Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.

Title page

Cooperative signaling between Homodimers of Metabotropic Glutamate Receptors 1&5.

Tatyana N. Sevastyanova and Paul J. Kammermeier, Department of Pharmacology and Physiology (TNS and PJK), University of Rochester Medical Center, Rochester, NY 14642.

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

Running title: Functional interaction of mGluR1 and mGluR5.

Corresponding Author:

Paul J. Kammermeier
Associate Professor
Department of Pharmacology and Physiology
University of Rochester Medical Center
601 Elmwood Avenue, Box 711
Rochester, NY 14642
Voice: 585.275.5606
Fax: 585.273.2652
paul_kammermeier@urmc.rochester.edu

# of pages: 36
# of figures: 8
# of references: 39
# of words in Abstract: 228
# of words in Introduction: 610
# of words in Discussion: 1411

List of non-standard abbreviations:
mGluR: metabotropic glutamate receptor
SCG: superior cervical ganglion
NAM: negative allosteric modulator
PAM:positive allosteric modulator
BAY36: (BAY36 7620) (3aS,6aS)-Hexahydro-5-methylene-6a-(2-naphthalenylmethyl)-1H-cyclopenta[c]furan-1-one
MPEP: 2-Methyl-6-(phenylethynyl)pyridine hydrochloride
MATIDA: (3-MATIDA) α-Amino-5-carboxy-3-methyl-2-thiopheneacetic acid
MNI-137: 4-(8-Bromo-2,3-dihydro-2-oxo-1*H*-1,5-benzodiazepin-4-yl)-2-pyridinecarbonitrile

Conflict of interest.
None

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

Abstract.

Metabotropic glutamate receptors (mGluRs) function as dimers. Recent work suggests that mGluR1 and mGluR5 may physically interact, but the nature and functional consequences of this relationship have not been addressed. In this study, the functional and pharmacological consequences of this interaction were investigated. Using heterologous expression of mGluR cDNA in rat sympathetic neurons from the superior cervical ganglion (SCG) and inhibition of the native calcium currents as an assay for receptor activation, a functional interdependence between mGluR1 and 5 was demonstrated. In neurons co-expressing these receptors, combining a selective mGluR1 competitive antagonist with either an mGluR1 or mGluR5 selective negative allosteric modulator (NAM: BAY36 7620 and MPEP, respectively) strongly occluded signaling by both receptors to an approximately equal degree. By contrast, in cells co-expressing mGluR1 and 2, combining the same mGluR1 competitive inhibitor with an mGluR1 or mGluR2 NAM yielded partial and full inhibition of the response, respectively, as expected for independently acting receptors. In neurons expressing mGluR1 and 5, the selective NAMs each strongly inhibited the response to glutamate, suggesting that these receptors do not interact as heterodimers, which would not be inhibited by selective NAMs. Finally, evidence for a similar mGluR1/5 functional dependence is shown in medium spiny striatal neurons. Together, these data demonstrate cooperative signaling between mGluR1 and 5 in a manner inconsistent with heterodimerization, and thus suggesting an interaction between homodimers.

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

Introduction.

Metabotropic glutamate receptors (mGluRs) are class C G protein coupled receptors with widespread expression in the nervous system. There are three groups of mGluRs (I-III) categorized by homology, pharmacology and G protein coupling (Schoepp, 2001). Until recently, most mGluRs, which form stable, covalently linked dimers (Romano *et al.*, 1996; Ray and Hauschild, 2000; Tsuji, 2000; Kunishima *et al.*, 2000; Romano *et al.*, 2001), were thought to primarily function as homodimers. Recent work has shown that some combinations of mGluRs can form heteromeric receptors (Doumazane *et al.*, 2011), including some, such as mGluR2 and 4, that form heterodimers in heterologous systems (Doumazane *et al.*, 2011) and in neurons (Kammermeier, 2012a; Yin *et al.*, 2014).

It is becoming increasingly apparent that a careful characterization of the pharmacology of heteromeric mGluRs is necessary if their potential as therapeutic targets is to be fully realized. While some mGluR heteromers seem to clearly form hetero*di*mers (Doumazane *et al.*, 2011), this may not necessarily be the case for every mGluR heteromer pair. Since the pharmacological consequences of heteromer formation are largely a consequence of the nature of the interaction, it will be important to know the stoichiometry of each heteromeric mGluR and what impact stoichiometry has on the resulting pharmacology.

In a recent paper, a physical association between the group I mGluRs, mGluR1 and 5, was implied using a fluorescence energy transfer assay (Doumazane *et al.*, 2011), but neither the functional consequences of this interaction, nor its pharmacological impact, have been determined. Further, heterodimerization of wild-type mGluR1 and mGluR5 has *not* been demonstrated, and has been ruled out in at least one study (Romano *et al.*, 1996). Assessing the functional role of heteromerization

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

between highly homologous receptors such as mGluR1 and 5 has been hampered by the lack of selective agonists (Kammermeier, 2012b). In a previous study, we have circumvented this problem by constructing an mGluR1 point mutant, mGluR1 Y74A, with an alanine for tyrosine substitution near the glutamate binding pocket (Kammermeier and Yun, 2005). This mutation shifts the sensitivity of the receptor for glutamate by ~ 100 fold, rendering glutamate a selective agonist for co-expressed wild type mGluRs. In that study, mGluR1 Y74A was co-expressed with wild-type mGluR1 in rat sympathetic neurons from the superior cervical ganglion (SCG) to assess the functional consequences of homodimerization. The 'heteromeric' receptor (the mGluR1 mutant/mGluR1 wild-type dimer) was found to exhibit a dose-response nearly identical to the mutant homodimer, which demonstrated that mGluR dimers require ligand in both subunits for strong activation. Fortuitously, this theoretically provided a functional assay for heterodimerization between mGluR1 Y74A and other mGluR subtypes. As with the mGluR1 Y74A/mGluR1 wild-type combination, a heterodimer between mGluR1 Y74A and another mGluR should exhibit a concentration-response nearly identical to the Y74A mutant alone. Thus in the present study, the same mutant was co-expressed with mGluR5, a closely related receptor that resides in close proximity with mGluR1 (Doumazane *et al.*, 2011). Beginning with this strategy, we show that these receptors do appear to interact functionally, and that this interaction gives rise to a functional receptor pool with a unique pharmacological profile with respect to its sensitivity to selective mGluR1 and mGluR5 compounds. However, the functional profile of the mGluR1/5 receptor pair appears distinct from that expected of a putative mGluR1/5 heterodimer in this functional assay. Thus, we interpret these results as evidence for an interaction between mGluR1 and mGluR5 homodimers. This is the first demonstration that mGluR1 and 5, when coexpressed in the same neuron, exhibit functional interdependence. Finally, we present evidence that a similar pharmacological profile in cultured medium spiny neurons of the rat striatum, suggesting

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

that the receptor interaction bears physiological relevance, possibly giving rise to a unique therapeutic target.

Materials and Methods.

SCG Neuron Isolation

Male Wistar rats were euthanized with CO_2 and decapitated, in accordance with the University Committee on Animal Research methods. All SCG dissections were performed in chilled HBSS. The ganglia were removed and incubated for 60 min at 37^0C in Earles's balanced salt solution (InVitrogen, Carlsbad, CA) containing 0.5 mg/ml trypsin (Worthington Biochemical, Freehold, NJ) and 11 mg/ml collagenase D (Roche Diagnostics, Indianapolis, IN). After the incubation, cells were centrifuged twice for 6 min at 700 RPM, and resuspended in Minimal Essential Medium (Thermo Fisher Scientific, Waltham, MA) containing 10% FBS, penicillin/streptomycin and GlutaMax (InVitrogen). Resuspended cells were plated onto 35mm tissue culture dishes coated with poly-L-lysine (Sigma Aldrich, St. Louis, MO), and stored at 5% CO_2 at 37^0C for 3-5 hours.

cDNA microinjection of SCG

Nuclear microinjection of plasmids was performed 2-4 hours following SCG neuron isolation with an Eppendorf 5247 microinjector and Injectman NI2 micromanipulator (Eppendorf North America, Hauppage, NY). Injections were made with a Sutter (Novato, CA) P-97 horizontal electrode puller using thin-walled, borosilicate glass (World Precision Instruments, Sarasota, FL). The plasmids used for the injections were stored at -20^0 C varying from 0.9 to 1.5 ug/ul stock solution in TE buffer (10mM Tris, 1mM EDTA, pH 8). Each receptor (e.g. mGluR1) was injected at 100-150 ng/µl with a reporter gene - "enhanced" green fluorescent protein (0.02 µg/µl; pEGFPC1; Clontech Laboratories, Mountain

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

View, CA). Identical amounts of cDNA were injected of each receptor when expressed alone or with another receptor. Depending on the quality of the SCG cell culture, 30-200 cells were injected in each 35mm cell culture dish. The injected cells were placed back in the 37^0C incubator and recordings made the following day.

Electrophysiological Recordings and Data Analysis

All patch-clamp experiments were performed at ~ 22^0C (room temperature). Recording electrodes were made from 8250 glass (WPI Inc.) using a Sutter P-97 horizontal puller. Pipette resistances were 0.8-3.0 MΩ, which resulted uncompensated series resistances of 1-5 MΩ. For each recording, series resistance compensation of 80% was used. Patch-clamp data was obtained using an EPC-7 (HEKA Elektronic, Lambrencht, Germany), or an Axon 200B (Molecular Devices, Sunnyvale, CA). Custom data acquisition software (courtesy Stephen R. Ikeda, NIAAA, Rockville, MD) was used on a Macintosh G4 computer (Apple Computer, Cupertino, CA) with an InstruTech A/D board (ITC-16 or ITC-18; Port Washington, NY; now Heka Elektronik) was used for data acquisition. Data were sampled at 10 µsec, low-pass filtered at 5 kHz, digitized, and stored for later analysis using IgorPro software (WaveMetricts, Lake Oswego, OR).

Recording Solutions and Pharmacological agents

The cell bath solution (external) contained (in mM): 155 tris hydroxymethyl aminomethane, 20 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 glucose, 10 CaCl2, and 0.0003 tetrodotoxin (TTX), pH 7.4. The pipette solution (internal) contained (in mM): 120 N- methyl-D-glucamine (NMG) methanesulfonate, 20 TEA, 11 EGTA, 10 HEPES, 10 sucrose, 1 CaCl2, 4 MgATP, 0.3 Na2GTP, and 14 tris creatine phosphate, pH 7.2. L- Glutamate, the endogenous ligand for mGluRs was obtained from Sigma; other mGluR selective compounds including 3-MATIDA, BAY36-

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

7620, MPEP and MNI-137 were obtained from Tocris Bioscience (Ellisville, MO). Application of the drugs was achieved by a custom, gravity-driven perfusion system positioned 50-150 µm from the cell, allowing rapid solution exchange (≤250 ms). Maximum current inhibition was defined as an equivalent of the degree of calcium current inhibition after addition of each drug compared to the last current measurement prior to the addition.

Culturing of striatal neurons and measurement of intracellular calcium.

MSN striatal neurons were cultured by a method derived for the culturing of hippocampal neurons reported previously (Kammermeier and Worley, 2007). Briefly, the striatum was dissected from P0-3 rats, enzymatically dissociated with about 25 U/ml Papain (Worthington Biochemical COrp., Lakewood, NJ) for 1 hour at 37°C, then gently triturated, spun at 800 RPM for 5 minutes and plated onto glass coverslips coated with Poly-lysine in 'growth medium.' Growth medium consisted of MEM (Gibco/InVitrogen, Carlsbad, CA) supplemented with FBS (5%), Penecillin/streptomycin, Na Pyruvate (1 mM), glucose (0.6%), B-27 (2%), mito-serum extender (0.1%), uridine (10 µM), and 5-fluorodeoxyuridine (2.5 µM) (Sigma-Aldrich, St. Louis, MO). Cultures were used after 1-2 weeks *in vitro*, as indicated.

