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Development and Antiparkinsonian Activity of VU0418506, a Selective Positive Allosteric Modulator of Metabotropic Glutamate Receptor 4 Homomers without Activity at mGlu_{2/4} Heteromers

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Abstract

Metabotropic glutamate receptor 4 (mGlu₄) is emerging as a potential therapeutic target for numerous central nervous system indications, including Parkinson's disease (PD). As the glutamate binding sites among the eight mGlu receptors are highly conserved, modulation of receptor activity via allosteric sites within the receptor transmembrane domains using positive and

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acchemneur-o.6b00036. Effect of increasing doses of VU0418506 on rotarod performance; signal strength when selective agonist is used for receptor activation (PDF)

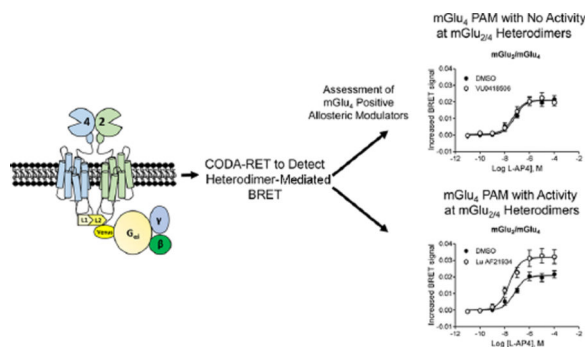
Author Contributions

C.M.N., C.K.J., X.L., M.B., A.T.G., A.L.B., A.L.R., M.T.L., J.S.D., J.A.J., P.J.C. conducted, analyzed, or provided input into interpretation of data from biological experiments. D.W.E., C.W.L., and C.R.H. performed the synthetic chemistry work. All authors contributed to the writing of the manuscript.

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negative allosteric modulators (PAMs and NAMs, respectively) has become a common strategy. We and others have used PAMs targeting mGlu₄ to show that potentiation of receptor signaling induces antiparkinsonian activity in a variety of PD animal models, including haloperidol-induced catalepsy and 6-hydroxydopamine-induced lesion. Recently, mGlu₄ has been reported to form heteromeric complexes with other mGlu receptor subtypes, such as mGlu₂, and the resulting heteromer exhibits a distinct pharmacological profile in response to allosteric modulators. For example, some mGlu₄ PAMs do not appear to potentiate glutamate activity when mGlu₂ and mGlu₄ are coexpressed, whereas other compounds potentiate mGlu₄ responses regardless of mGlu₂ coexpression. We report here the discovery and characterization of VU0418506, a novel mGlu₄ PAM with activity in rodent PD models. Using pharmacological approaches and Complemented Donor–Acceptor resonance energy transfer (CODA-RET) technology, we find that VU0418506 does not potentiate agonist-induced activity when mGlu₂ and mGlu₄ are heterodimerized, suggesting that the antiparkinsonian action of mGlu₄ PAMs can be induced by compounds without activity at mGlu_{2/4} heteromers.

Graphical Abstract



Keywords

Allosteric modulator; Parkinson's disease; metabotropic glutamate receptor

The primary pathological change underlying the motor symptoms associated with Parkinson's disease (PD) is death of dopaminergic neurons in the substantia nigra pars compacta (SNc) of the basal ganglia (BG).¹ Traditionally, therapeutic strategies for improving motor function in PD patients have relied on replacement of lost dopamine using L-DOPA or dopamine receptor agonists. However, increasing efforts have focused on developing nondopaminergic drug treatments for PD that normalize pathological changes in activity of BG output nuclei (the internal segment of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNr)) that occur after loss of dopaminergic neurons. We and others have used this underlying biology as rationale for the development of novel pharmacological agents with the potential to mimic the dramatic clinical efficacy observed using surgical ablation or deep brain stimulation of specific BG nuclei to reverse pathological changes in activity in the GPi (reviewed in refs 2 and 3).

In recent years, the mGlu₄ subtype of metabotropic glutamate (mGlu) receptor has emerged as a promising new therapeutic target for PD, and activation or potentiation of mGlu₄ has

been proposed to normalize activity in the BG motor circuit in PD patients.^{4,5} The mGlu₄ receptor is highly expressed on presynaptic GABAergic terminals projecting from the striatum to the GPe,^{6,7} the first synapse of the BG “indirect pathway”, where activation of this receptor reduces GABAergic transmission.⁸ Activity at the striato-GPe synapse increases excitatory drive from the subthalamic nucleus (STN) to the GPi/SNr. Excitation of the GPi/SNr through the indirect pathway balances the inhibitory input of striatal direct pathway neurons onto these output nuclei. Agonists and highly selective positive allosteric modulators (PAMs) of mGlu₄ reduce transmission at the striato-GPe synapse and have robust antiparkinsonian effects in rodent models.^{9–23} In addition, increasing evidence suggests that activation of mGlu₄ may have neuroprotective effects by reducing excessive excitatory drive from the STN to dopaminergic neurons,²⁴ as well as other neuroprotective actions in the SNc.

Unlike direct acting agonists, mGlu₄ positive allosteric modulators (PAMs) act at allosteric binding sites in the transmembrane domain to potentiate the response to glutamate; some mGlu₄ PAMs can also directly activate the receptor via an allosteric site.^{23,25} Multiple, structurally diverse mGlu₄ PAMs have been reported that provide high selectivity for mGlu₄, excellent pharmacokinetic properties, and robust efficacy in a range of rodent models of parkinsonian motor disability, as well as neuroprotective effects in models of toxin-induced dopaminergic cell loss.^{9–12,14–16,19,21,23} While the antiparkinsonian effects of mGlu₄ PAMs have been hypothesized to be mediated primarily by actions at the striato-GPe synapse, mGlu₄ is also localized in the striatum and recent studies raise the possibility that mGlu₄-mediated inhibition of transmission at corticostriatal synapses or other sites within the striatum could also contribute to the antiparkinsonian activity of mGlu₄ modulators.^{10,22,26,27}

Recent studies suggest that mGlu₄ can exist either as a constitutive homodimer or as a heterodimer with other mGlu receptor subtypes.^{28–30} We and others recently reported that mGlu₂ and mGlu₄ form a distinct complex when coexpressed in vitro and have provided evidence that the prototypical mGlu₄ PAM, *N*-phenyl-7-(hydroxyimino)cyclopropa[*b*]chromen-1*a*-carboxamide (PHCCC), does not result in observable potentiation of the activity of glutamate when mGlu₂ is coexpressed with mGlu₄.^{29,30} In contrast, two structurally distinct mGlu₄ PAMs that are thought to bind to an alternate allosteric site on mGlu₄,²⁵ VU0155041 and Lu AF21934, induce potentiation regardless of mGlu₂ expression, indicating a differential interaction of these two classes of compounds when mGlu₂ and mGlu₄ are coexpressed.³⁰

We have performed and reported a series of pharmacological, biochemical, and electrophysiology studies suggesting that mGlu₄ functions primarily as a heteromer at corticostriatal synapses.³⁰ For example, the compounds VU0155041 and Lu AF21934, ligands that potentiate responses when mGlu₂ and mGlu₄ are expressed together, activate and potentiate mGlu receptor-mediated inhibition of transmission at corticostriatal synapses, whereas PHCCC is without effect.^{10,22,30} In contrast, in the presence of agonist, PHCCC has robust effects on mGlu₄ modulation of transmission at multiple other synapses, including the striato-GPe synapse,²¹ suggesting that mGlu₄ is likely to function as an mGlu₄ homomer at this key synapse in the BG indirect pathway. These studies highlight the need to determine

whether mGlu₄ PAMs that may only potentiate the activity of mGlu₄ when expressed in homomeric form will induce full efficacy in reducing parkinsonian motor disability or whether it is important to optimize mGlu₄ PAMs that are also active at mGlu_{2/4} heteromers as PD therapeutics. PHCCC does not possess an ideal pharmacokinetic profile to test this hypothesis in vivo. We now report optimization of VU0418506³¹ as a novel mGlu₄ PAM with excellent pharmacokinetic properties, good brain penetration, and a profile that is consistent with activity only at homomeric mGlu₄ when assessed using a Complemented Donor-Acceptor resonance energy transfer (CODA-RET) approach.³² This technique relies upon measuring the signal induced by activating a defined receptor heteromer (in this case, mGlu_{2/4}); using this assay, we show that, in contrast to Lu AF21934, VU0418506 is not active at mGlu_{2/4} heteromers. Nonetheless, VU0418506 exhibits robust efficacy in rodent models of parkinsonian motor disability, suggesting that activity at mGlu_{2/4} heterodimers is not required for the antiparkinsonian effect of mGlu₄ PAMs.

