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Input-specific regulation of glutamatergic synaptic transmission in the medial prefrontal cortex by mGlu₂/mGlu₄ receptor heterodimers

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Abstract

Metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors that regulate various aspects of central nervous system processing in normal physiology and in disease. They are thought to function as disulfide-linked homodimers, but studies have suggested that mGluRs can form functional heterodimers in cell lines. Using selective allosteric ligands, ex vivo brain slice electrophysiology, and optogenetic approaches, we found that two mGluR subtypes—mGluR2 and mGluR4 (or mGlu₂ and mGlu₄)—exist as functional heterodimers that regulate excitatory transmission in a synapse-specific manner within the rodent medial prefrontal cortex (mPFC). Activation of mGlu₂/mGlu₄ heterodimers inhibited glutamatergic signaling at thalamo-mPFC synapses but not at hippocampus-mPFC or amygdala-mPFC synapses. These findings raise the possibility that selectively targeting these heterodimers could be a therapeutic strategy for some neurologic and neuropsychiatric disorders involving specific brain circuits.

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INTRODUCTION

The metabotropic glutamate (mGlu) receptors include eight major subtypes (mGlu₁-mGlu₈) that differentially regulate synaptic transmission and other aspects of brain function based on their signaling pathways and cellular and synaptic localization (1, 2). Selective ligands for individual mGlu receptor subtypes have the potential as treatments for multiple brain disorders, including schizophrenia, depression, anxiety, Alzheimer's disease, Parkinson's disease, and addiction, as well as others (3). Historically, the eight mGlu receptor subtypes have been thought to function primarily as obligate homodimers (4, 5). However, time-resolved fluorescence resonance energy transfer (trFRET) and single-molecule fluorescence resonance energy transfer (smFRET) studies have revealed that mGlu receptors can also form heterodimers in HEK293 cells (6, 7). These findings could be critically important for therapeutically targeting these receptors.

Recent efforts have been successful in establishing unique pharmacological profiles for heterodimers between at least two mGlu receptor subtypes, mGlu₂ and mGlu₄, relative to mGlu₂ and mGlu₄ homodimers using selective positive allosteric modulators (PAMs) [7,9-15]. These include selective mGlu₄ PAMs that potentiate mGlu₄ homodimers, but not mGlu₂ homodimers or mGlu₂/mGlu₄ (mGlu_{2/4}) heterodimers (8-10) and other mGlu₄ PAMs that potentiate responses to activation of either mGlu₄ homodimers or mGlu_{2/4} heterodimers (9, 10). Furthermore, novel nanobodies have been identified that act as selective PAMs at mGlu₂ homodimers but have no effects on mGlu₄ homodimers or mGlu_{2/4} heterodimers (11). Identifying pharmacological differences between mGlu_{2/4} heterodimers versus homodimeric forms of these receptors has enabled studies that provide evidence that mGlu_{2/4} heterodimers may play a role in regulation of transmission at corticostriatal (10) and lateral perforant path-dentate gyrus synapses (12). However, the pharmacological tools used in these previous studies relied on evaluation of synergistic effects of orthosteric agonists (12) or compounds that acted on a single mGlu receptor protomer, rather than having complementary compounds that have independent actions on each protomer of a heterodimer complex (10). Thus, results of these previous studies in native systems could be explained by mechanisms other than activation of functional mGlu_{2/4} heterodimers. Furthermore, none of these previous studies included tools that would differentiate between mGlu_{2/4} and mGlu_{3/4} in native systems.

Both mGlu₂ and mGlu₄ are expressed in the medial prefrontal cortex (mPFC). The effect of mGlu₂ activation has been extensively studied in the mPFC, where mGlu₂ inhibits excitatory synaptic transmission at the thalamo-mPFC synapse. In addition, group III mGlu receptor agonists also inhibit excitatory synaptic transmission in the mPFC (13), which raises the possibility that mGlu₂ and mGlu₄ are co-expressed at the thalamo-mPFC synapse and that the effects of mGlu₂ agonists in regulating this synapse are mediated by mGlu_{2/4} heterodimers. However, roles for mGlu₄ in the effects of the group III mGlu receptor agonist in mPFC have not been established, and it is not clear whether this effect is mediated by specific actions at the thalamo-mPFC synapse. If mGlu₂ and mGlu₄ act as heterodimers at thalamo-mPFC synapses, this could have important implications for possible strategies for treatment of schizophrenia and related disorders. Over activity at this synapse has been postulated to play an important role in the pathophysiology that may underlie some

symptoms associated with schizophrenia, including hallucinations and some aspects of cognitive dysfunction (14). Furthermore, serotonergic hallucinogens, such as 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI) increase spontaneous activity at this synapse, and this can be reversed by activation of mGlu₂ (15–18), and by newer atypical antipsychotic agents that have antagonist activity to the serotonin receptor 5-HT₂. Thus, it will be critical to determine whether the ability of mGlu₂ and mGlu₄ agonists to reduce transmission at this synapse is due to activation of an mGlu_{2/4} heterodimeric form of the receptors.

