Published in final edited form as:

Biol Psychiatry. 2013 March 15; 73(6): 501–509. doi:10.1016/j.biopsych.2012.09.012.

Unique signaling profiles of positive allosteric modulators of metabotropic glutamate receptor subtype 5 determine differences in *in vivo* activity

Jerri M. Rook¹, Meredith J. Noetzel¹, Wendy A. Pouliot³, Thomas M. Bridges¹, Paige N. Vinson¹, Hyekyung P. Cho^{1,2}, Y. Zhou¹, Rocco D. Gogliotti¹, Jason T. Manka¹, Karen J. Gregory^{1,4}, Shaun R. Stauffer^{1,2}, F. Edward Dudek³, Z. Xiang¹, Colleen M. Niswender¹, J. Scott Daniels¹, Carrie K. Jones¹, Craig W. Lindsley^{1,2}, and P. Jeffrey Conn¹

¹Department of Pharmacology and Vanderbilt Center for Neuroscience Drug Discovery, Vanderbilt University Medical Center, Nashville, TN 37212

²Department of Chemistry, Vanderbilt University Medical Center, Nashville, TN 37212

³Department of Physiology, University of Utah School of Medicine, Salt Lake City, UT 84108

⁴Drug Discovery Biology Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, VIC, Australia

Abstract

Background—Metabotropic glutamate receptor subtype 5 (mGlu₅) activators have emerged as a novel approach to the treatment of schizophrenia. Positive allosteric modulators (PAMs) of mGlu₅ have generated tremendous excitement and fueled major drug discovery efforts. Although mGlu₅ PAMs have robust efficacy in preclinical models of schizophrenia, preliminary reports suggest that these compounds may induce seizure activity. Prototypical mGlu₅ PAMs do not activate mGlu₅ directly but selectively potentiate activation of mGlu₅ by glutamate. This mechanism may be critical to maintaining normal activity-dependence of mGlu₅ activation and achieving optimal *in vivo* effects.

Methods—Using specially engineered mGlu₅ cell lines incorporating point mutations within the allosteric and orthosteric binding sites, as well as brain slice electrophysiology and *in vivo*

© 2012 Society of Biological Psychiatry. Published by Elsevier Inc. All rights reserved.

Corresponding Author: P. Jeffrey Conn, Department of Pharmacology & Vanderbilt Center for Neuroscience Drug Discovery, Vanderbilt University Medical Center, 1215D Light Hall, 2215-B Garland Ave, Nashville, TN, USA, 37232-0697, Telephone: 615-936-2478, Fax: 615-343-3088, jeff.conn@vanderbilt.edu.

Financial Disclosures:

Over the past two years, Dr. Conn has served as a consultant for Millipore Corp. (St. Charles, MO). He serves on the Scientific Advisory Board of Seaside Therapeutics (Boston, MA) and Karuna Pharmaceuticals (Boston, MA). Dr. Conn has received research funding from Janssen Pharmaceutica (Beerse, Belgium) and Seaside Therapeutics (Boston, MA) and is engaged in collaborations with both companies that have the potential of generating milestone and royalty payments. Dr. Conn, Dr. Lindsley, Dr. Bridges, Dr. Vinson, Dr. Stauffer, Dr. Niswender, Dr. Daniels and Dr. Jones receive salary support from Johnson and Johnson. Dr. Conn, Dr. Lindsley, Dr. Stauffer, Dr. Niswender, and Dr. Jones are inventors on multiple pending and issued patents that protect different classes of allosteric modulators of mGlu₅ and other GPCRs. Dr. Dudek has received financial support from Johnson Pharmaceutical Research Institute, Johnson-Ethicon, and Neurotherapeutics Pharma and has equity interest in EpiTel, Inc. Dr. Dudek has received financial support in the form of grants, gifts, and/or consulting fees from Johnson Pharmaceutical Research Institute, Johnson-Ethicon, and Neurotherapeutics Pharma. He has also received consulting fees and has equity interest in EpiTel, Inc. Drs. Rook, Noetzel, Pouliot, Cho, Zhou, Gogliotti, Manka, Gregory, and Xiang report no biomedical financial interests or potential conflicts of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

electroencephalography and behavioral pharmacology, we found that some mGlu₅ PAMs have intrinsic allosteric agonist activity in the absence of glutamate.

Results—Both *in vitro* mutagenesis and *in vivo* pharmacology studies demonstrate that VU0422465 is an agonist PAM that induces epileptiform activity and behavioral convulsions in rodents. In contrast, VU0361747, an mGlu₅ PAMs optimized to eliminate allosteric agonist activity, has robust *in vivo* efficacy and does not induce adverse effects at doses that yield high brain concentrations.

Conclusions—Loss of the absolute dependence of mGlu₅ PAMs on glutamate release for their activity can lead to severe adverse effects. The finding that closely related mGlu₅ PAMs can differ in their intrinsic agonist activity provides critical new insights that is essential for advancing these molecules through clinical development for treatment of schizophrenia.

Keywords

Glutamate; allosteric modulators; agonist; schizophrenia; seizure; convulsions; mGlu₅

Introduction

A large number of clinical and preclinical studies increasingly support the hypothesis that selective activators of a metabotropic glutamate receptor subtype 5 (mGlu₅) may provide a novel therapeutic approach for treatment of schizophrenia, cognitive disorders, and some genetic childhood developmental disorders. A major breakthrough that allowed mGlu₅ selective activators to emerge as a viable approach was discovery of highly selective positive allosteric modulators (PAMs) of this receptor. Discovery of selective allosteric modulators is providing fundamental advances in our understanding of GPCR signaling and modulation, causing a paradigm shift in studies of GPCR signaling and drug discovery (1). Unlike traditional agonists, GPCR PAMs do not activate the receptor directly but maintain dependence on normal signaling mechanisms, selectively increasing receptor responses to endogenous agonists. Theoretically, this unique property of PAMs could provide therapeutic advantages by maintaining the normal temporal and spatial requirements of receptor activation by natural agonists. However, at present, there has been no clear demonstration that PAMs of mGlu₅ or other GPCRs can elicit fundamentally different effects from traditional agonists in intact systems.