RESULTS

Development of VU0418506, a potent and selective mGlu₄ PAM. VU0418506 (Figure 1A³¹) is a novel mGlu₄ PAM with robust efficacy in cells expressing mGlu₄. The compound is highly selective for mGlu₄ relative to other mGlu receptor subtypes (with the exception of activity at mGlu₆, a receptor which is primarily expressed in the retina), has an excellent pharmacokinetic profile in multiple species, and achieves high brain exposure after oral administration. Further characterization reveals that VU0418506 is a potent PAM of human mGlu₄ activity assessed using two distinct assays (pEC₅₀ = 7.17 ± 0.07, mean ± SEM, 68 nM, calcium assay, Figure 1B, C, pEC₅₀ = 7.27 ± 0.08, EC₅₀ = 55.7 nM, GIRK/thallium flux assay, Figure 1D). Additionally, VU0418506 potentiates the activity of glutamate at the rat mGlu₄ receptor (pEC₅₀ = 7.34 ± 0.04, 46 nM, Figure 1D). The compound also does not exhibit direct allosteric agonist activity in these assays as assessed by the lack of response when compound is added alone (Figure 1B, “compound add” and Figure 1C, black circles). Increasing concentrations of VU0418506 progressively left-shifted the concentration-response curve (CRC) for glutamate at both the human and rat receptors, with maximal efficacy achieved at approximately 1 μM (Figure 1E, F); again, no increase in the baseline was observed, suggesting a lack of allosteric agonist activity in this assay.

As mGlu₄ has become a novel target that is being explored for the treatment of Parkinson's disease, we next assessed the ability of VU0418506 to reverse haloperidol-induced catalepsy (HIC), a rodent model of parkinsonian motor disability. Animals were treated with a 1.5 mg/kg dose of haloperidol; 1 h later, animals were administered increasing doses of VU0418506 and catalepsy was assessed 30 min after VU0418506 dosing. VU0418506 showed robust activity in this model after oral administration, with significant activity in reversing catalepsy at doses of 3 mg/kg and higher (Figure 2A). The maximal efficacy achieved was similar to that observed with a 56.6 mg/kg oral dose of an adenosine A_{2A} antagonist, which served as a positive control.³³ In addition to assessing reversal of catalepsy, we also quantified plasma and brain levels of VU0418506 to establish a pharmacokinetic/pharmacodynamic (PK/PD) relationship for VU0418506 in the HIC model. Plasma and brain samples were taken immediately after catalepsy assessment (*t* = approximately 40 min after dosing) to measure total brain concentrations of VU0418506.

Unbound brain levels were determined by multiplying total levels of drug by the unbound fraction assessed using a brain homogenate binding (BHB) assay (rat $f_u = 0.0045$). As shown in Figure 2B, 50% reversal of HIC was observed with a total brain concentration of 7.1 μM and an estimated unbound concentration of 34 nM. This shows an excellent correlation with the in vitro EC_{50} of VU418506 in potentiating rat mGlu₄ activity (46 nM).

To further establish a PK/PD relationship, we also assessed levels of VU0418506 in the cerebrospinal fluid (CSF), as this provides another assessment of “free” drug. In these studies, satellite animals were dosed and CSF samples were drawn at the same time points as plasma and brain samples were taken from the HIC studies (i.e., approximately 40 min postdose). As shown in Figure 2C, there was a good correlation between increasing CSF levels and in vivo activity in HIC; this is further elaborated in Figure 2D in which the log of the CSF concentration is plotted versus the % reversal in HIC, resulting in an in vivo EC_{50} value of 210 nM.

We also examined a time course of activity of VU0418506 in the reversal of HIC. In these studies, haloperidol was given at $t = 0$, a 30 mg/kg dose of VU0418506 was given at $t = 1.5$ h, and catalepsy was assessed 30, 60, 120, 240, and 360 min later (Figure 3A). Plasma and brain samples were taken from these same animals during this experiment, and this study revealed that sustained levels of VU0418506 were observed at least 6 h after dosing (Figure 3B), correlating with significant HIC reversals. As an initial assessment of effects on locomotor performance, we measured the effect of increasing doses of VU0418506 on rotorod performance (Supporting Information Figure 1). These studies showed that, at doses up to 100 mg/kg, we did not observe any impairment in the ability of rats to perform a rotorod challenge.

As an additional assessment of antiparkinsonian activity, we also performed studies in rats with unilateral 6-hydroxydopamine (6-OHDA) lesions and assessed the effects of VU0418506 on the reversal of forelimb asymmetry. Unilateral 6-OHDA injection into the medial forebrain bundle results in loss of use of the paw on the affected side, and an ability to restore equivalent use of both paws is considered a measure of antiparkinsonian activity. In these experiments, an acute dose of VU0418506 was administered and animals were assessed for asymmetry after 30 min. As can be seen in Figure 4A, a 2.5 mg/kg dose of L-DOPA completely reversed motor deficits assessed by measures of forelimb asymmetry. VU0418506 administered alone (10 and 30 mg/kg) also induced statistically significant reversals of forelimb asymmetry. Several mGlu₄ PAMs, including ADX88178, VU0346770 and Lu AF21934, have also been shown to potentiate the effects of subthreshold doses of L-DOPA in various 6-OHDA lesion models.^{10,14,16} When combined, doses of L-DOPA and VU0418506 that were inactive alone (0.75 and 3 mg/kg, respectively) now exhibited statistically significant efficacy (Figure 4B). Overall, this profile indicates that VU0418506 is a potent mGlu₄ PAM and exhibits a good pharmacokinetic profile with high brain penetration. Importantly, VU0418506 has robust antiparkinsonian activity in two rodent models and exhibits an excellent PK/PD relationship.

Finally, we compared the activity of VU0418506 and Lu AF21934 in cells coexpressing mGlu₂ and mGlu₄ using both traditional pharmacological approaches as well as CODA-

RET. While all mGlu₄ receptor PAMs shift the glutamate concentration response curve (CRC) in cells expressing mGlu₄ alone, our previous studies revealed that mGlu₄ PAMs appear to fall into two broad classes, one class that does not shift the glutamate CRC in cells coexpressing mGlu₂ and mGlu₄ (i.e., PHCCC), and a second class of compounds, represented by Lu AF21934, that can potentiate responses in these cells.³⁰ We evaluated the effects of VU0418506 in cells expressing mGlu₄ alone and mGlu₄ in combination with mGlu₂. While VU0418506 induced significant potentiation in cells only expressing mGlu₄ (Figure 1 and Table 1), this compound had no effect on the glutamate CRC in cells expressing mGlu₂ and mGlu₄ (Figure 5A). In contrast, and as reported previously,³⁰ Lu AF21934 induced a leftward shift in the glutamate CRC in cells expressing both mGlu₂ and mGlu₄ receptors (Figure 5B, Table 1). Overall, these data are consistent with the hypothesis that a distinct population of receptors is present in cells coexpressing mGlu₂ and mGlu₄ that is potentiated by Lu AF21934 but not by VU0418506; the simplest explanation for these findings is that this population is a heterodimer of mGlu₂ and mGlu₄, but this cannot be definitely established in cells coexpressing both receptors given the possibility of forming both homomers and heteromers.

To verify that VU0418506 can activate mGlu₄ homomers but not mGlu₂/mGlu₄ heterodimers, whereas Lu AF21934 can activate both homomers and heteromers, we turned to CODA-RET technology.³² This system relies on a luciferase complementation assay coupled with bioluminescence resonance energy transfer (BRET) to measure proximity. In these experiments, different subtypes of receptor (in this case mGlu₂ and mGlu₄) are labeled with N- and C-terminal fragments of a luminescent donor molecule (in Figure 6, termed L1 and L2). When two different receptor subunits form a heterodimer, these two halves complement, forming a functional luciferase molecule. In contrast, in a homodimer only a fragment of the donor molecule will be present and a luminescent signal will not be generated in response to substrate. In parallel, we express a defined G protein, in this case G_{αi}, labeled with the acceptor molecule, mVenus. Activation of the complemented luminescent heterodimeric receptor leads to recruitment of this acceptor-labeled G_{αi} protein; the resulting proximity of the complemented donor and the acceptor molecule leads to BRET.

Using this system, we coexpressed either mGlu₄_L1/mGlu₄_L2 or mGlu₄_L1/mGlu₂_L2 and tested the effects of PAMs with the selective group III mGlu receptor agonist L-(+)-2-Amino-4-phosphonobutyric acid (L-AP4, Figure 7). In contrast to its activity when mGlu₄_L1 and mGlu₄_L2 are coexpressed (Figure 7A), VU0418506 is unable to potentiate L-AP4 responses when mGlu₄_L1 and mGlu₂_L2 are coexpressed (Figure 7B). Lu AF21934, however, is able to potentiate responses in cells expressing both mGlu₄_L1/mGlu₄_L2 (Figure 7C) as well as mGlu₄_L1/mGlu₂_L2 combinations (Figure 7D). In control studies (Supporting Information Figure 2), complementing two mGlu₂ subunits generates a signal when the selective group II mGlu agonist, (2*S*,2'*R*,3'*R*)-2-(2',3'-dicarboxycyclopropyl)glycine (DCG-IV), is used for receptor activation. The signal induced by DCG-IV is unchanged in the presence of unlabeled mGlu₄. If cells under these same conditions (i.e., labeled mGlu₂ subunits and unlabeled mGlu₄) are activated using the group III mGlu agonist L-(+)-2-amino-4-phosphonobutyric acid (L-AP4), no signal is generated (Supporting Information Figure 2). These important control experiments indicate that a

BRET signal is only generated by activation of the complemented pair of receptors, and not by a higher order complex of receptors. Taken together, the CODA-RET data validate the hypothesis that mGlu₂/mGlu₄ heterodimers are potentiated by Lu AF21934 but not by VU0418506, whereas both compounds activate mGlu₄ homomers.