For ratiometric calcium imaging experiments, glass coverslips containing MSNs were loaded with Fura-2 AM for ~20 minutes then transferred into a perfusion chamber. Cells were perfused in HEPES-buffered physiological saline containing (in mM) 137 NaCl, 0.56 $MgCl_2$, 4.7 KCl, 1 Na_2HPO_4, 10 HEPES, 5.5 glucose, and 1.26 $CaCl_2$, pH 7.4. Imaging was performed using an inverted Nikon microscope through a 40X oil immersion objective lens (numerical aperture, 1.3). Fura-2 AM loaded cells were excited alternately with light at 340 and 380 nm by using a monochrometer-based illumination system (TILL Photonics, Pleasanton, CA), and the emission at 510 nm was captured by

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

using a digital frame transfer CCD camera. Images were captured every 2 s with an exposure of 40 ms and 4 by 4 binning. Analysis was performed by TILL Vision software. Amplitudes of responses were defines as peak fluorescence induced by each drug application minus baseline preceding the application. For reporting of inhibitor effects (Fig. 8C), decay of responses to DHPG (applied at the start and end of each experiment), were assumed to be linear and responses in the presence of inhibitors were compared to the predicted control response at the given time. DHPG, MATIDA, and BAY36 were obtained from Tocris.

Results.

mGluRs 1 and 5 interact functionally.

Neurons from the rat SCG provide a null-mGluR background upon which specific mGluRs can be expressed in a native neuronal environment (Ikeda, 1997; Kammermeier and Ikeda, 1999). To assess the potential functional interaction between mGluRs 1Y74A and 5, each receptor (or both) was made to express in isolated rat SCG neurons by intranuclear cDNA injection (Lu *et al.*, 2009), and the G protein mediated inhibition of the native calcium currents was used as an assay for receptor signaling. Representative experiments are shown in Fig. 1A. Application of glutamate to neurons expressing mGluR5b (Fig. 1A, *upper*) results in a rapid and reversible inhibition of calcium currents (Fig. 1B, *filled circles*), when currents are measured using a 25 msec test pulse to +10 mV from a holding potential of -80 mV (Fig. 1A, *upper right*). Likewise, in cells injected with mGluR1 Y74A (Fig. 1A *center,* B, *squares*), glutamate induced a strong inhibition of the current, but with a shifted response, such that inhibition was not seen below 100 µM, and the maximal inhibition was stronger, peaking at > 60%, consistent with results observed in previous work (Kammermeier and Yun, 2005). In that paper we showed that 1 mM glutamate produces a response that is about 90% saturating. These data are

consistent with a recent report on group I mGluRs and their coupling to neuronal calcium channels (Kammermeier and Ikeda, 1999).

When mGluR1 Y74A and mGluR5b were expressed together, the glutamate dose-response curve was surprising (Fig 1A *lower*, B, *diamonds*). Co-expression resulted in a high-efficacy response, similar to the mGluR1 Y74A mutant, but with a potency that was intermediate to the receptors expressed separately. When similar experiments were conducted using a different mGluR5 splice variant, mGluR5a, the results were similar (Fig 1C). In both cases, mGluR5 co-expression with mGluR1 Y74A resulted in an intermediate potency, high-efficacy glutamate response. These data are difficult to reconcile with a model in which the two receptors function independently.

To determine whether the functional interaction observed in these experiments was reciprocal, the corresponding mutation was made in mGluR5b. The resulting receptor, mGluR5b Y64A, was expressed alone or with mGluR1 (Fig. 2A). As with the Y-A mutation in mGluR1, the dose-response of the mutant mGluR5 was right shifted, although less severely than the mGluR1 mutant. Responses peaked at nearly 80% inhibition with an apparent EC_{50} of about 56 µM. Cells expressing mGluR1 alone had responses as expected, peaking at ~ 40% inhibition and with an apparent EC_{50} of about 1µM, consistent with previous reports (Kammermeier and Yun, 2005; Beqollari and Kammermeier, 2010). As with the mGluR1 Y74A/mGluR5 combination, when mGluR5 Y64A was co-expressed with mGluR1, responses showed strong efficacy and intermediate potency (EC_{50} of 16 µM) compared to each receptor alone. These data suggest that the interaction between mGluR1 and mGluR5 is at least qualitatively reciprocal.

We next tested whether mGluR1 Y74A (constructed from the mGluR1a variant), which appeared to dimerize with wild-type mGluR1a in our functional assay (Kammermeier and Yun, 2005), would interact with mGluR1b, which cannot form dimers with mGluR1a (Remelli *et al.*, 2008). Thus, our

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.

model predicts that the glutamate concentration-response of the mGluR1 Y74A/mGluR1b combination should mimic that of mGluR1 Y74A/mGluR5 (high-efficacy, intermediate potency) rather than that of mGluR1 Y74A/mGluR1a (high-efficacy, potency nearly identical to the YA mutant alone). Indeed, as shown in Fig. 2B when these receptors were expressed together, the partial glutamate-response curve indicates a high-efficacy response with a potency intermediate to that of mGluR1b and mGluR1 Y74A when expressed alone, validating our prediction. Thus, these data support the finding (Remelli *et al.*, 2008) that mGluR1a and 1b fail to dimerize.

It is possible that two overexpressed receptors with different potencies to the same agonist and sharing limited downstream signaling intermediates may yield a concentration-response that lies between that of each receptor when expressed alone. This type of interaction is likely to produce a concentration-response with a Hill slope observably less steep than the more potent receptor. This was not seen in the case of mGluR1 and 5. Nevertheless, to rule out this interpretation, mGluR2, known to act independently of mGluR1 (Doumazane *et al.*, 2011), was expressed in SCG neurons with mGluR1 Y74A as a negative control (Fig. 2C). The mGluR2 glutamate response has an EC_{50} in the low µM range with a maximal calcium current inhibition of about 50-60% (Fig. 2C, *filled circles*). The glutamate dose-response curve in the presence of both mGluR1 Y74A and mGluR2 was as expected if these receptors were functioning independently, and if intracellular signaling elements were not limiting. That is, an intermediate potency response was not observed, despite mGluR2 being a high efficacy receptor which may be expected to deplete intracellular signaling intermediates more so than mGluR5. At lower glutamate concentrations the responses were indistinguishable from those seen with mGluR2. At 1 mM, the response to the combined receptors was increased slightly to 82 ± 4% (n=5), suggesting a partially additive response. Note that a fully additive response is not expected because the inhibitory mechanism of the two receptors is partially shared (Kammermeier and Ikeda,

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

1999), and because the assay measures inhibition, which does not sum linearly in the very high efficacy range and which mechanistically cannot reach 100% (Bean, 1989). Thus, the functional interaction between mGluR1 and 5 exhibits selectivity consistent with the demonstrated close association between these receptors, but not between mGluR1 and mGluR2 (Doumazane *et al.*, 2011).

Pharmacological assessment of the mGluR1/5 interaction.

To begin to assess the nature of the cooperation between mGluR1 Y74A and mGluR5b (Fig.1Ba) or mGluR5 Y64A and mGluR1 (Fig. 2A) are coexpressed, the effects of the mGluR1 selective negative allosteric modulator (NAM), BAY36 7620 *((3aS,6aS)-Hexahydro-5-methylene-6a- (2-naphthalenylmethyl)-1H-cyclopenta[c]furan-1-one) ("BAY36")* (Carroll *et al.*, 2001), and the mGluR5 selective NAM, 2-Methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP), a commonly used, highly selective and efficacious allosteric antagonist at mGluR5 (Gasparini *et al.*, 1999) were examined (Fig. 3). A schematic of the experimental design illustrating the expressed receptors and compounds targeting them is shown above each panel in Fig. 3. Note that the upper, larger box used to illustrate each receptor represents the N-terminal orthosteric ligand binding site and the lower smaller box represents the heptahelical domain containing the allosteric site. Note also that these cartoons are not intended to convey receptor stoichiometry or even physical interaction, only to represent the receptors that were expressed in each experiment. Paired control experiments (recorded on the same days) for individually expressed receptors are shown in Supplemental Figure 1.

In the first set of experiments (Fig. 3A&B) a partial glutamate dose-response curve was constructed in the absence and presence of a NAM targeting the mutant (YA) subunit. Interestingly, strong inhibition was observed by both 1 μM MPEP (Fig. 3A) and 1 μM BAY36 (Fig. 3B) at glutamate

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

concentrations below the range at which the targeted receptors (mGluR5 YA and mGluR1 YA, respectively) are active. In addition, the overall shape of the curves in the presence of the inhibitors more closely resembled that of the mutant receptor when expressed alone, although with decreased efficacy. This was surprising because in each case the mutant receptor was the target of the inhibitor. As a control, we tested whether cells expressing mGluR1 Y74A and mGluR2, as in Fig. 2C were susceptible to inhibition by BAY36 at a glutamate concentration below that which the mGluR1 mutant was expected to be active. As expected, BAY36 failed to reduce the response to 10 μM glutamate in these cells. Calcium current was inhibited in these cells by 41±4% when 10 μM glutamate was applied alone and 40±4% when glutamate was applied in the presence of BAY36 (n=6). Next, similar experiments were performed but this time targeting the wild-type receptors with their respective NAMs (Fig. 3C&D). The results of these experiments were perhaps less surprising, with each inhibitor leaving a response quite similar to that of the un-targeted (mutant) receptor when expressed alone (see Supplemental Figure 1 for comparison). To some extent, these data should be interpreted with caution, particularly because BAY36 was a poor inhibitor of mGluR1 at [glutamate] above 100 μM, but the data with MPEP, a more reliable inhibitor, were similar. It is however intriguing that regardless of the receptor targeted by the NAM (mutant or wild-type), the potency of the resulting response was similar. However, at this point is is difficult to conceptualize a model for the interaction between these receptors without knowing to what extent the YA mutation alters the behavior of the receptors, or the functional consequences of their interaction. Thus, a series of experiments were conducted using selective antagonists in SCG neurons expressing wild-type mGluR1, mGluR5, or both receptors together.

Effects of selective NAMs on the function of wild-type mGluR1 and mGluR5 when coexpressed.

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

To determine whether coexpression of wild-type mGluR1 and mGluR5 in SCG neurons results in a receptor pool that interacts functionally, responses to a single concentration of glutamate (10 µM) were examined when applied alone or in the presence of saturating concentrations of 1 µM BAY36 or 1 µM MPEP in the same cells (Fig. 4). Control experiments illustrating the effects of these drugs on SCG neurons expressing mGluR1 or mGluR5 alone are shown in Supplemental Figure 2. In 8 cells examined, the average (± SEM) calcium current inhibition observed upon application of 10 µM glutamate was 44±4%. On average, the responses in the presence of BAY36 and MPEP were 17±4% and 21±4%, respectively. The average responses under each condition are shown in Fig. 4A. Consistent with the results described above (Fig. 3), each inhibitor alone produced a relatively strong inhibition of the basal glutamate response which was surprisingly super-additive compared with the responses in the presence of each antagonist, which summed to only about 86% of the control response. This suggests that the two receptors are not acting in a strictly independent manner.

Perhaps more surprising was the relationship between the specific responses in each cell compared with each other and with the paired control responses in each cell. Fig. 4B illustrates calcium current time courses and sample current traces from two sample cells. One example (Fig. 4B, *upper*) shows a cell in which the responses in the presence of each NAM were quite small compared to the control responses such that the sum of the responses was smaller than the control response. Another shows a cell in which the sum of the inhibited responses were larger than the control response (Fig. 4B *lower*). These data are interesting because if mGluR1 and 5 acted in a strictly independent manner, one would expect that the responses in each selective inhibitor be inversely proportional. That is, the larger the contribution of one receptor to the total response in a given cell, the smaller the expected contribution is of the other. In fact, the relationship between responses in the presence of BAY36 and MPEP in these cells was the opposite. Fig. 4C shows the absolute response (% calcium current

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

inhibition) of 10 µM glutamate in the presence of BAY36 plotted vs. the response in the presence of MPEP in the same cell. The data show a clear, statistically significant positive correlation (slope = +0.74±0.2 from a fit to a simple line function- *dotted line*, y=mx+b; r=0.78, $p < 0.05$ from Pearson correlation).

To verify the prediction that responses in the presence of selective inhibitors should be inversely correlated, similar experiments were analyzed in which mGluR1 and mGluR2 were coexpressed in SCG neurons and 10 µM glutamate was applied alone and with a selective mGluR1 inhibitor (either the competitive antagonist *α-Amino-5-carboxy-3-methyl-2-thiophe neacetic acid,* "MATIDA" (Moroni *et al.*, 2002) at 100 µM or 1 µM BAY36) as well as a selective mGluR2 inhibitor (the mGluR2 selective NAM, MNI-137 (Hemstapat *et al.*, 2007) at 3 µM). Since the maximal responses varied more widely in these cells, each response was normalized to that in 10 µM glutamate alone and was plotted as a 'fractional' response in the presence of the mGluR1 inhibitor (as indicated in Fig. 4D) vs. the response in the presence of MNI-137. These data were also fit to a line function and as predicted demonstrated a negative correlation (Fig. 4D, *dotted red line*). The data from Fig. 4C were also recalculated as fractional inhibition in each cell (BAY36 vs. MPEP) to illustrate that the positive correlation persisted (Fig. 4D, *solid line*). Control responses to these drugs in SCG neurons individually expressing each receptor are shown in Supplemental Figure 2. These data combined with those presented above suggest that mGluR1 and 5, when coexpressed in SCG neurons, gives rise to a pool of receptors that appear to function in an interdependent manner. To further elucidate the nature of this interaction, additional experiments are necessary.

