Differential activity of $mGlu_7$ allosteric modulators provides evidence for $mGlu_{7/8}$ heterodimers at hippocampal Schaffer collateral-CA1 synapses

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Glutamate acts at eight metabotropic glutamate (mGlu) receptor subtypes expressed in a partially overlapping fashion in distinct brain circuits. Recent evidence indicates that specific mGlu receptor protomers can heterodimerize and that these heterodimers can exhibit different pharmacology when compared to their homodimeric counterparts. Group III mGlu agonist-induced suppression of evoked excitatory potentials and induction of long-term potentiation at Schaffer collateral-CA1 (SC-CA1) synapses in the rodent hippocampus can be blocked by the selective $mGlu_7$ negative allosteric modulator (NAM), ADX71743. Curiously, a different $mGlu_7$ NAM, 6-(4-methoxyphenyl)-5-methyl-3-pyridin-4-ylisoxazonolo[4,5-c]pyridin-4(5H)-one, failed to block these responses in brain slices despite its robust activity at $mGlu_7$ homodimers *in vitro*. We hypothesized that this might result from heterodimerization of $mGlu_7$ with another mGlu receptor protomer and focused on $mGlu_8$ as a candidate given the reported effects of $mGlu_8$-targeted compounds in the hippocampus. Here, we used complemented donor acceptor-resonance energy transfer to study $mGlu_{7/8}$ heterodimer activation *in vitro* and observed that ADX71743 blocked responses of both $mGlu_{7/7}$ homodimers and $mGlu_{7/8}$ heterodimers, whereas 6-(4-methoxyphenyl)-5-methyl-3-pyridin-4-ylisoxazonolo[4,5-c]pyridin-4(5H)-one only antagonized responses of $mGlu_{7/7}$ homodimers. Taken together with our electrophysiology observations, these results suggest that a receptor with pharmacology consistent with an $mGlu_{7/8}$ heterodimer modulates the activity of SC-CA1 synapses. Building on this hypothesis, we identified two additional structurally related $mGlu_7$ NAMs that also differ in their activity at $mGlu_{7/8}$ heterodimers, in a manner consistent with their ability to inhibit synaptic transmission and plasticity at SC-CA1. Thus, we propose that $mGlu_{7/8}$ heterodimers are a key molecular target for modulating the activity of hippocampal SC-CA1 synapses.

Glutamate, the major excitatory neurotransmitter in the brain, acts at eight metabotropic glutamate (mGlu) receptors, all belonging to the G protein-coupled receptor (GPCR) family. These eight receptors are divided into three major groups based on sequence similarity, G protein coupling, and shared pharmacology (1, 2). The mGlu receptors are organized as disulfide-linked homodimers that can signal both in *cis* and in *trans* (3, 4); when co-expressed in heterologous cells, group I mGlu receptor ($mGlu_{1\ and\ 5}$) protomers can form heterodimers with each other (5) and group II ($mGlu_{2\ and\ 3}$) and III ($mGlu_{4,\ 6,\ 7\ and\ 8}$) can form heterodimers within and between groups (5–7), eliciting dramatic effects on receptor pharmacology (7–9). As expression of these receptors often overlaps in certain cell types in the brain, the significance of mGlu receptor heterodimerization on cellular physiology and pharmacology has emerged as a critical question for the mGlu receptor field. The observations that ligands can differentiate between homodimers and heterodimers and show distinct effects in different brain regions suggest that understanding mGlu heterodimer expression and pharmacology will provide opportunities for precision medicine approaches, wherein specific circuits might be selectively targeted for therapeutic purposes.

Much of the work to date on mGlu receptor heterodimers has focused on $mGlu_{2/4}$ heterodimers (6–10). In our evaluations of the pharmacology of structurally distinct mGlu receptor positive allosteric modulators (PAMs) initially identified as having activity at $mGlu_4$ receptor homodimers, we found that certain $mGlu_4$ PAMs potentiated responses at corticostriatal synapses, whereas others did not (7, 11). Since $mGlu_2$ is co-expressed with $mGlu_4$ at these synapses (12, 13), we hypothesized that differential PAM activity at $mGlu_{4/4}$

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homodimers and mGlu$_{2/4}$ heterodimers might underlie these electrophysiology results (7, 14). To test our hypothesis, we used complemented donor acceptor-resonance energy transfer (CODA-RET), an *in vitro* technique developed in our laboratory, to selectively measure signaling by defined heterodimers without contamination by homodimers expressed in the same cells (15). CODA-RET revealed that mGlu$_4$ PAMs were segregated into two categories: those that can enhance activity of the mGlu$_{2/4}$ heterodimer, and those that cannot (11), which mirrored their ability to potentiate responses at corticostriatal synapses (7). Furthermore, by pairing electrophysiological recordings at various cortical inputs with additional CODA-RET studies, we were able to establish a critical role for mGlu$_{2/4}$ heterodimers at projections from the thalamus to the medial prefrontal cortex (mPFC), but not at hippocampal–mPFC or amygdala–mPFC synapses (8), suggesting differences in expression of various homodimer and heterodimer pairs that could eventually be exploited therapeutically.