DISCUSSION

In recent years, multiple structurally diverse compounds, acting via a variety of mechanisms, have been reported as selective activators or potentiators of mGlu₄ and have demonstrated promise in the reversal of motor deficits and induction of neuroprotective effects in PD models.^{9–16,34,35} We now present a novel mGlu₄ PAM, VU0418506, with suitable properties as a brain penetrant, orally bioavailable, mGlu₄ tool compound. VU0418506 exhibits robust antiparkinsonian activity in two PD models, and we show that its effects correlate with compound exposure in the HIC model, generating a pharmacokinetic/pharmacodynamic relationship. As reported by Engers et al.,³¹ VU0418506 exhibits a desirable in vitro and in vivo PK profile across three species, good brain penetration, and is highly selective for mGlu₄.

Previous studies suggest that mGlu₄ can function as homomeric receptors or in heteromeric form with mGlu₂.²⁸ Furthermore, recent studies suggest that mGlu_{2/4} heterodimers are present at the corticostriatal synapse,³⁰ a key synapse involved in regulating motor function. Finally, two previously reported mGlu₄ PAMs, VU0155041 and Lu AF21934, potentiate activity in cells expressing mGlu₂ and mGlu₄ and also have robust efficacy in models of parkinsonian motor disability.^{10,23,30} Based on this latter finding, it is possible that mGlu₄ PAM action specifically at mGlu_{2/4} complexes could contribute to the antiparkinsonian effects of mGlu₄ PAMs. However, VU0418506 had no effect on agonist responses in cells in which mGlu₂ and mGlu₄ were coexpressed. Using a CODA-RET approach that specifically monitors activity of the heterodimeric receptor configuration, we show here that VU0418506 is inactive at mGlu_{2/4}, despite its robust antiparkinsonian activity in both HIC and 6-OHDA lesion models. These data suggest that activity at mGlu_{2/4} heteromers is not required to achieve antiparkinsonian activity of mGlu₄ PAMs. In addition to potential implications for the roles of mGlu₄ in regulating motor function, these data have important practical implications and suggest that it is not necessary to intentionally optimize mGlu₄ PAMs that have activity at mGlu_{2/4} heteromers to achieve antiparkinsonian efficacy; activity at only mGlu₄ homomers may also provide a selectivity advantage if heteromers of mGlu₄ with other mGlu_s are widespread in the CNS or highly expressed in peripheral tissues.

Our current studies also shed new light on the activity of PAMs when mGlu₂ and mGlu₄ are coexpressed. Our original hypothesis was that there was a preferential interaction between mGlu₂ and mGlu₄ compared to each homomeric combination as we had shown that transfection ratios of 1:10 (mGlu₂:mGlu₄) resulted in a total loss of potentiation induced by certain mGlu₄ PAMs.³⁰ However, theoretically, we would predict that there should be three populations of receptors in our cell line: mGlu_{2/2}, mGlu_{4/4} and mGlu_{2/4}. As glutamate is approximately 10-fold more potent at mGlu₂ compared to mGlu₄, we would be unable to detect potentiation of mGlu₄ homomers with weaker PAMs that do not shift the glutamate response at mGlu₄ by at least 10-fold. However, compounds with higher efficacies at mGlu₄

might induce measurable potentiation if the efficacy is sufficient to increase the potency of glutamate to a point that is left-shifted compared to the potency of glutamate at mGlu₂ or mGlu_{2/4}. To overcome these complexities of receptor coexpression, we turned to CODA-RET technology. This assay relies upon complementation and bioluminescence resonance energy transfer (BRET) and allows the detection of a signal induced only by a defined mGlu_{2/4} heterodimer without any contribution from homodimers that may be present. These data show that the heterodimer can be activated by the mGlu₄ selective agonist, L-AP4, and responds by recruiting and coupling with the cognate G_{αi} protein. These studies clearly show that VU0418506 does not potentiate agonist responses when mGlu₂ and mGlu₄ are heterodimerized (Figure 7B). In contrast, as predicted from our previous pharmacology studies, Lu AF21934 potentiates agonist-induced responses at an mGlu_{2/4} heterodimer using a CODA-RET readout.

The finding that VU0418506 does not potentiate mGlu_{2/4} responses could suggest that this mGlu₄ PAM fails to bind to the receptor when it is heterodimerized with mGlu₂. Alternatively, VU0418506 might act as a silent allosteric ligand (SALs) at this receptor complex; SALs are compounds that bind to allosteric sites on GPCRs without inhibiting or potentiating responses.^{36,37} While additional studies will be needed to address these mechanisms, the differentiation of Lu AF21934 and VU0418506 presented here is interesting in light of studies showing that Lu AF21934 engages an allosteric site that is distinct from the binding site of other mGlu₄ PAMs.²⁵ Further studies using a combination of CODA-RET, muta-genesis and/or radioligand binding will be required to differentiate between these intriguing possibilities.

In summary, we report here the discovery and characterization of VU0418506, a compound with activity in two antiparkinsonian rodent models. VU0418506 exhibits an excellent PK:PD relationship in an antiparkinsonian model. Based on our in vitro CODA-RET studies with defined homomers and heteromers, we show that VU0418506, while active at mGlu₄ homomers, fails to potentiate the activity of an mGlu₄ agonist at mGlu_{2/4} heterodimers. These findings are consistent with the hypothesis that the antiparkinsonian activity of mGlu₄ PAMs is mediated by actions at mGlu₄ homomeric receptors.

METHODS

Synthesis

Lu AF21934 was synthesized as described in ref 30. The A_{2A} antagonist (*N*-(6-(3,5-dimethyl-1*H*-pyrazol-1-yl)-2-(furan-2-yl)-pyrimidin-4-yl)-2-(4-methylpiperazin-1-yl)acetamide, compound **23**), previously disclosed by Neurocrine Biosciences, was used as a positive control and was synthesized as described.³⁸ VU0418506 was synthesized as described in ref 31.

Drugs

Haloperidol, L-DOPA methyl ester, 6-hydroxydopamine hydrobromide, apomorphine, and clozapine were obtained from Sigma (St. Louis, MO). Buprenorphine was obtained from Patterson Veterinary (Devens, MA). L-Glutamate was obtained from Tocris Biosciences

(Ellisville, MO). VU0418506 and LuAF21934 were suspended in an aqueous solution of 10% Tween 80. Benserazide and L-DOPA methyl ester were dissolved in 0.9% saline, while haloperidol was dissolved in 0.85% lactic acid (Sigma) and 0.5% Cremophor EL (Sigma), respectively. Except for L-DOPA, which is unstable at neutral pH, all drug formulations were adjusted to a pH of approximately 7 using 1 N sodium hydroxide. Drugs were administered in a volume of 1–2 mL/kg (i.p. and s.c.) or 10 mL/kg (p.o.).

Cell Line Establishment and Cell Culture

Cell lines for all non-CODA-RET experiments were established and cultured as described in refs 14 and 30. Briefly, human mGlu₄/G_{q15}/CHO cells were grown in 90% Dulbecco's modified Eagle's medium (DMEM), 10% dialyzed fetal bovine serum (FBS), 100 units/mL penicillin/streptomycin, 20 mM HEPES (pH 7.3), 1 mM sodium pyruvate, 20 µg/mL proline, 2 mM glutamine, 400 µg/mL G418 sulfate (Mediatech, Inc., Herndon, VA), and 5 nM methotrexate (Calbiochem, EMD Chemicals, Gibbstown, NJ). Cell culture reagents were purchased from Life Technologies (Carlsbad, CA) unless otherwise noted. Rat mGlu₂ or rat mGlu₄ coding sequences were cloned into the pIRESpuro3 vector, transfected into HEK/GIRK cells, and selected with puromycin. Polyclonal rat mGlu₂/HEK/GIRK and rat mGlu₄/HEK/GIRK cells were cultured in growth media as previously described in ref 30, supplemented with Non-Essential Amino Acids. Rat mGlu₄ was also subcloned into the pIREShyg3 vector, and the resulting plasmid was transfected into rat mGlu₂/HEK/GIRK cells; cells were then selected with 200 µg/mL hygromycin B. Polyclonal cells were cultured in growth media supplemented with 100 µg/mL hygromycin B.

