mGlu₅ Positive Allosteric Modulators Facilitate Long-Term Potentiation via Disinhibition Mediated by mGlu₅-Endocannabinoid Signaling

Zixiu Xiang, Xiaohui Lv, James Maksymetz, Branden J. Stanley, Ayan Ghoshal, Colleen M. Niswender, Craig W. Lindsley, and P. Jeffrey Conn

INTRODUCTION
The metabotropic glutamate (mGlu) receptor type 5 (mGlu₅) is a GTP-binding protein-coupled receptor that is widely expressed throughout the brain, and particularly abundant at postsynaptic sites in forebrain and limbic circuits that are essential for cognitive functions. In recent years, mGlu₅ has gained tremendous attention as a novel potential therapeutic target for treatment of multiple brain disorders that involve impaired cognitive function and psychiatric conditions, such as schizophrenia. Multiple mGlu₅ positive allosteric modulators (PAMs) based on distinct chemical scaffolds have been identified and shown to have cognition-enhancing and antipsychotic-like effects in a number of animal models. For instance, early studies revealed that mGlu₅ PAMs enhance performance in the Morris water maze, and reverse cognitive deficits in novel object recognition, T-maze-based and operant-based set-shifting tasks, and social novelty discrimination. Furthermore, multiple mGlu₅ PAMs reverse amphetamine-induced hyperlocomotor activity in rats, a rodent model predictive of antipsychotic-like efficacy. These results highlight the potential utility of mGlu₅ PAMs as a novel approach for reversing cognitive deficits and treating psychotic symptoms in patients suffering from major brain disorders.

In addition to causing these behavioral effects, mGlu₅ plays a critical role in regulating N-methyl-D-aspartate receptor (NMDAR)-dependent long-term potentiation (LTP) in the CA1 region of the hippocampus, a form of synaptic plasticity thought to be important for hippocampal-dependent learning and memory. For instance, genetic deletion or pharmacological inhibition of mGlu₅ reduces LTP at Shaffer collateral (SC)-CA1 synapse. However, it is not clear whether the potentiation of NMDAR currents is critical for the ability of mGlu₅ PAMs to enhance hippocampal LTP. We now report the characterization of effects of two structurally distinct mGlu₅ PAMs, VU-29 and VU0092273, on NMDAR currents and hippocampal LTP. As with other mGlu₅ PAMs that do not display observable bias for potentiation of NMDAR currents, VU0092273 enhanced both mGlu₅ modulation of NMDAR currents and induction of LTP at the hippocampal Schaffer collateral (SC)-CA1 synapse. In contrast, VU-29 did not potentiate mGlu₅ modulation of NMDAR currents but induced robust potentiation of hippocampal LTP. Interestingly, both VU-29 and VU0092273 suppressed evoked inhibitory postsynaptic currents (eIPSCs) in CA1 pyramidal cells, and this effect was blocked by the cannabinoid receptor type 1 (CB1) antagonist AM251. Furthermore, AM251 blocked the ability of both mGlu₅ PAMs to enhance LTP. Finally, both PAMs failed to enhance LTP in mice with the restricted genetic deletion of mGlu₅ in CA1 pyramidal cells. Taken together with previous findings, these results suggest that enhancement of LTP by mGlu₅ PAMs does not depend on mGlu₅ modulation of NMDAR currents but is mediated by a previously established mechanism in which mGlu₅ in CA1 pyramidal cells induces endocannabinoid release and CB1-dependent disinhibition.

KEYWORDS: positive allosteric modulators, mGlu₅, hippocampus, long-term potentiation, endocannabinoid

ABSTRACT: Metabotropic glutamate (mGlu) receptor type 5 (mGlu₅) positive allosteric modulators (PAMs) enhance hippocampal long-term potentiation (LTP) and have cognition-enhancing effects in animal models. These effects were initially thought to be mediated by potentiation of mGlu₅ modulation of N-methyl-D-aspartate receptor (NMDAR) currents. However, a biased mGlu₅ PAM that potentiates Gαq-dependent mGlu₅ signaling, but not mGlu₅ modulation of NMDAR currents, retains cognition-enhancing effects in animal models, suggesting that potentiation of NMDAR currents is not required for these in vivo effects of mGlu₅ PAMs. However, it is not clear whether the potentiation of NMDAR currents is critical for the ability of mGlu₅ PAMs to enhance hippocampal LTP. We now report the characterization of effects of two structurally distinct mGlu₅ PAMs, VU-29 and VU0092273, on NMDAR currents and hippocampal LTP. As with other mGlu₅ PAMs that do not display observable bias for potentiation of NMDAR currents, VU0092273 enhanced both mGlu₅ modulation of NMDAR currents and induction of LTP at the hippocampal Schaffer collateral (SC)-CA1 synapse. In contrast, VU-29 did not potentiate mGlu₅ modulation of NMDAR currents but induced robust potentiation of hippocampal LTP. Interestingly, both VU-29 and VU0092273 suppressed evoked inhibitory postsynaptic currents (eIPSCs) in CA1 pyramidal cells, and this effect was blocked by the cannabinoid receptor type 1 (CB1) antagonist AM251. Furthermore, AM251 blocked the ability of both mGlu₅ PAMs to enhance LTP. Finally, both PAMs failed to enhance LTP in mice with the restricted genetic deletion of mGlu₅ in CA1 pyramidal cells. Taken together with previous findings, these results suggest that enhancement of LTP by mGlu₅ PAMs does not depend on mGlu₅ modulation of NMDAR currents but is mediated by a previously established mechanism in which mGlu₅ in CA1 pyramidal cells induces endocannabinoid release and CB1-dependent disinhibition.