One of the most exciting aspects of targeting mGlu₅ for treatment of schizophrenia is that mGlu₅ PAMs have potential utility in treatment of all major symptom domains (positive, negative and cognitive symptoms) (1–4). Animal studies reveal that mGlu₅ PAMs have robust efficacy in rodent models predictive of antipsychotic efficacy and enhance multiple aspects of cognitive function (5–10). However, recent preliminary reports suggest that mGlu₅ PAMs may also have severe target-dependent adverse effects, including induction of behavioral convulsions (11–14), potentially preventing mGlu₅ PAMs from advancing to clinical development. Interestingly, traditional mGlu₅ agonists can also induce seizure activity (15, 16). This is especially important in light of recent studies suggesting that some mGlu₅ PAMs exhibit allosteric agonist activity when evaluated in overexpressing cell lines (11, 17); however, this has only been observed with artificially high levels of mGlu₅ expression (9). It is not known whether mGlu₅ PAMs that have been evaluated *in vivo* elicit functionally relevant agonist activity and the potential impact of allosteric agonist activity in the CNS has not been evaluated.

Given the recent findings of adverse effects of mGlu₅ PAMs and the potential impact of authentic allosteric agonist activity in mGlu₅ PAMs if it exists, we engineered an inducible cell line allowing assessment of novel mGlu₅ PAMs for allosteric agonist activity with

either high or low levels of mGlu₅ expression. Interestingly, we found that closely related mGlu₅ PAMs can exhibit fundamentally different profiles in terms of mGlu₅ allosteric agonist activity. Most notably, the mGlu₅ PAM VU0424465 has robust allosteric agonist activity in a low-expressing cell-based system and in multiple native systems. Moreover VU0424465 induces intense epileptiform activity in hippocampal slices and generalized seizure activity and behavioral convulsions in rats. In contrast, closely related compounds behave as pure mGlu₅ PAMs with no observable agonist activity in any cell line or native system. These compounds exhibit *in vivo* efficacy in animal models predictive of antipsychotic activity but do not induce seizure activity or observable adverse effects. Thus, subtle structural changes can lead to a molecular switch of pure mGlu₅ PAMs to robust mGlu₅ allosteric agonists, dramatically altering the effects of mGlu₅ PAMs in the central nervous system (CNS). This provides direct demonstration that maintaining activity-dependence of mGlu₅ activation with pure mGlu₅ PAMs can provide major advantages, avoiding adverse effect liabilities observed with mGlu₅ agonists. These critical new mechanistic insights reveal key advantages of mGlu₅ PAMs and the critical importance of chemically optimizing pure PAMs using native systems and cell lines that do not overexpress mGlu₅ to achieve efficacy without inducing adverse effects associated with direct receptor activation.

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS) and antibiotics were purchased from Invitrogen (Carlsbad, CA). DHPG and LY341495 were purchased from Ascent Scientific (Bristol, UK) and Tocris (Ellisville, MO), respectively. VU0422465 (18), VU0361747 (10), 5MPEP (19) and MTEP (20) were synthesized as described previously. Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were either analytical or HPLC grade.

Cell culture and mutagenesis

HEK293A cells lines stably expressing rat mGlu₅ were maintained, (9) and point mutations were introduced into rat mGlu₅ and polyclonal stable cell lines generated (21), as described previously.

Fluorescence-based calcium assays in rat mGlu₅ cells

Measurement of mGlu₅-mediated intracellular Ca²⁺ mobilization was performed using the Ca²⁺ sensitive dye, Fluo-4 and a Flexstation II, as described previously (9). Data were transformed and fitted using GraphPad Prism 5.0 (Graph-Pad Software, Inc., San Diego, CA).

Brain slice electrophysiology

Animals were cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were 5–6 weeks for LTD, and 24–30 days for epileptiform activity. LTD experiments were conducted as previously described (9). For epileptiform experiments 400 μ m transverse slices were made as described for LTD experiments, except that slices were transferred directly from cutting to room temperature ACSF (in mM: 124 NaCl, 5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 glucose, 2 CaCl₂, 1.2 MgCl₂) for 1 hr. Slices were transferred to the recording chamber and perfused with 30–32°C ACSF. Recording electrodes were placed in the cell body layer of CA3. Field potential recordings (spontaneous events) were acquired. mGlu₅ compounds or DHPG were applied using a perfusion system. Sampled data was analyzed offline using MiniAnalysis

(Synaptosoft Inc., Fort Lee, NJ) to determine the amplitude and inter-event interval of the spontaneous events and normalized to the predrug period.

***in vivo* electroencephalography**

EEG recordings and analysis were performed as described previously (22). Male Sprague-Dawley (225–250 g) rats were implanted with a single cortical EEG electrode and allowed to recovery for 7 days. Animals were placed in a Plexiglass chamber where continuous video and EEG data were collected for 24 hr. The EEG signals were amplified (5,000 × gain; high-pass filter 1 Hz; low-pass filter, 100 Hz), digitized at 500 Hz and acquired with AcqKnowledge software (Biopac Systems, Santa Barbara, CA). Compounds were diluted in 10% Tween 80 (pH 7.0) at a concentration of 1 mg/3 ml and injected intraperitoneally. The number of spikes from each EEG trace was counted with a threshold of 3 standard deviations from the mean baseline taken prior to the injection of the compound. Both positive and negative events were counted, and the mean and 95% confidence intervals (CI) were calculated for each group.