In addition to mGlu$_2$ and mGlu$_4$, Doumazane *et al.* (5) demonstrated that all group II and group III mGlu receptors can heterodimerize *in vitro*. The group III mGlu receptors primarily act as presynaptic autoreceptors and heteroreceptors (1); among this subgroup, mGlu$_7$ and mGlu$_8$ are co-expressed in many of the same brain regions, including the hippocampus (16). Using group III mGlu-specific agonists such as L-AP4, pharmacological profiles consistent with a role for both mGlu$_7$ and mGlu$_8$ at Schaffer collateral-CA1 (SC-CA1) synapses have been identified (17–19). In these studies, after L-AP4 application, field excitatory postsynaptic potentials (fEPSPs) were reduced, while paired pulse ratios increased, suggesting a presynaptic mechanism (17, 18, 20). Notably, of the other group III receptors, mGlu$_6$ expression is restricted to the retina (21), and although L-AP4 can activate mGlu$_4$, an mGlu$_4$-selective PAM and an mGlu$_4$-preferring agonist did not affect fEPSPs measured at SC-CA1 synapses (17). Additionally, use of a different and recently described group III agonist, LSP4-2022 (22), in a concentration range that is selective for mGlu$_4$ over mGlu$_7$ and mGlu$_8$, did not affect fEPSPs (18). While a group II mGlu receptor agonist can induce long-term potentiation (LTP) at SC-CA1 synapses, this effect has recently been shown to be mediated by a novel postsynaptic mechanism (23). Collectively, these results suggest that presynaptic responses at SC-CA1 synapses are most likely mediated by mGlu$_7$ and/or mGlu$_8$.

To explore this question further, we focused on an evaluation of multiple mGlu$_7$-selective negative allosteric modulators (NAMs) at SC-CA1 synapses (24–26). Two of these NAMs, ADX71743 and 6-(4-methoxyphenyl)-5-methyl-3-pyridin-4-ylisoxazonolo[4,5-c]pyridin-4(5H)-one (MMPIP), block responses in heterologous cells that express only mGlu$_7$ homodimers (24, 25), and ADX71743 has been reported to block agonist-mediated inhibition of fEPSPs at SC-CA1 synapses (25), an effect we replicated in the study by Klar *et al.* (18). In contrast, we showed that MMPIP did not block L-AP4-induced effect on fEPSPs at this same synapse (27). Here, we show that this divergence in activity between ADX71743 and MMPIP also extends to LTP. Using CODA-RET, we now show that ADX71743 blocks agonist-mediated responses at mGlu$_{7/8}$ heterodimers, whereas MMPIP is without effect. We extend these findings to two highly structurally related mGlu$_7$ NAMs, VU6010608 and VU6010953 (26, 28), which are structurally identical except for different alkoxy substitutions; these two compounds also show differential activity at mGlu$_7$ homodimers and mGlu$_{7/8}$ heterodimers that match their profiles in blocking LTP at SC-CA1 synapses. These studies suggest that the complexity of mGlu receptor assembly has widespread implications for receptor pharmacology and, by extension, therapeutic targeting.

Results

The mGlu$_7$ NAM MMPIP does not block LTP at SC-CA1

We have previously identified a divergence in pharmacology of two mGlu$_7$ NAMs, ADX71743 and MMPIP (Fig. 1*A*), in blocking L-AP4's effect on fEPSPs at SC-CA1 (18, 25, 27), despite both compounds blocking mGlu$_7$ homodimer-mediated responses *in vitro* (Fig. 1*B*). We have also previously shown that ADX71743 blocks the induction of LTP at SC-CA1 synapses (18). In contrast, but consistent with its lack of effect on L-AP4-mediated inhibition of fEPSPs (27), MMPIP does not block LTP at SC-CA1 (Fig. 1, *C* and *D*).