Effect of combining selective competitive and non-competitive antagonists.

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

To gain further insight into the functional interaction between mGluR1 and 5 and to determine whether this interaction was similar between wild-type receptors as the interaction we observed between wild-type and YA mutant mGluRs (Figs. 1-3), responses to glutamate in the presence of the selective NAMs BAY36 and MPEP were examined in the presence of the mGluR1 selective competitive antagonist MATIDA. In theory, these data should be somewhat comparable to those in Fig. 3, since MATIDA as a competitive antagonist, should have a similar effect as the YA mutation in that it should shift potency of mGluR1 rightward. Indeed, Supplemental Figure 3 shows that MATIDA does selectively right-shift mGluR1 potency compared to mGluR5. Interestingly, combining MATIDA with either BAY36 or MPEP (Fig. 5A&B) results in a glutamate concentration response curve that appears quite similar to the NAM inhibited curves using the YA mutants (Fig. 3). These data suggest that the mGluR1/mGluR5 receptor complex responds in a qualitatively similar way to the mGluR1 YA/mGluR5 or the mGluR5 YA/mGluR1 complexes. Perhaps more revealing is that the responses were nearly identical when MATIDA was combined with either BAY36, and mGluR1 NAM, or MPEP, an mGluR5 NAM. Clearly, if mGluR5 was acting independently from mGluR1, co-application of BAY36 and MATIDA should not hinder its activity, and much larger responses should be seen at 10 and 100 µM glutamate. Together, these data suggest that the mGluR YA mutants appear to accurately mimic the interactions between wild-type receptors, and provide additional support for the conclusion that mGluR1 and 5 function in a mutually dependent manner.

To gain a better understanding of the mechanism underlying the mGluR1/5 interaction, it may be helpful to examine the pooled effects of various combinations of antagonists against a single concentration of glutamate. Thus, Fig. 6A illustrates average calcium current inhibitory responses in SCG neurons expressing both mGluR1 and 5 to 10 µM glutamate applied alone or with the indicated combinations of the inhibitors BAY36 (1µM), MPEP (1 µM), and MATIDA (100 µM). Effects of these

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

compounds on SCG neurons expressing mGluR1 or mGluR5 separately are shown in Supplemental Figure 2. As shown in Fig. 6A, all of the inhibitors significantly reduced the magnitude of calcium current inhibition by 10 μM glutamate. Combining the competitive antagonist MATIDA with either NAM produced particularly strong reductions in the glutamate response that were statistically indistinguishable. This strong reduction in the response was perhaps expected for the MATIDA+MPEP condition, since both expressed receptors were targeted with saturating concentrations of selective inhibitors (Fig. S2), but the strong reduction in the response in the presence of MATIDA+BAY36 cannot be reconciled with a model in which mGluR1 and mGluR5 act independently since the inhibition of the glutamate response was almost completely occluded and was in fact comparable to the response of 5±0.3% (n=3) observed in the presence of the two NAMs, BAY36 and MPEP. Further, the fact that each inhibitor when applied separately produced a significant but only partial reduction in the response demonstrates that both mGluR1 and mGluR5 are clearly expressed in these neurons. Combining both of the NAMs, BAY36 and MPEP, also strongly inhibited the responses to all receptor combinations as expected. The response to 10 μM glutamate in BAY36 and MPEP in cells expressing mGluR1, mGluR5 and both were 3±4% (n=3), 0±0.4% (n=3), and 5±0.3% (n=3), respectively.

To validate the predictions about the behavior of independently acting receptors in this experiment, analogous experiments were performed in SCG neurons expressing mGluR1 and mGluR2. Here, the mGluR2 selective NAM MNI-137 was used (rather than MPEP) to inhibit that receptor specifically. The efficacy and selectivity of the compounds in cells expressing each receptor separately are shown in Supplemental Figure 2. As expected, the selective inhibitors MATIDA and MNI-137 both significantly but partially reduced the glutamate response (Fig. 6B), confirming the expression of each receptor. By contrast to the results with mGluR1 and 5, combining MATIDA with BAY36 only partially

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

reduced the response, but a very strong reduction was seen when MATIDA was combined with MNI-137, precisely as expected for independently acting receptors. Together, these data support a model in which mGluR1 and mGluR5 function in a mutually dependent fashion when coexpressed in the same neuron, such that the heptahelical (7TM) domains of both receptors must be free of allosteric inhibitors for the receptor complex to produce substantive G protein activation.

Model for mGluR1/5 interaction: receptors partially activate in trans.

The data above strongly suggest that signaling by mGluR1 and mGluR5, when expressed in the same cell, is interdependent but the mechanistic details remain unclear. With these data however, some hypotheses can begin to be formed. Combining a wild type group I mGluR subtype with a YA mutant is advantageous in that this mutation shifts glutamate sensitivity 50-100 fold, which renders glutamate a fairly selective agonist for the wild type subunit. Another consistent effect of this mutation is that it causes the affected receptor to generate much higher efficacy responses, at least when inhibition of calcium currents in SCG neurons are used as the assay for signaling. The mechanistic basis for this increase in efficacy is unclear, but it may provide a fortuitous means to discriminate signaling between the two expressed receptor subtypes. A reexamination of the data in Fig. 1 could suggest a model in which two major effects are apparent: *First*, glutamate binding to the wild type receptor (mGluR5 in Fig. 1) may produce G protein coupling via the YA mutant receptor. This hypothesis arises from the emergence of a high efficacy response at lower glutamate concentrations than is apparent when the YA receptor is expressed alone. This hypothesis suggests that ligand binding in mGluR1 can induce some G protein activation in mGluR5, and *vice versa*, is not solidly supported with those data alone, but will be tested more thoroughly below. The *second* effect is that association with the YA mutant receptor seems to shift the potency of the wild type receptor to the

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

right. This is suggested by the lack of responses below 100 µM when the receptors are coexpressed. The nature of this effect is unclear and may be caused by either a reduced affinity for glutamate in the wild type receptor or the inability to trans-activate at lower gluatmate levels, perhaps until at least some of the associated receptor (the YA mutant) is bound to ligand. Distinguishing between these possibilities may be difficult and is beyond the scope of this study, but this effect is likely not apparent when combining wild type mGluR1 with wild type mGluR5, since both receptors have similar affinities for glutamate, and indeed most agonists (Kammermeier, 2012b), and similar EC_{50}s in this assay (see Supplemental Figure 3).

To begin to test whether activation can occur *in trans* between mGluRs 1 and 5, inhibition of the M-type potassium current (Brown and Adams, 1980) in SCG neurons was used rather than calcium current inhibition as an assay. Inhibition of the M-current occurs via depletion of PIP_2 by phospholipase C upon G_q activation (Suh and Hille, 2002). This pathway is simpler than calcium channel inhibition in SCG neurons, which has both $G_{i/o}$ (via Gβγ) and $G\alpha_q$ components (Kammermeier and Ikeda, 1999). For this experiment, two mutant receptors were used. The first was mGluR5 Y64A, which can activate $G\alpha_q$ and modulate the M-current, but only at glutamate concentrations above the tens of µM range (Fig. 7A, B). The second was a C-terminal deletion mutant of mGluR1 (termed “mGluR1 dCT”), which shows plasma membrane expression and function, and can activate $G\alpha_{i/o}$, but not $G\alpha_q$ (Kammermeier, 2010) and cannot therefore modulate the M-current (Fig. 7A, B). As expected, in SCG neurons expressing mGluR1 dCT, application of glutamate up to 300 µM did not produce any detectable inhibition of the M-current. At 3 and 300 µM glutamate, M-current was inhibited by -3±5% (n=6) and -7±3% (n=10), respectively (Fig. 7A, B; negative inhibition indicates enhancement of the current, shown on the bar graph as 0% inhibition). In neurons expressing only mGluR5 Y64A, M-current inhibition was substantial, but only at the highest glutamate

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

concentration tested. In these cells, inhibition by 3 and 300 µM glutamate was -4±3% (n=9) and 72±5% (n=12), respectively. Thus at 3 µM glutamate, neither receptor was capable of modulating the M-current when expressed alone, although since mGluR1 dCT can induce calcium current inhibition at this concentration (Kammermeier, 2010) it can be assumed that glutamate binds this receptor when present at 3 µM. Finally, when mGluR1 dCT and mGluR5 Y64A were coexpressed in the same cells, inhibition was indeed observed at low glutamate concentrations. In these cells, inhibition by 3 and 300 µM glutamate was 18±7% (n=12) and 54±6% (n=12), respectively. Thus, when two mutant receptors that cannot separately induce M-current inhibition at 3 µM glutamate are coexpressed in SCG neurons, significant inhibition of the current is observed upon application of 3 µM glutamate. These data can be interpreted as supportive of the hypothesis predicting group I mGluR activation *in trans*. At 3 µM glutamate, ligand binds only to mGluR1 dCT, but the observed M-current inhibition is suggestive of G protein activation at the intracellular side of mGluR5 Y64A, the only mGluR present that can cause $G\alpha_q$ activation.

Evidence for an mGluR1-mGluR5 interaction in natively expressed receptors.

The data above provided compelling evidence that mGluR1 and 5 interact in a mutually dependent manner when heterologously expressed in SCG neurons. To determine whether this interaction is physiologically relevant, it was necessary to observe it in neurons that natively express both receptors. To achieve this, medium spiny neurons (MSNs) from the striatum of neonatal rats were isolated and grown in culture for approximately two weeks. These cells were chosen because several studies have reported that they express both mGluR1 and mGluR5 ((Shigemoto *et al.*, 1992; 1993; Pisani *et al.*, 2001; Gubellini *et al.*, 2004). To maximize the throughput of the experiments, intracellular calcium responses were examined in Fura-2-AM loaded cells in response to group I

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

mGluR selective compounds. The experimental protocol was similar to a subset of the experiments described in Fig. 6A (above), but utilizing the group I mGluR agonist DHPG to activate the receptors rather than glutamate to avoid activation of ionotropic glutamate receptors and group II & III mGluRs. Thus, 50 µM DPHG was applied alone (at the start and end of each experiment), and in the presence of BAY36, MATIDA, and BAY36 + MATIDA together (1 µM each). Notably, neurons grown in culture for less than a week failed to respond to DHPG (not shown). This was unsurprising as group I mGluR expression is developmentally regulated, and is not expected to be highly expressed before postnatal day 7 (Jokel *et al.*, 2001). Fortuitously, many striatal neurons isolated from P0-3 rats did respond to DHPG when allowed to grow in culture for 2 weeks (Fig. 8). From these neurons, two distinct populations were evident: those that exhibited a clear reduction in the DHPG response in the combined presence of the two inhibitors, BAY36 and 3-MATIDA (≤70% of the control response, n=11 cells; “Group A”), and those that did not (≥80% of the control response; n=11 cells, “Group B”; Fig. 8A, B). Neurons in Group B appeared to express only mGluR5, since they were insensitive to all combinations of the mGluR1 inhibitors. The magnitude of DHPG responses in these cells were weak compared to those in Group A (Fig. 8A), which may indicate that expression of mGluR5 precedes mGluR1 in MSNs cultured from young rats. Interestingly, DHPG responses in neurons of Group A were significantly, but not strongly, reduced by each inhibitor alone, but were potently reduced by co-application of both (Fig. 8C). The weak effect of each mGluR1 inhibitor alone suggests that in Group A cells, mGluR5 is present and responsible for much of the agonist-induced intracellular calcium response, but the strong inhibition of this effect with combined orthosteric and allosteric mGluR1 inhibitors is difficult to reconcile with any model in which mGluR1 and 5 act independently. Further, these results are consistent with the model in that occlusion of mGluR1 signaling appears to strongly

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

blunt even signaling that's primarily mediated by mGluR5, as judged by the weak inhibition by each antagonist applied separately.