Here, we characterized a highly selective negative allosteric modulator (NAM) that can act at the mGlu₂ protomer, but not mGlu₃, to inhibit activation of the mGlu_{2/4} heterodimer by an orthosteric agonist acting selectively at the mGlu₄ protomer. This selective NAM, along with the previously available mGlu₄ PAMs that can differentiate mGlu₄ homomer from heteromer, allowed for a more definitive evaluation of potential functional roles of mGlu_{2/4} heterodimers at identified synapses in the CNS than was previously possible. With these tools, we uncovered exciting new evidence that mGlu₂ and mGlu₄ receptors operate as functional heterodimers to reduce transmission in a synapse-specific manner.

RESULTS

L-AP4 has different effects on optogenetically isolated thalamo-, hippocampo-, and amygdala-mPFC synaptic responses

The mPFC receives synaptic inputs from multiple cortical and subcortical regions, including the thalamus (particularly medial thalamic structures, such as mediodorsal (MD) and the midline/intralaminar nuclei), ventral hippocampus, and basolateral amygdala (19–26). To determine whether mGlu₄ activation differentially modulates synaptic transmission at thalamo-cortical synapses and at synapses from other synaptic inputs to mPFC, we used optogenetic approaches to selectively activate the synaptic input of the mPFC from these regions. Optically evoked EPSCs (oEPSCs) were recorded from layer V pyramidal cells in coronal brain slices at a holding potential of –70mV and elicited by a blue light. Bath application of the group III mGlu agonist L-2-amino-4-phosphonobutyric acid (L-AP4; 30 μM) caused a robust inhibition of oEPSCs on thalamo-mPFC synapses measured at a time point when the drug was still present, right before drug washout (Fig. 1A). However, at the same time point, it only had a slight and non-significant acute effect on hippocampo-mPFC synaptic responses (Fig. 1B) and no effect on amygdala-mPFC synapses (Fig. 1C). These results suggest that L-AP4-induced acute inhibition of excitatory synaptic transmission in the mPFC is input specific, with a significant effect only on thalamo-mPFC synapses (Fig. 1, A to C). We note that L-AP4 did appear to induce a delayed-onset, prolonged inhibition at hippocampo-mPFC synapses (Fig. 1B). At around 14 to 15 min, the inhibition of oEPSCs was statistically significant, but there was no significant change in paired-pulse ratio (PPR; Fig. 1B), suggesting a postsynaptic mechanism was involved in the delayed inhibition. At other synapses, acute and more long-term depression of synaptic transmission are mechanistically distinct (27), and, for the current studies, we focused on mGlu receptors involved in acute regulation of excitatory synaptic transmission.

The effect of L-AP4 on thalamo-mPFC synaptic responses is mediated by mGlu₄

L-AP4 activates all group III mGlu receptor subtypes (mGlu_{4,6,7,8}), though mGlu₆ receptor expression is limited to the retina (28, 29). Thus, we used a panel of pharmacological tools to determine the specific group III mGlu receptor subtype(s) involved in the effect of L-AP4 on thalamo-mPFC synaptic responses. L-AP4 inhibited oEPSC amplitudes in a concentration-dependent manner at thalamo-mPFC synapses in layer V pyramidal cells of prefrontal cortical slices (Fig. 2A). LSP4-2022, an agonist that is selective for mGlu₄ at the 5 μ M concentration used (30), mimicked the inhibitory effect of L-AP4 at this synapse (Fig. 2B), while the mGlu₈ agonist (S)-3,4-dicarboxyphenylglycine (DCPG; 1 μ M) (31) did not (Fig. 2C). In addition, the mGlu₇ NAM ADX71743 (3 μ M) (32) did not block the L-AP4 induced inhibition of oEPSCs (Fig. 2D). These results suggest that L-AP4-induced inhibition of excitatory synaptic transmission at thalamo-mPFC synapses is mediated by mGlu₄. Similar results were obtained in electrically evoked EPSCs in mPFC layer V pyramidal cells with L-AP4 in conjunction with LSP4-2022, DCPG, and ADX71743 (fig. S1, A to E). This was consistent with the optogenetic studies and suggests the effect of mGlu₄ on thalamo-cortical EPSCs is clearly observed with electrical stimulation of multiple excitatory synapses.

mGlu₂ and mGlu₄ can function as heterodimers in the modulation of thalamo-mPFC synaptic transmission

