined interact with the allosteric site labeled by R214127 (Pagano et al., 2000; Litschig et al., 1999). Furthermore, some previously characterized mGluR1 PAMs (represented by Ro 67-7476 and VU-71) have been postulated to share a homologous binding pocket with MPEP in mGluR5 based on critical amino acids implicated in a detailed mutagenesis study (Pagano et al., 2000; Knoflach et al., 2001; Hemstapat et al., 2006). However, competition binding assays revealed that two other PAMs Ro 67-7476 and VU-71 do not displace the binding of radio labeled R214127 (Hemstapat et al., 2006). Thus, we are only at the beginnings of

understanding the diversity of allosteric modulatory site on individual mGluR and other GPCR subtypes.

While the current studies suggest that CPPHA does not act by direct interactions with the MPEP site, our studies also provide an important hint that the ligands at the MPEP site can regulate effects of ligands at the CPPHA site indirectly. This is based on the finding that the neutral MPEP site ligand, 5MPEP, non-competitively inhibits CPPHA action. This is consistent with our previous observation of CPPHA potentiation is blocked by another neutral allosteric modulator, DCB (3,3'-dichlorobenzaldazine) (Chen et al., 2004). On the surface, this may imply that ligands at the MPEP site and the CPPHA site can allosterically regulate one another. However, if CPPHA could allosterically regulate MPEP ligand affinity and vice versa, radioligand binding studies should reveal a non-competitive effect of CPPHA on MPEP site binding.

The finding that mGluR5 PAMS can be identified that act on distinct allosteric sites raises the interesting question of whether PAMs that act at these sites will have identical effects on mGluR5 function. In the simplest view, these compounds would induce similar increases in activity of mGluR5 regardless of the signaling pathway or cell population involved. However, a growing body of evidence suggests that different traditional orthosteric agonists can differentially activate different signaling pathways of a single GPCR, a phenomenon referred to as agonist receptor trafficking (Brink et al., 2000; Gazi et al., 2003; Berg et al., 1998). Based on this, it is possible that allosteric potentiators of mGluRs could differentially regulate coupling of these receptors to different signaling pathways. Further, it is conceivable that PAMs that act at different sites could differentially regulate coupling of mGluR5 to different pathways. mGluR5

can couple to multiple signaling pathways and physiological responses. For instance, we reported that mGluR5 in cortical astrocytes activates PI hydrolysis and phosphorylation of extracellular signal regulated kinase (ERK2) by completely independent mechanisms (Peavy et al., 2001; 2002). Furthermore, we and a number of other investigators have found that activation of mGluR5 can have a wide variety of effects on different neuronal populations, including cell depolarization, modulation of different potassium currents, potentiation of NMDA receptor currents, and a variety of other responses (see Valenti et al., 2002). It is likely that these responses are mediated by different signaling mechanisms and could be differentially regulated. Interestingly, we recently reported that that the MPEP site PAM, DFB, and and CPPHA have subtly different effects on mGluR5-mediated responses in astrocytes (Zhang et al., 2005). Both PAMs potentiate mGluR5-mediated increases in intracellular calcium with similar profiles. This is consistent with the similarities of effects of CPPHA and CDPPB on calcium mobilization shown in the present manuscript. Interestingly, DFB and CDPPB have different effects on coupling of mGluR5 to ERK1/2 phosphorylation in these same cells (Zhang et al., 2005). DFB induces a leftward shift of the agonist concentration response curve for ERK1/2 phosphorylation similar to that seen with calcium mobilization. However, CPPHA had a small agonist effect when added alone and potentiated the effect of low concentrations of agonist but inhibited the effect of high concentrations of agonist on the ERK1/2 phosphorylation response. When considered in the light of the present findings, this raises the possibility that ligands at these two allosteric sites may differentially regulated coupling of mGluR5 to these two signaling pathways. This is also interesting in light or previous studies showing that mGluR5 PAMs that act at the MPEP site (ie.

CDPPB) have behavioral effects in animal models that can predict antipsychotic-like efficacy. It is not known whether compounds that act at the CPPHA site will have this effect. In future studies, it will be important to systematically compare effects of mGluR5 PAMs that act at these different allosteric sites in native systems and *in vivo*.

# CHAPTER IV

## MGLUR5 PAMS FACILITATES LTP INDUCTION IN THE RAT HIPPOCAMPAL CA1 REGION

### Introduction

Metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors (GPCRs) that play important roles in modulating neuronal excitability and synaptic transmission at glutamatergic synapses (Conn and Pin, 1997; Schoepp et al., 1999; Coutinho and Knopfel, 2002). Group I mGluRs (mGluR1 and mGluR5) represent one of three major subgroups of these receptors and are localized postsynaptically in multiple brain regions. Recent studies provide compelling evidence that selective ligands for group I mGluRs have potential as therapeutic agents for a range of neurological and psychiatric disorders, including disorders involving impaired cognitive function (Kinney et al., 2005; Campbell et al., 2004; Moghaddam, 2004; Marino et al 2002; Anwyl 1999).

A prominent role of group I mGluRs, and especially the mGluR5 subtype, is regulation of activity of the NMDA subtype of ionotropic glutamate receptor (see Marino and Conn, 2002). A large number of studies suggest that mGluR5 and NMDA receptors are closely associated signaling partners and that mGluR5 plays a critical role in maintaining normal NMDA receptor function. Based on this, it has been postulated that increased activity of mGluR5 could enhance NMDA receptor-dependent forms of synaptic plasticity and cognitive function. Consistent with this, mGluR5 knockout mice display impairments in NMDA receptor-mediated hippocampal long-term potentiation (LTP) and NMDA-dependent memory tasks, such as the Morris water maze and

contextual information in the fear-conditioning test (Lu et al., 1997). Also, mGluR5 selective antagonist MPEP blocks theta burst stimulation (TBS)-induced LTP in area CA1 in hippocampal slices (Francesconi et al., 2004; Shalin et al., 2006) and *in vivo* (Manahan-Vaughan and Braunewell, 2005). Finally, the group 1 mGluR agonist DHPG primes LTP induction at relatively low concentrations (Cohen et al., 1998; Raymond et al., 2000). However, multiple studies suggest that activation of mGluR5 may also play a critical role in induction of an NMDA receptor-independent form of long-term depression (LTD). DHPG induces LTD in hippocampal area CA1 (Gasparini et al. 1999; Huber et al., 2001) and this response is absent in mGluR5 null mice and slices incubated with the mGluR5 selective antagonist MPEP (Faas et al. 2002; Gasparini et al. 1999; Hou and Klann 2004; Huang and Hsu 2006; Huang et al. 2004 and Huber et al., 2001). These actions of mGluR5 activation on both LTP and LTD could have opposing effects on transmission in area CA1 and the precise roles of synaptically activated mGluR5 in regulating hippocampal synaptic plasticity are not entirely clear.

A critical aspect of the majority of studies of mGluR5 involvement in LTD, is that these studies rely on exogenous application of group I mGluR agonists and may not reflect a response to activation of mGluR5 by synaptically released glutamate. We have now developed novel compounds that act as potent and highly selective positive allosteric modulators (PAMs) of mGluR5 (O'Brien, et al., 2003; 2004; Lindsley et al., 2004; Kinney et al., 2005; de Paulis et al., 2006; Chen et al, 2007). These compounds do not activate mGluR5 alone but selectively potentiate the receptor's response to glutamate. This provides an exciting new tool that allows selective potentiation mGluR5 activation by glutamate with afferent stimulation. We have now determined the effect of selective

potentiation of the mGluR5 response to afferent stimulation on rat hippocampal CA1 plasticity. Interestingly, our studies suggest that mGluR5 potentiation enhances induction of hippocampal LTP in response to stimuli that are at the threshold for inducing LTP under normal conditions.

## Material and Methods

### Hippocampal slices PI hydrolysis assay.

Agonist-induced PI hydrolysis was measured in rat hippocampal slices using a modification of method outlined by Berridge et al. (1982) as previously described (Conn and Sanders-Bush, 1986). Briefly, cross-chopped (350 μM X 350 μM) slices of male (6-9 weeks) Sprague-Dawley rat hippocampus were incubated with 95% O2 5% $CO_2$ bubbled Krebs buffer (108 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$ and 10 mM Glucose). The tissue was allowed to recover for 30 minutes with shaking at 37ºC. Following tissue recovery, the tissue was combined, washed with warm Krebs buffer, and 25 μL of gravity packed slices were incubated with 175 μL Krebs containing 0.5 μCi [3H]myo-inositol for 45 minutes. VU-29 or vehicle controls were added and incubated for 15 minutes, followed by the addition of 10 mM LiCl and incubation for an additional 15 minutes. Finally, DHPG was added followed by an additional 45 minute incubation. The reaction was terminated by the addition of 900 μL of chloroform:methanol (1:2). The aqueous and organic phases were separated by addition of 300 μL chloroform and 300 μL water, vortexing, and allowing the phases to separate by gravity. The aqueous phase was added to anion exchange columns (AG 1-X8 Resin, 100-200 mesh, formate form, BIO-RAD) and [3H]inositol phosphates were eluted and measured by liquid scintillation counting.