KEYWORDS: positive allosteric modulators, mGlu₅, hippocampus, long-term potentiation, endocannabinoid
CA1 synapses of the hippocampus in ex vivo slice preparations\(^1\)\(^7\)\(^{−}\)\(^9\) and in freely moving rats\(^2\)\(^0\)\(^2\)\(^1\). Furthermore, the mGlu1/5 agonist, DHPG, primes LTP induction\(^2\)\(^2\)\(^,\)\(^2\)\(^3\) and multiple mGlu5 PAMs can facilitate induction of LTP at SC-CA1 synapses\(^6\),\(^1\)\(^6\),\(^2\)\(^4\).

Multiple studies have revealed that activation of mGlu5 can positively modulate NMDAR currents in multiple neuronal populations\(^2\)\(^5\)\(^−\)\(^2\)\(^8\) and abundant evidence suggests that NMDAR signaling plays a critical role in hippocampal LTP and in regulating cognitive function\(^2\)\(^9\)\(^−\)\(^3\)\(^1\). Furthermore, NMDAR hypofunction can contribute to pathophysiological changes underlying cognitive disruption, schizophrenia, and other brain disorders\(^3\)\(^2\)\(^−\)\(^3\)\(^4\). These studies raise the possibility that mGlu5 PAMs enhance synaptic plasticity and cognitive function by potentiating mGlu5-dependent regulation of NMDAR signaling. However, we now report that VU-29 displays stimulus bias and does not potentiate mGlu5 coupling to NMDAR currents in CA1 pyramidal cells. This suggests that the ability of mGlu5 PAMs to enhance hippocampal LTP is not dependent on the ability of these compounds to potentiate mGlu5-induced increases in NMDAR currents. Interestingly, recent studies suggest that, in addition to potentiating NMDAR currents, mGlu5 in CA1 pyramidal cells can also reduce inhibitory synaptic transmission by a mechanism that involves release of an endocannabinoid (eCB) from CA1 pyramidal cells and subsequent activation of CB1 eCB receptors (CB1R) on inhibitory terminals to reduce synaptic inhibition\(^3\)\(^7\),\(^3\)\(^8\). This disinhibition provides another potential mechanism by which mGlu5 PAMs could also enhance hippocampal LTP. Consistent with this, we performed a series of studies that suggest VU-29 and VU0092273 enhance LTP at SC-CA1 synapses by a mechanism involving mGlu5-induced disinhibition in CA1 pyramidal cells mediated by eCB signaling.