In addition to mGlu₄, previous studies suggest that mGlu₂ agonists can also inhibit transmission at thalamo-mPFC synapses (15, 17, 18). To determine whether mGlu₂ and mGlu₄ can function as heterodimers in the modulation of synaptic transmission at thalamo-mPFC synapses, we took advantage of pharmacological reagents that selectively modulate signaling by mGlu_{2/4} heteromers relative to homomeric forms of the receptors. First, we used Lu AF21934, an mGlu₄ PAM that is efficacious at both mGlu₄ homodimers and mGlu_{2/4} heterodimers (10), to determine if it could potentiate the effect of a sub-maximal concentration of L-AP4 on oEPSCs at thalamo-mPFC synapses in layer V pyramidal cells of prefrontal cortical slices. In the absence of Lu AF21934, 3 μ M L-AP4 inhibited oEPSCs by ~20%. However, 10 μ M Lu AF21934 significantly potentiated the response to L-AP4, and the same concentration of L-AP4 inhibited oEPSCs by ~50% in the presence of the mGlu_{2/4} PAM (Fig. 2E). In contrast, 30 μ M N-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC), an mGlu₄ PAM that is only active at mGlu₄ homodimers but not mGlu_{2/4} heterodimers (10), failed to potentiate the L-AP4 induced inhibition of oEPSCs (Fig. 2E). Similar to PHCCC, another mGlu₄ PAM, VU0418506 (3 μ M), that has no efficacy at mGlu_{2/4} heterodimers (9), failed to potentiate the effect of L-AP4 on oEPSCs (Fig. 2E). Together, these results suggest that functional mGlu_{2/4} heterodimers might be present at thalamo-mPFC synapses and play an important role in the regulation of glutamate release at this synapse. Curiously, these results also argue against a functional role for mGlu_{4/4} homodimers at this synapse.

The mGlu₂ NAM MRK-8-29 differentiates mGlu₄ homodimers from mGlu_{2/4} heterodimers

To more definitively test the hypothesis that mGlu_{2/4} heterodimers are involved in regulation of transmission at the thalamo-mPFC synapse, it would be important to have a selective

NAM that acts at the mGlu₂ protomer to block mGlu_{2/4} activity. If a NAM acting on the mGlu₂ protomer blocks responses to orthosteric agonists acting at the mGlu₄ protomer, this combined with the heterodimer PAMs (which act at the mGlu₄ protomer) used above would provide a clear demonstration that the two protomers must interact to induce mGlu_{2/4}-mediated responses at the thalamo-mPFC synapse. We previously reported the characterization of MRK-8-29 as a novel mGlu₂-selective NAM that has no effects on responses to L-AP4 at mGlu₄ or on other mGlu receptor subtypes expressed as homodimers (33–36). To evaluate MRK-8-29 as a potential mGlu_{2/4} NAM, we determined the effects of this compound on responses to L-AP4 in human embryonic kidney (HEK) cell lines expressing only mGlu₄ or both mGlu₂ and mGlu₄ (and presumably, therefore, at least some mGlu_{2/4} heterodimers) by assessing effects on G-protein-gated inwardly rectifying potassium (GIRK) channels using a well-established thallium flux assay (37). MRK-8-29 had no effect on L-AP4-induced thallium influx in the cell line expressing mGlu₄ alone (Fig. 3A and table S1), but inhibited L-AP4-induced thallium influx in a concentration-dependent manner, reduced the L-AP4 maximal response and shifted the concentration-response-curves (CRC) to the right in the cell line expressing both mGlu₂ and mGlu₄ (Fig. 3B, table S1).

While studies with native mGlu₂ and mGlu₄ are critical for assessing effects of MRK-8-29 on native receptors, cell lines expressing mGlu₂ and mGlu₄ include homodimers of each receptor as well as mGlu_{2/4} heterodimers (9). Thus, the ability of MRK-8-29 to inhibit L-AP4-induced activation of mGlu_{2/4} heterodimers was further confirmed by the complemented donor-acceptor resonance energy transfer (CODA-RET) assay, which allows selective detection of a signal from heterodimer activation without contamination of the signal from homodimeric receptors (9, 38). Using this technique, we confirmed that MRK-8-29 does not inhibit L-AP4-induced responses in cultured HEK-293T cells expressing mGlu_{4/4} homodimers (Fig. 3C; table S2), but concentration-dependently inhibited the L-AP4-induced responses in the cells expressing mGlu_{2/4} heterodimers with reduced maximal responses and rightward shift of the L-AP4 CRC (Fig. 3D; table S2). The effects of MRK-8-29 on the L-AP4 induced response at mGlu₄ homodimers and mGlu_{2/4} heterodimers are illustrated schematically (Fig. 3E).

L-AP4-induced inhibition of synaptic responses at thalamo-mPFC synapse is blocked by mGlu₂ NAM MRK-8-29

Having established the effect of MRK-8-29 on mGlu_{2/4} heterodimers, we used this compound to further test the hypothesis that L-AP4-induced inhibition of oEPSCs at the thalamo-mPFC synapse is mediated by activation of mGlu_{2/4}. In the presence of MRK-8-29 (30 μM), the ability of L-AP4 (30 μM) to inhibit oEPSCs at thalamo-mPFC synapse in prefrontal cortical slices was greatly diminished (Fig. 3F). Notably, L-AP4 acts only on the mGlu₄ protomer, whereas MRK-8-29 acts selectively at the mGlu₂ protomer to inhibit mGlu_{2/4} heterodimer signaling. Taken together with the finding that heterodimer, but not homodimer-selective, mGlu₄ PAMs potentiate this response, these data provide strong support for the hypothesis that functional mGlu_{2/4} heterodimers are involved in inhibition of transmission at thalamo-mPFC synapses.