**Electrophysiology**.

Brains were removed from young adult (6-9 weeks) male Sprague-Dawley rats and 400 μM transverse slices were prepared using a vibratome (Leica Microsystems, Nussloch, Germany. Drugs were diluted to the appropriate concentrations in either DMSO (< 0.1%) or aCSF (125 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1.25 mM $NaH_2PO_4$, 2 mM $CaCl_2$, 25 mM glucose, and 25 mM $NaHCO_3$, equilibrated with 95% $O_2$/5% $CO_2$) and applied to the slice at a rate of 2 mL/min. Extracellular field potentials were recorded from slices that recovered in an interface chamber perfused with oxygenated aCSF (2 mL/min) for at least 1.5 hrs at 31°C. Bipolar stimulating electrodes were placed in the stratum radiatum near the CA3-CA1 border in order to stimulate the Schaffer collaterals. Recording electrodes were pulled with a Flaming/Brown micropipette puller (Sutter Instruments) to a resistance of 3-5MΩ, filled with aCSF and placed in the stratum radiatum of area CA1. Field potential recordings were acquired using an A-M systems 1800 amplifier and pClamp 9.0 software. The 40% maximal stimulus intensity was determined prior to each experiment. Baseline stimulation was presented at 0.05 Hz. LTP was induced by either one train or four trains of theta burst stimulation (TBS)(threshold TBS: nine bursts of four pulses at 100 Hz; 230 ms interburst interval; 10 HZ TBS: nine bursts of four pulses at 100 Hz; 100 ms interburst interval).

**Cell Based Calcium Fluorescence Measurement.**

Secondary rat cortical astrocytes were prepared as described previously (Chen et al., 2007; Peavy et al., 2001; Zhang et al., 2005). Rat mGluR1d was transfected into HEK293A cells using Lipofectamine as described previously (Chen et al., 2007). Rat mGluR2 and human mGluR4 were co-expressed with $G_{qi5}$, which enables coupling to the

calcium mobilization as reported by Galici et al. (2006). Cells were loaded with calcium-sensitive dye according to the manufacturer's instructions (Calcium 3 kit; Molecular Devices, Sunnyvale, CA) after incubation in glutamate/glutamine-free medium (DMEM and 10% dialyzed fetal bovine serum) for 5 h. Compound A (1 ml) from Calcium 3 kit was dissolved in 20 ml of 1x Hanks' balanced salt solution (HBSS; Invitrogen) containing 2.5 mM probenecid (Sigma), adjusted to pH 7.4. Cells were loaded for 50 min at 37°C with 5% carbon dioxide. Dye was then carefully removed, and cells were washed with HBSS containing probenecid. Cells were maintained in the same buffer at room temperature for the following assay. For calcium fluorescence measurement of rat cortical astrocytes, allosteric modulators were added 5 min before the addition of agonist manually. Agonist was added at a speed of 52 μl/s, and calcium flux was measured using Flexstation II (Molecular Devices) at 25°C. All of the peaks of the calcium response were normalized to the maximum response to a saturated dose of glutamate (10 μM). The submaximal concentration ($EC_{20}$ value) of glutamate was determined for every separate experiment, allowing for a response varying from 10 to 30% of the maximum peak.

## Results

### VU-29 potentiates DHPG-induced phophoinositide hydrolysis in the rat hippocampal slices.

In previous studies, cell culture and recombinantly expressed mGluRs were used to demonstrate the mGluR5 PAM activity of VU-29 (Chen et al., 2007). Prior to using this compound for studies of the role of mGluR5 in hippocampal LTP, we verified that this compound potentiates mGluR5-mediated responses in hippocampal slices by determining the effect of this compound on mGluR5-mediated increases in PI hydrolysis. Consistent with previous studies (Sacaan et al., 1998; Johnson et al., 1999; Gasparini et al., 1999), DHPG induced a concentration-dependent increase in PI hydrolysis in rat hippocampal slices (Figure 4-1A). Consistent with its effect on mGluR5-mediated calcium mobilization in cell lines, VU-29 (500 nM) had no effect on the baseline PI hydrolysis but induced a leftward shift in the DHPG concentration response curve (CRC) and enhanced the maximum response (Figure 4-1A; Student's t-test, $p<0.05$) (Figure 4-1A). We then determined the effect of VU-29 on the response to a single concentration of DHPG that induced and approximate EC20 PI hydrolysis response (3 μM). At this concentration, DHPG (3 μM) induced a small but significant increase in PI hydrolysis compared with vehicle control (Figure 4-1B; Student's t-test, $P<0.05$), which was significantly potentiated by VU-29 (5 μM) (Figure 4-1B; Student's t-test, $P<0.05$) but not by the neutral mGluR5 ligand, 5MPEP (100 μM) (Figure 4-1B; Student's t-test). Also consistent with previous studies in cell culture systems, the potentiation of DHPG-induced PI hydrolysis in hippocampal slices by VU-29 was completely blocked by 5MPEP (Figure 4-1B; Student's test, $P<0.05$).

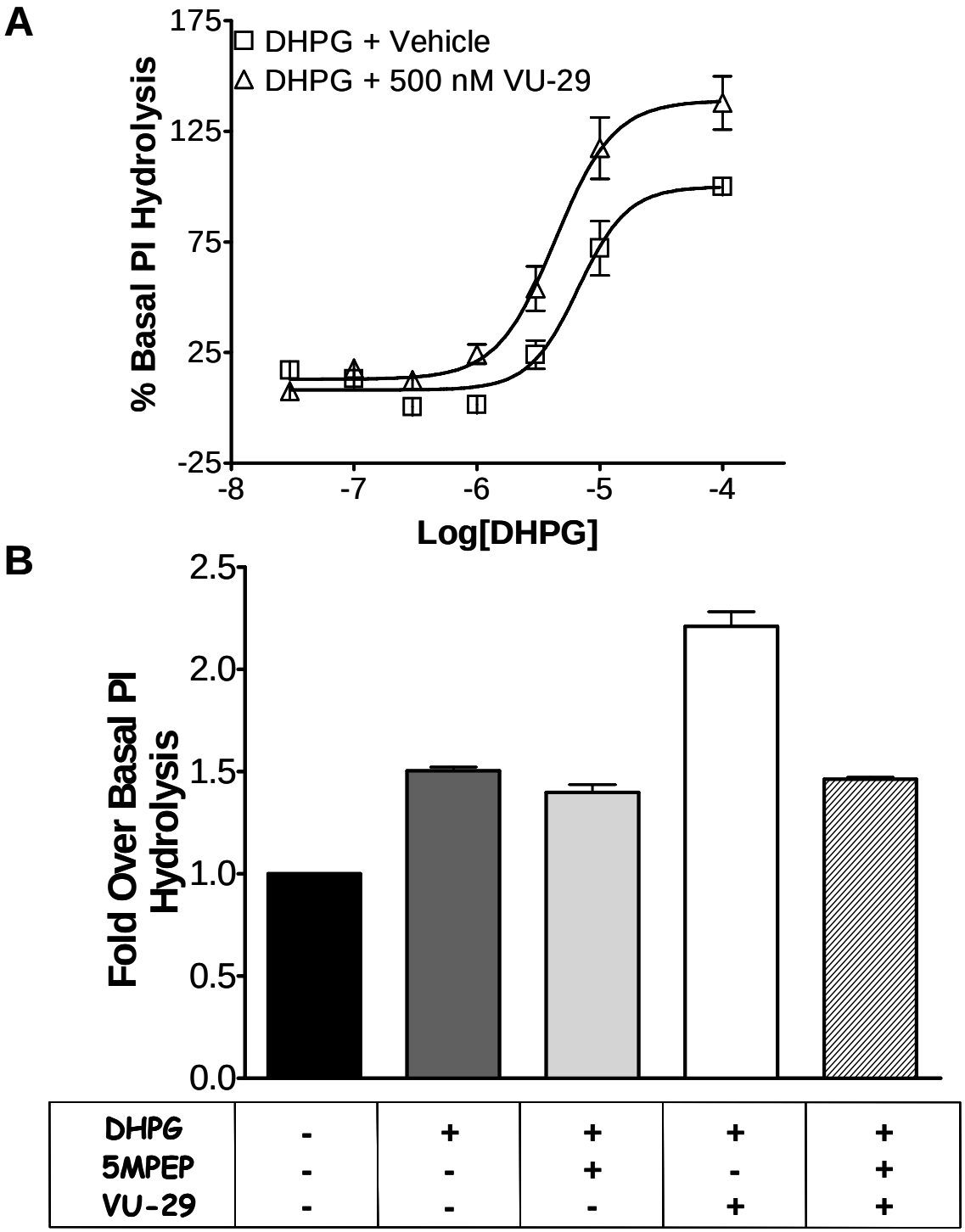

A
175
125
75
25
-25
-8
-7
-6
-5
-4
DHPG + Vehicle
DHPG + 500 nM VU-29
% Basal PI Hydrolysis
Log[DHPG]
B
2.5
2.0
1.5
1.0
0.5
0.0
Fold Over Basal PI Hydrolysis
DHPG - + + + +
5MPEP - - + - +
VU-29 - - - + +