ADX71743 and MMPIP differentially inhibit mGlu$_{7/8}$ heterodimers

Given that ADX71743 and MMPIP share the ability to block the activity of mGlu$_7$ homodimers *in vitro*, it was unexpected to observe such distinct effects on electrophysiological measures at SC-CA1. To explore the potential for differential activity of the two NAMs at mGlu$_{7/7}$ homodimers and mGlu$_{7/8}$ heterodimers, we used CODA-RET (Fig. 2, *A*–*C*) (15). To carry out this assay, mGlu$_7$ and mGlu$_8$ were fused at their C-termini with split luciferase fragments. These two fragments (L1 and L2) are incapable of producing bioluminescence when expressed alone, but when brought into proximity, they complement to form a functional enzyme capable of generating bioluminescence (29, 30). By monitoring bioluminescence resonance energy transfer (BRET) between the complemented luciferase (donor), which identifies the pair of mGlu protomers, and monomeric Venus (mVenus) (acceptor)-labeled $G_{\alpha i}$ subunits, we can selectively measure signaling by defined heterodimers (Fig. 2*B*) or homodimers (Fig. 2, *A* and *C*). Using CODA-RET, we found that in cells expressing mGlu$_{7/7}$ homodimers, the potency of DL-AP4 was 100-fold lower than that observed for mGlu$_{7/8}$ heterodimers (Fig. 2, *D* and *E*). Furthermore, in cells expressing mGlu$_{7/7}$ homodimers, both ADX71743 and MMPIP antagonized agonist-induced responses (Fig. 2*D*). Conversely, in cells expressing mGlu$_{7/8}$ heterodimers, only ADX71743 blocked the CODA-RET signal (Fig. 2*E*). As expected, ADX71743 was inactive at mGlu$_{8/8}$ homodimers (Fig. 2*F*), consistent with its reported specificity for mGlu$_7$ (25).

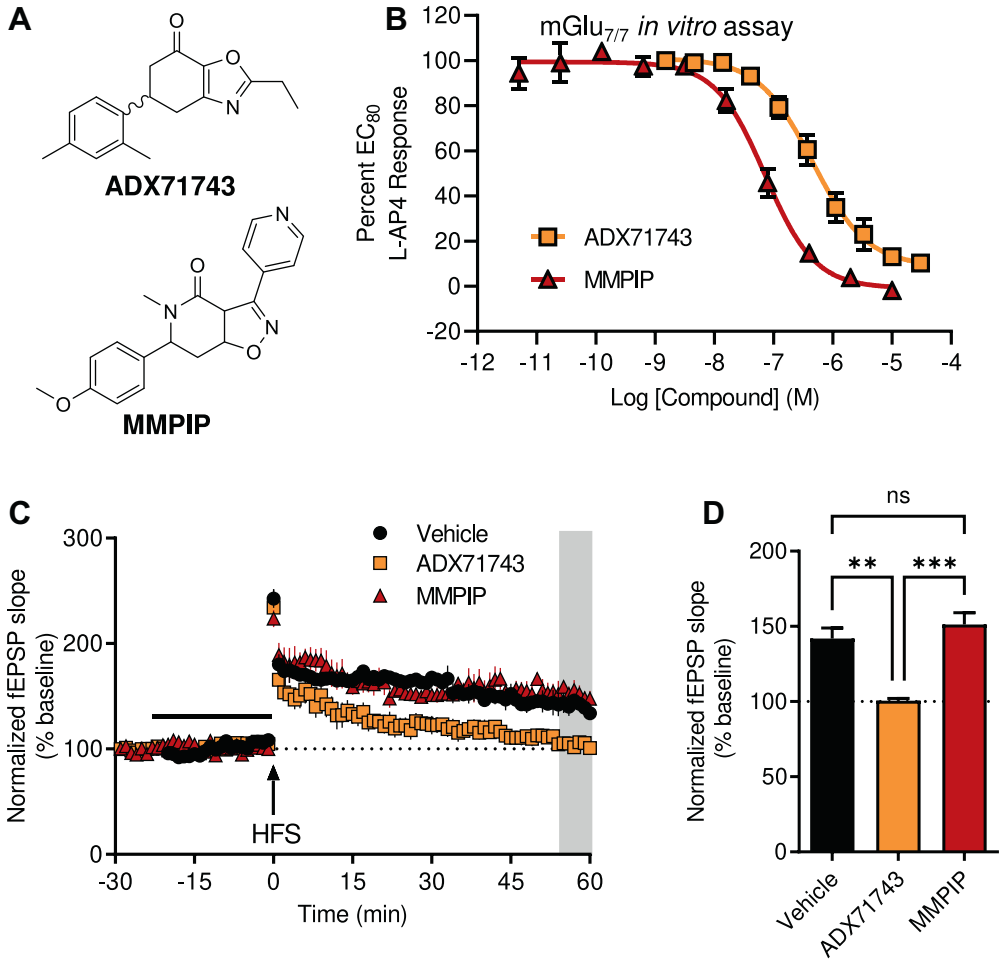

Figure 1. Despite robust blockade of mGlu$_{7/7}$ homodimer activity *in vitro*, MMPIP does not block LTP at SC-CA1 synapses. *A*, the structures of ADX71743 and MMPIP are shown. *B*, increasing concentrations of the mGlu$_7$ NAMs ADX71743 (*orange*) and MMPIP (*dark red*) were applied to HEK293 cells expressing rat mGlu$_7$ and the promiscuous G protein, G$_{\alpha 15}$. Both compounds were able to inhibit L-AP4-induced calcium responses with mean IC$_{50}$s of 460 and 72 nM, respectively (mean ± SEM, n = 5 or 3 independent determinations in triplicate, respectively). *C*, electrophysiology experiments showing the effect of vehicle (*black*), 3 μM ADX71743 (*orange*), or 10 μM MMPIP (*dark red*) applied to brain slices 20 min prior (*black bar*) to the induction of LTP at SC-CA1 synapses using an HFS protocol. Data represent the mean ± SEM of 3 to 5 slices per condition. Data for ADX71743 originally appeared in the study by Klar *et al.* (18). *D*, the average of the last 5 min of recording in C (*gray box*) is plotted for vehicle (*black*), ADX71743 (*orange*), and MMPIP (*dark red*). One-way ANOVA with Tukey's post hoc test, $^{**}p < 0.01$, $^{***}p < 0.001$. HFS, high-frequency stimulation; LTP, long-term potentiation; NAM, negative allosteric modulator; SC-CA1, Schaffer collateral-CA1.