Behavioral manifestations of seizure activity

Rats received treatment of 56.6 mg/kg VU0361747, 10 mg/kg VU0422465 or 10 mg/kg VU0422465 + 3 or 10 mg/kg MTEP. Compounds were formulated in 10% Tween 80 (pH 7.0) at a concentration of 1 mg/3 ml and injected intraperitoneally. Animals were monitored continuously and scored for behavioral manifestations of seizure activity in 5 min periods, once every 5 min for the first 15 min, then once every 15 min up to 1 h, and every 30 minutes up to 2 h. Behavioral manifestations of seizures were scored using a modified Racine scoring system (23). Briefly, a score of 0 represents no behavior alterations; score 1, immobility, mouth and facial movements, or facial clonus; score 2, head nodding, tail extension; score 3, forelimb clonus, repetitive movements; score 4, rearing and tonic clonic seizure; and score 5, continuous rearing and falling, severe generalized tonic clonic seizure. Rats were then euthanized and brains removed and processed for compound exposure levels.

Statistical Analysis

Data are reported as mean ± SEM. Analysis of LTD experiments was performed using a one-way analyses of variance (ANOVA with Tukey post-hoc test. Statistical analysis of epileptiform electrophysiological experiments was performed using a one-way ANOVA with a Dunnett's post-hoc test. For effect of drug treatment and time on behavioral convulsions a two-way, repeated measures ANOVA was used. Differences between treatment groups and within treatment groups over time were identified using Tukey post-hoc tests. Comparison of spike rate (mean + 95% CI) from cortical EEG recordings between vehicle and VU0424452- or VU0361747-treated groups was performed using a Student's t test. For all test, statistical significance was considered $p < 0.05$.

Results

Optimization of mGlu₅ PAMs with allosteric agonist activity

We previously reported discovery of a novel mGlu₅ PAM series, finding that some compounds behaved as pure PAMs whereas others exhibited allosteric agonist activity (9, 10). Interestingly, this allosteric agonist activity was only observed in cell lines expressing high levels of mGlu₅ and not in lower-expressing cell lines or in native systems, suggesting that agonist activity can be an artifact of over expression of the receptor and not indicative of physiological effects or *in vivo* efficacy (9). We sought to develop VU0360172-based mGlu₅ PAMs that possessed allosteric agonist activity in low mGlu₅-expressing cell lines. Chemical optimization efforts led to VU0424465, the most potent and efficacious compound

within this scaffold (Supplemental Figure S1). Novel compounds derived from this scaffold were evaluated for agonist activity in previously described rat HEK293 cells expressing low levels of mGlu₅ (9) similar to those seen in native tissues and most representative of physiological systems. Consistent with our previous report (9), these mGlu₅ PAMs did not display agonist activity in inducing calcium mobilization in cells expressing low levels of mGlu₅ but did potentiate the response to an EC₂₀ concentration of glutamate, as demonstrated by VU0361747 (Figure 1A), a compound behaving as a pure PAM regardless of mGlu₅ expression levels (9). Interestingly, the newly discovered compound, VU0424465, exhibited robust agonist activity and induced calcium mobilization in the absence of glutamate (EC₅₀ = 171 ± 15 nM, maximum efficacy 65% compared to glutamate) (Figure 1C). Historical PAMs, such as CDPPB, have been reported to possess minimal agonist activity in various cell-based assays. In the mGlu₅ low-expressing cell line, the maximum agonist effect observed was 13% of the glutamate response with an EC₅₀ of 5.8 μM (data not shown). While previously reported PAMs, such as CDPPB, provided the first tool compounds to assess *in vivo* efficacy in behavioral models (6, 7, 24, 25), the magnitude of agonist activity coupled with its poor solubility and brain penetration made it necessary to optimize new compounds that possessed ideal physicochemical and pharmacokinetic properties to achieve adequate unbound brain concentrations to assess the effects of allosteric agonist activity *in vivo*. VU0424465 potentiated glutamate-induced calcium mobilization, displaying a potency of 1.5 ± 0.8 nM (Figure 1B) and induced a 6.6-fold shift in the glutamate concentration-response curve (data not shown). Additional studies assessing the ability of 10 μM VU0424465 to modulate glutamate activity at other mGlu subtypes, demonstrated that this compound is highly selective for mGlu₅ (Supplemental Figure S2A).

Agonist activity of VU0424465 mediated by interaction with an allosteric site

To assess whether VU0424465 interacts with the previously identified allosteric binding site, we evaluated its ability to inhibit binding of [³H]methoxy-PEPy to the MPEP allosteric binding site on mGlu₅. VU0424465 exhibited high affinity at this site with a K_i value of 11.8 ± 0.1 nM (Supplemental Figure S2B). Additionally, pharmacological studies revealed that the mGlu orthosteric antagonist LY341495 (100 μM – 1 mM) did not shift VU0424465 agonist activation of mGlu₅ (Figure 2A). VU0424465 also did not inhibit binding of the group I orthosteric binding site radioligand [³H]quisqualate to rat mGlu₅-expressing cell membranes (data not shown). These results suggest that VU0424465 does not act as a traditional orthosteric site agonist. In contrast, the neutral allosteric MPEP-site ligand, 5MPEP (30 μM – 300 μM), induced concentration-dependent parallel rightward shifts of the VU0424465 agonist concentration-response curve with no effect on the maximum response (Figure 2A). These data suggest that VU0424465-induced activation of mGlu₅ is mediated by a competitive interaction with the MPEP allosteric binding site.