Figure 4-1: VU-29 potentiates DHPG-induced PI hydrolysis in rat hippocampal slices. **A**. 500nM VU-29 significantly potentiated the dose response curve of DHPG-induced PI hydrolysis in the rat hippocampal slices. In the presence of VU-29 the EC50 of DHPG was 4.3 ± 1.1 μM, significantly lower than vehicle control of 6.7 ± 1.8 μM. Additionally, the maximum response was also enhanced 1.38 ± 0.13 folds. **B**. 3 μM DHPG increased baseline PI hydrolysis, which was significantly enhanced by pre-incubation of 5 μM VU-29 but not altered by 100 μM 5MPEP using rat hippocampal slices. 5MPEP blocked the potentiation caused by VU-29. (n=5, done in triplicate). Error bars represent S.E.M. (This figure was contributed by Dr. Douglas J. Sheffler)

**VU-29 potentiates threshold TBS-induced LTP in rat hippocampal CA1 region.**

To determine the effect of VU-29 on TBS-induced LTP, field EPSPs (fEPSPs) were recorded from SC-CA1 synapses. Application of 500 nM VU-29 had no effect on the baseline synaptic responses as measured by initial fEPSP slope (Figure 4-2A). In the absence of VU-29, a single train of threshold TBS induced a slight potentiation of EPSPs (115 $\pm$ 6% of control at 45 min post TBS). Interestingly, this same TBS protocol induced robust LTP in slices incubated with VU-29 (500 nM) for 20 min prior to application of TBS (152 $\pm$ 8% of control at 45 min post TBS) (Figure 4-2B; Student's T-test, $P<0.05$). 5MPEP (100 μM) completely blocked TBS-induced LTP in the presence of VU-29 (92 $\pm$ 14% of control at 45 min post TBS) (Figure 4-3A), suggesting that the action of VU-29 is due to actions on mGluR5. In contrast, 5MPEP (100 μM) had no effect on induction of LTP by 10 HZ TBS protocol which is sufficient to induce robust LTP in the absence of VU-29 (192 $\pm$ 19% of control at 45 min post TBS in the absence of 5MPEP; 185 $\pm$ 13% of control 45 min post TBS in the presence of 5MPEP) (Figure 4-3B). This suggests that the effect of 5-MPEP is specific to the VU-29 potentiation and excluded the possibility that 5MPEP blocked LTP induction through blockade of signaling components required for LTP induction other than mGluR5. VU-29 did not alter pared pulse facilitation, fiber valley amplitude and input-output curve compared with vehicle controls (data not shown).

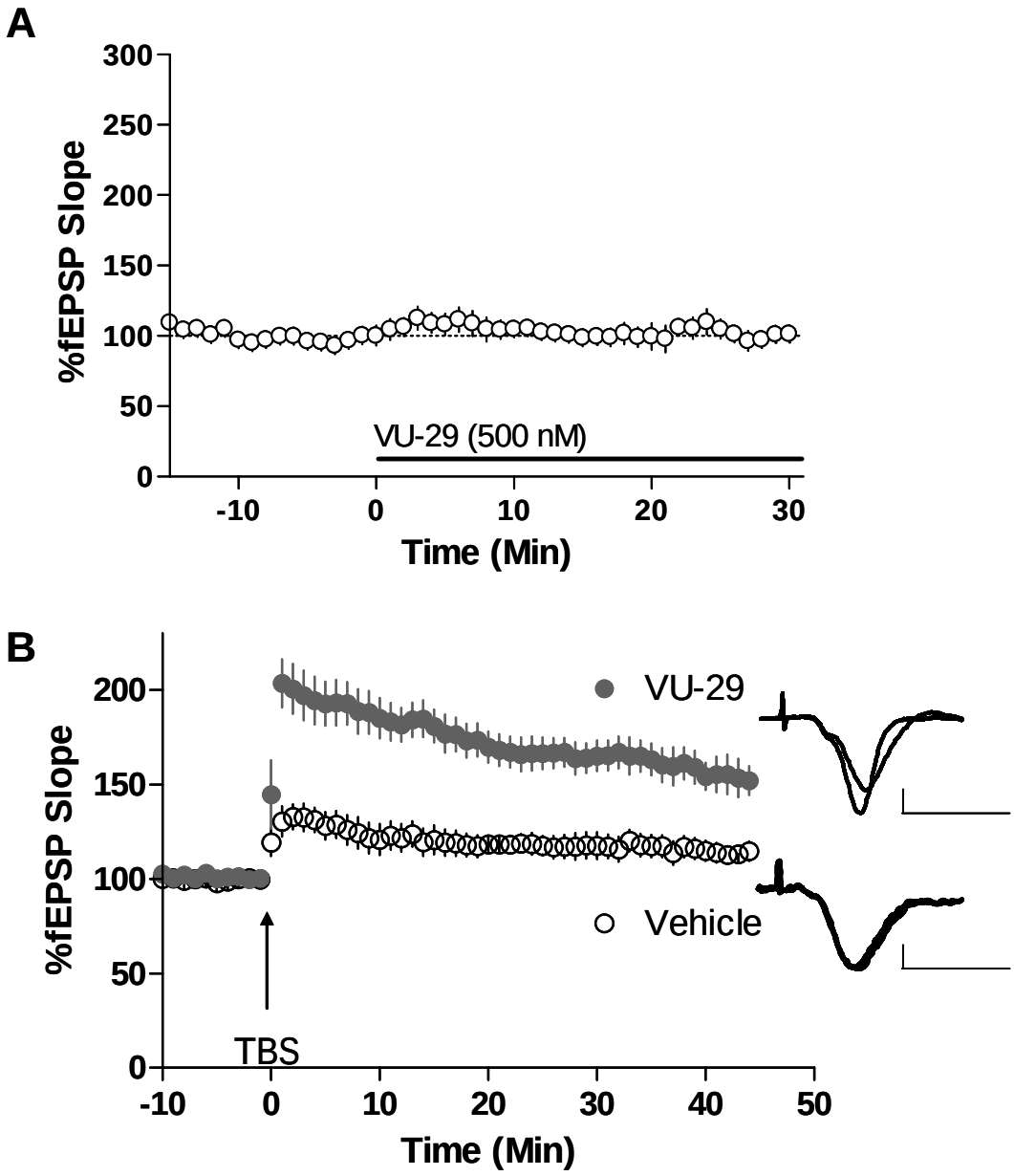



Figure 4-2: The mGluR5 PAM VU-29 facilitates the induction of LTP in area CA1 of the hippocampus. **A.** A 30 min incubation of 500 nM VU-29 did not alter the baseline fEPSP recorded from rat hippocampal CA1 region (n=8). **B.** In control slices, threshold TBS induced marginal potentiation of fEPSP. In the presence of VU-29 the same stimulation (20 min pre-incubation) yielded a significant potentiation (n=12; Student's T-test, $P<0.05$). Error bars represent S.E.M. (This figure was contributed by Dr. Jessica L. Banko partially)