Activation of mGlu_{2/4} heterodimers inhibits DOI-induced increases in sEPSCs in mPFC layer V pyramidal cells

We and others have previously shown that 5-HT₂ receptor activation with serotonergic hallucinogens (including DOI) induces an increase in frequency of spontaneous EPSCs (sEPSCs) in layer V pyramidal cells (15, 17, 18, 39, 40). Furthermore, lesions of the medial thalamus reduce the ability of 5-HT₂ receptor activation to induce increases in sEPSCs (17), suggesting that the thalamo-mPFC synapse is the primary synapse that increases glutamate release in response to 5-HT₂ receptor activation. The increase in sEPSCs can be suppressed by activation of mGlu₂ (15, 16) or group III mGlu receptors (13, 39, 40). These previous studies, along with the present findings described above, raise the possibility that functional mGlu_{2/4} heterodimers at thalamo-mPFC synapses might be engaged in regulation of 5-HT₂ receptor-mediated increases in sEPSCs in layer V pyramidal cells. To test this hypothesis, we used L-AP4 in combination with the mGlu_{2/4} NAM MRK-8-29 to determine if MRK-8-29 could reverse the L-AP4 effect on DOI-induced sEPSCs. Bath application of 10 μ M DOI, a 5-HT₂ receptor agonist, significantly increased the frequency of sEPSCs in layer V pyramidal cells of prefrontal cortical slices (Fig. 4A). In the presence of L-AP4 (3 μ M), the same concentration of DOI (10 μ M) had no effect on sEPSC frequency (Fig. 4B), suggesting that activation of group III mGlu receptors, likely mGlu₄, is able to inhibit sEPSCs induced by 5-HT₂ receptor activation. In addition, the inhibitory effect of L-AP4 on DOI-induced sEPSCs was antagonized by the mGlu₂ NAM, MRK-8-29 (Fig. 4C). Together, these data demonstrated that L-AP4 inhibited DOI-induced sEPSCs (Fig. 4B and D), which could be reversed by the mGlu₂ NAM MRK-8-29 that is efficacious at mGlu_{2/4} heterodimers (Fig. 4C and D).

To further confirm the role of mGlu_{2/4} in the DOI-induced increase in sEPSCs, we used two mGlu₄ PAMs, Lu AF21934 that is active at Glu_{2/4} heterodimers (10) and VU0418506 that is selective for mGlu₄ homodimers and is inactive at mGlu_{2/4} heterodimers (9), to determine if they had differential effects on L-AP4 modulation of DOI-induced sEPSCs. Bath application of a low concentration of L-AP4 (0.3 μ M) did not block DOI-induced sEPSCs (fig. S2A). However, in the presence of 10 μ M Lu AF21934, the same low concentration of 0.3 μ M L-AP4 was able to suppress DOI-induced sEPSCs, compared to 0.3 μ M L-AP4 alone (fig. S2, B and D). In contrast, in the presence of VU0418506 (3 μ M), the low concentration of L-AP4 was not able to suppress DOI-induced sEPSCs, compared to 0.3 μ M L-AP4 alone (fig. S2, C and D). These results, combined with the studies of eEPSCs outlined above, suggest that activation of mGlu_{2/4} heterodimers can reduce both evoked and DOI-induced EPSCs at the thalamo-mPFC synapse.

DISCUSSION

Since the initial reports suggesting that mGlu₄ and mGlu₂ can form heterodimers in cell lines and transfected neurons (6, 8), emerging evidence has suggested the presence of functional mGlu_{2/4} heterodimers in native brain tissues. Previous studies identified two synapses that may be regulated by mGlu_{2/4} heterodimers. These include corticostriatal synapses (10) and hippocampal lateral perforant path-dentate gyrus synapses (12). However, these studies relied on showing synergistic effects of orthosteric agonists (12) or on the use

of agents acting solely on the mGlu₄ protomer to differentiate between mGlu_{2/4} heteromers and mGlu₄ homomers (10). The previous lack of allosteric modulators that can differentiate between mGlu₂ and mGlu₃, as well as compounds that modulate mGlu_{2/4} activity by actions at the mGlu₂ protomer, allows for alternate interpretations that may not require activity of mGlu_{2/4} heteromeric receptors. To more definitively establish a role for mGlu_{2/4} heterodimers, it is important to demonstrate that ligands acting at either the mGlu₂ or mGlu₄ protomer of a heteromeric complex have effects that are consistent with their actions on the heteromeric receptor. In the present studies, we took advantage of the highly selective allosteric modulators that act on mGlu₄ protomers and have distinct pharmacological profiles for mGlu₄ homodimers and mGlu_{2/4} heterodimers. In addition, we report the discovery that MRK-8-29 is a novel mGlu_{2/4} NAM that acts on the mGlu₂ protomer, but not mGlu₃, to inhibit responses to L-AP4, which provides a major advance in more firmly establishing a clear role for mGlu_{2/4} and not mGlu_{3/4} heterodimers at an identified synapse in the CNS than did any previous studies. Furthermore, each of these tools has been characterized for activity at mGlu_{2/4} heteromeric receptors using CODA-RET, which allows definitive demonstration of activities at defined homomeric and heteromeric receptors.