Calcium Mobilization Assays

Potency experiments were performed as described in ref 14. Human mGlu₄/G_{q15}/CHO cells (30 000 cells/20 µL/well) were plated in black-walled, clear-bottomed, TC treated, 384-well plates (Greiner Bio-One, Monroe, North Carolina) in DMEM containing 10% dialyzed FBS, 20 mM HEPES, 100 units/mL penicillin/streptomycin, and 1 mM sodium pyruvate (Plating Medium). The cells were grown overnight at 37 °C in the presence of 5% CO₂. The next day, the medium was removed and replaced with 20 µL of 1 µM Fluo-4, AM (Invitrogen, Carlsbad, CA) prepared as a 2.3 mM stock in DMSO and mixed in a 1:1 ratio with 10% (w/v) pluronic acid F-127 and diluted in Assay Buffer (Hank's Balanced Salt Solution (HBSS), 20 mM HEPES and 2.5 mM Probenecid (Sigma-Aldrich, St. Louis, MO)) for 45 min at 37 °C. Dye was removed and replaced with 20 µL of Assay Buffer. For concentration-response curve experiments, compounds were serially diluted 1:3 into 10 point concentration response curves in DMSO, transferred to daughter plates using an Echo acoustic plate reformatter (Labcyte, Sunnyvale, CA) and diluted in Assay Buffer to a 2× final concentration. Ca²⁺ flux was measured using the Functional Drug Screening System 6000 (FDSS6000, Hamamatsu, Japan). After establishment of a fluorescence baseline for 2 s (2 images at 1 Hz; excitation, 470 ± 20 nm; emission, 540 ± 30 nm), 20 µL of test compounds were added to the cells, and the response was measured. After 142 s later, 10 µL (5×) of an EC₂₀ concentration of glutamate was added to the cells, and the response of the cells was measured. 147 s after this add, an EC₈₀ concentration of glutamate was added (assay is schematically shown in Figure 1B). Calcium fluorescence was recorded as fold over basal fluorescence and raw data were normalized to the maximal response to glutamate.

Potency (EC_{50}) and maximum response (% Glu Max) for compounds were determined using a four parameter logistical equation in GraphPad Prism (La Jolla, CA). For efficacy experiments, a constant amount of compound was applied 142 s prior to the addition of a full glutamate concentration-response curve and the left shift of the EC_{50} of the curves was calculated as “fold shift”, which represents compound efficacy.

Thallium Flux Assays

Thallium flux assays were performed according to methods described in ref 23 with minor modifications. For dye loading, media was exchanged with Assay Buffer (HBSS containing 20 mM HEPES, pH 7.4) using an ELX405 microplate washer (BioTek), leaving 20 μ L/well, followed by addition of 20 μ L/well 2 \times FluoZin-2 AM (330 nM final) indicator dye (Life Technologies, prepared as a DMSO stock and mixed in a 1:1 ratio with pluronic acid F-127) in Assay Buffer. After a 1 h incubation at room temperature, dye was exchanged with Assay Buffer, leaving 20 μ L/well. Thallium flux was measured at room temperature using a Functional Drug Screening System 7000 (FDSS 7000, Hamamatsu). Baseline readings were taken (2 images at 1 Hz; excitation, 470 ± 20 nm; emission, 540 ± 30 nm), and test compounds (2 \times) were added in a 20 μ L volume and incubated for 140 s before the addition of 10 μ L of Thallium Buffer with or without agonist (5 \times). Data were collected for an additional 2.5 min and analyzed using Excel (Microsoft Corp, Redmond, WA) as previously described,²³ and the concentration-response curves were fitted to a four-parameter logistic equation to determine potency estimates using GraphPad Prism:

$$y = \text{bottom} + \frac{\text{top} - \text{bottom}}{1 + 10^{(\log EC_{50} - A) \text{HillSlope}}}$$

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

CODA-RET Assays: Constructs for Expression Vectors and Transfection

cDNAs for rat mGlu₂ and mGlu₄ were N-terminally tagged with a hemagglutinin (HA) epitope tag using standard molecular biology procedures. cDNAs encoding the L1 (residues 1-229) or L2 (residues 230-311) fragments of Renilla Luciferase 8 (RLuc8, a gift from Sam Gambhir, Stanford) were fused in frame to the C terminus of mGlu₄ and mGlu₂ following the linker “GSPP-ARAT” in the pcDNA3.1 vector. The following G protein constructs were also used: G_{αi}-mVenus with the mVenus inserted at position 60, untagged G_{β1}, and untagged G_{γ2}. The integrity of all the constructs was confirmed with sequencing analysis. Cultured HEK-293T cells were transfected with a constant amount of plasmid cDNA using polyethylenimine (Polysciences Inc.) in a 1:1 ratio in 10 cm dishes. The ratio of transfected plasmids was optimized to maximize the luminescence of the complemented donor as well as the dynamic range of the BRET response to L-AP4. In the case of mGlu₄ homomers, the ratio of mGlu₄-L1, mGlu₄-L2, G_{αi}-mVenus, G_{β1} and G_{γ2} was 4:4:1:1:1 (for a 10 cm dish, 4, 4, 1, 1, and 1 μ g respectively). For the mGlu₂ and mGlu₄ heteromer, the ratio of mGlu₄-L1, mGlu₂-L2, G_{αi}-mVenus, G_{β1}, and G_{γ2} was 4:8:1:1:1 (for a 10 cm dish, 4, 8, 1, 1, and 1 μ g

respectively). Cells were maintained in culture with DMEM supplemented with 10% FBS. Experiments were performed 48 h after transfection.

CODA-RET Assay

Cells were harvested, washed twice, and resuspended in PBS. Approximately 200 000 cells per well were distributed in 96-well plates and stimulated by indicated drugs dissolved in prewarmed PBS for 5 min at 37 °C. A concentration of 5 μ M coelenterazine H (the substrate for luciferase) was added to each well (Dalton Pharma Services). Two minutes after the addition of coelenterazine H, the fluorescence and luminescence was quantified (Pherastar, BMG Labtech) and the BRET signal was determined by calculating the ratio of the emission of mVenus (510–540 nm) over the emission of Rluc8 (485 nm).

Haloperidol-Induced Catalepsy

Catalepsy was assessed in Sprague–Dawley rats using a horizontal bar placed 6 cm from the testing surface as described in and performed.¹⁴ The forepaws of each rat were placed on the bar with the body positioned at an angle of $\sim 45^\circ$ to the testing surface. The latency in seconds required for the rat to remove one or both forepaws from the bar was manually measured with a cutoff time of 30 s. Rats were randomly assigned to treatment groups and injected with haloperidol (1.5 mg/kg, i.p.) and returned to home cages for 60 min. Vehicle, VU0418506 (1–100 mg/kg, p.o.), or the adenosine A2A antagonist (*N*-(6-(3,5-dimethyl-1*H*-pyrazol-1-yl)-2-(furan-2-yl)pyrimidin-4-yl)-2-(4-methylpiperazin-1-yl)acetamide, 56.6 mg/kg, p.o.) was administered and catalepsy was assessed 30 min later. We have previously demonstrated that this dose of adenosine A2A antagonist referred to as compound **23**³³ produces a robust reversal of haloperidol-induced catalepsy that serves as positive control for this assay. For the time course study, haloperidol was always administered 1.5 h prior to catalepsy assessment. For the 30 min group, haloperidol was administered 1 h prior to compound; for the 60 min group, haloperidol was given 30 min before compound; for the 120 min group, compound was given 30 min before haloperidol, for the 240 min group, compound was given 2.5 h before haloperidol, and for the 360 min group, compound was given 4.5 h before haloperidol.

Rotarod Test

The effects of VU0418506 on motor performance were evaluated using a rotarod as described in ref 14 (Med Associates, Inc., St. Albans, VT). All rats were given an initial training trial of 120 s, followed by two additional training trials of 85 s, approximately 10 min apart, using a rotarod (7.5 cm in diameter) rotating at a constant speed of 20 rpm. After initial training trials, a baseline trial of 120 s was conducted, and any rats that did not reach the 120 s criterion were excluded from the study. Rats were then treated with vehicle or VU0418506 (10, 30, 56.6, or 100 mg/kg, p.o.) and tested 30 min later. The time each animal remained on the rotarod was recorded and animals that did not fall off of the rotarod were given a maximal score of 120 s.

6-OHDA Lesions-Induced Forelimb Asymmetry

Unilateral 6-OHDA Lesions. In these studies, rats were first assessed in a baseline forelimb asymmetry assay to ensure that there was no inherent bias in paw use prior to lesions. Animals were then injected with buprenorphine (0.03 mg/kg, s.c.) and anesthetized with isoflurane before being placed in a stereotaxic frame. Unilateral lesions of the nigrostriatal dopamine projections were made by injecting 6-hydroxydopamine (6-OHDA) into the left medial forebrain bundle (AP -3.6 mm, ML 1.8 mm, DV -8.6 mm).³⁹ Briefly, a 5 μ L microsyringe with an outer diameter of 0.47 mm (Fisher Scientific, Pittsburgh, PA) was lowered to the injection site where 4 μ L of 6-OHDA hydrobromide (3.6 μ g/ μ L in ice-cold saline containing 0.015% ascorbic acid) were injected over a period of 4 min. The sham lesion procedure involved lowering the injection cannula 2 mm dorsal to the lesion site without delivering an intracerebral injection. After suturing the scalp rats were injected with 5% dextrose in Ringer's solution (3 mL s.c.) to prevent postsurgical dehydration. In order to minimize weight loss encountered in some 6-OHDA-lesioned rats, the regular chow was supplemented with Bacon Softies (Bio-Serv, Frenchtown, NJ).