To further confirm that VU0424465 behaves as an allosteric agonist, individual single point mutations, substituting amino acids known to be essential for orthosteric ligand binding (R68E and D304A) and activation at mGlu₅ (C240E) were introduced into rat mGlu₅ (26). Each mutation abolished mGlu₅ activation by two orthosteric agonists, glutamate and DHPG ((*S*)-3,5-Dihydroxyphenylglycine) (Figure 2B). However, robust agonist activity of VU0424465 was maintained at each of the mGlu₅ orthosteric mutations (Figure 2B). Mutation of A809V results in a loss of function of ligands that act at the mGlu₅ MPEP allosteric binding site (27), while potentiation by the non-MPEP site PAM, CPPHA, is lost with the F585I mutation (28). Therefore, agonism by glutamate and DHPG, as well as VU0424465, was evaluated at these mGlu₅ allosteric site mutations. Glutamate and DHPG agonist activity was maintained in cells expressing mGlu₅-F585I and mGlu₅-A809V. Likewise, VU0424465 induced significant activation at mGlu₅-F585I (Figure 2C). However, VU0424465-induced agonist activity was completely abolished in cells expressing mGlu₅-

A809V (Figure 2C). Altogether, these data strongly support the hypothesis that VU0424465 acts as an allosteric agonist that binds to the allosteric MPEP site.

VU0424465-induced agonist activity in native systems

Previous reports suggest that allosteric agonist activity of mGlu₅ PAMs in cell-based systems may be an artifact of artificially high receptor expression levels (9). VU0424465 is the first mGlu₅-selective agonist not based on the glutamate structure that has agonist activity in cell lines expressing low levels of mGlu₅. To determine whether VU0424465 possesses agonist activity in native systems, we assessed its ability to evoke calcium mobilization responses in rat cortical astrocytes and established electrophysiological responses to mGlu₅ activation in rat brain slices. In contrast to previously characterized mGlu₅ PAMs, VU0424465 alone induced a small, but significant, concentration-dependent increase in calcium mobilization in rat cortical astrocytes (data not shown). The VU0424465 maximal response was $16 \pm 0.8\%$ ($p < 0.05$) of the glutamate maximal response, suggesting that VU0424465 is a partial agonist in this system.

A well-characterized response to mGlu₅ activation in the CNS is mGlu₅ agonist-induced long-term depression (LTD) of transmission at the Schaffer collateral – CA1 synapse in the hippocampus. Orthosteric agonists, such as DHPG (group I mGlu agonist), evoke a prolonged LTD response. In contrast, previously evaluated mGlu₅ PAMs do not induce LTD alone but potentiate the response to DHPG (5, 9). Unlike previous PAMs, treatment with VU0424465 (10 μ M) alone induced a pronounced LTD response, with depression of the field excitatory postsynaptic potential (fEPSP; $38.3 \pm 8.7\%$ of baseline) that persisted for at least 55 min after compound washout (Figure 3A). DHPG and VU0424465 induced maximum depressions of synaptic responses to -0.7 ± 1.3 and 38.0 ± 3.4 percent of baseline fEPSPs, respectively. Thus, VU0424465 exhibits strong agonist activity in hippocampal slices (Figure 3B). In contrast, addition of the pure mGlu₅ PAM VU0361747 (9) alone had no effect on fEPSP slope ($103.0 \pm 2.0\%$ of baseline; Figure 3B), consistent with our previous report (9).

VU0424465-induced epileptiform activity in area CA3 in the rat hippocampus

The agonist activity of VU0424465 observed in astrocytes and induction of hippocampal LTD is the first demonstration that a compound classified as an agonist-PAM in *in vitro* assays has agonist activity in native CNS preparations. For PAMs targeting mGlu₅, this may be especially important since orthosteric agonists of group I mGlu, such as DHPG, can induce epileptiform seizure activity in hippocampal slices (29–31). Thus, we performed studies to determine whether VU0424465 can induce epileptiform activity in CA3 pyramidal neurons in hippocampal slices in a manner similar to that seen with non-selective group I mGlu receptor agonists. In parallel, VU0361747, a pure mGlu₅ PAM with no agonist activity in any recombinant or native system was evaluated (9). Consistent with previous reports, application of 50 μ M DHPG induced robust epileptiform activity, as evidenced by the decreased inter-event interval of spontaneous field population spikes observed utilizing field potential recordings in the pyramidal cell body layer of area CA3 in hippocampal slices ($42.9 \pm 4.6\%$ of baseline inter-event interval). DHPG had no significant effect on the amplitude of population spikes ($125.6 \pm 17.2\%$ of baseline). Interestingly, application of VU0424465 (10 μ M) also induced robust epileptiform activity with a decrease in the inter-event interval ($51.7 \pm 7.8\%$ of baseline) and no effect on amplitude ($105.9 \pm 3.7\%$ of baseline) of spontaneous population spikes similar to that observed with DHPG. In contrast, treatment of slices with 10 μ M of the pure PAM, VU0361747, had no significant effect on either the inter-event interval ($86.5 \pm 11.7\%$ of baseline) or amplitude ($99.4 \pm 3.9\%$ of baseline) of spontaneous firing (Figure 4).

To confirm VU0424465 effects were mediated by mGlu₅, we determined the effect of the mGlu₅ allosteric antagonist MTEP on the response to VU0424465. Slices were treated with MTEP (10 μ M) for 10 min prior to the co-addition of 10 μ M MTEP and 10 μ M VU0424465 for an additional 10 min. In the presence of MTEP, VU0424465 had no significant effect on the inter-event interval (98.8 ± 11.8 % of baseline) or amplitude (106.8 ± 10.8 % of baseline) of spontaneous population spikes in area CA3 (Figure 4). When MTEP was added alone, a slight increase in the inter-event interval was observed (143.0 ± 14.4 % baseline), with no effect on amplitude (100.1 ± 5.4 % of baseline) of spontaneous firing. Collectively, these results suggest that VU0424465 induces epileptiform activity in CA3 pyramidal neurons and this activity is dependent on agonism of mGlu₅.