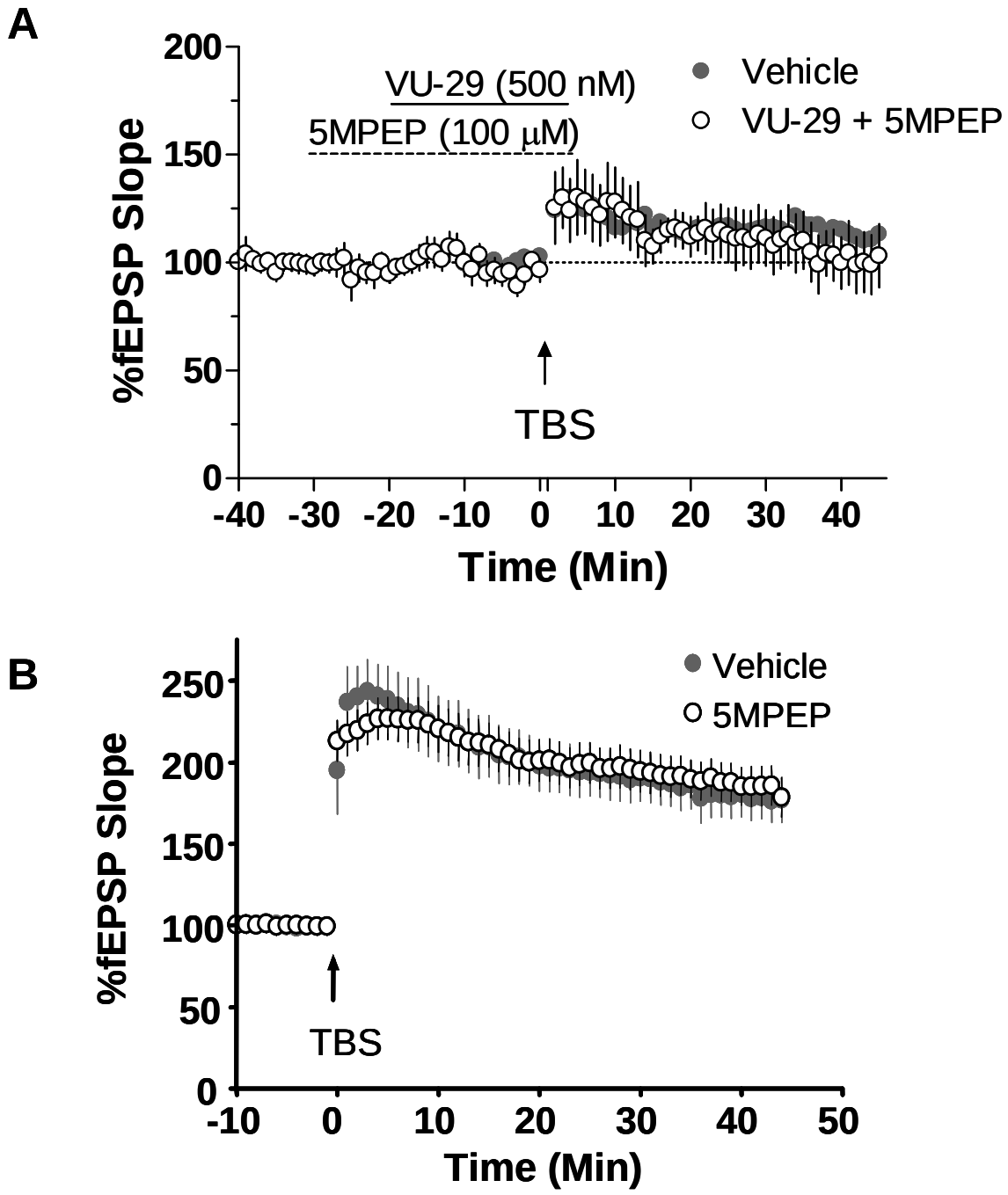



Figure 4-3. The VU-29-LTP is blocked by 5MPEP. A. Pre-incubation of 100 μM 5MPEP completely inhibited VU-29 facilitated TBS induced LTP back to vehicle control (n=8; Student's t-test). B. 10 HZ TBS-induced LTP was not altered by the pre-incubation of 100 μM 5MPEP (n=8; Student's t-test). Error bars represent S.E.M. (This figure was contributed by Dr. Jessica L. Banko partially)

**VU-29 does not potentiate LTP induced by suprathreshold TBS or alter established saturated LTP.**

The finding that selective potentiation mGluR5 responses enhances threshold TBS LTP suggests that mGluR5 can play an important role in regulating synaptic plasticity in response to stimulus trains that are just below the threshold needed to induce a robust LTP response. It is possible that this represents a potentiation of the normal processes underlying TBS LTP. Alternatively, mGluR5 activation could induce another form of LTP that is superimposed on the normal potentiation induced by threshold TBS. If the latter is the case, VU-29 may be expected to potentiate the LTP response to a suprathreshold TBS protocol. Furthermore, it is possible that VU-29 could further potentiate saturated LTP that has been established. Finally, if potentiation of mGluR5 induces competing processes leading to both induction of LTP and LTD, it is possible that VU-29 could actually decrease the magnitude of LTP induced by a suprathreshold stimulation or facilicate depotentiation of a saturated LTP response. To address this, we determined the effect of VU-29 on the LTP response to suprathreshold TBS stimulation. Interestingly, VU-29 had no effect on the LTP response when a stimulus protocol was used (4 trains of 10 HZ TBS) that induces robust LTP in the absence of VU-29 (Figure 4-4A) (178 $\pm$ 9%%potentiation at 30 min post TBS in the absence of VU-29; 176 $\pm$ 9%% potentiation in the presence of VU-29). We then applied VU-29 to slices in which a maximal LTP response had been established to determine whether VU-29 would alter the response to TBS stimulation in under conditions of saturated LTP. As with the response to a single suprathreshold TBS protocol, VU-29 had no effect on the response to suprathreshold LTP in slices in which LTP had been saturated (Figure 4-4B). These indicated VU-29 facilitated LTP utilized the same signaling machineries as normal

**Induction of LTP in the presence of VU-29 is dependent on activation of NMDA receptors and src family kinases.**

Previous studies suggest that induction of LTP at the SC-CA1 synapse by suprathreshold TBS is dependent on activation of both NMDA receptors and src family kinases (Collingridge et al., 1987; Lu et al., 1998; Salter et al., 2004). Also, previous studies suggest that activation of mGluR5 potentiates NMDA receptor currents and that mGluR5-induced activation of src family kinases is important for this response (Doherty et al., 1997, 2000; Jia et al., 1998; Awad et al., 2000; Mannaioni et al., 2001; Benquet et al., 2002; Gerber et al., 2007). Thus, it is likely that potentiation of synaptically activated mGluR5 by VU-29 enhances induction of normal NMDA receptor-dependent LTP. However, it is also possible that VU-29 leads to induction of a distinct form of LTP that is independent of NMDA receptor and src family kinase activation. To control for this possibility to verify that TBS LTP is dependent on activation of the same signaling pathways in VU-29 treated slices, we determined the effects of the NMDA receptor antagonist, D-AP5 and the src family kinase inhihbitor PP1 on TBS-induced LTP in the presence of VU-29 (Figure 4-5). Both D-AP5 (50 μM) and PP1 (20 μM) completely blocked induction of LTP by the combination of VU-29 and threshold TBS (Figure 4-5).