Apomorphine-Induced Rotation

Three weeks after surgery rats were tested for apomorphine-induced rotation.⁴⁰ Briefly, rats were injected with apomorphine (0.05 mg/kg, s.c.), connected to a tether, and immediately placed into a cylinder-style rotometer (AccuScan, Columbus, OH). In this assay, animals were administered apomorphine at a dose that only activates supersensitive dopamine receptors (on the lesioned side), which induced turning behavior that was used as a functional assessment of dopamine depletion. The number and direction of full 360° turns was automatically recorded over a period of 30 min. The total number of turns (contralateral + ipsilateral) and the net number of contralateral turns (contralateral-ipsilateral) were calculated. Animals passing criteria were then assessed to ensure forelimb asymmetry had been achieved; animals with >50 apomorphine-induced rotations/30 min and an asymmetry score of < -60 were progressed to drug studies.

Forelimb Asymmetry Assay

6-OHDA lesion-induced forelimb asymmetry was assessed using the cylinder test.⁴¹ This assay measures the rats' forelimb use during spontaneous exploration of a Plexiglas cone. Briefly, rats were gently placed into a clear Plexiglas cone (bottom diameter 21 cm, top diameter 37 cm [CleverSys, Reston, VA]) and their behavior was recorded over a period of 10 min by a video camera placed underneath the Plexiglas cone using Pinnacle Studio software (Pinnacle Systems, Mountain View, CA). Video recordings were analyzed offline by an observer blinded to both lesion status and drug treatment. The VLC media player (<http://www.videolan.org/vlc/download-windows.html>) was used to replay videos at various playback speeds (0.125–1.0 \times real-time speed) in order to facilitate detailed analysis of forepaw placement during rearing and landing.

Forelimb usage was analyzed as outlined by Lundblad et al.⁴² In order to avoid scoring incidental contact with the cylinder wall only weight-bearing contacts as evidenced by splayed digits were scored. Subsequent wall contacts were only counted after a rat completely withdrew its paw(s) from the wall resulting in their weight being exclusively

supported by their hindlimbs. All floor landings from a rearing or grooming position onto either an individual forelimb or onto both forelimbs simultaneously were scored. The following parameters were used to calculate a composite measure of forelimb usage termed Forelimb Asymmetry (FLA) Index Score according to the formula below:

$$\text{FLA Index Score} = 100 \times \frac{(L_{\text{impaired}} - L_{\text{unaffected}}) + (W_{\text{impaired}} - W_{\text{unaffected}})}{(L_{\text{impaired}} + L_{\text{unaffected}}) + (W_{\text{impaired}} + W_{\text{unaffected}})}$$

where $W_{\text{unaffected}}$ is number of wall contacts of the unaffected (ipsilateral) forelimb, W_{impaired} is number of wall contacts of the impaired (contralateral) forelimb, $L_{\text{unaffected}}$ is number of landings on the unaffected (ipsilateral) forelimb, and L_{impaired} is number of landings on the impaired (contralateral) forelimb.

The FLA Index Score provides continuous data ranging between -100 (100% preference of the unaffected limb) and +100 (100% preference of the impaired limb), with a score of zero indicating a lack of forepaw preference.

Lesion Verification

After completion of the behavioral studies, lesioned animals were sacrificed and dopamine content in the lesioned and intact striatal side were determined by HPLC with electrochemical detection (as done in ref 14). Animals with less than 95% striatal dopamine depletion were excluded from statistical analysis. Rats were decapitated under deep isoflurane anesthesia, and the brains extracted. Tissue punches of the dorsolateral striatum (ipsi- and contralateral to the lesion) were frozen on dry ice and tissue concentrations of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), and homo-vanillic acid (HVA) were analyzed by HPLC with electrochemical detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
CNS	central nervous system
DOI	2,5-dimethoxy-4-iodoamphetamine

GIRK	G protein inwardly rectifying potassium
GPCR	G protein-coupled receptor
HTS	high-throughput screening
PHCCC	<i>N</i> -phenyl-7-(hydroxyimino)cyclopropa[<i>b</i>]chromen-1a-carboxamide
L-AP4	L-2-amino-4-phosphonobutyric acid
DCG-IV	(2 <i>S</i> ,2' <i>R</i> ,3' <i>R</i>)-2-(2',3'-dicarboxycyclopropyl)glycine
mGlu	metabotropic glutamate receptor
NAM	negative allosteric modulator
PAM	positive allosteric modulator
SAR	structure-activity relationship

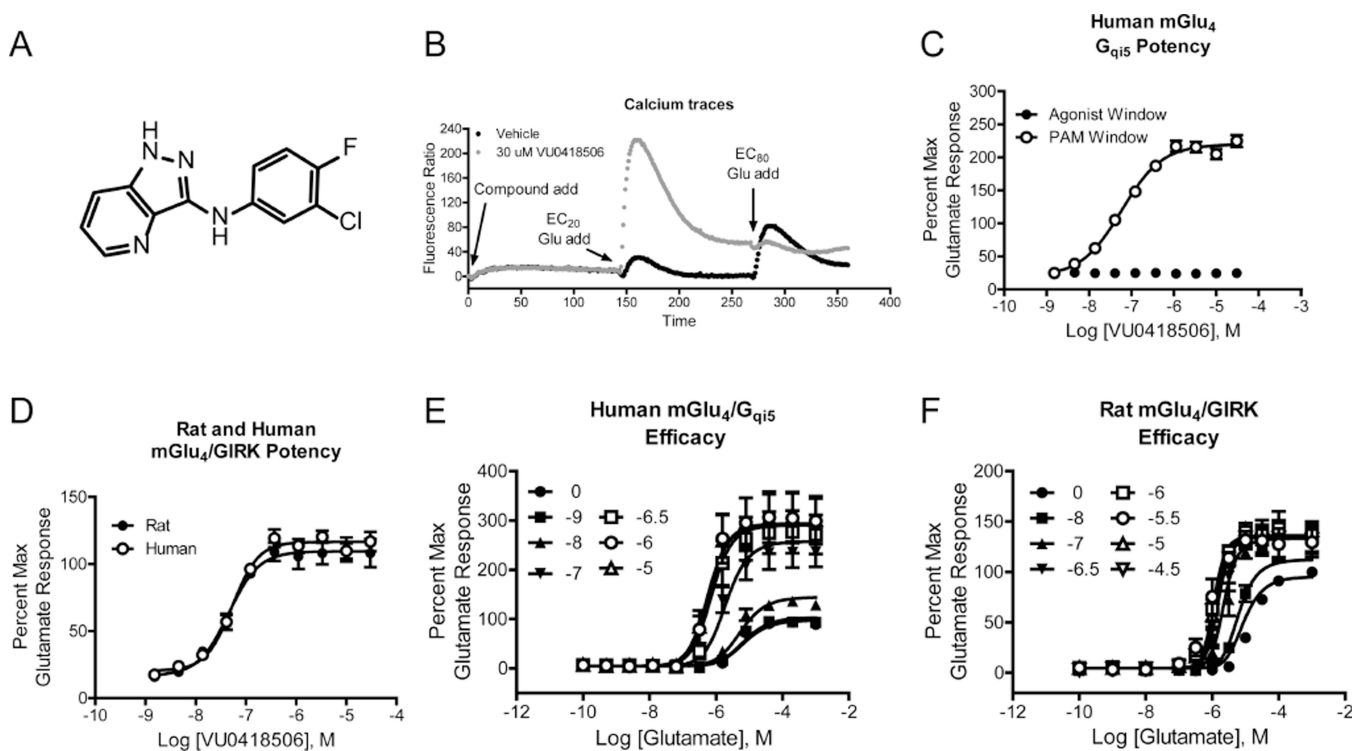
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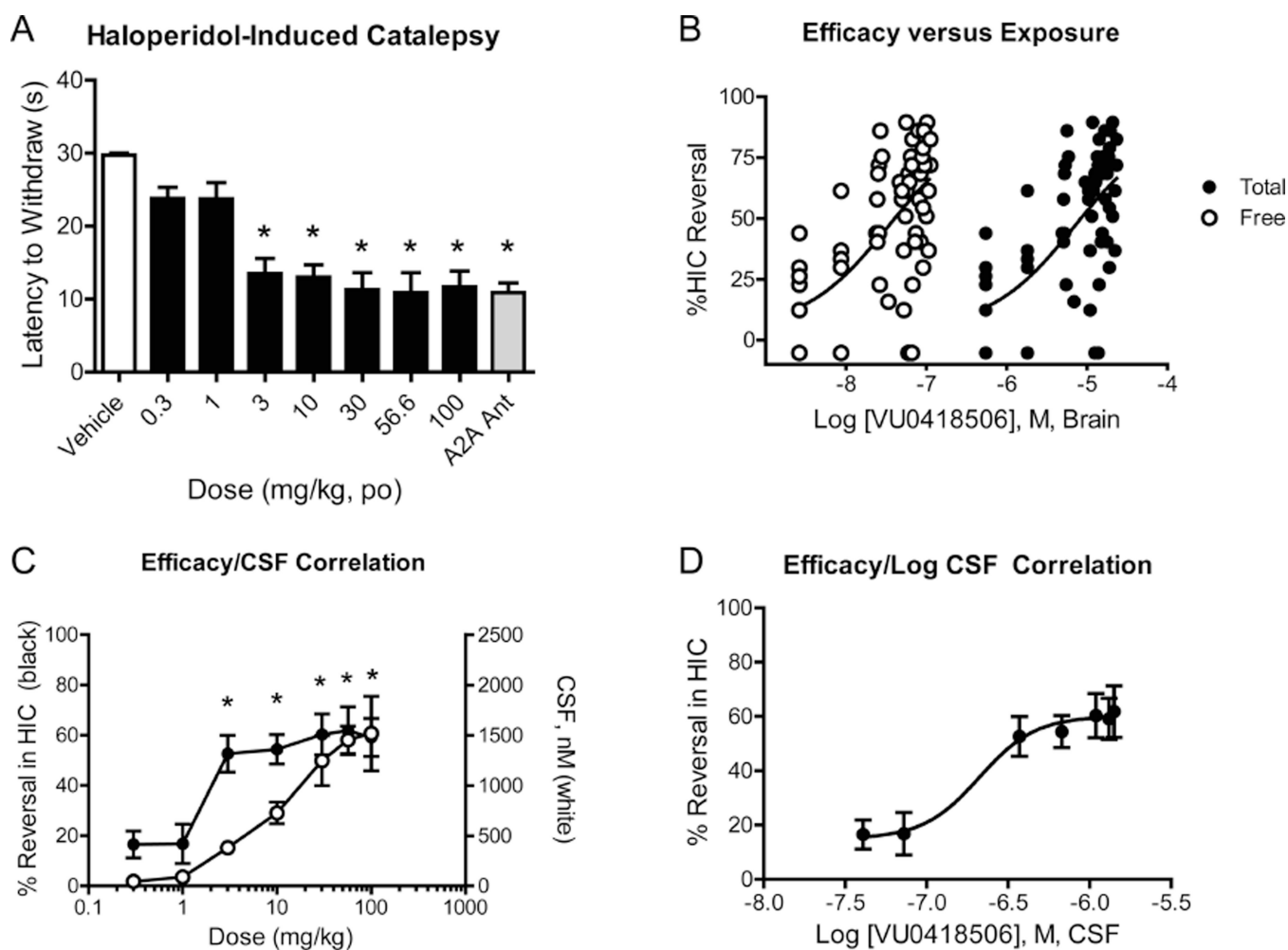
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**Figure 1.**