Using these novel molecular probes in conjunction with electrophysiology and optogenetic studies, we provide strong evidence that mGlu_{2/4} heterodimers selectively inhibit transmission at the thalamo-mPFC synapse, a key synapse that plays a critical role in multiple aspects of mPFC function. Specifically, we used a range of mGlu₄ PAMs that show distinct pharmacologic profiles and found that the homodimer-selective mGlu₄ PAMs PHCCC and VU0418506 do not potentiate L-AP4-mediated inhibition of thalamo-mPFC oEPSCs. In contrast, Lu AF21934, which is efficacious at both mGlu₄ homodimers and mGlu_{2/4} heterodimers, potentiates the L-AP4 effect. Furthermore, the mGlu_{2/4} NAM MRK-8-29, which shows no efficacy at mGlu₄ homodimers, inhibited L-AP4-induced inhibition of oEPSCs at this synapse. Consistent with these findings, previous anatomical studies suggest that both mGlu₂ and mGlu₄ are co-expressed at thalamo-mPFC synapses (41–44). The findings that the homodimer-selective mGlu₄ PAMs PHCCC and VU0418506 failed to potentiate L-AP4 induced inhibition of thalamo-mPFC synaptic transmission suggested that there are probably no functional mGlu₄ homodimers present at this synapse. However, the current studies could not rule out the possibility of the presence of functional mGlu₂ homodimers at thalamo-mPFC synapses, which remains to be determined in future studies.

It is particularly interesting that the role of mGlu_{2/4} heterodimers is synapse-specific and active at thalamo-mPFC synapses, but not at synapses in the same neuronal population from projections originating in the hippocampus or amygdala. Based on this, mGlu_{2/4} heterodimers could selectively modulate specific functions of the mPFC associated with specific synapses. For instance, the thalamic input to mPFC is critically engaged in many different cognitive processes, such as regulation of goal-directed behaviors, working memory, and social dominance behavior (45–48). In contrast, the information flow between the ventral hippocampus and mPFC is important for spatial working memory, fear learning and anxiety-related behaviors (49–51) and amygdala-mPFC synaptic interactions have been implicated in regulation of emotional process, stress, fear learning and anxiety-related behaviors (52–54). The exquisite synapse specificity of mGlu_{2/4} is very exciting and

provides a novel opportunity to exert highly selective modulation of activity at a specific input to a critical neuronal population. It is especially interesting in light of previous studies suggesting that modulation of activity at thalamo-mPFC synapses plays a major role in actions of hallucinogenic drugs and inhibition of transmission at this synapse could provide a benefit in schizophrenia patients (13, 15–17, 55, 56). Thus, the mGlu_{2/4} heterodimer at thalamo-mPFC synapses could be a novel therapeutic target for treatment of positive symptoms of schizophrenia. In addition, integrity of the mediodorsal thalamic prefrontal cortical loop is essential for cognitive control and motivation (57, 58), and interruption of integrity of the MD thalamic nucleus and PFC has been implicated in psychiatric symptoms related to cognitive deficits and negative symptoms of schizophrenia (59, 60). However, it remains to be determined whether and how the mGlu_{2/4} heterodimer at thalamo-mPFC synapses could contribute to treatment of cognitive disturbance and negative symptoms of schizophrenia. Nonetheless the potential relevance of the current findings to potential strategies for treatment of schizophrenia highlights the exciting breakthrough of identifying a mechanism that may enable highly synapse-specific pharmacological manipulations in a given brain structure.

Previous studies with animal models have shown that Lu AF21934, a mGlu₄ PAM with activity at mGlu_{2/4}, can have effects that are relevant for treatment of symptoms associated with schizophrenia, including reversal of hyperactivity induced by MK-801 or amphetamine, and head twitches induced by DOI (39). In light of our current understanding, it is possible that some of these previously reported effects of Lu AF21934 could be mediated by actions on mGlu_{2/4}. In future studies, it will be important to fully evaluate the behavioral effects of mGlu₄ and mGlu₂ homomer-selective allosteric modulators relative to mGlu_{2/4} modulators to develop a more complete understanding of the potential utility of selectively targeting homomeric versus heteromeric forms of these receptors.

Our findings here along with previous reports altogether suggest that selectively targeting heteromeric forms of the receptors could provide a novel approach to precisely modulate specific inputs and functional responses. The findings that mGlu₂ and mGlu₄ only form heterodimers at selective synapses in the CNS raise the exciting possibility that developing novel therapeutics that targets these heterodimeric receptors might provide a unique therapeutic profile for treatment of brain disorders.

MATERIALS AND METHODS

Animals

For ex vivo electrophysiology studies, both male and female C57BL/J6 mice (7 to 14 weeks old) were used. As we did not note any obvious differences between male and female mice in electrophysiological responses to pharmacological agents, the data from both sexes were pooled and analyzed together. Mice were group-housed in an animal facility maintained at 23 °C with 12-hour light/dark cycles and food and water available *ab libitum* in their home cages. All 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*.