**RESULTS**

**Differential Effects of VU-29 and VU0092273 on Potentiation of NMDAR Currents in Hippocampal CA1 Pyramidal Cells.** mGlu5 is a closely associated signaling partner with the NMDAR and its activation has been shown to potentiate NMDAR currents in hippocampal CA1 pyramidal cells\(^1\)\(^6\),\(^2\)\(^7\),\(^2\)\(^8\). Interestingly, we recently reported that some
mGlu5 PAMs can induce stimulus bias in mGlu5 signaling and potentiate mGlu5-induced calcium mobilization and ERK1/2 phosphorylation without potentiating the effect of mGlu5 activation on NMDAR currents in CA1 pyramidal cells. To determine whether mGlu5-dependent potentiation of NMDAR currents is critical for the ability of mGlu5 PAMs to enhance hippocampal LTP, we examined the effects of a structurally unique mGlu5 PAM, VU-29, that has been shown to potentiate hippocampal LTP, on mGlu5-dependent modulation of NMDAR currents. VU-29 has an EC50 of 9 nM at mGlu5 in cell line Ca2+ mobilization assays, and at a concentration of 1 μM it does not potentiate responses to activation of other representatives from the major groups of mGlu receptors, mGlu1, mGlu2, or mGlu4. Very close analogues of VU-29 have been evaluated at all eight mGlu receptor subtypes and have been shown to be selective for mGlu5. We next performed studies to confirm that VU-29 and VU0092273 enhance threshold theta burst stimulation (TBS) LTP at the Schaffer collateral (SC)-CA1 synapse in rat hippocampal slices under the conditions employed for studies of NMDAR modulation.6,16,24 Threshold TBS (one train of nine bursts of four pulses at 100 Hz, with 230 ms interburst interval) induced a slight potentiation of fEPSP slope measured at 45 min after TBS (106.8 ± 3.5% of baseline, n = 7), whereas pre-treatment of slices with VU-29 (0.1 μM) or VU0092273 (1 μM) resulted in a robust LTP in response to the threshold TBS, compared to that following threshold TBS alone (137.9 ± 9.0% of baseline with VU-29, n = 11; or 137.2 ± 4.0% of baseline with VU0092273, n = 13; one way ANOVA, F(2, 34) = 9.005, p < 0.001, with Dunnett’s post-test). As noted, application of the mGlu5 PAM VU-29 or VU0092273 alone had no effect on NMDA-evoked currents when applied at a concentration of 5 μM.14 Of note, we did not assess the effects of VU-29 and VU0092273 on synaptically evoked NMDAR-mediated responses. However, in a previous study, we evaluated effects of other mGlu5 PAMs on both NMDA-induced responses and NMDAR EPSCs. These previous studies showed similar effects of PAMS on the two responses.6

**mGlu5 PAMs VU-29 and VU0092273 Enhance LTP at SC-CA1 Synapses in Rats.** We next performed studies to confirm that VU-29 and VU0092273 enhance threshold theta burst stimulation (TBS) LTP at the Schaffer collateral (SC)-CA1 synapse in rat hippocampal slices under the conditions employed for studies of NMDAR modulation.6,16,24 Threshold TBS (one train of nine bursts of four pulses at 100 Hz, with 230 ms interburst interval) induced a slight potentiation of fEPSP slope measured at 45 min after TBS (106.8 ± 3.5% of baseline, n = 7), whereas pre-treatment of slices with VU-29 (0.1 μM) or VU0092273 (1 μM) resulted in a robust LTP in response to the threshold TBS, compared to that following threshold TBS alone (137.9 ± 9.0% of baseline with VU-29, n = 11; or 137.2 ± 4.0% of baseline with VU0092273, n = 13; one way ANOVA, F(2, 34) = 9.005, p < 0.001, with Dunnett’s post-test). As noted, application of the mGlu5 PAM VU-29 or VU0092273 alone had no effect on fEPSP slope, which is consistent with our previous results.6,16

**VU-29 and VU0092273 Inhibit eIPSCs in CA1 Pyramidal Cells.** The finding that VU-29 can enhance hippocampal LTP without potentiating mGlu5 effects on NMDAR currents raises the important question of the mechanism by which mGlu5 PAMs enhance hippocampal LTP. It is well established that induction of LTP at SC-CA1 synapses can be regulated by GABA_A receptor-mediated
inhibition through controlling postsynaptic responses in CA1 pyramidal cells. 40,41 Reduction of inhibitory GABAergic synaptic transmission by endocannabinoid (eCB) receptor type 1 (CB1R) activation facilitates induction of LTP at SC-CA1 synapses,37,42 and mGlu5 activation is known to regulate inhibitory transmission by increasing eCB release from CA1 pyramidal cells.37,38 This raises the possibility that the potentiation of LTP by VU-29 and VU0092273 is through a disinhibition mechanism mediated by eCB-CB1R signaling. To test this, we first examined the effects of VU-29 and VU0092273 on inhibitory synaptic transmission in CA1 pyramidal cells. Evoked inhibitory postsynaptic currents (eIPSCs) were recorded at a holding potential of −70 mV using patch pipettes containing a high concentration of Cl− in the intracellular solution and elicited by a stimulating electrode placed in the stratum radiatum near the recorded cell in the presence of ionotropic glutamate receptor antagonists CNQX (20 μM) and AP-5 (50 μM). In such a recording condition, eIPSCs were inward currents. The bath application of VU-29 (0.1 μM) or VU0092273 (1 μM) significantly inhibited the IPSC amplitude (85.3 ± 4.9% of baseline with VU-29, n = 6; 84.7 ± 3.5% of baseline with VU0092273, n = 7; Figure 3A,B, *p < 0.05). When coapplied with CB1 receptor antagonist AM251 (2 μM), neither VU-29 (0.1 μM) nor VU0092273 (1 μM) inhibited evoked IPSCs in CA1 pyramidal cells (100.8 ± 4.1% with VU-29 and AM251, n = 6; 108.6 ± 7.5% of baseline with VU0092273 and AM251, n = 7; Figure 3C,D, p > 0.5).