Seizure activity and behavioral convulsions in rats following administration of VU0424465

In addition to their ability to induce epileptiform activity in hippocampal slices, previous studies reveal that intracerebroventricular injection of Group I mGlu agonists such as DHPG can induce behavioral convulsions in rats (15, 16). Given that VU0424465 has mGlu₅ agonist activity and induces epileptiform activity in slices, we assessed the ability of VU0424465 to induce behavioral convulsions in rats. Male Sprague Dawley rats (275–300g) were administered VU0424465 (0.1 – 10 mg/kg, i.p., 10% Tween 80) and behavioral responses were monitored continuously for 2 hr. Behavioral convulsions were measured using a modified Racine score (0–5). Systemic administration of VU0424465 induced a dose-dependent increase in observed behavioral manifestations of seizure activity with peak responses observed approximately 1 hr after injection (Figure 5). A dose-response analysis revealed that measurable responses (stage 1 on Racine scale) could be observed with doses as low as 0.1 mg/kg (i.p.) and that 3 mg/kg VU0424465 was capable of inducing fully generalized stage 5 behavioral convulsions (Figure 5). In contrast, administration of a much higher dose of the pure PAM VU0361747 (56.6 mg/kg, i.p.) induced no measureable behavioral disturbances at any time point (Figure 5). The mGlu₅ allosteric antagonist, MTEP (3 or 10 mg/kg, i.p.) induced a dose-dependent blockade of the behavioral response to VU0424465 (10 mg/kg) with complete blockade of VU0424465-induced behavioral convulsions at 10 mg/kg MTEP (Supplemental Figure S3). These data are consistent with the *in vitro* data and suggest that the behavioral effects are mediated by activation of mGlu₅. Consistent with these behavioral manifestations, continuous video-electroencephalographic (EEG) monitoring from a cortical surface electrode revealed that 10 mg/kg VU0424465 induced intense behavioral and generalized electrographic seizure activity developing into electrographic status epilepticus (Figure 6B). In contrast 56.6 mg/kg VU0361747 did not induce any measureable behavioral or electrographic response (Figure 6C). These data do not rule out the possibility that VU0361747 induces focal seizure activity in another brain region not detected by the cortical electrode. However, they provide clear evidence that this pure PAM does not induce generalized seizure activity that manifest as behavioral convulsions.

To assess the brain exposure of VU0424465 and VU0361747 after systemic administration, brains were obtained at 2 hr from animals receiving 10 mg/kg VU0424465 and 56.6 mg/kg VU0361747 and analyzed for unbound drug concentrations. The free fraction of VU0424465, as determined by brain homogenate binding, is 0.007. A 10 mg/kg dose of VU0424465, which induces seizure activity *in vivo* and maximal behavioral convulsions, corresponded to an unbound brain concentration of VU0424465 of 22.0 ± 1.5 nM (Supplemental Figure S4). Interestingly, the unbound brain concentration of VU0361747 achieved following administration was more than 50 times higher (1107.2 ± 34.1 nM; Supplemental Figure S3) than those following administration of VU0424465 with the fraction unbound in the brain being 0.0956 as previously reported (9). Thus, the pure mGlu₅ PAM did not induce observable seizure activity or behavioral convulsions despite achieving

high brain concentrations well above those required for maximal potentiation of mGlu₅ responses and that are 50 times higher than those of VU0424465 that induce maximal fully generalized seizure activity.

Discussion

Positive allosteric modulation of mGlu₅ has emerged as an exciting new therapeutic strategy for major brain disorders, including schizophrenia, cognitive impairments, and some genetic childhood developmental disorders and they are being rapidly advanced in drug development efforts (1–4). It has been postulated that targeting allosteric sites to avoid direct activation of the receptor could lower the risk of adverse effects that may occur than with agonist-induced overstimulation. This may be especially important for mGlu₅ activators since previous studies suggest that agonists of group I mGlu₅ induce seizure activity resulting in excitotoxicity and neuronal damage (15, 32). Previous antagonist studies suggest that mGlu₅ activation participates in the induction of seizure activity (16). Additionally, multiple genetic developmental disorders involving epilepsy implicate disruptions in mGlu₅ signaling as a possible cause of pathogenesis (2). Therefore, seizure activity is an adverse effect that might be expected for a traditional mGlu₅ agonist but hoped to be avoided with allosteric modulation. Utilizing pharmacological and mutagenesis techniques, the present studies demonstrate that some mGlu₅ PAMs can act as functional agonists via an allosteric binding site on mGlu₅. This agonist activity is observed in cell lines with low mGlu₅ expression levels similar to receptor densities observed in native preparations, as well as in multiple native systems, suggesting the agonist activity is not an artifact of mGlu₅ over expression in cell-based systems. Furthermore, differences in the *in vitro* profile of different mGlu₅ PAMs can translate into fundamentally different *in vivo* effects, with mGlu₅ allosteric agonists inducing intense seizure activity and behavioral convulsions. This provides the first direct evidence that pure allosteric potentiators that do not possess agonist activity in either *in vitro* systems or *in vivo* can avoid severe adverse effects observed with agonists. These results suggest that the presence or absence of allosteric agonist activity in a given mGlu₅ PAM series could be a major differentiating factor for novel mGlu₅ PAMs in terms of adverse effect liability.