The primary differences between the current results observed in cultured MSNs and those obtained from patch-clamp studies in SCG neurons are that 1) inhibition by MATIDA or BAY36 applied separately was weaker than was observed in SCG neurons, and 2) the response in the presence of both BAY36 and MATIDA appeared slightly larger (as a fraction of the total response) than in SCG neurons. These discrepancies can both be explained if these MSNs express higher levels of mGluR5 than mGluR1 such that a fraction of mGluR5 signaling is coordinated with mGluR1 signaling, as in SCG neurons, but another subset acts independently. Indeed, despite the evidence that MSNs express both mGluR1 and mGluR5 (Shigemoto *et al.*, 1992; 1993; Pisani *et al.*, 2001; Gubellini *et al.*, 2004), some data indicate that the group I mGluR mediated calcium responses in these cells are primarily mediated via mGluR5 (Voulalas, 2005). Note that if mGluR1 and 5 signal entirely independently, no additional reduction in the DHPG response would be expected with these inhibitors applied together vs. each applied alone, since both target mGluR1. In addition, since the doses of each inhibitor applied should be saturating, and in SCG neurons completely block mGluR1 signaling when expressed alone, application of both inhibitors is not expected to produce an additive inhibition. Indeed, in this experiment, the block seen with both drugs was far greater than even an additive effect. Thus, these data strongly suggest that mGluR1 and 5, whether heterologously expressed in peripheral neurons or natively expressed in central neurons, function in a mutually dependent manner, which suggests that efficient signaling of mGluR1 or mGluR5 requires the partner receptor be potentially activatable. Further, they imply that when these receptors are expressed at native, presumably lower, expression levels they still exhibit signs of mutually dependent interaction that was described in more detail using heterologously expressed receptors as described above.

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

Discussion.

The data presented here demonstrate a novel functional interdependence between mGluR1 and mGluR5. Using mutagenesis combined with selective pharmacology, it was demonstrated that combining mGluR1 and mGluR5 gives rise to a receptor with a unique pharmacological profile that cannot be adequately explained by the two receptors acting independently, nor by a heterodimerization model as assessed by functional and pharmacological assays. The data in Figs. 4&6 is the strongest evidence that wild type mGluR1 and 5 can signal in a mutually dependent way. These data show that when the receptors are co-expressed, the observed reductions in glutamate signaling by the mGluR1-targeted NAM BAY36 and the mGluR5 targeted NAM MPEP are positively correlated, by contrast to expectations for independently acting receptors, such as that observed using selective antagonists in cells expressing mGluR1 and mGluR2. In addition, co-application of the mGluR1-selective competitive antagonist MATIDA with each selective NAM resulted in a similarly strong inhibition of the glutamate signal. If these receptors function independently, the inhibitors BAY36 and MATIDA should not produce additive reductions in the glutamate response (since both are saturating concentrations targeting the same receptor), and a clear difference in the response should be evident in the presence of BAY36+MATIDA vs. MPEP+MATIDA. However, neither of these predictions were borne out. The fact that each antagonist partially, but not completely, inhibited glutamate signaling provides a positive control for expression of *both* receptors. Together, these data provide strong evidence that mGluR1 and 5 can signal through an interdependent pathway to at least some degree when co-expressed in the same neuron. Finally, the data from cultured striatal MSNs (Fig. 8) provides compelling evidence that the described mGluR1/5 functional interaction is physiologically relevant when these receptors are co-expressed natively in a central neuron.

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

Due to the nature of the interaction and the lack of highly selective orthosteric agonists (Kammermeier, 2012b), it is not difficult to see why this functional interdependence has gone unnoticed. First, because the receptors share a high degree of homology, when they are natively expressed each has a similar efficacy and potency (to glutamate and DHPG) for coupling to various effectors. Also, because of the nature of the interaction, a very specific combination of inhibitors was necessary to detect it and most studies using these inhibitors were aimed at identifying which group I mGluR subtypes are involved in various functions rather than at deciphering an interaction between receptors. However, there are some indications from the literature suggesting the validity of our model. For example, Volk et al. (Volk *et al.*, 2006) showed that while mGluR-mediated long term depression of hippocampal excitatory post-synaptic potentials (mGluR-LTD) was prevented with co-application of the mGluR5 allosteric inhibitor MPEP and the mGluR1 orthosteric inhibitor LY367385 (similar to the data presented here in Fig. 4A), the transient short term depression persisted. Similar results were reported by Hou and Klann (2004) (Hou and Klann, 2004). These responses may be akin to the small effects we see in the presence of MATIDA and MPEP, which together strongly reduce mGluR1/5 signaling, but less strongly than when each receptor is expressed separately (see Figs. 6 and S2). However, it should be noted that there are examples of neurons that appear to co-express mGluR1 and 5 but exhibit separable function. Examples include GABAergic neurons in the substantia nigra (Marino *et al.*, 2001) and CA1 pyramidal neurons (Mannaioni *et al.*, 2001). These discrepancies can be explained in a few ways. While Marino showed that mGluR1 and 5 are both expressed postsynaptically in substantia nigral neurons, they did so with electron microscopy. It is possible that each receptor was mutually expressed in different subset of these neurons. However, it is also possible that some neurons actively segregate these receptors by restricting their subcellular localization to separate compartments such that coupling to certain effectors is achieved primarily by

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

one receptor. This could be done with specific association with scaffolds like Homer proteins (Brakeman *et al.*, 1997), which interact with all known mGluR5 splice variants, but not with mGluR1b or mGluR1c (Tu *et al.*, 1998).

While we have shown a functional interaction between mGluR1 and mGluR5 signaling, and recent studies have shown that these receptors may interact physically (Doumazane *et al.*, 2011), the precise nature of this interaction cannot be determined at this time. Doumazane et al., (2011) used a FRET approach to examine proximity between several mGluRs and their data suggested that the group I mGluRs can likely physically interact, as can all of the group II and III mGluRs tested in their assay. However, the stoichiometry of only one pair, mGluR2 and 4, were examined in more detail. These were determined to form heterodimers (Doumazane *et al.*, 2011). Our more recent study (Kammermeier, 2012a) has confirmed that the mGluR2/4 interaction is consistent with a heterodimer interaction. Further, Yin et al. (Yin *et al.*, 2014) have recently identified native mGluR2/4 heterodimers in prefrontal cortical neurons. When coexpressed in SCG neurons (Kammermeier, 2012a), mGluR2 and 4 formed a pharmacologically unique receptor that could not be strongly activated by specific orthosteric agonists, but was fully activated by glutamate and by combining mGluR2 and mGluR4-targeted selective agonists (DCG-IV and L-AP4, respectively). This was consistent with a heterodimer model, because mGluR dimers can only be substantially activated by ligand binding in both subunits. Further, mGluR2/4 appeared to be insensitive to inhibition by the mGluR2 selective NAM Ro64-5229, consistent with previous findings showing that mGluR dimers cannot be inhibited by a NAM that can bind only one subunit in the dimer (Hlavackova *et al.*, 2005). It should be stressed that the mGluR1/5 interaction described here appears *inconsistent* with the expected dogmatic mGluR dimer interaction for two reasons. *First*, in the SCG system, mGluR1/5 heterodimers are expected to be inactive unless ligand can activate both subunits. It is apparent that this is not the case in the mutant/wild type

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

experiments described in Figs. 1 & 2 as well as the MATIDA experiments in Fig. 6. In Figs. 1&2, the heterodimer model would predict that the concentration-response curve would closely resemble the lower potency receptor when both receptors were co-expressed (the mutant receptors in Figs. 1 & 2). In Fig. 6, MATIDA would be expected to produce very strong block of a putative mGluR1/5 heterodimer, since it should prevent ligand binding at one dimer subunit. *Second*, at least two studies have demonstrated that mGluR dimers are not inhibited by NAMs when the compound can interact with only one subunit (Hlavackova *et al.*, 2005; Kammermeier, 2012a). Hlavackova et al. showed that chimeric mGluR1 dimers engineered to contain only one MPEP binding site per dimer were uninhibited by MPEP (Hlavackova *et al.*, 2005), and we showed that mGluR2/4 heterodimers expressed in SCG neurons were unaffected by the mGluR2 selective NAM Ro64-5229 (Kammermeier, 2012a).

It should also be noted that in one recent paper (Goudet *et al.*, 2005), heterodimers of mGluR1 and 5 were observable. However, that study examined chimeric mGluRs in which mGluR1 and mGluR5 were fused to the C-termini of $GABA_B$ receptors. Those receptors were specifically engineered to form heterodimers so that the authors could control receptor subunit composition, and the experiments were as such not designed to determine whether mGluR1 and 5 heterodimerize. Indeed, our data reveal with mGluR1a and mGluR1b, which differ only in their C-terminal tails, reveal that the identity of the C-terminus of group I mGluRs may be an important determinant of dimerization. While mGluR1a (wild-type) appears to form dimers with mGluR1a Y74A in our assay (Kammermeier and Yun, 2005), mGluR1b does not. This conclusion is supported by a recent biochemical study demonstrating that mGluR1a and 1b cannot efficiently dimerize (Remelli *et al.*, 2008).

Thus, the data presented in the current study suggest that mGluR1 and 5 interact functionally, but likely do not at least *predominantly* form heterodimers. In this model, we postulate that mGluR1

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

homodimers interact with mGluR5 homodimers when coexpressed in the same cells, such that efficient G protein coupling requires that the heptahelical domains of both receptors be uninhibited by NAMs to function with full efficacy. At this time however, our data can only rule out that the receptors are not *primarily* heterodimers. It remains unclear whether some portion of the mGluR1/5 interaction consists of *bona fide* heterodimers. Thus, additional work will be necessary to further elucidate the stoichiometry of these receptors. At this time however, the best interpretation of our data is that mGluR1 homodimers exhibit functional cooperation with mGluR5 homodimers.

Acknowledgments.

We thank D.I. Yule (University of Rochester) for assistance and guidance with the calcium imaging experiments (Fig. 8), and A. V. Smrcka and G. G. Tall (University of Rochester) for helpful discussions.

Authorship Contributions

Participated in research design: Kammermeier and Sevastyanova

Conducted experiments: Kammermeier and Sevastyanova

Contributed new reagents or analytic tools: Kammermeier

Performed data analysis: Kammermeier and Sevastyanova

Wrote or contributed to writing the manuscript: Kammermeier

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

References

Bean BP (1989) Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature* **340**:153–156.

Beqollari D, and Kammermeier PJ (2010) Venus fly trap domain of mGluR1 functions as a dominant negative against group I mGluR signaling. *J Neurophysiol* **104**:439–448.

Brakeman PR, Lanahan AA, O'Brien R, Roche K, Barnes CA, Huganir RL, and Worley PF (1997) Homer: a protein that selectively binds metabotropic glutamate receptors. *Nature* **386**:284–288.

Brown DA, and Adams PR (1980) Muscarinic suppression of a novel voltage-sensitive K+ current in a vertebrate neurone. *Nature* **283**:673–676.

Carroll FY, Stolle A, Beart PM, Voerste A, Brabet I, Mauler F, Joly C, Antonicek H, Bockaert J, Müller T, Pin JP, and Prézeau L (2001) BAY36-7620: a potent non-competitive mGlu1 receptor antagonist with inverse agonist activity. *Mol Pharmacol* **59**:965–973.

Doumazane E, Scholler P, Zwier JM, Eric T, Rondard P, and Pin J-P (2011) A new approach to analyze cell surface protein complexes reveals specific heterodimeric metabotropic glutamate receptors. *The FASEB Journal* **25**:66–77.

Gasparini F, Lingenhöhl K, Stoehr N, Flor PJ, Heinrich M, Vranesic I, Biollaz M, Allgeier H, Heckendorn R, Urwyler S, Varney MA, Johnson EC, Hess SD, Rao SP, Sacaan AI, Santori EM, Velicelebi G, and Kuhn R (1999) 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu5 receptor antagonist. *Neuropharmacology* **38**:1493–1503.

Goudet C, Kniazeff J, Hlavackova V, Malhaire F, Maurel D, Acher F, Blahos J, Prézeau L, and Pin J-P (2005) Asymmetric functioning of dimeric metabotropic glutamate receptors disclosed by positive allosteric modulators. *J Biol Chem* **280**:24380–24385.

Gubellini P, Pisani A, Centonze D, Bernardi G, and Calabresi P (2004) Metabotropic glutamate receptors and striatal synaptic plasticity: implications for neurological diseases. *Prog Neurobiol* **74**:271–300.

Hemstapat K, Da Costa H, Nong Y, Brady AE, Luo Q, Niswender CM, Tamagnan GD, and Conn PJ (2007) A Novel Family of Potent Negative Allosteric Modulators of Group II Metabotropic Glutamate Receptors. *Journal of Pharmacology and Experimental Therapeutics* **322**:254–264.