In vitro pharmacological profile of VU0418506, a potent mGlu₄ PAM. (A) Structure of VU0418506. (B) Calcium traces for a representative experiment performed in the presence and absence of 30 μM VU0418506. In this “triple addition” assay, a baseline read is taken for 2 s and compound or vehicle is added (designated with arrow and “compound add”). After approximately 2 min, a concentration of glutamate that elicits a 20% maximal response (“EC₂₀ add”) is added. This addition detects the activity of potentiators. In the final addition (“EC₈₀ add”), a concentration of glutamate that elicits an EC₈₀ response is added. In this chimeric G protein assay, we anticipate that glutamate does not induce full coupling; inclusion of a PAM further stabilizes the active receptor state, resulting in a response in the presence of the PAM that is greater than the maximal response elicited by a saturating concentration of glutamate. As shown in the “compound add” section of the trace, VU0418506 does not elicit activity in this assay in the absence of glutamate. (C) Potency of VU0418506 in the absence (black symbols) and presence (white symbols) of an EC₂₀ concentration of glutamate at human mGlu₄ using the G_{q15}-mediated calcium assay reflected in panel (B). The pEC₅₀ is 7.24 ± 0.04 (mean ± SEM), EC₅₀ = 59.6 nM, N = 10 determinations performed in triplicate. (D) Potency of rat and human mGlu₄ using native coupling to GIRK channels as a readout. For rat mGlu₄, the pEC₅₀ is 7.34 ± 0.04 (mean ± SEM), EC₅₀ = 46.6 nM, N = 3 determinations performed in triplicate. For human mGlu₄, the pEC₅₀ is 7.27 ± 0.08 (mean ± SEM), EC₅₀ = 55.7 nM, N = 3 determinations performed in singlicate. (E, F) Increasing concentrations of VU0418506 were added to human mGlu₄/G_{q15} cells or rat mGlu₄/GIRK cells approximately 2 min prior to the addition of increasing concentrations of glutamate. Data represent three independent determinations performed in duplicate.

**Figure 2.**

VU0418506 exhibits antiparkinsonian activity in reversing haloperidol-induced catalepsy at unbound brain and CSF concentrations at or above the *in vitro* EC₅₀ at rat mGlu₄. (A) A 1.5 mg/kg dose of haloperidol was given to rats, followed 1 h later by increasing doses of VU0418506 given orally; after 30 min, catalepsy was assessed. 56.6 mg/kg, dosed orally, of the adenosine A_{2A} antagonist compound **23**³³ (gray bar) was used as a positive control. **p* < 0.0001, one-way ANOVA with Bonferroni post-test. Data represent mean ± SEM, *N* = 9–10 animals. (B) Total (black circles) and predicted unbound/free (white circles) brain levels were determined. Calculated EC₅₀ values were 7.1 μM (total) and 34 nM (unbound/free). Data represent mean ± SEM, *N* = 9–10 animals. (C) CSF levels of VU0418506 were determined after oral dosing and correlated with HIC reversal. Statistically significant reversals in HIC (data from panel (A)) were observed when CSF concentrations exceeded in the *in vitro* EC₅₀ at rat mGlu₄. (D) Concentration–response curve of CSF concentration versus response, with a calculated EC₅₀ value of 210 nM.

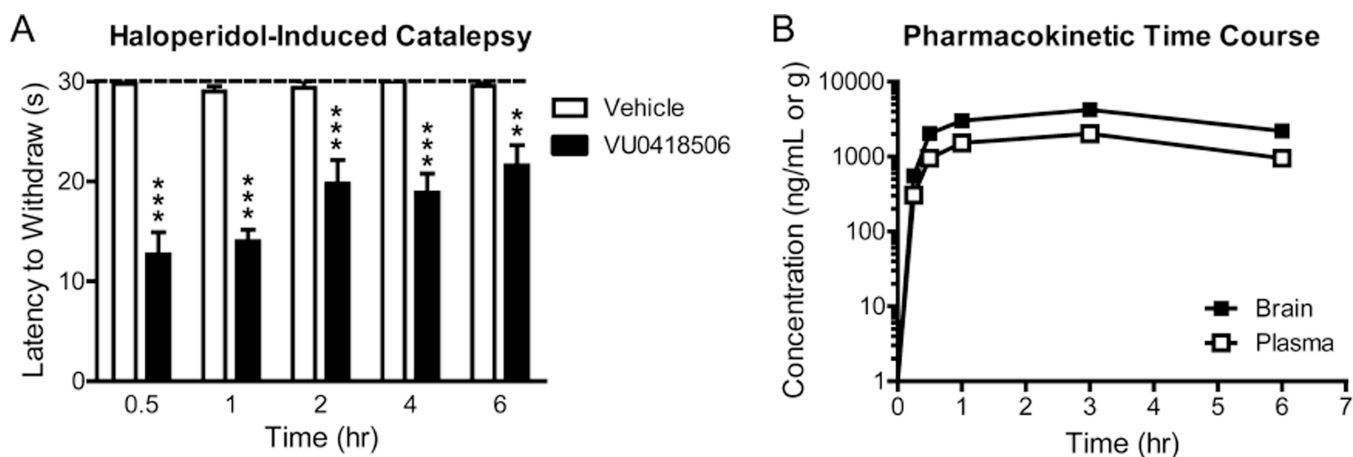


Figure 3.

VU0418506 exhibits antiparksonian activity out to 6 h after administration, paralleling exposure levels. (A) Animals were administered vehicle or a 30 mg/kg dose of VU0418506 and efficacy in reversing catalepsy was assessed 0.5, 1, 2, 4, or 6 h after dosing. Haloperidol was given 1.5 h before each catalepsy measurement as described in Methods. ** $p < 0.01$, *** $p < 0.0001$. Data represent mean \pm SEM, $N = 8$ –10 animals. (B) Plasma (white squares) and brain (black squares) exposures were calculated up to 6 h after a 30 mg/kg dose of VU0418506. Data represent the average calculated from two animals.