Another key finding is that minor alterations to the chemical structure of pure mGlu₅ PAMs results in a “molecular switch”, changing the *in vitro* profile of the compound from pure potentiation of orthosteric agonist activation to robust allosteric agonist activity. Such molecular switches in the *in vitro* efficacy profile of a series of mGlu₅ PAMs can introduce major complications in drug discovery efforts to optimize novel efficacious compounds with the absence of adverse effect liability. These results illustrate the need to employ cell lines that express relatively low mGlu₅ levels to allow unambiguous identification of allosteric agonists versus pure PAMs in discovery efforts. While preliminary studies suggest that VU0422465 is highly metabolically stable and does not appear to generate active metabolites, it is conceivable that structural changes through hepatic metabolism could yield metabolites that have different pharmacological effects from the parent compound. In addition to molecular switches from PAM to allosteric agonist shown here, we have previously reported closely related compounds can display PAM versus NAM activity (33). Thus, it is important to carefully monitor and evaluate the activities of major metabolites of parent mGlu₅ PAMs to ensure the parent compound is responsible for primary *in vivo* effects observed. Finally, an important consideration is that some patient populations may be especially sensitive to mGlu₅ PAMs. For instance, mGlu₅ PAMs have been raised as a possible approach to treatment of tuberous sclerosis (2), a genetic childhood developmental disorder. One prominent symptom of tuberous sclerosis is epileptic seizure activity. While the present results provide a possible path that may avoid exacerbation of seizure activity in treating these patients, it is conceivable that pure PAMs will have effects in patient

populations that have underlying epileptic disorders that are not observed in the majority of patients. Thus, it will be critical to carefully evaluate multiple properties of mGlu₅ PAMs, including metabolites and possible adverse effect liability in specific patient populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

JMR and MJN contributed equally to the work included in the manuscript. The authors would like to thank Kiran Gogi and Ryan Morrison for their technical expertise. This work is supported by the National Institutes Health (R01 MH062646, R01 MH074953, U01 MH087965 (PJC); U54 MH084659 (CWL); F32 MH088234 (JMR); F32 NS071746 (MJN)) and Johnson & Johnson. Infrastructure for the EEG recordings from the Dudek laboratory (WP and FED) was supported by the CounterACT Program, National Institutes of Health Office of the Director (NIH OD), and the National Institute of Neurological Disorders and Stroke (NINDS), Grant Number NO1-NS-4-2359.

References

1. Conn PJ, Christopoulos A, Lindsley CW. Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat Rev Drug Discov*. 2009; 8:41–54. [PubMed: 19116626]
2. Auerbach BD, Osterweil EK, Bear MF. Mutations causing syndromic autism define an axis of synaptic pathophysiology. *Nature*. 2011; 480:63–68. [PubMed: 22113615]
3. Conn PJ, Lindsley CW, Jones CK. Activation of metabotropic glutamate receptors as a novel approach for the treatment of schizophrenia. *Trends Pharmacol Sci*. 2009; 30:25–31. [PubMed: 19058862]
4. Krueger DD, Bear MF. Toward fulfilling the promise of molecular medicine in fragile X syndrome. *Annu Rev Med*. 2011; 62:411–429. [PubMed: 21090964]
5. Ayala JE, Chen Y, Banko JL, Sheffler DJ, Williams R, Telk AN, et al. mGluR5 positive allosteric modulators facilitate both hippocampal LTP and LTD and enhance spatial learning. *Neuropsychopharmacology*. 2009; 34:2057–2071. [PubMed: 19295507]
6. Darrah JM, Stefani MR, Moghaddam B. Interaction of N-methyl-D-aspartate and group 5 metabotropic glutamate receptors on behavioral flexibility using a novel operant set-shift paradigm. *Behav Pharmacol*. 2008; 19:225–234. [PubMed: 18469540]
7. Kinney GG, O'Brien JA, Lemaire W, Burno M, Bickel DJ, Clements MK, et al. A novel selective positive allosteric modulator of metabotropic glutamate receptor subtype 5 has in vivo activity and antipsychotic-like effects in rat behavioral models. *J Pharmacol Exp Ther*. 2005; 313:199–206. [PubMed: 15608073]
8. Liu F, Grauer S, Kelley C, Navarra R, Graf R, Zhang G, et al. ADX47273 [S-(4-fluoro-phenyl)-{3-[3-(4-fluoro-phenyl)-[1,2,4]-oxadiazol-5-yl]-piperidin-1-yl}-methanone]: a novel metabotropic glutamate receptor 5-selective positive allosteric modulator with preclinical antipsychotic-like and procognitive activities. *J Pharmacol Exp Ther*. 2008; 327:827–839. [PubMed: 18753411]
9. Noetzel MJ, Rook JM, Vinson PN, Cho HP, Days E, Zhou Y, et al. Functional impact of allosteric agonist activity of selective positive allosteric modulators of metabotropic glutamate receptor subtype 5 in regulating central nervous system function. *Mol Pharmacol*. 2012; 81:120–133. [PubMed: 22021324]
10. Rodriguez AL, Grier MD, Jones CK, Herman EJ, Kane AS, Smith RL, et al. Discovery of novel allosteric modulators of metabotropic glutamate receptor subtype 5 reveals chemical and functional diversity and in vivo activity in rat behavioral models of anxiolytic and antipsychotic activity. *Mol Pharmacol*. 2010; 78:1105–1123. [PubMed: 20923853]
11. Conn, PJ.; Stauffer, SR.; Zhou, S.; Manka, J.; Williams, R.; Noetzel, MJ.; Gregory, KJ.; Vinson, P.; Niswender, CM.; Jones, CK.; Steckler, T.; MacDonald, G.; Lindsley, CW. Allosteric modulators of metabotropic glutamate receptor 5 for treatment of Schizophrenia. 7th International Metabotropic Glutamate Receptors Meeting; Oct 6, 2011; Taormina, Italy. 2011.