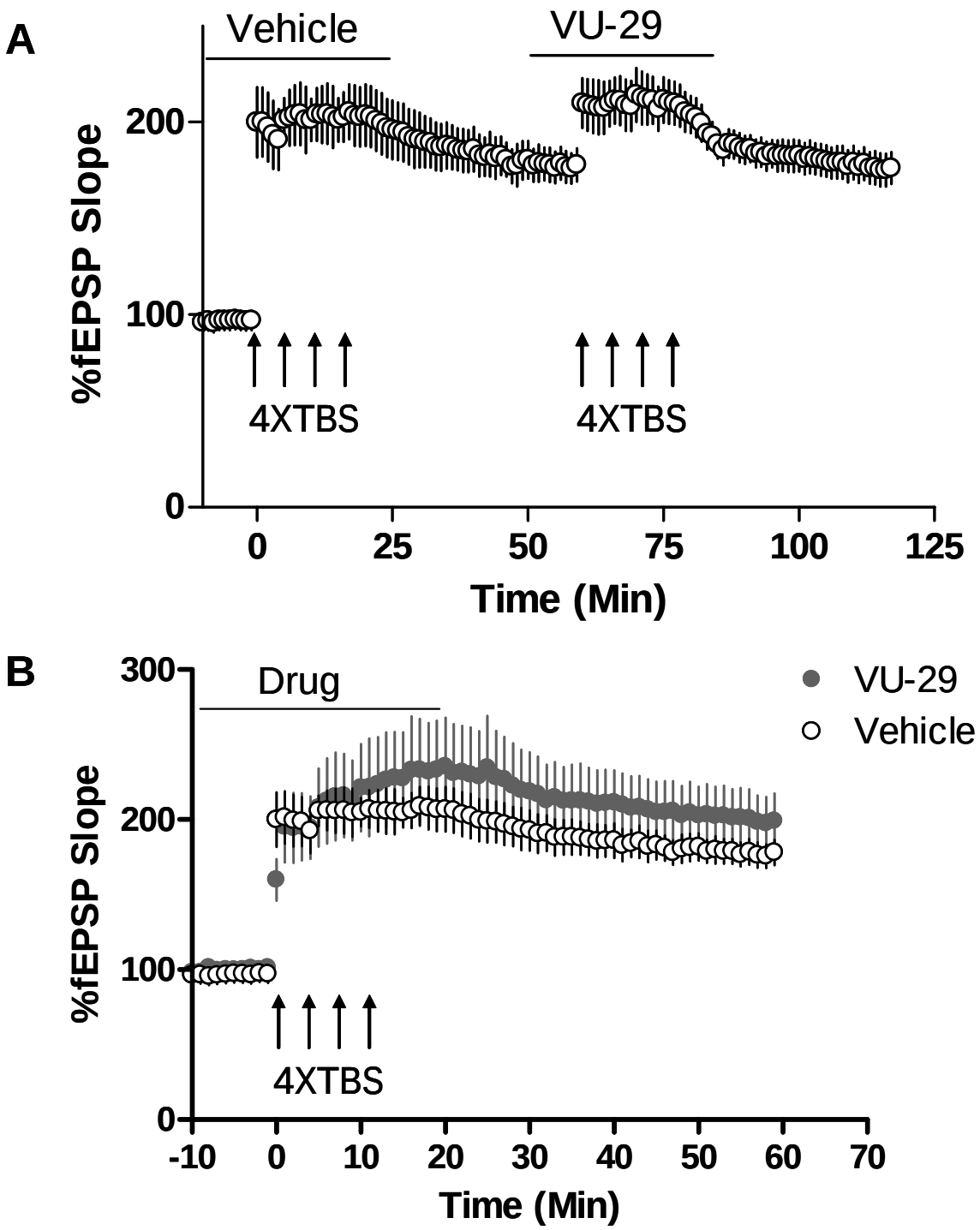

A
Vehicle
VU-29
%fEPSP Slope
200
100
0
4XTBS
4XTBS
0
25
50
75
100
125
Time (Min)
B
Drug
VU-29
Vehicle
%fEPSP Slope
300
200
100
0
4XTBS
-10
0
10
20
30
40
50
60
70
Time (Min)

Figure 4-4: VU-29 facilitated LTP shares similar mechanisms as TBS-induced LTP in area CA1 of the hippocampus. A. Saturated TBS-induced LTP occluded 500nM VU-29 facilitated LTP. LTP was induced by 4 trains of 10 HZ TBS. After 30 minutes, the slices were incubated with 500 nM VU-29, followed by another 4 trains of 10 HZ TBS, which did not overcome the potentiation induced by the first 4 trains of TBS (n=8; Student's t-test). B. 500 nM VU-29 did not alter saturated TBS-induced LTP. In control slices, a 4X 100 HZ TBS-induced saturated potentiation. The same stimulation in the presence of VU-29 yielded a potentiation with no significant difference compared with control (n=5; Student's t-test). Error bars represent S.E.M.

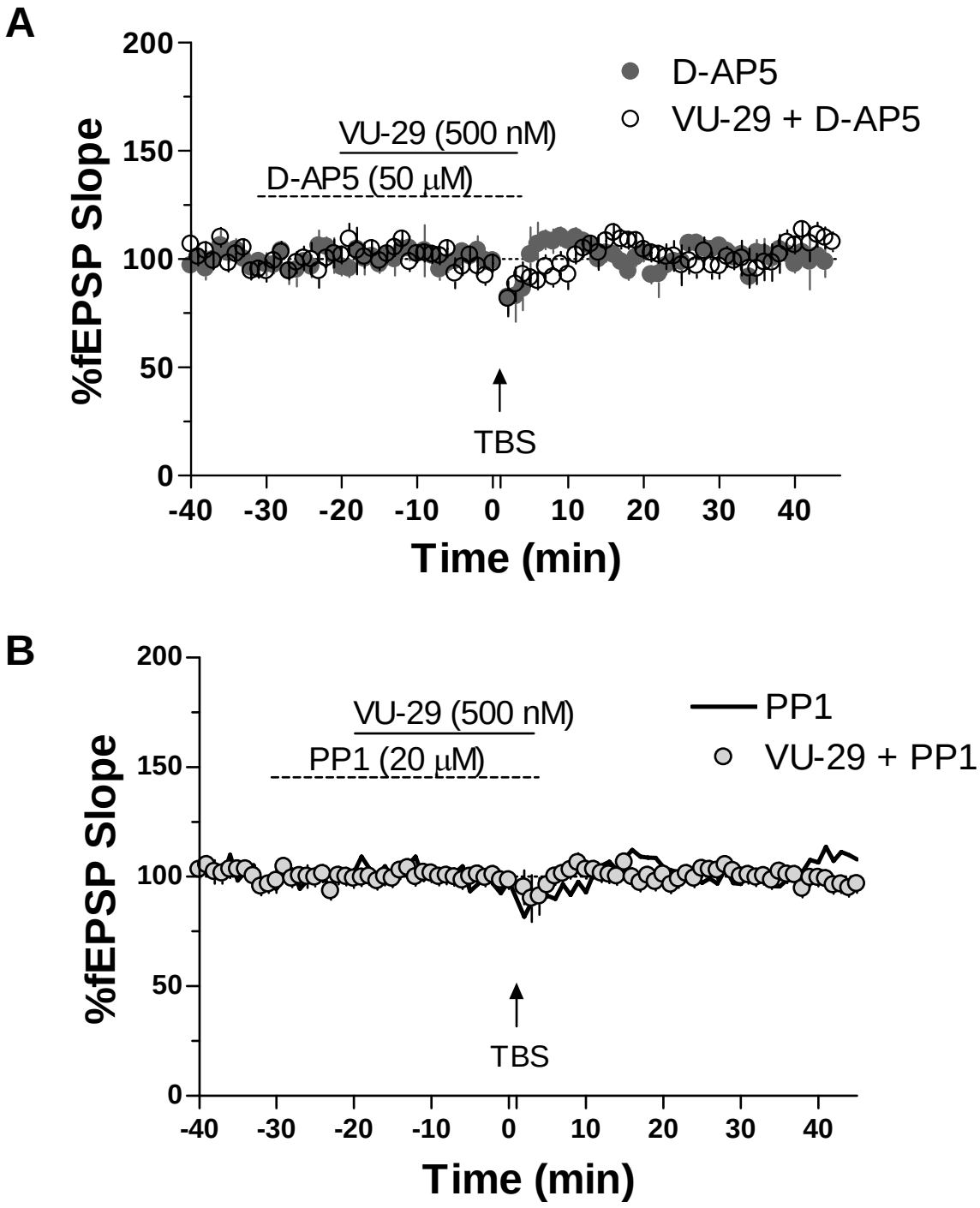



Figure 4-5: NMDA Receptor antagonist and Src family kinase inhibitor block VU-29 facilitated LTP of fEPSP in rat hippocampal CA1 region. 50 μM NMDA receptor antagonist, D-AP5 (A), or 20 μM Src family kinase inhibitor, PP1 (B), completely blocked TBS-induced LTP in the presence or absence of VU-29 (n=8; Student's T-test). Error bars represent S.E.M. (This figure was contributed by Dr. Jessica L. Banko partially)

**A structurally distinct mGluR5 PAM has similar effects to VU-29 on threshold TBS-induced LTP.**

Epping-Jordan et al., (2005) recently presented a preliminary report of a novel mGluR5-selective PAM, termed Addex-47273 that is structurally unrelated to VU-29. Unfortunately, ADX-47273 was only presented in abstract form. However, if mGluR5 PAM activity of this compound could be verified, this would provide an excellent additional tool to provide further evidence that selective potentiation of mGluR5 potentiates LTP and that the actions of VU-29 are mediated by potentiation of mGluR5. Thus, we established a protocol for synthesis of ADX-47273 and characterized this compound to verify that it is a selective mGluR5 PAM. In previous studies, we have rigorously characterized DHPG-induced calcium mobilization in secondary cultured rat cortical astrocytes and found that this response is exclusively mediated by mGluR5 (Peavy et al., 2002) and that mGluR5 PAMS induce a robust potentiation of this response (Zhang et al., 2005; Rodriguez et al., 2005; Hemstapat et al 2006; Chen et al., 2007). ADX-47273 induced a robust potentiation of glutamate-induced calcium mobilization in cortical astrocytes in a manner similar to that previously reported for VU-29 and other mGluR5 PAMS (Figure 4-6). Thus, ADX-47273 induced a concentration-dependent potentiation of the response to an EC20 – EC30 concentration of glutamate (300 nM) with an EC50 value of 108 ± 41 nM (Figure 4-6). Furthermore, ADX-47273 (10 μM) did not affect the glutamate concentration response curves of mGluR1, mGluR2 and mGluR4 (Figure 4-7; Student's T-test), suggesting that this compound is selective as a PAM for mGluR5 relative to these other mGluR subtypes. Consistent with the effects of VU-29, (Figure 4-2B), ADX-47273 (10 μM) induced a significant increase in threshold TBS-

induced LTP of fEPSPs in the rat hippocampal CA1 region (Figure 4-8) (134 $\pm$ 7% potentiation at 30 min post TBS in vehicle; 173 $\pm$ 9% potentiation at 45 min post TBS in ADX-47373).