Hlavackova V, Goudet C, Kniazeff J, Zikova A, Maurel D, Vol C, Trojanova J, Prézeau L, Pin J-P, and Blahos J (2005) Evidence for a single heptahelical domain being turned on upon activation of a dimeric GPCR. *EMBO J* **24**:499–509.

Hou L, and Klann E (2004) Activation of the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway is required for metabotropic glutamate receptor-dependent long-term depression. *Journal of Neuroscience* **24**:6352–6361.

Ikeda SR (1997) Heterologous expression of receptors and signaling proteins in adult mammalian

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.

References

sympathetic neurons by microinjection. *Methods Mol Biol* **83**:191–202.

Jokel ES, Garduno ER, Ariano MA, and Levine MS (2001) Metabotropic Glutamate Receptors mGluR1α and mGluR2/3 Display Dynamic Expression Patterns in Developing Rat Striatum. *Dev Neurosci* **23**:1–6.

Kammermeier PJ (2010) C-terminal deletion of metabotropic glutamate receptor 1 selectively abolishes coupling to Galphaq. *European Journal of Pharmacology* **627**:63–68.

Kammermeier PJ (2012a) Functional and pharmacological characteristics of metabotropic glutamate receptors 2/4 heterodimers. *Mol Pharmacol* **82**:438–447.

Kammermeier PJ (2012b) The orthosteric agonist 2-chloro-5- hydroxyphenylglycine activates mGluR5 and mGluR1 with similar efficacy and potency. *BMC Pharmacol* **12**:6.

Kammermeier PJ, and Ikeda SR (1999) Expression of RGS2 alters the coupling of metabotropic glutamate receptor 1a to M-type K+ and N-type Ca2+ channels. *Neuron* **22**:819–829.

Kammermeier PJ, and Worley PF (2007) Homer 1a uncouples metabotropic glutamate receptor 5 from postsynaptic effectors. *Proc Natl Acad Sci USA* **104**:6055–6060.

Kammermeier PJ, and Yun J (2005) Activation of metabotropic glutamate receptor 1 dimers requires glutamate binding in both subunits. *J Pharmacol Exp Ther* **312**:502–508.

Kunishima N, Shimada Y, Tsuji Y, Sato T, Yamamoto M, Kumasaka T, Nakanishi S, Jingami H, and Morikawa K (2000) Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. *Nature* **407**:971–977.

Lu VB, Williams DJ, Won Y-J, and Ikeda SR (2009) Intranuclear microinjection of DNA into dissociated adult mammalian neurons. *Journal of Visualized Experiments : JoVE*, doi: 10.3791/1614.

Mannaioni G, Marino MJ, Valenti O, Traynelis SF, and Conn PJ (2001) Metabotropic glutamate receptors 1 and 5 differentially regulate CA1 pyramidal cell function. *Journal of Neuroscience* **21**:5925–5934.

Marino MJ, Wittmann M, Bradley SR, Hubert GW, Smith Y, and Conn PJ (2001) Activation of group I metabotropic glutamate receptors produces a direct excitation and disinhibition of GABAergic projection neurons in the substantia nigra pars reticulata. *Journal of Neuroscience* **21**:7001–7012.

Moroni F, Attucci S, Cozzi A, Meli E, Picca R, Scheideler MA, Pellicciari R, Noe C, Sarichelou I, and Pellegrini-Giampietro DE (2002) The novel and systemically active metabotropic glutamate 1 (mGlu1) receptor antagonist 3-MATIDA reduces post-ischemic neuronal death. *Neuropharmacology* **42**:741–751.

Pisani A, Gubellini P, Bonsi P, Conquet F, Picconi B, Centonze D, Bernardi G, and Calabresi P (2001) Metabotropic glutamate receptor 5 mediates the potentiation of N-methyl-D-aspartate responses in medium spiny striatal neurons. *Neuroscience* **106**:579–587.

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

References

Ray K, and Hauschild BC (2000) Cys-140 is critical for metabotropic glutamate receptor-1 dimerization. *J Biol Chem* **275**:34245–34251.

Remelli R, Robbins MJ, and McIlhinney RAJ (2008) The C-terminus of the metabotropic glutamate receptor 1b regulates dimerization of the receptor. *J Neurochem* **104**:1020–1031.

Romano C, Miller JK, Hyrc K, Dikranian S, Mennerick S, Takeuchi Y, Goldberg MP, and O'malley KL (2001) Covalent and noncovalent interactions mediate metabotropic glutamate receptor mGlu5 dimerization. *Mol Pharmacol* **59**:46–53.

Romano C, Yang WL, and O'malley KL (1996) Metabotropic glutamate receptor 5 is a disulfide-linked dimer. *J Biol Chem* **271**:28612–28616.

Schoepp DD (2001) Unveiling the functions of presynaptic metabotropic glutamate receptors in the central nervous system. *J Pharmacol Exp Ther* **299**:12–20.

Shigemoto R, Nakanishi S, and Mizuno N (1992) Distribution of the mRNA for a metabotropic glutamate receptor (mGluR1) in the central nervous system: An in situ hybridization study in adult and developing rat. *J Comp Neurol* **322**:121–135.

Shigemoto R, Nomura S, Ohishi H, Sugihara H, Nakanishi S, and Mizuno N (1993) Immunohistochemical localization of a metabotropic glutamate receptor, mGluR5, in the rat brain. *Neuroscience Letters* **163**:53–57.

Suh B-C, and Hille B (2002) Recovery from muscarinic modulation of M current channels requires phosphatidylinositol 4,5-bisphosphate synthesis. *Neuron* **35**:507–520.

Tsuji Y (2000) Cryptic dimer interface and domain organization of the extracellular region of metabotropic glutamate receptor subtype 1. *Journal of Biological Chemistry* 8.

Tu JC, Xiao B, Yuan JP, Lanahan AA, Leoffert K, Li M, Linden DJ, and Worley PF (1998) Homer binds a novel proline-rich motif and links group 1 metabotropic glutamate receptors with IP3 receptors. *Neuron* **21**:717–726.

Volk LJ, Daly CA, and Huber KM (2006) Differential roles for group 1 mGluR subtypes in induction and expression of chemically induced hippocampal long-term depression. *J Neurophysiol* **95**:2427–2438.

Voulalas PJ (2005) Metabotropic Glutamate Receptors and Dopamine Receptors Cooperate to Enhance Extracellular Signal-Regulated Kinase Phosphorylation in Striatal Neurons. *Journal of Neuroscience* **25**:3763–3773.

Yin S, Noetzel MJ, Johnson KA, Zamorano R, Jalan-Sakrikar N, Gregory KJ, Conn PJ, and Niswender CM (2014) Selective actions of novel allosteric modulators reveal functional heteromers of metabotropic glutamate receptors in the CNS. *Journal of Neuroscience* **34**:79–94.

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes.

Work described in this manuscript was supported by National Institutes of Health, National Institute of General Medical Sciences [R01 GM101023].

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

Legends for Figures

Figure 1. Functional interaction between mGluR5 and mGluR1 Y74A. A, Calcium current amplitude time courses (*left*) and sample representative current traces (*right*) illustrating the effect of various concentrations of Glu (as indicated) in SCG neurons expressing mGluR5b (*upper*), mGluR1 Y74A (*center*), and both receptors (*lower*). The voltage protocol, a 25 msec step to +10 mV- near the peak current- from a holding potential of -80 mV, for the current traces is shown in A (*upper right*). B&C, Average (±SEM) responses at a range of [Glu]s from SCG neurons expressing mGluR5 alone (circles), mGluR1 Y74A alone (squares), or both receptors together (diamonds). B shows a set of experiments using the mGluR5b splice variant, and C shows experiments using the mGluR5a splice variant. Numbers of cells for each point are give in the text. Solid and hatched lines show the results of fits to the Hill equation.

Figure 2. The interaction between mGluR1 and mGluR5 is reciprocal, and mGluR1 does not interact functionally with mGluR2. A, Average (±SEM) Glu dose response relationship of calcium current inhibition in SCG neurons in cells expressing mGluR1 alone (filled squares), mGluR5b Y64A alone (filled circles), or both receptors together (open squares). Fits to the Hill equation are shown as solid or hatched lines, yielding EC_{50} values of 1 µM, 56 µM, and 16 µM for mGluR1, mGluR5 Y64A, and both receptors together, respectively. B, Average (±SEM) Glu dose response relationship of calcium current inhibition in SCG neurons in cells expressing mGluR1aY74A alone (filled squares), mGluR1b Y64A alone (filled diamonds), or both receptors together (open diamonds). Fits to the Hill equation are shown as solid or hatched lines, yielding EC_{50} values of 351 µM, 2 µM, and 118 µM for mGluR1aYA, mGluR1b, and both receptors together, respectively (assuming maximal responses of

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

60% for mGluR1aYA and 66% for both receptors together). C, Average (±SEM) Glu dose response relationship of calcium current inhibition in SCG neurons in cells expressing mGluR1 Y64A alone (filled squares), mGluR2 alone alone (filled circles), or both receptors together (open squares). Fits to the Hill equation are shown as solid lines, yielding EC_{50} values of 5 µM, 980 µM for mGluR2, mGluR1 Y74A, respectively. Due to the complex shape, the data from cells with both receptors coexpressed was not fit. D, Schematic illustrating pharmacologically relevant domains of a generic mGluR, including the large extracellular region containing the orthosteric binding site shown as a large rectangle, and the heptahelical domain containing the binding sites for allosteric compounds shown as a smaller rectangle. This type of cartoon will be used in subsequent figures.

Figure 3. Selective NAMs strongly inhibit signaling of combined mGluR YA/mGluR WT signaling. A, Neurons coexpressing mGluR5 Y64A and mGluR1 are strongly inhibited by 1µM MPEP. Average (±SEM) calcium current inhibition produced by indicated [glutamate] in cells coexpressing mGluR5YA with mGluR1 in the absence (filled triangles; n=5) and presence (open triangles; n=4) of 1 µM MPEP. B, Average (±SEM) calcium current inhibition produced by indicated [glutamate] in cells coexpressing mGluR5 with mGluR1YA in the absence (filled squares; n=5) and presence (open squares; n=6) of 1 µM BAY36. C, Average (±SEM) calcium current inhibition produced by indicated [glutamate] in cells coexpressing mGluR5YA with mGluR1 in the absence (filled diamonds; n=12 at 1 mM, n=7 at 100 µM) and presence (open diamonds; n=12 at 10 µM, n=5 at all other [Glu]s) of 1 µM BAY36. D, Average (±SEM) calcium current inhibition produced by indicated [glutamate] in cells coexpressing mGluR5 with mGluR1YA in the absence (filled diamonds; n=6) and presence (open diamonds; n=5 at 10 µM, n=6 at all other [Glu]s) of 1 µM MPEP.

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

Figure 4. Inhibition of the glutamate response by mGluR1 and mGluR5 selective NAMs in SCG neurons expressing mGluR1 and mGluR5. A, Average (±SEM) calcium current inhibition by 10 µM glutamate in the absence of inhibitors ("Glu") and in the presence of either 1 µM BAY36 ("+BAY36") or 1 µM MPEP ("+MPEP"). Asterisk indicates that the control response is statistically distinguishable from the responses in either inhibitor ($p<0.05$, ANOVA), NS indicates that the responses in BAY36 and MPEP are not significantly different. B, Calcium current amplitude time courses for sample cells showing strong inhibition by both BAY36 and MPEP (B *upper*) and weak inhibition by both inhibitors (B *lower*). Graphs indicate the time and duration of each drug application. The order of application of the inhibitors (BAY36 and MPEP) was alternated from cell to cell to avoid systematic errors, although responses did not appear to consistently desensitize. Insets show sample control current traces (larger black traces), currents inhibited by 10 µM glutamate alone (smaller black traces), and glutamate inhibited currents in the presence of BAY36 (blue) or MPEP (red). Scale bars represent 0.1 nA and 5 msec (B *upper*) and 0.5 nA and 5 msec (B *lower*). C, Correlation between the magnitude of glutamate responses in the presence of BAY36 and in MPEP in SCG neurons coexpressing mGluR1 and 5, as in A and B. Data were fit to a line function ($y=mx+b$), with a slope +0.74 and a r value of 0.78 ($n=8$), yielding a statistically significant positive correlation ($p<0.05$, Pearson correlation). D, Positive correlation of the fractional inhibition (inhibition in each drug divided by that of 10 µM glutamate alone in the same cell) in the presence of BAY36 vs. that in MPEP in mGluR1/5 expressing cells (black circles, dotted line, from the same data illustrated in C) and in the presence of BAY36 vs. that in MNI-137 (blue open squares) or MATIDA vs. that in MNI-137 (red squares) in cells expressing mGluR1 and 2. Fit to a line of these combined data illustrating the expected negative correlation is shown as a red dotted line.