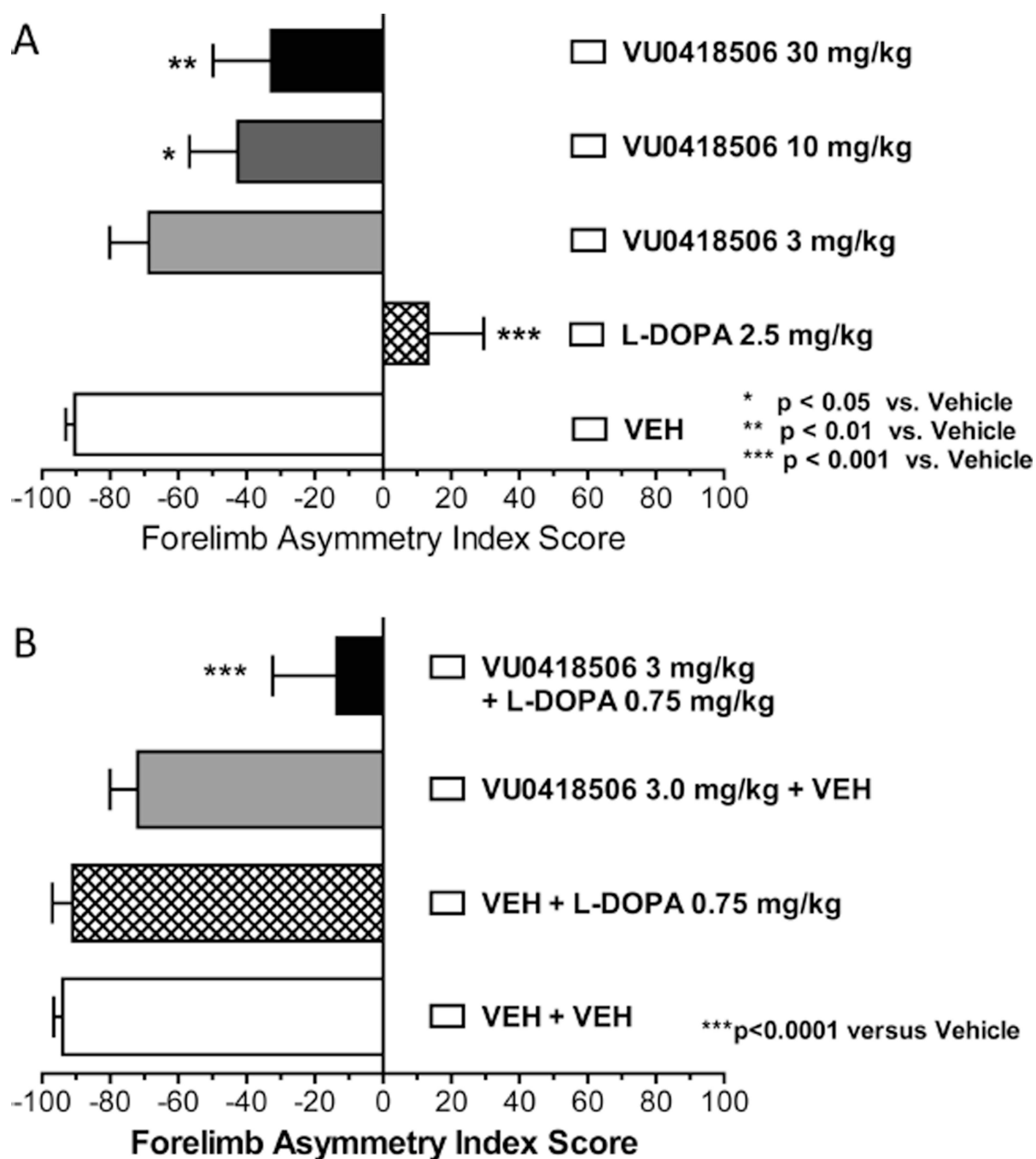


Figure 4.

VU0418506 reverses forelimb asymmetry deficits, both alone and with a subthreshold dose of L-DOPA, in a 6-OHDA model of Parkinson's disease. (A) 2.5 mg/kg dose of L-DOPA (hatched bar), as well as 10 and 30 mg/kg VU0418506 (gray and black bars, respectively), significantly reverse forelimb asymmetry deficits (p -values shown in figure, one-way ANOVA with Dunnett's multiple comparison test). (B) Doses of 0.75 mg/kg L-DOPA and 3 mg/kg VU0418506 are ineffective alone, but, when paired together, significant reversals of forelimb asymmetry phenotypes are observed ($p < 0.0001$ versus vehicle, one-way ANOVA

with Dunnett's multiple comparison test). Dose groups for both studies represent mean \pm SEM from 6 to 18 animals.

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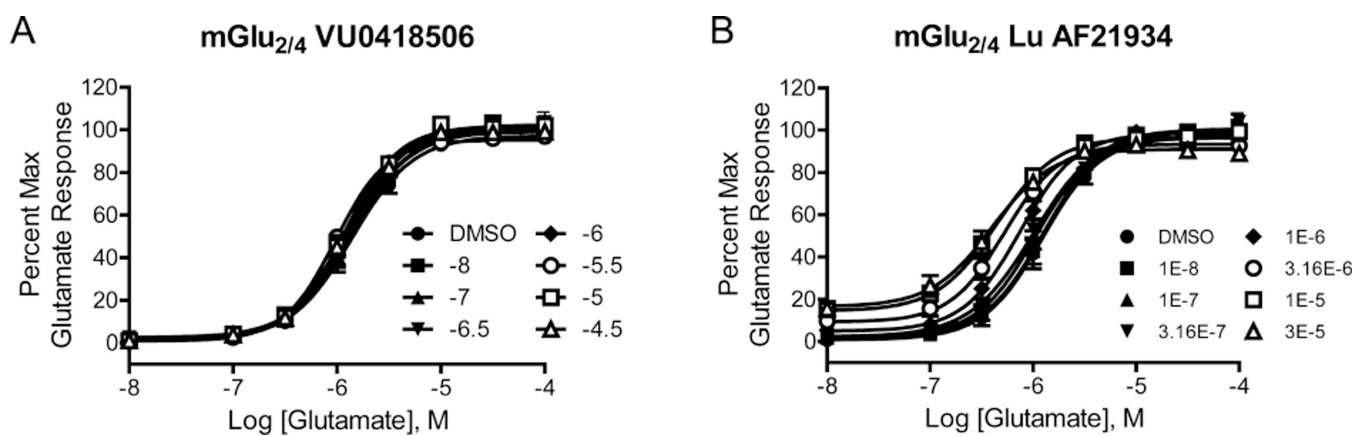


Figure 5. Differential interactions of (A) VU0418506 and (B) Lu AF21934 with mGlu₄ in the presence and absence of mGlu₂. Increasing concentrations of compound were applied prior to the application of increasing concentrations of glutamate and concentration-response curves were assessed. Data represent three independent experiments performed in duplicate.

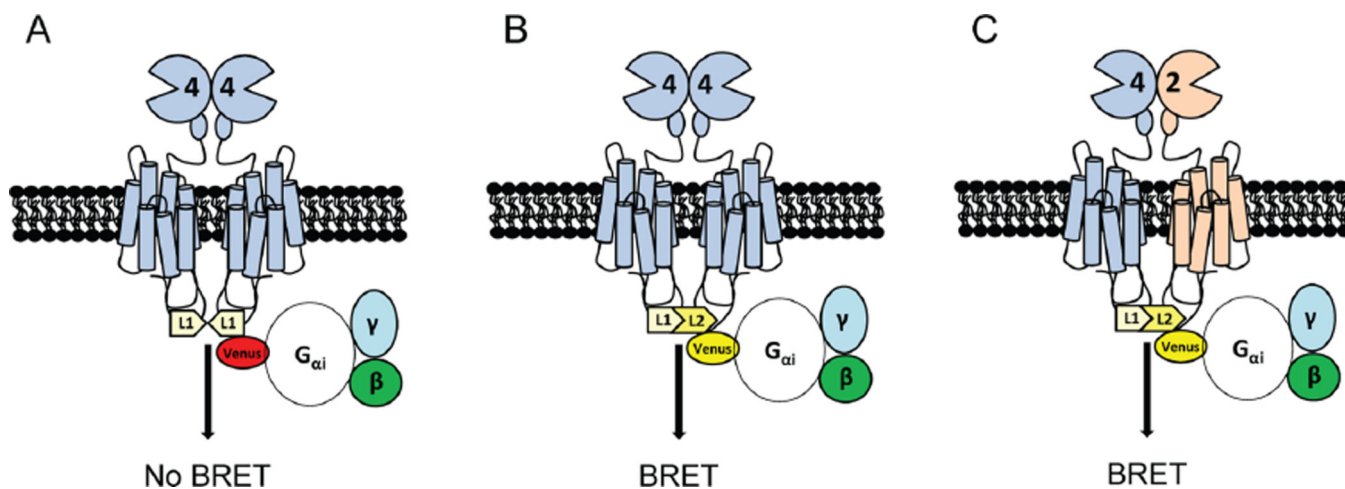


Figure 6.

Schematic of CODA-RET technology to detect activity of heteromeric mGlu_{2/4}. (A) When expressed alone, mGlu₄ or mGlu₂ (mGlu₄ shown) form obligate homodimers due to disulfide linkages. If only mGlu₄ receptors labeled with the L1 fragment of luciferase are expressed, no complementation between homomers occurs and no luminescence or BRET signal is detected. (B) If mGlu₄ receptors tagged with L1 and L2 are coexpressed, luciferase is complemented and a BRET signal is produced upon G protein recruitment. (C) If mGlu₄ tagged with L1 and mGlu₂ tagged with L2 are expressed, complementation occurs, resulting in a BRET signal with G protein. This allows detection of a signal from heteromer activation without contamination of the signal by homomeric mGlu₂ or mGlu₄.