Two highly similar NAMs, VU6010608 and VU6010953, show differential blockade of LTP at SC-CA1 synapses that is consistent with activity at mGlu$_{7/8}$ heterodimers

During our medicinal chemistry campaign to optimize allosteric modulators of mGlu$_7$, we recently identified VU6010608 and VU6010953, compounds that differ structurally by a single alkoxy substitution and possess highly similar *in vitro* profiles in cells expressing mGlu$_7$ homodimers (26, 28) (Fig. 3*A*). We have previously shown that VU6010608 blocks high-frequency stimulation (HFS)–induced LTP at SC-CA1 (26), and we show here that it was also effective in blocking LTP induced using an alternate stimulation protocol, theta burst stimulation (TBS) (Fig. 3, *B* and *C*). To our surprise, the highly related VU6010953 compound failed to block TBS-induced LTP at the same synapses (Fig. 3, *B* and *C*). An examination of the profile of these two compounds using CODA-RET showed that both completely blocked responses to mGlu$_{7/7}$ homodimers (Fig. 3*D*), but, like ADX71743 and MMPIP, they diverged in their activity at mGlu$_{7/8}$ heterodimers (Fig. 3*E*), with VU6010953 unable to inhibit activity of the heterodimer, consistent with its lack of effect on LTP. VU6010608 was inactive at mGlu$_{8/8}$ in CODA-RET (Fig. 3*F*), confirming its reported preference for mGlu$_7$ (26).

Discussion

Glutamate exerts critical actions at a variety of mGlu receptors that are differentially expressed in various circuits throughout the brain, making them highly attractive targets for novel therapeutics. However, such efforts can be complicated by the expression of the same receptor in multiple brain regions, making it challenging to avoid off-target effects. GPCR heterodimers have long been touted as potential targets to enhance the specificity of drug action, but there has been relatively little evidence for their expression *in vivo*. Emerging evidence for the expression and activity of mGlu$_{2/4}$ heterodimers at certain synapses, but not others (7–10, 31), has