Drugs and virus

L-AP4 was obtained from Abcam (Cambridge MA). DOI was obtained from Sigma-Aldrich (St Luis, MO). DCPG was obtained from Tocris (Minneapolis, MN). ADX71743, VU0418506, Lu AF21934, LSP4-2022, PHCCC and MRK-8-29 were synthesized in house at Vanderbilt Center for Neuroscience Drug Discovery. AAV5/CaMKIIa-hChR2 (H134R)-EYFP-WPRE (61) (titer $> 4 \times 10^{12}$ vg/ml) was obtained from University of North Carolina Vector Core.

Stereotaxic surgery.—A subset of mice underwent stereotaxic surgeries. Adeno-associated virus serotype 5 (AAV5) carrying fusion genes for ChR2 and EYFP under the CaMKIIa promoter (AAV5/CaMKIIa-hChR2 (H134R)-EYFP-WPRE, UNC Vector Core) were bilaterally injected into the mediodorsal thalamic nucleus, ventral hippocampus or basolateral amygdala. Mice (5 to 7 weeks old) were anaesthetized with 2-3% isoflurane and placed on a motorized stereotaxic apparatus (Neurostar, Tübingen, Germany). A small craniotomy was made on the skull over the injection location. Viral particles were injected through a 28-gauge needle using a Hamilton syringe placed on a Microinjection Robot mounted on the Neurostar motorized stereotaxic apparatus at a rate of 0.2 μ L/min. Injection volumes were 200-500nl each location. The following coordinates relative to Bregma were used (in mm), for thalamus, the mediodorsal nuclei were targeted, A/P: -1.5, M/L: ± 0.36 , D/V: -3.5; for the ventral hippocampus, A/P: -3.4, M/L: ± 3.2 , D/V: -4.0; for the basolateral amygdala, A/P: +1.2, M/L: ± 3.2 , D/V: -4.9. Three to seven weeks following surgery, brains were removed for ex vivo slice electrophysiology recordings.

Brain slice preparation

Coronal brain slices (300 μ m) containing the mPFC were prepared from mice using a procedure similar to those described previously (15, 34). Specifically, mice were anesthetized with isoflurane and perfused transcardially with ice-cold, oxygenated (95% O₂/5% CO₂) cutting solution. Brains were removed from the skull and submerged in cold, oxygenated cutting solution, which was composed of (in mM): 220 glucose, 2.5 KCl, 8 MgSO₄, 0.5 CaCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 D-glucose. Coronal brain slices (300 μ m) containing the prefrontal cortex were cut using a Leica VT1200S microtome (Leica Microsystems Inc), incubated at 32 °C for 30 min in artificial cerebrospinal fluid (ACSF) with addition of 12 mM N-acetyl-L-cysteine (pH adjusted to 7.3-7.4 with KOH and osmolality to 300-310 with deionized water), and then maintained at room temperature afterward until transferred to a recording chamber. The chamber was continuously superfused with oxygenated regular ACSF at a rate of ~ 2 mL/min. The ACSF contained (in mM): 126 NaCl, 2.5 KCl, 2.0 CaCl₂, 1.0 MgSO₄, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 D-glucose.

Ex vivo electrophysiology

Whole cell recordings were made from visually identified layer V pyramidal cells in mPFC slices under an Olympus BX50WI upright microscope (Olympus, Lake Success, NY). Recording electrodes were prepared from borosilicate glass (Sutter Instruments, Novato, CA) using a Narishige puller (model PP-830; Narishige International USA, East Meadow,

NY) and had resistance of 3–4 M Ω when filled the following electrode solution (in mM): 123 K-gluconate, 7 KCl, 1 MgCl₂, 0.025 CaCl₂, 10 HEPES, 0.1 EGTA, 2 ATP and 0.2 GTP at a pH of 7.3 and osmolarity of ~295 mOsm. The cell was voltage-clamped at –70 to –75 mV, closed to the calculated equilibrium potential of Cl[–] such that GABA_A receptor mediated inhibitory currents were undetectable under this recording condition. For electrically evoked excitatory postsynaptic currents (eEPSCs), a concentric bipolar electrode (Frederick Haer Company, Bowdoinham, ME) was placed at layer 2/3. For optically evoked EPSCs (oEPSCs), a blue light (470nm LED, CoolLED) was delivered through a 40x water immersion objective lens on the Olympus BX50WI upright microscope. The electrophysiological signal was amplified using an Axon Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA), low-pass filtered at 3 kHz, digitized at 20 kHz and acquired using a Clampex10/DigiData1440A system (Molecular Devices). Recordings with change in access resistance greater than 20% that affected EPSC kinetics were excluded from the final analysis.