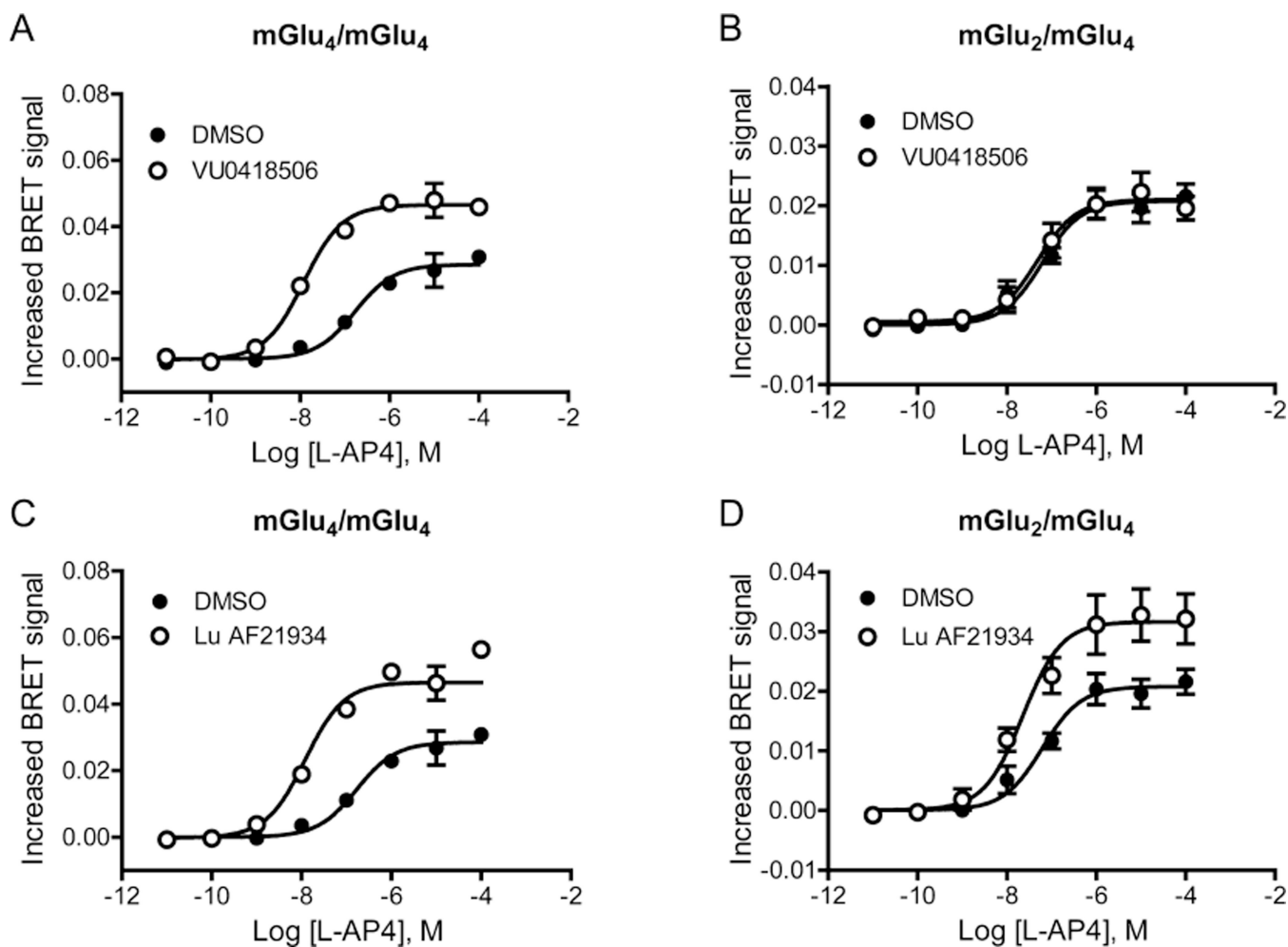


Figure 7.

CODA-RET technology reveals that VU0418506 is ineffective in the mGlu_{2/4} heteromeric conformation while Lu AF21934 induces potentiation. (A, C) As illustrated in Figure 6, receptor constructs were generated in which mGlu₄ was labeled with either L1 or L2 of Renilla luciferase as described in Methods and expressed in cells with mVenus-labeled G_{ai} G protein as well as unlabeled G_{β1} and G_{γ2}. Both VU0418506 and Lu AF21934 were able to potentiate responses by shifting the curve to the left ((A) pEC₅₀ DMSO, 6.75 ± 0.07, mean ± SEM, pEC₅₀ VU0418506, 7.91 ± 0.06, **p* = 0.0039; max response DMSO 0.029 ± 0.001, max response VU0418506, 0.047 ± 0.0004, **p* = 0.0005; (C) pEC₅₀ DMSO, 6.75 ± 0.07, pEC₅₀ Lu AF21934, 7.70 ± 0.01, **p* = 0.0026; max response DMSO, 0.029 ± 0.001, max response Lu AF21934, 0.050 ± 0.003, **p* = 0.0043). (B, D) In contrast, in cells coexpressing labeled mGlu₂ and mGlu₄ with mVenus-labeled G_{ai} G protein, VU0418506 was unable to potentiate responses, while Lu AF21934 induced potentiation ((B) pEC₅₀ DMSO, 7.27 ± 0.19, mean ± SEM, pEC₅₀ VU0418506, 7.31 ± 0.17, **p* = 0.274; max response DMSO, 0.021 ± 0.002, max response VU0418506, 0.021 ± 0.002, **p* = 0.196; (D) pEC₅₀ DMSO, 7.27 ± 0.19, pEC₅₀ Lu AF21934, 7.66 ± 0.11, **p* = 0.077; max response

DMSO, 0.021 ± 0.002 , max response Lu AF21934, 0.032 ± 0.004 , $*p = 0.0043$). All t tests were one tailed, paired t tests. Data represent 3–4 experiments performed in triplicate.

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Table 1Profiles of mGlu₄ PAMs in Cells Expressing mGlu₄ Alone versus Cells Expressing mGlu₂ and mGlu₄^a

	VU0418506			Lu AF21934	
	mGlu ₄	pEC ₅₀	fold shift	pEC ₅₀	fold shift
DMSO	4.80 ± 0.06			4.81 ± 0.04	
-8	5.08 ± 0.07	1.9 ± 0.12 ^c		4.85 ± 0.03	1.1 ± 0.03
-7	5.55 ± 0.17 ^b	6.0 ± 1.34		4.99 ± 0.07 ^b	1.5 ± 0.12
-6.5	5.67 ± 0.14 ^b	7.7 ± 1.44 ^c		5.00 ± 0.04 ^b	1.6 ± 0.09 ^c
-6	5.93 ± 0.12 ^b	14.0 ± 1.78 ^c		5.14 ± 0.07 ^b	2.1 ± 0.17
-5.5	6.07 ± 0.21 ^b	20.8 ± 5.82		5.32 ± 0.07 ^b	3.2 ± 0.28 ^c
-5	5.87 ± 0.13 ^b	12.2 ± 2.29 ^c		5.39 ± 0.06 ^b	3.8 ± 0.21 ^c
-4.5	5.96 ± 0.15 ^b	15.1 ± 2.90 ^c		5.43 ± 0.08 ^b	4.2 ± 0.42
	mGlu _{2/4}	pEC ₅₀	fold shift	pEC ₅₀	fold shift
DMSO	5.86 ± 0.07			5.91 ± 0.08	
-8	5.86 ± 0.08	1.0 ± 0.03 ^c		5.91 ± 0.11	1.0 ± 0.07
-7	5.87 ± 0.05	1.0 ± 0.05		5.99 ± 0.06	1.2 ± 0.07
-6.5	5.89 ± 0.07	1.1 ± 0.04 ^c		6.00 ± 0.09 ^b	1.2 ± 0.05 ^c
-6	5.94 ± 0.06	1.2 ± 0.06 ^c		6.13 ± 0.09 ^b	1.7 ± 0.04
-5.5	6.02 ± 0.02	1.5 ± 0.18		6.29 ± 0.06 ^b	2.4 ± 0.13 ^c
-5	5.93 ± 0.07	1.2 ± 0.04 ^c		6.36 ± 0.08 ^b	2.8 ± 0.07 ^c
-4.5	5.94 ± 0.06	1.2 ± 0.04 ^c		6.41 ± 0.08 ^b	3.2 ± 0.07

^aN = 3 independent experiments performed in duplicate.^bp < 0.05. For pEC₅₀ comparison, the potency in the presence of compound was compared to the respective DMSO control for either mGlu₄ or mGlu_{2/4} using repeated measures ANOVA with a Bonferroni post-test to compare the potency of glutamate in the presence of increasing concentrations of the PAM with DMSO vehicle control.^cp < 0.05. For the fold shift of the glutamate concentration-response curve, responses were compared for a given concentration of PAM between mGlu₄ and mGlu_{2/4} using a paired, two-tailed *t* test.