12. Lindsley, CW. 'Molecular switches' on mGluR allosteric ligands that modulate modes of pharmacology and/or subtype selectivity. 7th International Metabotropic Glutamate Receptors Meeting; Oct 5, 2011; Taormina, Italy. 2011.
13. Lindsley, CW.; MacDonald, G. Development of novel agents for the treatment of schizophrenia. 243rd ACS National Meeting; San Diego, CA. March 25, 2012; 2012. p. Abstract MEDI-443
14. Parmentier-Batteur, S.; Hutson, PH.; Menzel, K.; Mattson, BA.; Uslander, JH.; Forest, T.; Zhang, XF.; O'Brien, JA.; Magliaro, BC.; Huszar, SL.; Hugues, A.; Lodge, KE.; Jacobson, MA.; Stump, CA.; Tynebor, RM.; Anthony, NJ.; Williams, TA. Preclinical profile of a novel metabotropic glutamate receptor 5 positive allosteric modulator: from efficacy to mechanism-based neurotoxicity. 7th International Metabotropic Glutamate Receptors Meeting; Oct 5, 2011; Taormina, Italy. 2011.
15. Tizzano JP, Griffey KI, Schoepp DD. Receptor subtypes linked to metabotropic glutamate receptor agonist-mediated limbic seizures in mice. *Ann N Y Acad Sci.* 1995; 765:230–235. discussion 248. [PubMed: 7486609]
16. Wong RK, Bianchi R, Chuang SC, Merlin LR. Group I mGluR-induced epileptogenesis: distinct and overlapping roles of mGluR1 and mGluR5 and implications for antiepileptic drug design. *Epilepsy Curr.* 2005; 5:63–68. [PubMed: 16059439]
17. Stauffer, SR. Identification of allosteric agonists of mGlu5: a mechanism for adverse effect liability and implications for potentiator scaffold selection and design. 243rd ACS National Meeting; San Diego, CA. 2012. p. Abstract MEDI-25
18. Williams R, Manka JT, Rodriguez AL, Vinson PN, Niswender CM, Weaver CD, et al. Synthesis and SAR of centrally active mGlu5 positive allosteric modulators based on an aryl acetylenic bicyclic lactam scaffold. *Bioorg Med Chem Lett.* 2011; 21:1350–1353. [PubMed: 21315585]
19. Rodriguez AL, Nong Y, Sekaran NK, Alagille D, Tamagnan GD, Conn PJ. A close structural analog of 2-methyl-6-(phenylethynyl)-pyridine acts as a neutral allosteric site ligand on metabotropic glutamate receptor subtype 5 and blocks the effects of multiple allosteric modulators. *Mol Pharmacol.* 2005; 68:1793–1802. [PubMed: 16155210]
20. Cosford ND, Tehrani L, Roppe J, Schweiger E, Smith ND, Anderson J, et al. 3-[(2-Methyl-1,3-thiazol-4-yl)ethynyl]-pyridine: a potent and highly selective metabotropic glutamate subtype 5 receptor antagonist with anxiolytic activity. *J Med Chem.* 2003; 46:204–206. [PubMed: 12519057]
21. Gregory KJ, Noetzel MJ, Rook JM, Vinson PN, Stauffer SR, Rodriguez AL, et al. Investigating mGlu5 Allosteric Modulator Cooperativity, Affinity and Agonism: Enriching Structure-function Studies and Structure-activity Relationships. *Mol Pharmacol.* 2012
22. Lehmkuhle MJ, Thomson KE, Scheerlinck P, Pouliot W, Greger B, Dudek FE. A simple quantitative method for analyzing electrographic status epilepticus in rats. *J Neurophysiol.* 2009; 101:1660–1670. [PubMed: 19129295]
23. Racine RJ. Modification of seizure activity by electrical stimulation. II. Motor seizure. *Electroencephalogr Clin Neurophysiol.* 1972; 32:281–294. [PubMed: 4110397]
24. Chen Y, Nong Y, Goudet C, Hemstapat K, de Paulis T, Pin JP, et al. Interaction of novel positive allosteric modulators of metabotropic glutamate receptor 5 with the negative allosteric antagonist site is required for potentiation of receptor responses. *Mol Pharmacol.* 2007; 71:1389–1398. [PubMed: 17303702]
25. Won H, Lee HR, Gee HY, Mah W, Kim JI, Lee J, et al. Autistic-like social behaviour in Shank2-mutant mice improved by restoring NMDA receptor function. *Nature.* 2012; 486:261–265. [PubMed: 22699620]
26. Wellendorph P, Brauner-Osborne H. Molecular basis for amino acid sensing by family C G-protein-coupled receptors. *Br J Pharmacol.* 2009; 156:869–884. [PubMed: 19298394]
27. Pagano A, Ruegg D, Litschig S, Stoehr N, Stierlin C, Heinrich M, et al. The non-competitive antagonists 2-methyl-6-(phenylethynyl)pyridine and 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester interact with overlapping binding pockets in the transmembrane region of group I metabotropic glutamate receptors. *J Biol Chem.* 2000; 275:33750–33758. [PubMed: 10934211]
28. Chen Y, Goudet C, Pin JP, Conn PJ. N-{4-Chloro-2-[(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)methyl]phenyl}-2-hydroxybenzamide (CPPHA) acts through a novel site as a positive

- allosteric modulator of group 1 metabotropic glutamate receptors. *Mol Pharmacol*. 2008; 73:909–918. [PubMed: 18056795]
29. Bianchi R, Chuang SC, Zhao W, Young SR, Wong RK. Cellular plasticity for group I mGluR-mediated epileptogenesis. *J Neurosci*. 2009; 29:3497–3507. [PubMed: 19295155]
30. Merlin LR, Bergold PJ, Wong RK. Requirement of protein synthesis for group I mGluR-mediated induction of epileptiform discharges. *J Neurophysiol*. 1998; 80:989–993. [PubMed: 9705485]
31. Zhao W, Bianchi R, Wang M, Wong RK. Extracellular signal-regulated kinase 1/2 is required for the induction of group I metabotropic glutamate receptor-mediated epileptiform discharges. *J Neurosci*. 2004; 24:76–84. [PubMed: 14715940]
32. Conn PJ, Pin JP. Pharmacology and functions of metabotropic glutamate receptors. *Annu Rev Pharmacol Toxicol*. 1997; 37:205–237. [PubMed: 9131252]
33. Stauffer SR. Progress toward Positive Allosteric Modulators of the Metabotropic Glutamate Receptor Subtype 5 (mGlu5). *ACS Chem Neurosci*. 2011; 2:450–470. [PubMed: 22860171]

Figure 1.