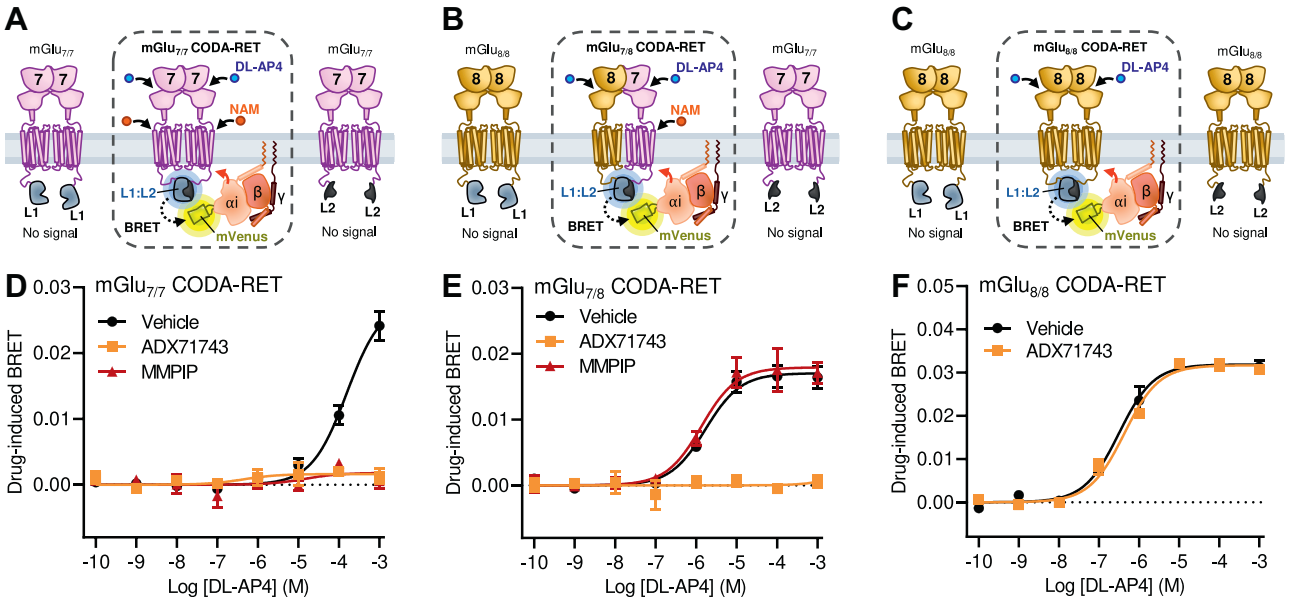

Figure 2. ADX71743 and MMPIP differentially inhibit mGlu$_{7/8}$ heterodimers. *A–C*, schematics of the CODA-RET approach. The defined (*B*) mGlu$_{7/8}$ heterodimer and (*A*) mGlu$_{7/7}$ and (*C*) mGlu$_{8/8}$ homodimers are shown with complemented split fragments (L1 and L2) of the luciferase donor (L1:L2), leading to luminescence and BRET-based CODA-RET *via* the G$_{\alpha i}$-fused mVenus upon receptor activation. As shown, homodimers that are formed by protomers fused to noncomplementing luciferase fragments will not luminesce and, therefore, do not contribute to the BRET signal. Note that while the split fragments are all conceptually denoted here as L1 or L2, we used split RLuc8 for (*A*) and (*B*) and split Nanoluc for (*C*), as described in Experimental procedures. *D–F*, CODA-RET results showing DL-AP4-concentration response curves in the presence of 50 μM of the indicated mGlu$_7$ NAMs for (*D*) mGlu$_{7/7}$ homodimers, (*E*) mGlu$_{7/8}$ heterodimers, and (*F*) mGlu$_{8/8}$ homodimers, respectively. Note that the mGlu$_7$ preferring NAM ADX71743 is active at mGlu$_{7/7}$ and mGlu$_{7/8}$ but inactive at mGlu$_{8/8}$, as expected, whereas MMPIP is only active at mGlu$_{7/7}$. Error bars represent the mean ± SEM for at least three independent experiments performed in triplicate. BRET, bioluminescence resonance energy transfer; CODA-RET, complemented donor acceptor-resonance energy transfer; NAM, negative allosteric modulator.

created an opportunity to differentiate homodimers and heterodimers pharmacologically, providing exciting precedent for this approach.

We show here that the pharmacology of select mGlu$_7$ receptor ligands at the SC-CA1 synapse is not consistent with that of an mGlu$_7$ homodimer, as ADX71743 inhibits both group III agonist-induced effects on fEPSPs and LTP, whereas MMPIP, which is a fully efficacious NAM at mGlu$_7$ homodimers *in vitro*, is completely without activity at SC-CA1 in brain slices. We hypothesized that this might result from heterodimerization of mGlu$_7$ with another presynaptic partner. mGlu$_6$ is restricted in expression to the retina (21), and, because the observed pharmacology in previous electrophysiology experiments argues against the involvement of mGlu$_4$ (17, 18), we turned to the other widely expressed presynaptic group III mGlu receptor, mGlu$_8$, as a potential mGlu$_7$ partner in the SC-CA1 area of the hippocampus. Given that the mGlu$_8$ receptor is expressed in the hippocampus and that the mGlu$_8$ agonist DCPG has been shown to act in this region (17, 19), we hypothesized that mGlu$_{7/8}$ heterodimerization might explain these native tissue findings. Our CODA-RET results were completely consistent with this hypothesis, as ADX71743 acted as a NAM at mGlu$_{7/7}$ and mGlu$_{7/8}$ heterodimers, whereas MMPIP was active at mGlu$_{7/7}$ but without effect at mGlu$_{7/8}$ receptors. Remarkably, we also found that members of a distinct structural scaffold could also differentiate mGlu$_{7/7}$ homodimers and mGlu$_{7/8}$ heterodimers, with VU6010953 inactive at mGlu$_{7/8}$ heterodimers as assessed by CODA-RET and ineffective at blocking LTP at SC-CA1 synapses. In contrast, VU6010608, which differs from VU6010953 only by a single alkoxy moiety, was active both *in vitro* at mGlu$_{7/8}$ heterodimers and in brain slices. That such a small difference in the structure of these NAMs produced such a profound change in their activity is quite extraordinary. The impact on mGlu receptor pharmacology controlled by a single alkoxy moiety suggests an enormous potential richness in the pharmacology of these targets, which must be explored by new, more focused structure–activity relationship studies as well as structural experiments comparing homodimeric and heterodimeric combinations to begin to understand how allosteric propagation of receptor activity can differ so profoundly between various receptor combinations. mGlu receptor compounds characterized to date have been identified by their activity at mGlu receptor homodimers. Thus, while compounds can be identified serendipitously as also active at mGlu receptor heterodimers as we have done here, by design, ligands will also be active at the receptor homodimer combination used for their original identification. Future efforts to identify heterodimer-selective compounds will require rescreening of existing libraries of compounds using, for example, a CODA-RET heterodimer configuration, and then counter-screening against homodimers to remove compounds that act at both.