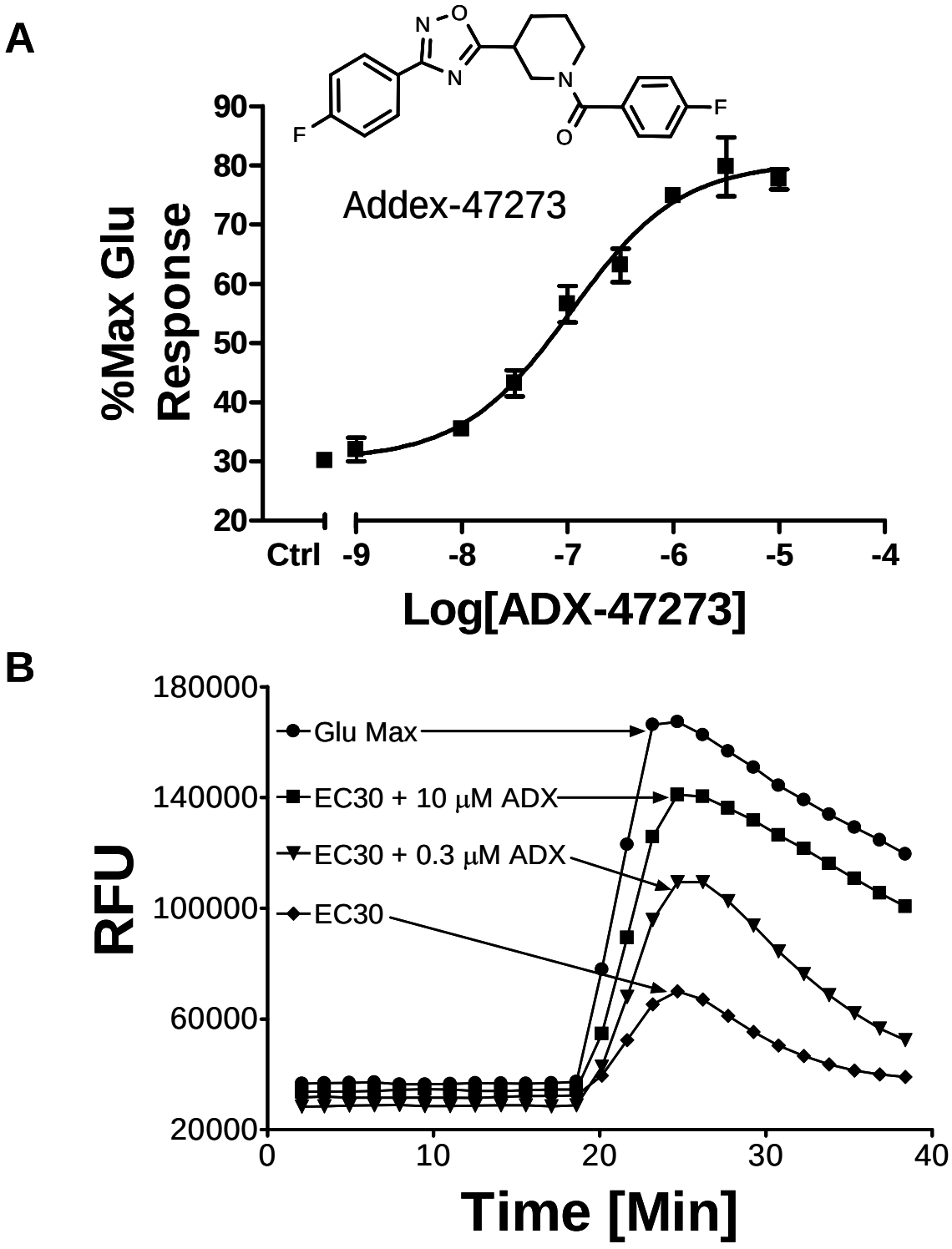

A
Addex-47273
%Max Glu Response
90
80
70
60
50
40
30
20
Ctrl
-9
-8
-7
-6
-5
-4
Log[ADX-47273]
B
RFU
180000
140000
100000
60000
20000
Glu Max
EC30 + 10 μM ADX
EC30 + 0.3 μM ADX
EC30
0
10
20
30
40
Time [Min]

Figure 4-6: ADX-47273 is a novel mGluR5 selective PAM from a different structural family. Using secondary cultured rat cortical astrocytes, intracellular calcium mobilization responses were measured in response to an $EC_{20-30}$ concentration of glutamate in the absence or presence of different concentrations of Addex-47273. The $EC_{20-30}$ concentration for glutamate was determined each day which was about 300 nM. Data were normalized to the maximum response of each reaction determined by 10 μM glutamate. The potency of ADX-47273 was determined to be 108 ± 41 nM. A shows the CRC of ADX-47273. B shows the Calcium mobilization traces of a representative experiment. Concentration-response curves were generated from three independent experiments each performed in duplicate. Error bars represent S.E.M.

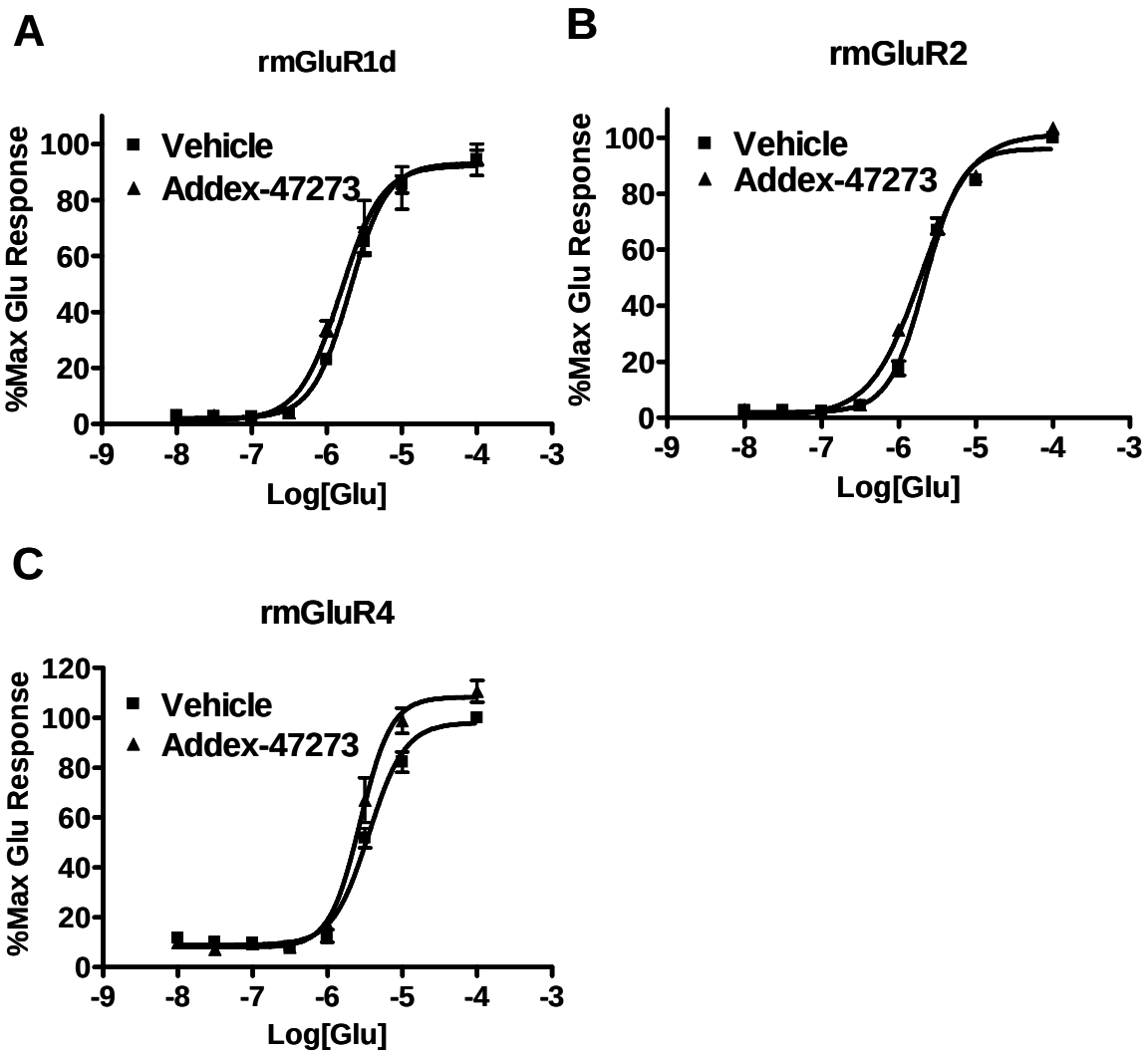



Figure 4-7: ADX-47273 does not potentiate mGluR1-, -2-, or -4-mediated responses. A concentration of Addex-47273 that maximally potentiated mGluR5 responses (10 μM) neither potentiated nor antagonized mGluR1- (A), mGluR2- (B), and mGluR4-mediated (C) responses in transiently transfected HEK 293A cells as measured using the calcium mobilization assay. Concentration-response curves were generated from three to four independent experiments performed in duplicate (Student's T-test). Error bars represent S.E.M.