VU0424465, an mGlu₅ positive allosteric modulator optimized for direct agonist activity, induces calcium mobilization in the absence of glutamate. (A) Representative raw calcium traces following the addition of 30 μ M VU0424465 (compound; 3–143 seconds; agonist) and followed by an EC₂₀ of glutamate (143–240 s); PAM) and EC₈₀ of glutamate (240–350 seconds); control trace represents the response to an EC₂₀ and EC₈₀ of glutamate following the addition of vehicle alone. Static ratio represents relative fluorescence units normalized to initial values. VU0424465, but not VU0361747, clearly induces a concentration-dependent release of calcium when added alone. Potencies were determined by adding a concentration-response curve of VU0424465 (B) followed by an EC₂₀ of glutamate (PAM) or (C) in the absence of glutamate (agonist). VU0424465 potentiated the calcium mobilization evoked by glutamate in a concentration-dependence manner. The PAM EC₅₀ of VU0424465 is 1.5 nM, while the agonist potency is 171 nM with a 65% maximum glutamate response. Data represent the mean $\pm$ S.E.M. of 5 independent experiments performed in duplicate. For these studies, rat mGlu₅-expressing cell lines were utilized for full *in vitro* characterization of novel compounds to correlate *in vitro* data to *in vivo* studies in rats; similar potency/efficacy profiles were observed in human mGlu₅-expressing cell lines.

Figure 2.

VU0424465-induced agonist activity is mediated via the mGlu₅ allosteric MPEP site. **(A)** VU0424465-induced calcium mobilization was not inhibited by 1 mM LY341495, an mGlu₅ orthosteric antagonist (mGlu₅ IC₅₀ 8.2 μ M). Increasing concentrations of 5MPEP, a neutral MPEP site ligand, induced a parallel rightward shift in the VU0424465 agonist concentration-response curve with no effect on the maximum response, suggesting a competitive interaction of VU0424465 with the allosteric mGlu₅ MPEP site. **(B)** Multiple single point mutations that eliminate calcium mobilization by the orthosteric agonists, glutamate and DHPG, did not abolish VU0424465-induced agonist activity. **(C)** Introduction of the single point mutation A809V, which abolishes activity at the mGlu₅ allosteric MPEP-site, eliminated agonist activity evoked by VU0424465. This mutation had no effect on orthosteric agonist activity of glutamate or DHPG (data not shown). The single point mutation, F585I, known to inhibit activity of the allosteric PAM CPPHA, had no effect on VU0424465 agonist activity. Data represent the mean $\pm$ S.E.M. of 3 independent experiments performed in duplicate.

Figure 3.

VU0424465 demonstrates true agonist activity in native systems. **(A)** Bath application of 10 μ M VU0424465 (solid line) for 10 minutes resulted in the induction of LTD measured 55 minutes after compound washout ($n=6$). **(B)** Maximum suppression of the fEPSP response measured as percent baseline. DHPG and VU0361747 were quantified from Noetzel et. al., 2012 in the absence of glutamate (agonist) (9). Data represent mean $\pm$ S.E.M.

Figure 4.

The mGlu₅ agonist-PAM VU0424465, but not the pure PAM VU0361747, induces epileptiform activity in CA3 in the hippocampal formation. Inter-event interval and amplitude of spontaneous firing were measured using field recordings in CA3 of the hippocampal formation. (A) Application of 50 μ M DHPG or 10 μ M VU0424465 for 10 minutes induced a robust decrease in the inter-event interval of spontaneous firing while having no significant effect on the amplitude ($n=8$). In contrast, application of 10 μ M VU0361747 had no effect on either the inter-event interval or amplitude of spontaneous firing ($n=8$). In the presence of 10 μ M MTEP, 10 μ M VU0424465 had no effect on the inter-event interval or amplitude of spontaneous firing ($n=7$). Error bars represent S.E.M. (B) Sample traces taken pre-drug (control) and after administration of compound. (C) Cumulative probability plots for the compounds of interest.

Figure 5.

The mGlu₅ agonist-PAM VU0424465, but not the pure PAM VU0361747, induces dose-dependent increases in behavioral convulsions. Rats treated with increasing doses of VU0424465 (0.1 – 10 mg/kg) exhibited dose-dependent seizure activity as measured by the modified Racine scale (0–5). Rats receiving a high dose of VU0361747 (56.6 mg/kg) did not display observable behavioral seizure activity. Compounds were diluted in 10% Tween 80 (pH 7.0, 1 mg/3 ml) and injected intraperitoneally. Data represent mean ± S.E.M (n=5).

Figure 6.

Representative cortical EEG recordings from (A) vehicle, (B) VU0424465 and (C) VU0361747-treated rats demonstrate that only the agonist-PAM VU0424465 induces epileptiform activity. (1–3) Compounds were diluted in 10% Tween 80 (pH 7.0, 1 mg/3 ml) and injected intraperitoneally. Traces below are temporally expanded portions of the EEG recording located within the boxes. Both behavioral and electrographic seizures were observed in VU0424465-treated animals (n=4). Seizure activity was quantified by the analysis of the rate of EEG spiking. The * denotes the time of drug administration. The arrow indicates the occurrence of a behavioral seizure with no EEG correlate. The # designates changes in the EEG due to movement artifact. (D) Comparison of spike rate (mean + 95% CI) between vehicle and VU0424465-treated animals revealed that after 4 h, EEG spiking greatly increased in the VU0424465-treated animals. (E) Comparison of spike rate (mean + 95% CI) showed that no difference in EEG spiking between vehicle and VU0361747-treated animals was observed (n=4). ^ $p < 0.05$, two-tailed t-test.