Analysis of electrophysiology data

Clampfit 10 (Molecular Devices) was used for analyzing evoked EPSCs; MiniAnalysis (Synaptosoft Inc) for detecting and analyzing spontaneous EPSCs; Excel (Microsoft) for data management; and Prism 5.0 (GraphPad Software) for statistical analysis. Data were presented as mean $\pm$ SEM and median with interquartile interval. Wilcoxon matched-pairs test was used for testing the change after drug application; Mann-Whitney test for comparing changes between two groups; one-way ANOVA plus Bonferroni adjusted post-test for comparing changes in more than two settings.

Thallium flux assays

Cell culture for thallium flux assays were established and cultured as described previously (9, 10, 62). Rat mGlu₂ or rat mGlu₄ was cloned into the pIRESpuro3 vector, transfected into human embryonic kidney (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 (63), supplemented with nonessential 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 μ g/ml hygromycin B. Polyclonal cells were cultured in growth media supplemented with 100 μ g/ml hygromycin B.

Thallium flux assays were then performed according to methods described previously (37) 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, (Invitrogen, prepared as a DMSO stock and mixed in a 1:1 ratio with pluronic acid F-127) in assay buffer. After 1 hour 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 (Hamamatsu). Baseline readings were taken (2 images at 1 Hz; excitation, 470 $\pm$ 20 nm; emission, 540 $\pm$ 30 nm), and test compounds (2X) 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 (5X). Data were collected for an

additional 2.5 min and analyzed using Excel (Microsoft) as previously described (63), and the concentration–response curves were fitted to a four-parameter logistic equation to determine potency estimates using GraphPad Prism as follows:

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

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 pEC_{50} is the negative logarithm of the molar concentration of compound required to generate a response halfway between the top and bottom.

CODA-RET assays

CODA-RET assays were performed in cultured HEK-293T cells that were transfected with rat mGlu₂ and mGlu₄ cDNAs tagged with a hemagglutinin (HA) epitope tag at the N-terminal using standard molecular biology procedures as previously described (9). Specific procedures were as follows: 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 “GSPPARAT” in the pcDNA3.1 vector. The following G-protein constructs were also used: G_{ai}-mVenus with the mVenus inserted at position 91, 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 homodimers, the ratio of mGlu₄-L1, mGlu₄-L2, G_{ai}-mVenus, G_{β1} and G_{γ2} was 4:4:1:1:1 (for a 10 cm dish, 4, 4, 1, 1, and 1 μg respectively). For the mGlu₂ and mGlu₄ heterodimer, the ratio of mGlu₄-L1, mGlu₂-L2, G_{ai}-mVenus, G_{β1}, and G_{γ2} was 8:4:1:1:1 (for a 10 cm dish, 8, 4, 1, 1, and 1 μg respectively). Cells were maintained in culture with DMEM supplemented with 10% FBS. Forty-eight hours after transfection, cells were harvested, washed twice, and resuspended in PBS. Approximately 200,000 cells per well were distributed in 96-well plates and stimulated by the indicated drugs dissolved in prewarmed PBS for 5 min at 37 °C. A concentration of 5 μM 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). This technique combines luciferase complementation and bioluminescence resonance energy transfer (BRET) to directly measure the signal induced by activating a defined receptor heterodimer (mGlu_{2/4} in the present studies). A modified *Renilla reniformis* luciferase (RLuc8) is split into N-terminal (L1) and C-terminal (L2) fragments which have no function alone but complement to form a functional luciferase when in close proximity. The fragments are fused to the C tail of the target receptors, here mGlu₂ and mGlu₄, to control the complemented protomers. In a homodimer, only one

fragment of the donor molecule will be present, and no signal will be generated in response to substrate. By monitoring the BRET signal between a complemented pair of defined protomers (as a donor) and mVenus labeled G_α subunit (as an acceptor), we can selectively determine G protein recruitment by the activated heterodimer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Effects of L-AP4 on optogenetically evoked glutamatergic transmission at thalamo-, hippocampo-, and amygdala-mPFC synapses.

(A to C) Schematic diagram showing the injection of AAV5-CaMKIIa-hChR2(E123T/T159C)-EYFP into the mediodorsal thalamic nucleus (MDT; A), ventral hippocampus (vHPC; B), or basolateral amygdala (BLA; C), each with a representative image of EYFP-tagged ChR2 expression in the injection site 3 to 5 weeks after viral injection (calibration bar, 200 μm), and a second schematic (right) depicting the acquisition of ex vivo whole-cell recordings from mPFC layer V pyramidal cells in response to light stimulation of ChR2:EYFP expressed axons. Bottom panels show the effect of L-AP4 on both oEPSCs over time and paired-pulse ratios (PPR) at each synapse type. Data are mean ± SEM from N = 8 neurons from total of 7 mice (A), and 7 neurons from 5 (B) or 4 (C) mice. Synapse-specific oEPSCs versus baselines were compared by Wilcoxon matched-pairs tests: ** $P < 0.01$ (A), $P > 0.1$ (B), and $P > 0.9$ (C). PPRs were also compared by Wilcoxon matched-pairs tests: ** $P < 0.01$ (A), $P > 0.9$ (B), and $P > 0.2$ (C). Abbreviations: dHPC = dorsal hippocampus, CTX = cortex, vCA1 = ventral CA1, MB = midbrain, CP = caudal putamen. Calibration bars for oEPSC traces: 100 pA/20 ms (A), 50 pA/20 ms (B), and 20 pA/20 ms (C). The gray-shaded vertical bar in the time course indicates the time points used to be averaged for statistical comparisons to baseline. For reference but not marked in the figure is the statistical comparison of oEPSCs and PPR at 14–15 min after L-AP4 application at

hippocampo-mPFC synapses (B): $P < 0.05$ and $P > 0.9$, respectively, by Wilcoxon matched-pairs test.