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5. Effect of combined inhibition by MATIDA and selective NAMs on the partial glutamate concentration response of mGluR1/5. A, Effect of glutamate (1-100 µM) applied alone (black circles) in SCG neurons expressing mGluR1 and mGluR5, and in the presence of 100 µM MATIDA and 1 µM BAY36 (open circles). All drug applications were paired in the same cells (n=6 for all data points). B, Effect of glutamate (10-100 µM) applied alone (black diamonds) in SCG neurons expressing mGluR1 and mGluR5, and 1-100 µM glutamate in the presence of 100 µM MATIDA and 1 µM BAY36 (open diamonds). All drug applications were paired in the same cells (n=3 for all data points).

Figure 6. Average effects of selective inhibitors on coexpressed mGluRs vs. 10 µM glutamate. A, Average (±SEM) response in SCG neurons expressing mGluR1 and mGluR5 to 10 µM glutamate applied alone or with the indicated inhibitors. Number of cells in each group shown in parentheses. Drug concentrations were: 100 µM MATIDA, 1 µM BAY36, 1µM MPEP. Asterisk denotes statistical difference from the control group ($p<0.05$, ANOVA). B, Average (±SEM) response in SCG neurons expressing mGluR1 and mGluR2 to 10 µM glutamate applied alone or with the indicated inhibitors. Number of cells in each group shown in parentheses. Drug concentrations were: 100 µM MATIDA, 1 µM BAY36, 3µM MNI-137. Asterisk denotes statistical difference from the control group ($p<0.05$, ANOVA).

Figure 7. Activation of mGluR5 Y64A *in trans* by mGluR1 dCT at 3 µM glutamate. A, Sample representative traces illustrating M-current from SCG neurons expressing mGluR1 dCT alone (*upper*), mGluR5 Y64A alone (*center*), and both receptors together (*lower*). Each panel shows control, uninhibited currents (*black*) superimposed with current in the presence (*gray*) of 3 µM (*left*) or 300 µM (*right*) Glu, as indicated. B *upper*, Cartoon illustrating receptor coexpression conditions. B

Molecular Pharmacology Fast Forward. Published on August 11, 2014 as DOI: 10.1124/mol.114.093468
This article has not been copyedited and formatted. The final version may differ from this version.
MOL #93468

lower, Average (±SEM) glutamate induced M-current inhibition in SCG neurons expressing the indicated receptors. Number of cells in each group shown in parentheses. Asterisks indicate $p < 0.05$ (ANOVA) vs. mGluR1 dCT only and mGluR5 Y64A only controls for the same [glutamate].

Figure 8. Mutually dependent signaling in striatal MSNs natively expressing both mGluR1 and mGluR5. A, Sample time courses of Fura2 ratiometric fluorescence changes, indicative of cytoplasmic calcium changes, in an MSN that appeared to express both mGluR1 and mGluR5 (*upper*) and an MSN that appeared to express only mGluR5 (*lower*). 50 µM DHPG was used as the agonist alone or in the presence of BAY36 (1 µM), MATIDA (100 µM), or both, as indicated. Note that the control response to DHPG alone did decrease somewhat during the course of the experiment, as indicated by the response to DHPG at the end of each experiment. B, Distribution of MSN responses to MPEP (*upper*, degree of inhibition of the DHPG response), and magnitude of the initial DHPG response (*lower*) in the MSNs that responded to DHPG. Responses were taken as the peak amplitude minus baseline (absolute fluorescence ratio values). C, Average calcium responses (±SEM) expressed as a percentage of the DHPG responses in each cell (corrected for decay of the control response) in the two observed subtypes of MSNs, as indicated.

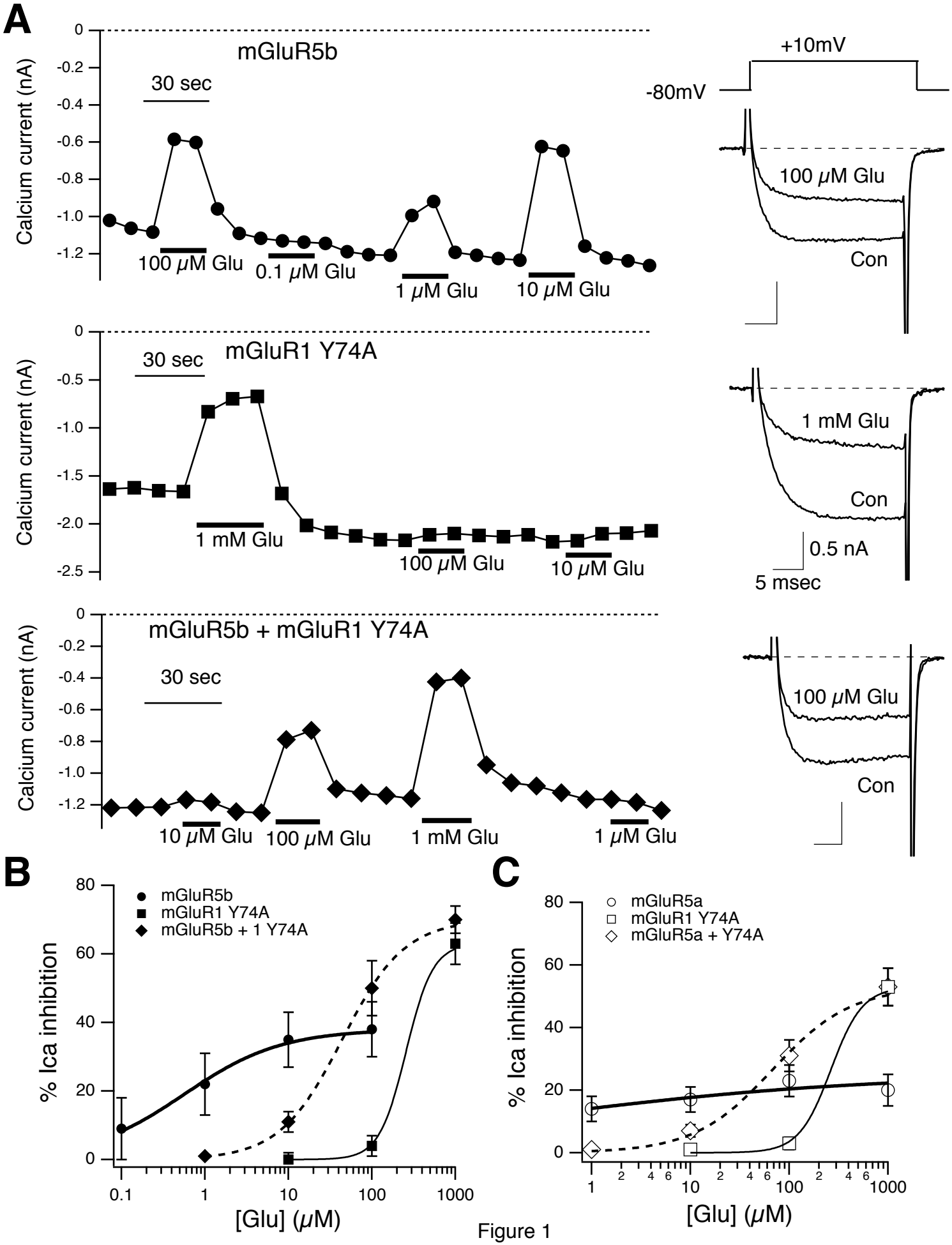
A
mGluR5b
30 sec
Calcium current (nA)
100 μM Glu
0.1 μM Glu
1 μM Glu
10 μM Glu
+10mV
-80mV
100 μM Glu
Con
mGluR1 Y74A
1 mM Glu
100 μM Glu
10 μM Glu
0.5 nA
5 msec
mGluR5b + mGluR1 Y74A
10 μM Glu
100 μM Glu
1 mM Glu
1 μM Glu
B
mGluR5b
mGluR1 Y74A
mGluR5b + 1 Y74A
% Ica inhibition
[Glu] (μM)
C
mGluR5a
mGluR1 Y74A
mGluR5a + Y74A
% Ica inhibition
[Glu] (μM)

Figure 1

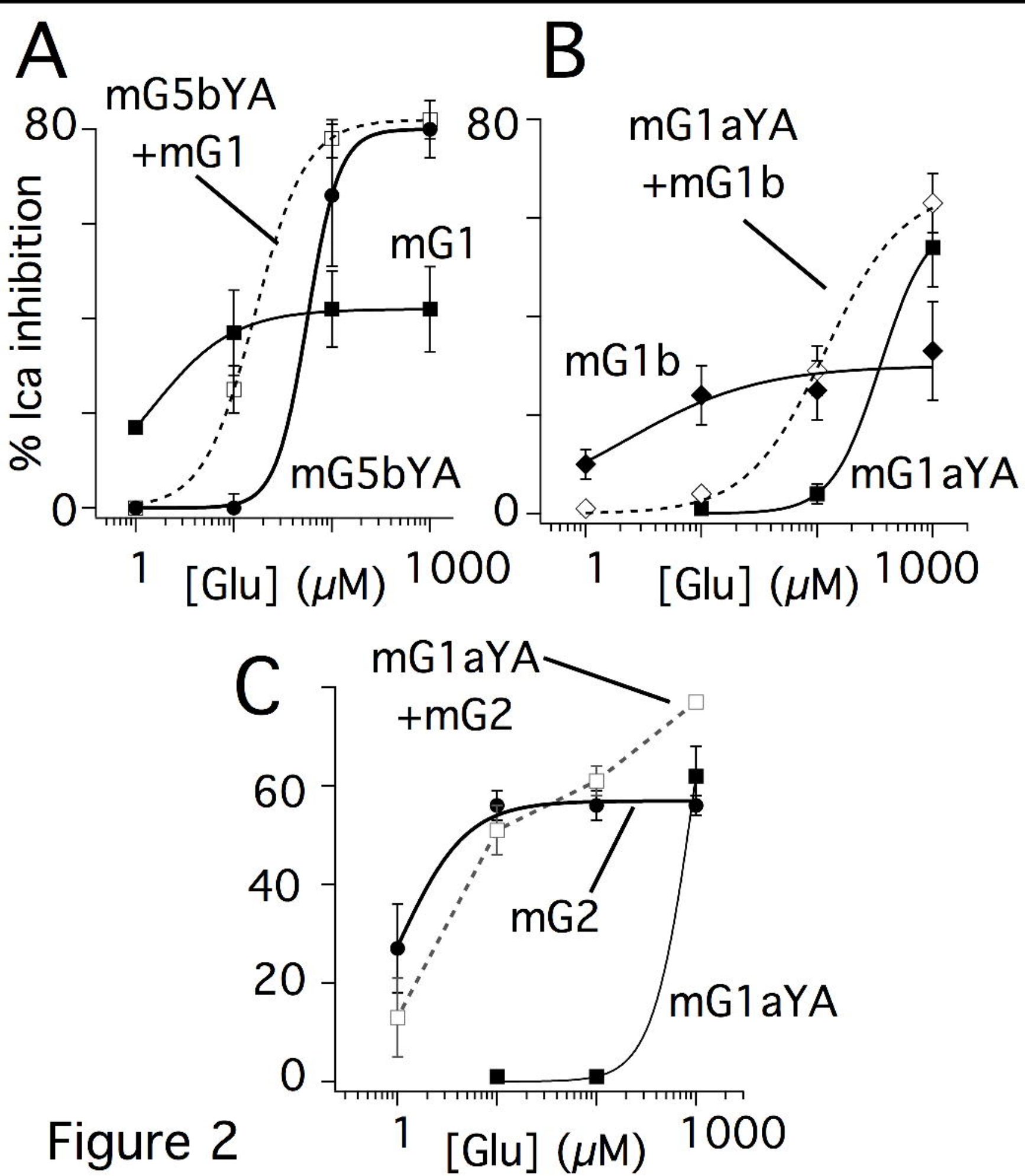
A
mG5bYA
+mG1
mG1
mG5bYA
80
0
% Ica inhibition
1
[Glu] (μM)
1000
B
mG1aYA
+mG1b
mG1b
mG1aYA
80
0
1
[Glu] (μM)
1000
C
mG1aYA
+mG2
mG2
mG1aYA
60
40
20
0
1
[Glu] (μM)
1000