Our findings strongly support the presence of mGlu$_{7/8}$ heterodimers in modulating activity at hippocampal SC-CA1 synapses. Our previous finding that mGlu$_7$ is required for the induction of LTP at these synapses (18) suggests the potential for an mGlu$_{7/8}$ heterodimer to contribute to hippocampal synaptic plasticity, learning, and memory. Historically,

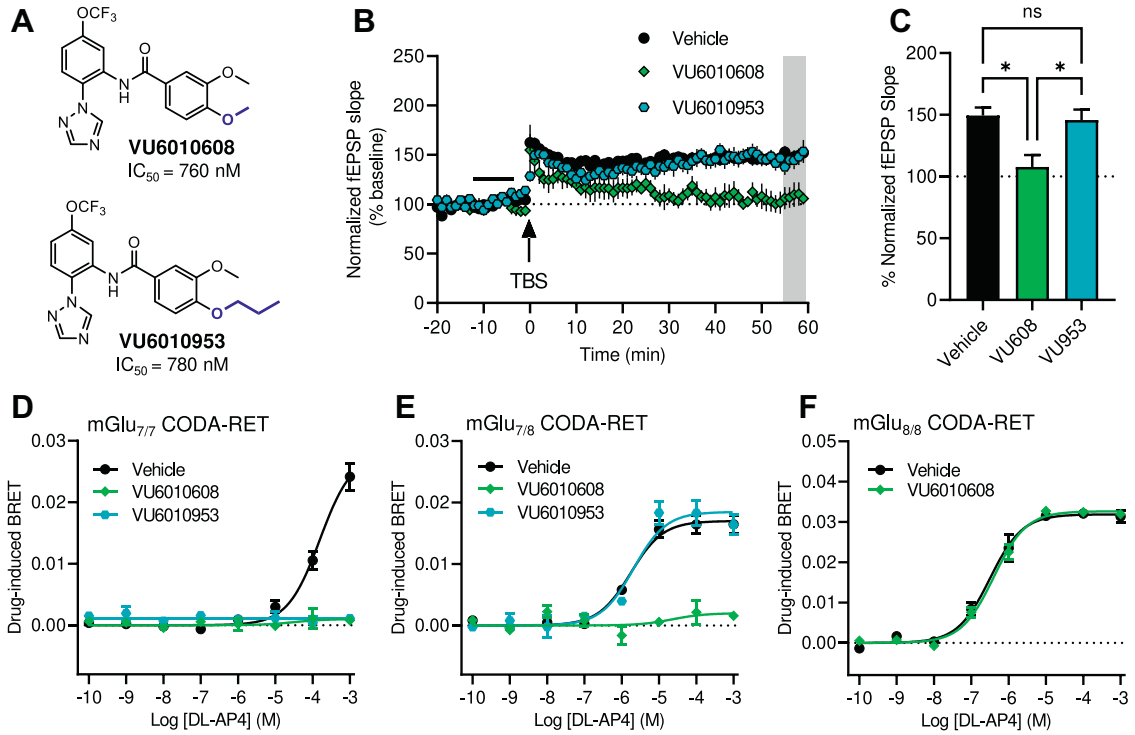

Figure 3. The NAMs VU6010608 and VU6010953 diverge in blocking responses at SC-CA1 synapses and at mGlu$_{7/8}$ heterodimers as assessed by CODA-RET. *A*, the structures of VU6010608 and VU6010953 are shown, with the different alkoxy substitutions shown in *blue* (VU6010608: methoxy; VU6010953: propoxy), along with their activities (IC_{50} values from references (26, 28)) in blocking rat mGlu$_7$-G$_{\alpha15}$-mediated calcium responses. *B*, electrophysiology experiments showing that bath application of 10 μM VU6010608 (*green diamonds*) to brain slices for 10 min prior to TBS (*black bar*) blocked LTP at SC-CA1 synapses compared to vehicle (*black circles*). In contrast, application of 30 μM VU6010953 (*cyan hexagons*) did not block TBS-induced LTP at the same synapses. *C*, the average of the last 5 min of recording in *B* (*gray box*) is plotted for vehicle (*black*), VU6010608 (VU608, *green*), and VU6010953 (VU953, *cyan*). Data represent the mean ± SEM of 4 to 15 slices per condition analyzed using one way ANOVA with a Tukey's post hoc test; *$p < 0.05$. *D–F*, CODA-RET results showing DL-AP4-concentration response curves in the presence of 50 μM of the indicated mGlu$_7$ NAMs for (*D*) mGlu$_{7/7}$ homodimers, (*E*) mGlu$_{7/8}$ heterodimers, and (*F*) mGlu$_{8/8}$ homodimers. Note that the NAM VU6010608 is active at mGlu$_{7/7}$ and mGlu$_{7/8}$ but inactive at mGlu$_{8/8}$, as expected, whereas VU6010953 is only active at mGlu$_{7/7}$. Error bars represent the mean ± SEM for at least three independent experiments performed in triplicate. CODA-RET, complemented donor acceptor-resonance energy transfer; LTP, long-term potentiation; NAM, negative allosteric modulator; SC-CA1, Schaffer collateral-CA1; TBS, theta burst stimulation.