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Fig. 2. Effects of L-AP4 and mGlu₄ PAMs with different pharmacologic profiles on glutamatergic transmission at thalamo-mPFC synapse.

(A) Time courses (*left*) and dot plot (*right*) summarizing the effects of L-AP4 at different concentration on oEPSCs at thalamo-mPFC synapses (N = 12 neurons from 10 mice for 3 μM L-AP4, 8 neurons from 7 mice for 30 μM L-AP4 [data are from Fig. 1A], and 5 neurons from 3 mice for 100 μM L-AP4. *P < 0.05 and **P < 0.01 by one-way ANOVA, [F(2,22)=7.30, P = 0.0037] with Bonferroni's post-test. (B) The effect of LSP4-2022 on oEPSCs at thalamo-mPFC synapses. N = 6 neurons from 5 mice; *P < 0.05 by Wilcoxon matched-pairs test. (C) The effect of mGlu₈ agonist DCPG on oEPSCs. N = 5 neurons from 3 mice; P > 0.4 by Wilcoxon matched-pairs test. (D) The effect of mGlu₇ NAM ADX71743 with 30 μM L-AP4 on oEPSCs (N = 5 neurons from 3 mice) compared to the effect of 30 μM L-AP4 alone (same data as in Fig. 1A). P > 0.4 by Mann-Whitney test. (E and F) The

effect of mGlu₄ PAM Lu AF21934, PHCCC or VU0418506 with 3 μ M L-AP4 on oEPSCs (N = 6 neurons from 4 mice, 7 per 5, and 5 per 4, respectively) compared to the effect of 3 μ M L-AP4 on oEPSCs (same data as in panel A). *P < 0.05 by one-way ANOVA [F(3,26) = 4.14, P = 0.0159] with Dunnett's post-test. Calibration bars for oEPSC traces: 100pA/10ms, 100pA/10ms, 40pA/10ms (A); 100pA/10ms (B, C); 200pA/10ms (D); 100pA/10ms (E).

Fig. 3. Effect of mGlu₂ NAM MRK-8-29 on L-AP4 induced inhibition of thalamo-mPFC synaptic transmission.

(**A and B**) The effects of MRK-8-29 on L-AP4 CRC in HEK cells expressing mGlu₄ homodimers (**A**) or mGlu_{2/4} heterodimers (**B**) by GPCR assay. (**C and D**) The effects of MRK-8-29 on L-AP4 CRC in HEK cells expressing mGlu₄ homodimers (**C**) or mGlu_{2/4} heterodimers (**D**) by CODA-RET assay. (**E**) The schematic diagrams illustrate the effect of MRK-8-29 on L-AP4 induced response at mGlu₄ homodimers (left) or mGlu_{2/4} heterodimers (right). (**F**) The effect of 30 μM MRK-8-29 with L-AP4 on oEPSCs (N = 7

neurons from 5 mice) compared to the effect of 30 μ M L-AP4 alone (same data as in Fig. 1A). *** $P < 0.001$ by Mann-Whitney test. Calibration bars for oEPSC traces: 100pA/10ms.

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Fig. 4. Effects of L-AP4 and MRK-8-29 on 5-HT₂ agonist DOI induced increase in sEPSCs in mPFC layer V pyramidal cells.

(A to C) Sample traces (top), cumulative probability plots of inter-event interval (IEI) of sEPSCs (middle) from three representative cells and summary plot of normalized group data (bottom) before and during application of 10 μ M DOI (A), before and during application of 10 μ M DOI in the presence of 3 μ M L-AP4, and before and during application of 10 μ M DOI in the presence of 30 μ M MRK-8-29 and 3 μ M L-AP4. Calibration bars for sEPSC traces: 20 pA/1 s (A and B), and 30 pA/1 s (C). Normalized sEPSC frequency after application of

10 μ M DOI verse baseline under different conditions were compared using Wilcoxon matched-pairs tests: * $P < 0.05$, $N = 7$ neurons from 5 mice (A, bottom); $P > 0.1$, $N = 5$ per 4 (B, bottom); * $P < 0.05$, $N = 6$ per 4 (C, bottom). **(D)** Cumulative probability plots summarizing DOI-induced sEPSC frequency change in control, in the presence of L-AP4 and in the combination of L-AP4 and MRK-8-29. * $P < 0.05$ and ** $P < 0.01$ by one-way ANOVA [$F(2,15)=7.113$, $P = 0.0067$] with Bonferroni's post-test.