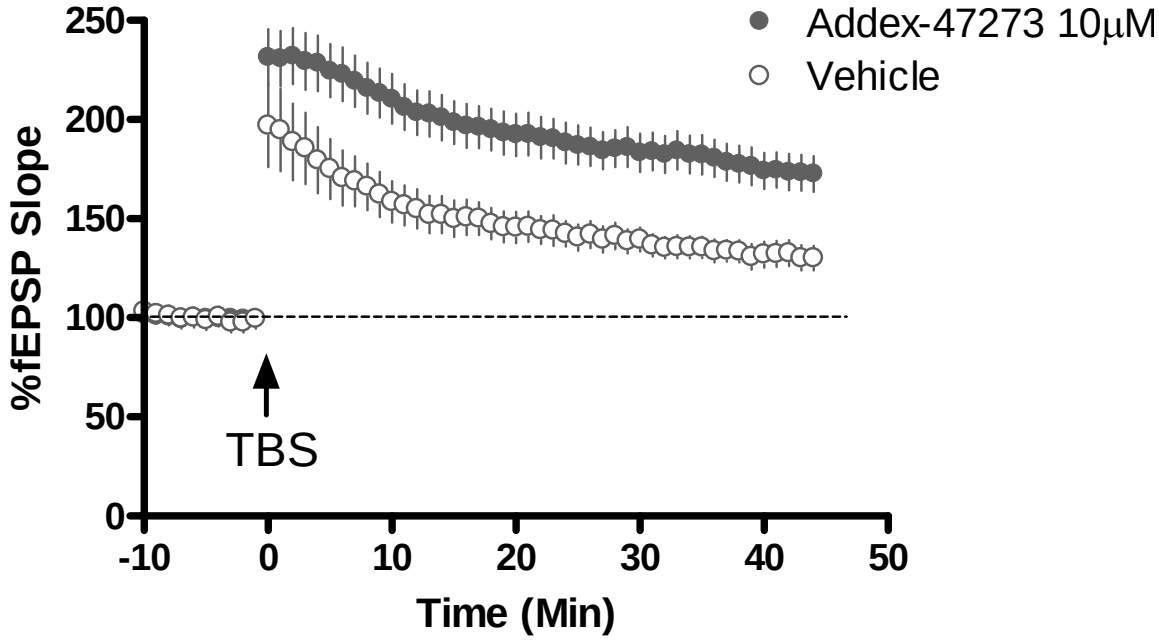



Figure 4-8: ADX-47273 facilitates threshold TBS-induced LTP in rat hippocampal CA1 region. In control slices, TBS delivered in the presence of vehicle resulted in a marginal potentiation of the fEPSP. The same threshold TBS delivered in the presence of 10 μM ADX-47273, induced a significantly larger potentiation of the fEPSP up to 45 minutes after stimulation (n=8; Student's T-test, $P<0.05$). Error bars represent S.E.M.

## Discussion

The present studies provide compelling evidence that selective mGluR5 PAMs enhance induction of LTP by a TBS protocol that is not sufficient to induce robust LTP in the absence of mGluR5 potentiation. This effect was seen with two structurally distinct mGluR5 PAMs and blocked by a compound belonging to a third structural class that has been developed to selectively block the actions of mGluR5 allosteric modulators on mGluR5. This adds to a large literature that has implicated group I mGluRs as playing a role in synaptic plasticity in hippocampal area CA1 and is consistent with previous findings that mGluR5 knockout mice display impaired LTP in area CA1 (Lu et al., 1997) and that mGluR5 antagonists block TBS-induced LTP in this region (Francesconi et al., 2004; Shalin et al., 2006; Manahan-Vaughan and Braunewell, 2005). Furthermore, bath application of the group 1 mGluR selective agonist DHPG primes hippocampal LTP induction at relatively low concentrations (Cohen et al., 1998; Raymond et al., 2000).

However, despite these previous studies, the impact of mGluR5-selective PAMs on afferent stimulation-induced LTP was difficult to predict prior to the present studies. For instance, other studies have reported that mGluR5 antagonists do not block LTP induced by high frequency titanic stimulation (Bortolotto et al., 2005; Fitzjohn et al., 1998). Additionally, higher concentrations of DHPG induce LTD in CA1 region (Gasparini et al. 1999; Huber et al., 2001), which is absent in mGluR5 null mice and could be blocked by MPEP from some studies (Faas et al. 2002; Gasparini et al. 1999; Hou and Klann 2004; Huang and Hsu 2006; Huang et al. 2004 and Huber et al., 2001). Based on this, it was difficult to predict whether selective potentiation of activation of

mGluR5 by afferent stimulation would enhance or reduce LTP, or would lead to induction of LTD by administration of stimuli that are subthreshold for induction of LTP.

While the present studies provide strong evidence in support of a role for mGluR5 in hippocampal TBS-induced LTP, they do not rule out the possibility that mGluR5 can also participate in induction of afferent stimulation-induced LTD under some conditions or stimulus protocols. It is conceivable that activation of mGluR5 by different stimulus protocols or in different physiological settings determines whether this receptor is more likely to lead to long term potentiation or depression or synaptic transmission. However, we saw not evidence that the mGluR5 PAM could promote long term depression of synaptic transmission under any of the stimulus protocols used here. This includes modest TBS that does not induce robust LTP, suprathreshold TBS that induces robust LTP, and application of intense TBS in slices in which LTP was already saturated.

While a role for mGluR5 in induction of LTD by afferent stimulation in the hippocampus remains a viable possibility, it is important to note that the large majority of studies implicating mGluR5 in induction of hippocampal LTD have relied on measuring responses to exogenous application of DHPG to hippocampal slices rather than activation of mGluR5 by afferent stimulation. Early studies suggested that a nonselective group I mGluR antagonist can block one form of low frequency stimulation-induced LTD at the SC-CA1 synapse (Oliet et al., 1997; Nicoll et al., 1998). However, the mGluR5-selective antagonist MPEP does not reduce afferent stimulation-induced LTD (Volk et al., 2006), suggesting that the effect of the more non selective antagonist are not likely mediated by blockade of mGluR5. Furthermore, DHPG activates both mGluR1 and mGluR5 and recent studies suggest that DHPG-induced LTD can only be inhibited by combined

blockade of both mGluR1 and mGluR5, suggesting that both receptors are important for mediating the response to exogenous agonist application (Volk et al., 2006). Thus, further studies are needed to develop a more complete understanding of the relative roles of mGluR1 and mGluR5 in both agonist- and afferent stimulation-induced LTD.

The mechanism by which activation of mGluR5 enhances hippocampal LTP is not known. Activation of this receptor has multiple actions that could contribute to this response. The most obvious possibility is that this is mediated by the known ability of mGluR5 to potentiate currents through the NMDA receptor (Doherty et al., 1997, 2000; Jia et al., 1998; Awad et al., 2000; Mannaioni et al., 2001). TBS-induced LTP at the SC-CA1 synapse is known to be dependent on NMDA receptor activation and potentiation of NMDA receptor function by mGluR5 provides an obvious mechanism that could contribute to this response. Consistent with this, TBS LTP in the presence of VU-29 remains NMDA receptor dependent. Also, the response is blocked by PP1, an inhibitor of src family kinases that are known to be critical for mGluR5 potentiation of NMDA receptor currents in CA3 pyramidal cells (Benquet et al., 2002). However, it is also possible that other actions of mGluR5 activation participate in regulation of hippocampal LTP. Activation of mGluR5 has a number of effects in area CA1 including blockade of AHP-type potassium currents in CA1 pyramidal cells (Mannaioni et al., 2001), depression of inhibitory synaptic transmission (Mannaioni et al., 2001), and acute potentiation of depolarization-induced glutamate release (Herrero et al., 1998; Rodríguez-Moreno et al., 1998; Fiacco et al., 2004). Also, mGluR5 activation induces depolarization of multiple other neuronal populations (Awad et al., 2000; Valenti et al., 2002). While mGluR1 is the primary group I mGluR subtype responsible for depolarization CA1

pyramidal cells recorded in the soma, it is possible or likely that mGluR5 could contribute to depolarization in dendrites and this could also contribute to the overall actions of mGluR5 activation. Finally, mGluR5 is also localized in astrocytes in the hippocampus where it activates PI hydrolysis and phosphorylation of extracellular signal regulated kinase (ERK2) (Peavy et al., 2001; 2002). Recently it has been shown that astrocytes could play a key role in regulating synaptic plasticity by releasing the NMDA receptor co-agonist D-Serine (Yang et al., 2003). Activation of mGluRs in astrocytes induces D-serine release (Kim et al., 2005, Mothet et al., 2005 and Schell et al., 1995), raising the possibility that this could also contribute to the effects of mGluR5 PAMs. In total, these combined effects of mGluR5 activation in the hippocampal formation would dramatically increase excitatory drive, neuronal excitability, and NMDA receptor activation in CA1 pyramidal cells, which could prime the cells for efficient induction of LTP.