Figure 2

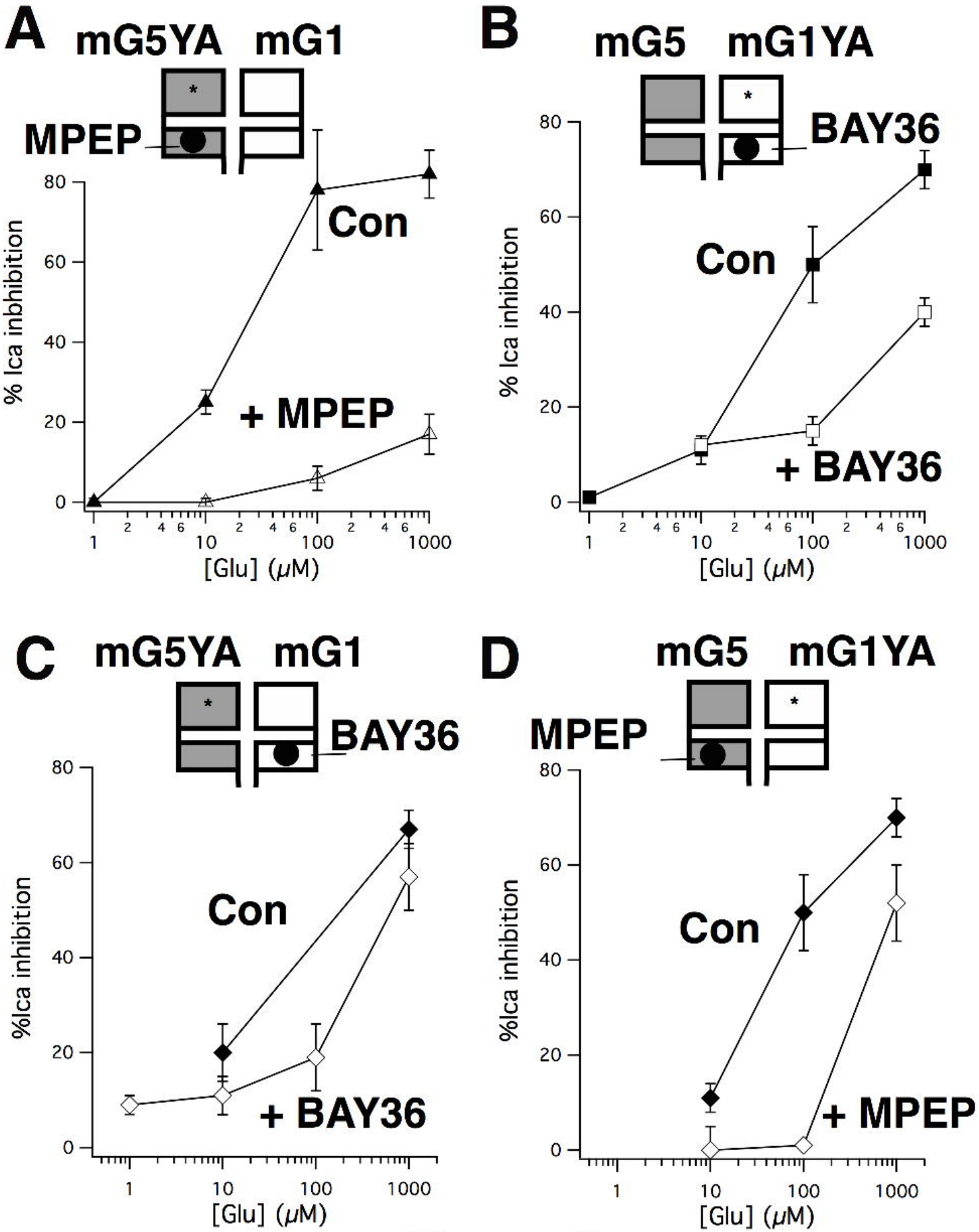
A
mG5YA mG1
MPEP
Con
+ MPEP
% Ica inbhibition
[Glu] (μM)
B
mG5 mG1YA
BAY36
Con
+ BAY36
% Ica inhibition
[Glu] (μM)
C
mG5YA mG1
BAY36
Con
+ BAY36
%Ica inhibition
[Glu] (μM)
D
mG5 mG1YA
MPEP
Con
+ MPEP
%Ica inhibition
[Glu] (μM)

Figure 3

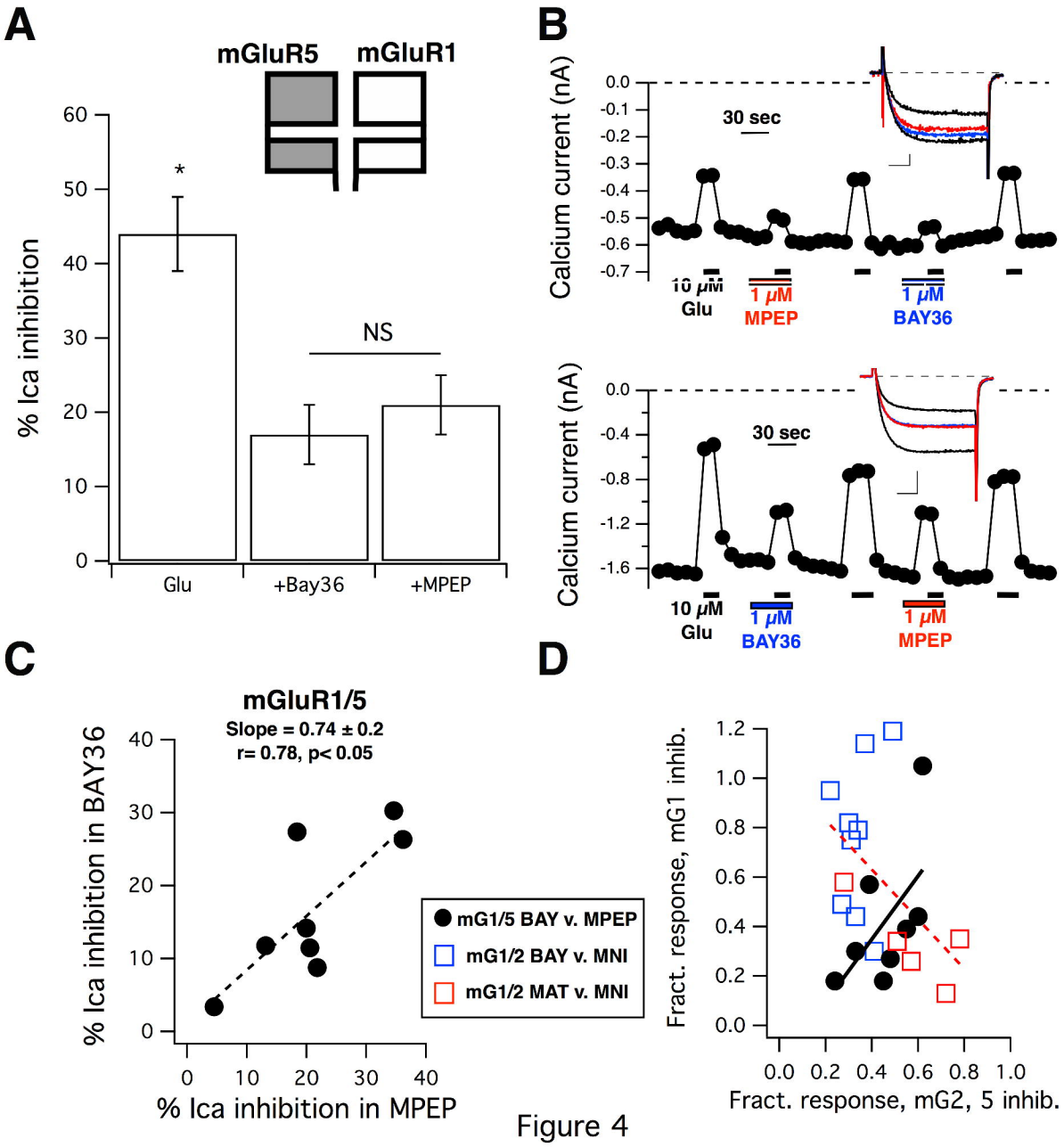
A
mGluR5
mGluR1
% Ica inhibition
60
50
40
30
20
10
0
*
NS
Glu
+Bay36
+MPEP
B
Calcium current (nA)
0.0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.6
-0.7
30 sec
10 μM Glu
1 μM MPEP
1 μM BAY36
Calcium current (nA)
0.0
-0.4
-0.8
-1.2
-1.6
30 sec
10 μM Glu
1 μM BAY36
1 μM MPEP
C
mGluR1/5
Slope = 0.74 ± 0.2
r= 0.78, p< 0.05
% Ica inhibition in BAY36
40
30
20
10
0
0
10
20
30
40
% Ica inhibition in MPEP
mG1/5 BAY v. MPEP
mG1/2 BAY v. MNI
mG1/2 MAT v. MNI
D
Fract. response, mG1 inhib.
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
1.0
Fract. response, mG2, 5 inhib.

Figure 4

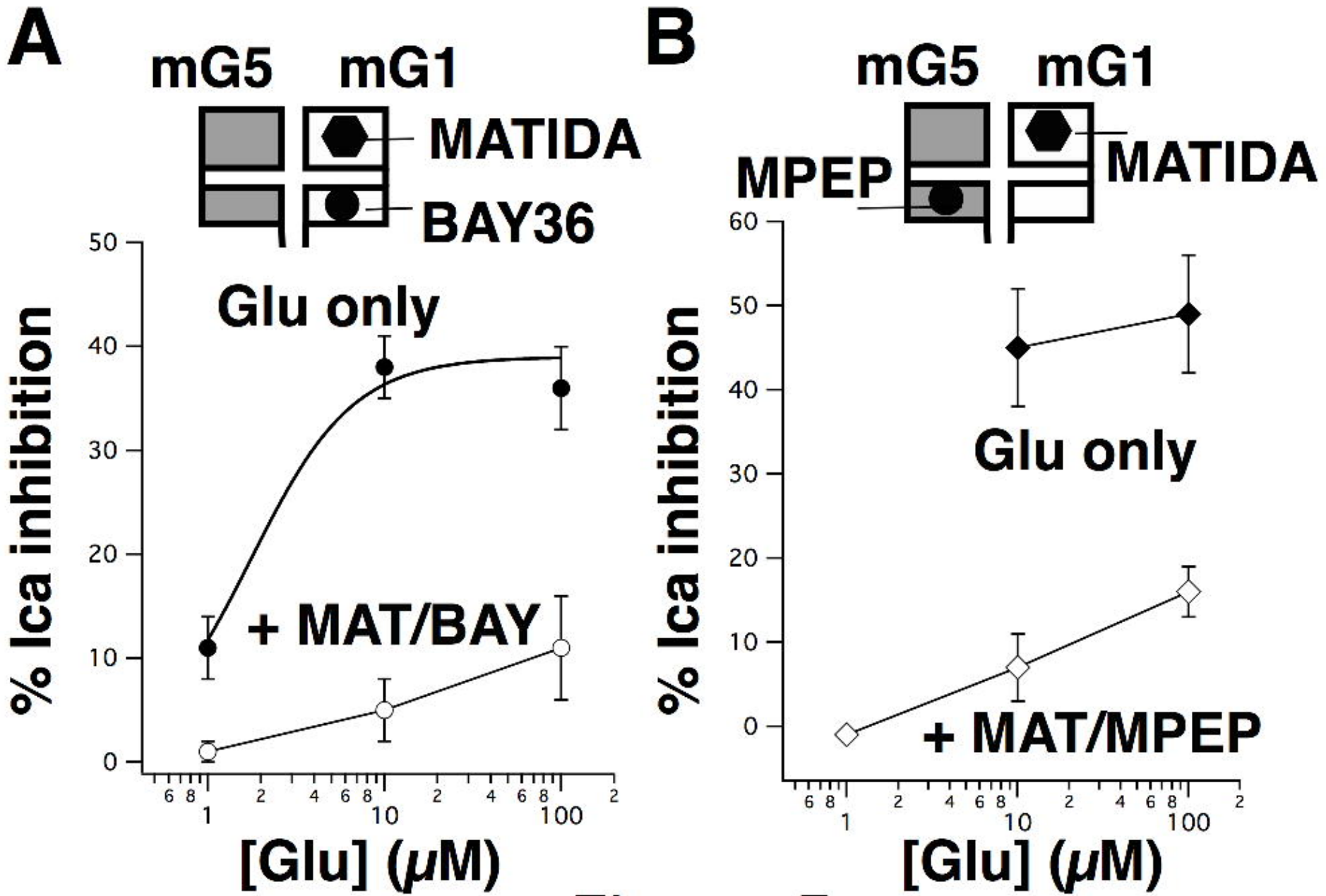
A
mG5
mG1
MATIDA
BAY36
Glu only
+ MAT/BAY
% Ica inhibition
[Glu] (μM)
B
mG5
mG1
MPEP
MATIDA
Glu only
+ MAT/MPEP
% Ica inhibition
[Glu] (μM)