mGlu$_7$ has been proposed to act as an "emergency brake" due to its low affinity for glutamate (1); the confirmation that mGlu$_7$-containing heterodimers exhibit dramatic left-shifts in agonist potency (32), however, suggests that this property may be specific to mGlu$_{7/7}$ homodimers. We and others have shown that mGlu$_7$ knockout animals, as well as animals modeling a loss-of-function mutation in mGlu$_7$ found in patients with neurodevelopmental disorders (33), exhibit seizures that involve the hippocampus (34–37). Moreover, an agonist with mGlu$_7$ activity has been shown to protect mice from the development and manifestation of seizures (38), and mGlu$_7$ activation or potentiation has been considered as a novel strategy for the treatment of intellectual disability and epilepsy (reviewed in the study by Fisher *et al.* (35)). It is also noteworthy that the NAM ADX71743 has been shown to elicit seizures in animals (39); in contrast, MMPIP does not exacerbate seizures induced by electrical shock or potentiate pentylenetetrazole-induced seizures (40). The intriguing observation that these two NAMs differ at the level of the mGlu$_{7/8}$ heterodimer suggests that future studies could explore the possibility that mGlu$_{7/8}$ heterodimers mediate seizure activity, requiring further evaluation of how reductions or loss of mGlu$_7$ in mice and humans causes seizure activity. Additionally, the finding that all of the group II and group III mGlu receptors can heterodimerize (5) suggests that it will now be essential to evaluate the profile of these two compounds, as well as other mGlu$_7$ PAMs and NAMs, at various heterodimeric combinations using CODA-RET to provide additional context to pharmacological profiles observed at native tissue locations in which mGlu$_7$ is co-expressed with other mGlu receptors. Based on our findings presented here, we anticipate that an evaluation of existing mGlu$_7$ and mGlu$_8$ orthosteric and allosteric ligands for activity at mGlu$_{7/8}$ heterodimers will shed new light on the ideal pharmacological profile of therapeutic candidates.

Experimental procedures

Compounds

L-AP4, DL-AP4, MMPIP, and glutamate were purchased from Tocris. LSP4-2022 and ADX71743 were synthesized in house using methods reported in the study by Klar *et al.* (18). VU06010608 and VU6010953 were synthesized in house using methods reported in the study by Reed *et al.* (26, 28).

Calcium assays

Calcium assays in which rat mGlu$_7$ was coupled to calcium mobilization *via* the promiscuous G protein G$_{\alpha15}$ were used to

determine *in vitro* potency and efficacy and were conducted as described in (18, 26, 41–43).

Construction and transfection of expression vectors for CODA-RET assays

cDNAs for rat $mGlu_7$ and $mGlu_8$ were N-terminally tagged with a hemagglutinin epitope tag using standard molecular biology procedures. cDNAs encoding the split fragments of *Renilla* Luciferase 8, L1 (residues 1–229), or L2 (residues 230–311), were fused in frame to the C-terminus of $mGlu_7$ and $mGlu_8$ following the linker "GSPPARAT" in the pcDNA3.1 vector. (RLuc8 was a gift from Sam Gambhir, Stanford.) cDNAs encoding the split fragments of Nano luciferase (Promega), LgBit (residues 1–158) or HiBit (residues 159–169: VSGWRLFKKIS), were fused in frame to the C-terminus of $mGlu_8$ following the linker "GSPPARAT" in the pcDNA3.1 vector. The following G protein constructs were also used: $G_{\alpha i}$-mVenus with the mVenus inserted at position 91, untagged $G_{\beta 1}$, and untagged $G_{\gamma 2}$. The integrity of all the constructs was confirmed with sequencing analysis. Cultured Human Embryonic Kidney 293T (HEK293T) cells were transfected with a constant amount of plasmid cDNA using polyethylenimine (Polysciences Inc) in a 1:2 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 DL-AP4. For CODA-RET experiments on $mGlu_7$ homodimers, the ratio of $mGlu_7$-L1, $mGlu_7$-L2, Gαi-mVenus, $G_{\beta 1}$, and $G_{\gamma 2}$ was 4:4:2:1:1 (for a 10-cm dish, 4, 4, 2, 1, and 1 μg, respectively). For CODA-RET experiments on $mGlu_{7/8}$ heterodimers, the ratio of $mGlu_8$-L1, $mGlu_7$-L2, $G_{\alpha i}$-mVenus, $G_{\beta 1}$, and $G_{\gamma 2}$ was 8:4:2:1:1 (for a 10-cm dish, 8, 4, 2, 1, and 1 μg, respectively). For CODA-RET experiments on $mGlu_8$ homodimers, the ratio of $mGlu_8$-LgBit, $mGlu_8$-HiBit, Gαi-mVenus, $G_{\beta 1}$, and $G_{\gamma 2}$ was 4:4:6:1:1 (for a 10-cm dish, 4, 4, 6, 1, and 1 μg, 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 1× Dulbecco's Phosphate Buffered Saline. Approximately 300,000 cells per well were distributed in 96-well plates and stimulated by the indicated drugs dissolved in prewarmed 1× Dulbecco's Phosphate Buffered Saline for 15 min at 37 °C. A concentration of 5 μM coelenterazine H (the substrate used for both complemented RLuc8 and NanoLuc) was added to each well (Dalton Pharma Services). Two minutes after the addition of coelenterazine H, the fluorescence and luminescence were quantified (Pherastar, BMG Labtech) and the BRET signal was determined by calculating the ratio of the emission of mVenus (535 nm) over the emission of RLuc8 or NanoLuc (475 nm).