In addition to implications for the mechanisms underlying hippocampal LTP and a possible role of mGluR5 in this process, these studies have important implications related to the possible development of mGluR5 PAMs as therapeutic agents. Discovery of selective PAMS for mGluR5 and other G-protein-coupled receptors has generated a great deal of interest in the possibility of developing this class of small molecules as a novel approach for treatment of CNS disorders (Christopoulos et al., 2002; May et al., 2004; Brauner-Osborne et al., 2007). For mGluR5, a large number of anatomy, electrophysiology, and behavioral studies with mGluR5 antagonists led to the hypothesis that activation of mGluR5 could have potential utility as a novel approach to treatment of schizophrenia (Marino and Conn, 2002). Unfortunately, it has been difficult to develop

agonists that selectively activate mGluR5 and have properties that are desirable in drug-like molecules. Furthermore, direct-acting receptor agonists can have problems associated with excessive receptor activation and strong desensitization that lead to adverse effects and tolerance with chronic administration. Discovery of highly selective mGluR5 PAMs such as VU-29 provided a major breakthrough and a novel approach to increasing activity of this receptor. Consistent with the hypothesis that these compounds have potential as novel antipsychotic agents, we found that a close analog of VU-29, termed CDPPB, has antipsychotic-like effects in two rodent models that are used to predict efficacy of novel agents at reversing the positive symptoms (ie psychosis) seen in schizophrenic patients (Kinney et al., 2005). Furthermore, Moghaddam et al. (2004) reported that CDPPB has effects on cell firing in the cortex that are opposite to those of psychomimetic agents. These data are consistent with the possibility that mGluR5 PAMs could prove to have antipsychotic efficacy. The present studies raise the possibility that, in addition to efficacy in treatment of positive symptoms of schizophrenia, mGluR5 PAMs could also have effects that may enhance impaired cognitive function. This could provide a major benefit relative to existing antipsychotic agents and raises the possibility that these compounds should also be evaluated for efficacy in models of other diseases that involve impaired cognitive function. Consistent with this, Balschun et al (2006) recently reported that icv injection of the mGluR5 PAM DFB, induces a marked improvement in spatial alternation retention when it was tested 24 hr after training. These data are consistent with the present findings and suggest that mGluR5 PAMs can enhance at least one form of hippocampal-dependent memory. In addition, exciting advances with allosteric potentiators of other mGluR subtypes suggest that these

compounds often have robust behavioral effects that are similar to those of direct-acting orthosteric agonists. For instance, we recently developed PHCCC as an allosteric potentiator of mGluR4 and found that this compound has antiparkinsonian effects in rodent models that are similar to those of the traditional agonist L-AP4 (Valenti et al., 2003; Marino et al., 2003). Furthermore, we (Galici et al., 2006) and others (Johnson et al., 2003; 2005) found that novel allosteric potentiators of mGluR2 have robust behavioral effects similar in rodent models that predict anxiolytic and antipsychotic activity. In future studies, it will be important to expand our understanding of the behavioral effects of mGluR5 PAMs and further evaluate the potential utility of these compounds in measures of cognitive function.

# CHAPTER V

## SUMMARY

Exciting progress has been made in the discovery of selective positive allosteric modulators of mGluR5. These compounds may provide a novel approach that could be useful in the treatment of certain central nervous system disorders, such as schizophrenia. However, because of their relatively low potencies, previously described mGluR5 PAMs are not useful for functional studies in native preparations. In addition, their pharmacological properties are not clear, including their sites of action and interactions with other mGluR5 allosteric modulators. It has been suggested that two allosteric potentiators, DFB and CDPPB, act by binding to the same allosteric site as the negative allosteric modulators of mGluR5 such as MPEP, because both of them reduce binding to the MPEP site. However, CPPHA, another mGluR5 PAM from a distinct structural family, does not bind to this site. Moreover, DFB reduces MPEP site binding only partially and CDPPB has a much lower affinity to MPEP site than its potency as mGluR5 PAM. All of these bring the site of action in mGluR5 into question.

We have synthesized a series of CDPPB analogs and report that these compounds bind to the MPEP site with affinities that are closely related to their potencies as mGluR5 potentiators. Furthermore, allosteric potentiation is antagonized by a neutral ligand at the MPEP site and reduced by a mutation of mGluR5 that eliminates MPEP binding. Together, these data suggest that interaction with the MPEP site is important for allosteric potentiation of mGluR5 by CDPPB and related compounds. In addition, whole-cell

patch-clamp studies in midbrain slices reveal that a highly potent analog of CDPPB, VU-29, selectively potentiates mGluR5 but not mGluR1-mediated responses in midbrain neurons, whereas a previously identified allosteric potentiator of mGluR1 has the opposite effect.

The finding that the binding to the MPEP site is important for the action of VU-29 suggests that CPPHA might act at a distinct site, especially when combined with the fact that it does not to bind to the MPEP site. As the second part of my graduate study, we have reported that CPPHA potentiates mGluR5 responses by a mechanism that is distinct from that of VU-29. VU-29- and CPPHA-induced potentiation of mGluR5 responses are blocked by a neutral ligand at the MPEP alloteric site termed 5MPEP. However, increasing concentrations of 5MPEP induce parallel rightward shifts in the VU-29 concentration response curve (CRC) whereas 5MPEP inhibits CPPHA potentiation in a non-competitive manner. Consistent with this, a mutation (A809V/mGluR5) that reduces binding of ligands to the MPEP site eliminates the effect of VU-29 but has no effect on the response to CPPHA. Conversely, a mutation that eliminates the effect of CPPHA does not alter the response to VU-29. CPPHA is also a PAM at mGluR1. Interestingly, the corresponding mutation of F585I/mGluR5 in mGluR1 (F599I/mGluR1) eliminates CPPHA's effect without altering the potentiation of a known PAM of mGluR1 (Ro 67-7476). Likewise, another mutation (V757L/mGluR1) abolishes potentiation of Ro 67-7476 has no effect on CPPHA. Finally, CPPHA does not displace binding of a radioligand for the previously characterized mGluR1 allosteric antagonist. Together, these data suggest that CPPHA acts at a novel allosteric site on both mGluR1 and 5 to potentiate responses to activation of these receptors.

mGluR5 has been implicated as playing an important role in hippocampal synaptic plasticity. However, the precise roles of mGluR5 in different forms of hippocampal synaptic plasticity remain unclear. In the third part of my study, we took the advantage of the novel pharmacological tools, mGluR5 selective PAMs, to determine the effect of selectively increasing activation of mGluR5 in response to stimulation of glutamatergic afferents at the Schaffer collateral – CA1 (SC-CA1) synapse in the hippocampus. The selective mGluR5 PAM, VU-29, potentiated DHPG-induced PI hydrolysis in rat hippocampal slices and this effect was blocked by 5MPEP, an mGluR5-selective neutral allosteric site ligand. Thus, VU-29 and 5MPEP have effects in hippocampal slices that are consistent with those observed in recombinant systems. Interestingly, VU-29 did not affect basal synaptic transmission at the SC-CA1 synapse but significantly enhanced threshold theta burst stimulation (TBS)-induced long-term potentiation (LTP). This LTP induction was completely blocked by 5MPEP and was mimicked by ADX-47273, a structurally distinct mGluR5-selective PAM, providing further support for a critical role of mGluR5. In addition, VU-29 potentiated LTP was blocked by the NMDA receptor antagonist, D-AP5 or by the src kinase inhibitor, PP1, suggesting that it shares a common mechanism with suprathreshold TBS-induced LTP. Interestingly, VU-29 had no effect on LTP induced by suprathreshold TBS stimuli or on saturated LTP. These data suggest that selective potentiation of synaptically activated mGluR5, facilitates threshold TBS-induced LTP in the rat hippocampal CA1 region, and raise the possibility that these agents could be used as potential cognition enhancing agents.

In summary, we have identified two distinct crucial sites for the action of two different families of mGluR5 PAMs respectively and shown their differential pharmacological properties. It has been shown that DFB and CPPHA could regulate mGluR5-mediated ERK1/2 phosphorylation differentially. Our finding is crucial to understand these different actions of mGluR5 PAMs and could orient future development of mGluR allosteric modulators. Meanwhile we have reported the first evidence that mGluR5 PAMs potentiates threshold TBS-induced LTP in rat hippocampal CA1 region. This is helpful to understand the fundamental role of mGluR5 in neuronal plasticity and suggest mGluR5 PAMs could have the potential to be cognition enhancing reagents. There are however still questions remaining about this phenomenon. For instance, the mechanism of action is still unclear. Also, the effect of mGluR5 PAMs on LTD has not been addressed. These are all future directions for our lab to pursue.

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