Figure 5

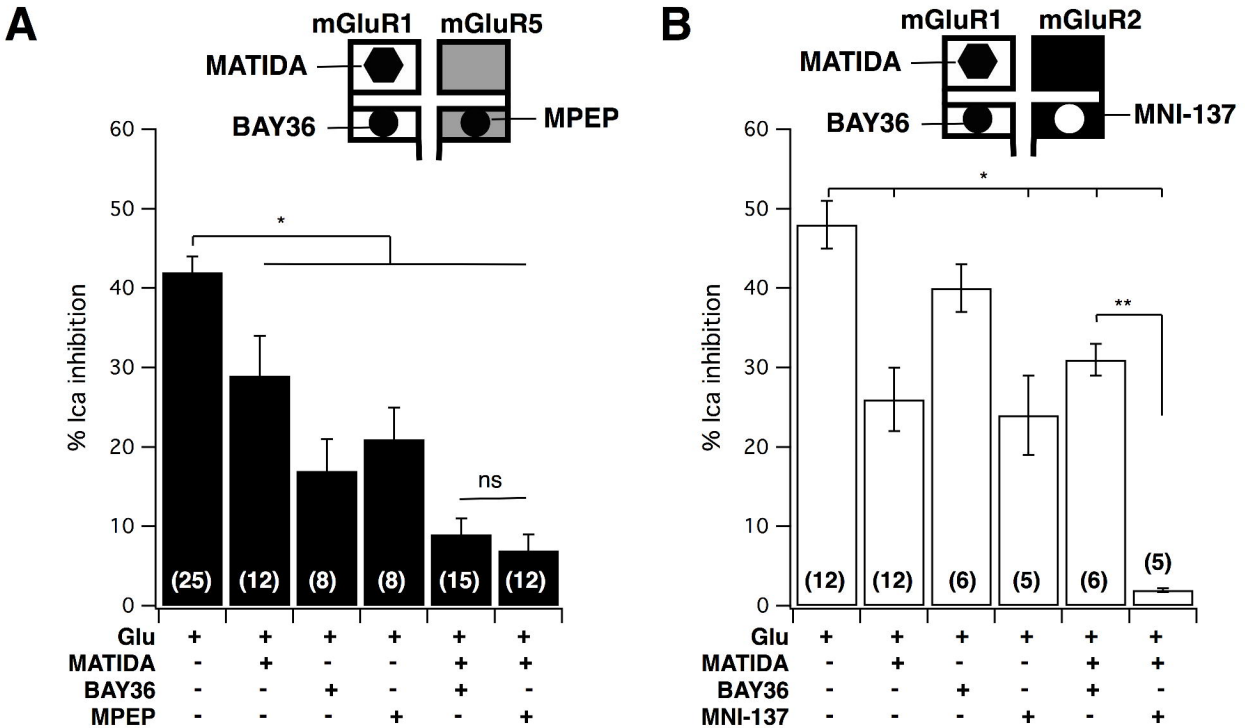
A
mGluR1
mGluR5
MATIDA
BAY36
MPEP
% Ica inhibition
60
50
40
30
20
10
0
*
ns
(25)
(12)
(8)
(8)
(15)
(12)
Glu + + + + + +
MATIDA - + - - + +
BAY36 - - + - + -
MPEP - - - + - +
B
mGluR1
mGluR2
MATIDA
BAY36
MNI-137
% Ica inhibition
60
50
40
30
20
10
0
*
**
(12)
(12)
(6)
(5)
(6)
(5)
Glu + + + + + +
MATIDA - + - - + +
BAY36 - - + - + -
MNI-137 - - - + - +

Figure 6

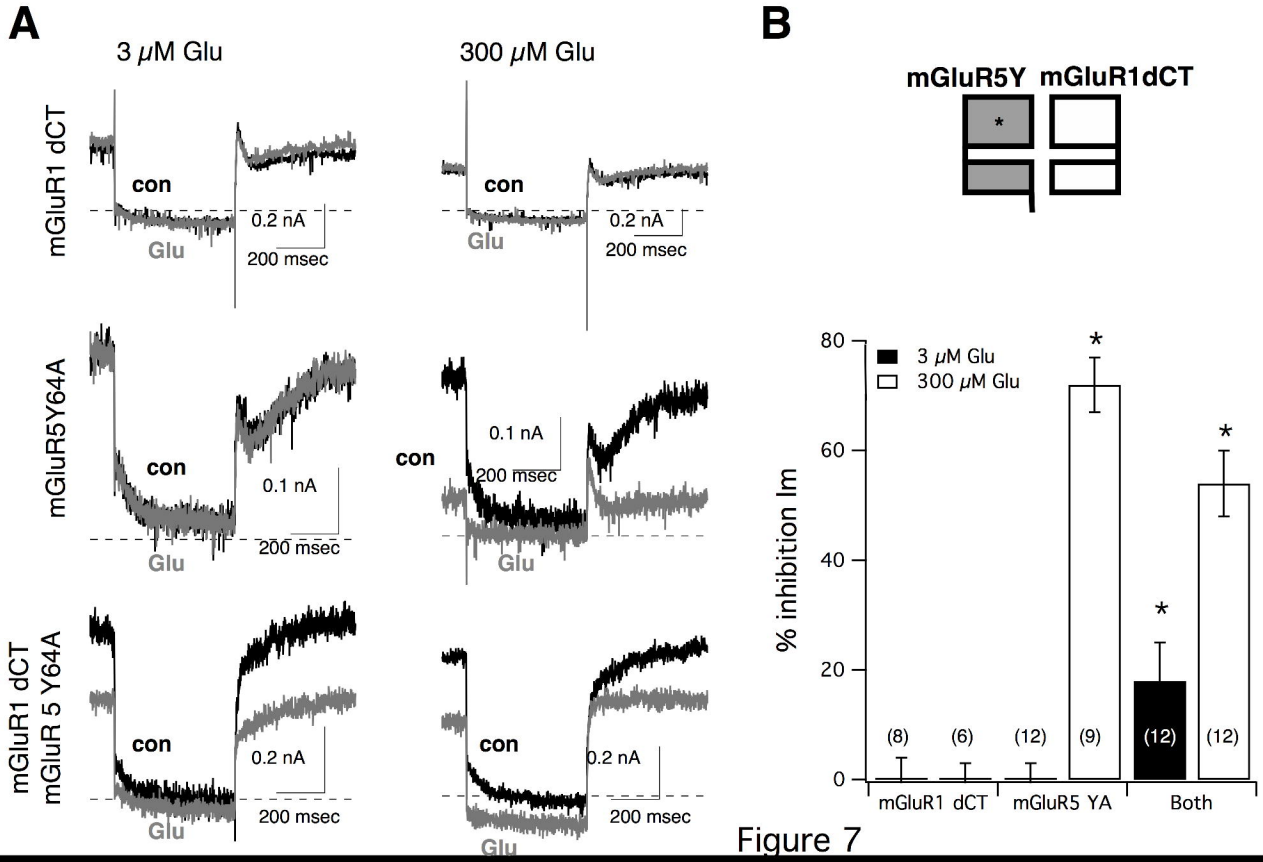
A
3 μM Glu
300 μM Glu
mGluR1 dCT
mGluR5Y64A
mGluR1 dCT
mGluR 5 Y64A
con
Glu
0.2 nA
200 msec
0.1 nA
200 msec
B
mGluR5Y
mGluR1dCT
*
3 μM Glu
300 μM Glu
% inhibition Im
80
60
40
20
0
(8)
(6)
(12)
(9)
(12)
(12)
mGluR1 dCT
mGluR5 YA
Both

Figure 7

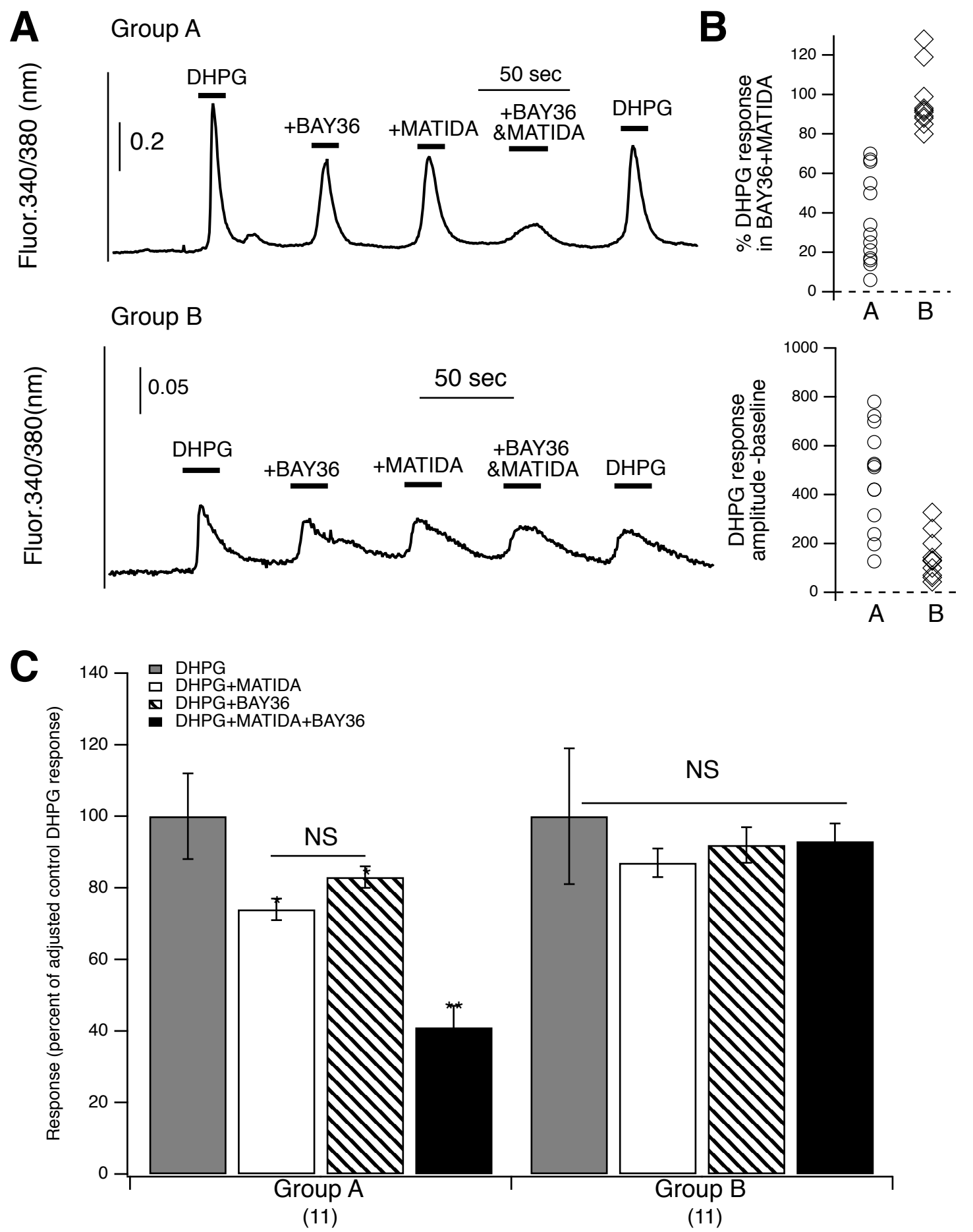
A
Group A
Fluor.340/380 (nm)
0.2
DHPG
+BAY36
+MATIDA
50 sec
+BAY36
&MATIDA
DHPG
Group B
Fluor.340/380(nm)
0.05
50 sec
DHPG
+BAY36
+MATIDA
+BAY36
&MATIDA
DHPG
B
% DHPG response
in BAY36+MATIDA
120
100
80
60
40
20
0
A
B
DHPG response
amplitude -baseline
1000
800
600
400
200
0
A
B
C
DHPG
DHPG+MATIDA
DHPG+BAY36
DHPG+MATIDA+BAY36
Response (percent of adjusted control DHPG response)
140
120
100
80
60
40
20
0
NS
NS
*
*
**
Group A
(11)
Group B
(11)

Figure 8