Electrophysiology

Animals were group housed with food and water available *ad libitum*. Animals were kept under a 12-h light/dark cycle with lights on from 6:00 AM to 6:00 PM, and slices were prepared during the light phase. All of the experimental procedures were approved by the Vanderbilt University Animal Care and Use committee and followed the guidelines set forth by the *Guide for the Care and Use of Laboratory Animals*. Six- to eight- week-old male C57BL6/J mice (Jackson Laboratories) were anesthetized with isofluorane, and the brains were removed and submerged in ice-cold cutting solution (in mM: 230 sucrose, 2.5 KCl, 8 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 10 D-glucose, 26 $NaHCO_3$). Coronal slices containing the hippocampus were cut at 400 μm using a Compresstome (Precisionary Instruments). Slices were transferred to a holding chamber containing NMDG-HEPES recovery solution (in mM: 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 D-glucose, 5 sodium ascorbate, 2 thiourea, 3 sodium pyruvate, 10 $MgSO_4$, 0.5 $CaCl_2$, pH 7.3–7.4, 305 mOsm) for 10 min at 32 °C. Slices were then transferred to a room temperature holding chamber for at least 1 h containing artificial cerebrospinal fluid (ACSF) (in mM: 126 NaCl, 1.25 NaH_2PO_4, 2.5 KCl, 10 D-glucose, 26 $NaHCO_3$, 2 $CaCl_2$, 1 $MgSO_4$) supplemented with 600 μM sodium ascorbate for slice viability. All buffers were continuously bubbled with 95% O_2/5% CO_2. Subsequently, slices were transferred to a 32 °C submersion recording chamber where they were perfused with ACSF at a rate of 2 ml/min. Paired-pulse fEPSPs were recorded from the stratum radiatum of CA1 and evoked by electrical stimulation (100 μs duration, every 20 s) through a concentric bipolar stimulating electrode placed near the CA3-CA1 border. Input–output curves were generated for each slice, and the stimulation intensity was adjusted to 40 to 50% of the maximum response. After 10 min of baseline recordings, $mGlu_7$ NAMs or vehicle were bath applied for 10 to 20 min.

LTP was induced by either HFS or TBS. HFS comprised two trains of 100 Hz stimulation (1 s duration, 20 s intertrain interval). TBS consisted of four trains of nine bursts, with each burst containing four pulses at 100 Hz and interburst interval of 100 ms and intertrain interval of 10 s. Data were digitized using a Multiclamp 700B, Digidata 1322A, and pClamp 10 software (Molecular Devices) and were analyzed offline using Clampfit 10.2 (Molecular Devices). For analysis, the slopes from three sequential sweeps were averaged. To test the effects of various treatments on the slope, all slopes were normalized to the averaged slopes during the predrug period (10-min baseline) and were presented as the percent of baseline. All drugs were diluted in ACSF and bath applied.

Data availability

All data are contained within the manuscript and are available upon request from Colleen M. Niswender (colleen.niswender@vanderbilt.edu) and Jonathan A. Javitch (Jonathan.Javitch@nyspi.columbia.edu).

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Abbreviations—The abbreviations used are: ACSF, artificial cerebrospinal fluid; BRET, bioluminescence resonance energy transfer; CODA-RET, Complemented Donor Acceptor-Resonance Energy Transfer; fEPSPs, field excitatory postsynaptic potentials; GPCR, G protein-coupled receptor; HFS, high frequency stimulation; ITI, inter-train interval; LTP, long-term potentiation; mGlu, metabotropic glutamate receptor; mPFC, medial prefrontal cortex; mVenus, monomeric venus; NAM, negative allosteric modulator; NanoLuc, Nano luciferase; PAM, positive allosteric modulator; PBS, phosphate buffered saline; RLuc8, *Renilla* luciferase 8; SC-CA1, Schaffer Collateral-CA1; TBS, theta burst stimulation.

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