These results indicate that both VU-29 and VU0092273, two structurally distinct mGlu5 PAMs, are able to suppress inhibitory synaptic transmission in CA1 pyramidal cells, and this effect is mediated by CB1R activation.

**Figure 3.** mGlu5 PAMs VU-29 and VU0092273 inhibit evoked IPSCs (eIPSCs) in CA1 pyramidal cells via activation of CB1 receptors. (A,B) Bath application of VU-29 (0.1 μM, A) or VU0092273 (1 μM, B) inhibits IPSCs in CA1 pyramidal cells evoked by a stimulating electrode placed in the stratum radiatum of the CA1 region. Left panels in A,B, time courses of normalized eIPSC amplitude during baseline and application of VU-29 (A) and VU0092273 (B) (*p < 0.05, Wilcoxon matched pairs signed rank test, n = 6–7). (C,D) In the presence of the CB1R antagonist AM251 (2 μM), VU-29 (0.1 μM, C) or VU0092273 (1 μM, D) failed to inhibit evoked IPSCs in CA1 pyramidal cells. Left panels in C and D: time courses of normalized eIPSC amplitude during baseline and application of VU-29 with AM251 (C) and VU0092273 with AM251 (D). Right panels in parts C and D: bar graphs summarizing the effects of VU-29 (C) and VU0092273 (D) on eIPSC amplitude in the presence of AM251 (p > 0.5, Wilcoxon matched pairs signed rank test, n = 6–7). Averaged sample traces: black, baseline; gray, during application of compound(s). Calibration for sample traces: (A and B) 50 pA/50 ms; (C) 100 pA/50 ms; (D) 100 pA/40 ms.

Involvement of CB1R Signaling in the mGlu5 PAM Mediated-Enhancement of LTP at SC-CA1 Synapses. To directly determine whether eCB-CB1R signaling is involved in VU-29 and VU0092273-induced potentiation of LTP at SC-CA1 synapse, the CB1R antagonist AM251 (2 μM) was coapplied with VU-29 (0.1 μM) or VU0092273 (1 μM) prior to threshold TBS. We found that, in the presence of AM251, neither VU-29 nor VU0092273 was able to enhance the threshold TBS-induced LTP measured at 45 min after threshold TBS (98.5 ± 6.6% of baseline in AM251 and VU-29, n = 6, compared to 137.9 ± 9.0% of baseline in VU-29 alone, n = 11; p < 0.01; Figure 4A,B; 112.5 ± 6.6% of baseline in AM251 and VU0092273, n = 8, compared to 137.2 ± 4.0% of baseline in VU0092273 alone, n = 13; p < 0.01; Figure 4C,D). Together with the data showing that the CB1R antagonist AM251 blocked mGlu5 PAM-induced inhibition of evoked IPSCs (Figure 3), these results support the notion that enhancement of LTP by both biased mGlu5 PAM VU-29 and an mGlu5 PAM that does not display observable bias for calcium mobilization relative to potentiation of NMDAR currents, VU0092273, shares a common mechanism involving mGlu5-eCB-CB1R signaling.
VU-29 and VU0092273 Enhance LTP at SC-CA1 Synapses in Mice. mGlu5 is expressed in pyramidal cells, inhibitory interneurons, and astrocytes in the CA1 region of the hippocampus. Previous studies have demonstrated that mGlu5 in CA1 pyramidal cells is critically involved in priming stimulation-induced facilitation of LTP at SC-CA1 synapses as well as in long-term depression of inhibitory synaptic transmission (iLTD) in CA1 pyramidal cells. Thus, if this is the mechanism by which mGlu5 PAMs act, the effect of mGlu5 PAMs on LTP should be absent in hippocampal slices from mice in which mGlu5 is selectively deleted from pyramidal cells. Before determining if enhancement of LTP by the mGlu5 PAMs is mediated by mGlu5 activation in CA1 pyramidal cells, we repeated the mGlu5 PAM facilitation of LTP experiment in wild-type (WT) mice to confirm that mice have responses to the mGlu5 PAMs that are similar to those observed in rats. As expected, both VU-29 (0.1 μM) and VU0092273 (0.1 μM) enhanced the LTP induced by threshold TBS at SC-CA1 synapses in mice measured at 50 min after the threshold TBS (137.6 ± 6.3% of baseline with VU-29, n = 7, p < 0.05; 142.3 ± 6.0% of baseline with VU0092273, n = 7, p < 0.005; compared to 116.5 ± 4.3% with threshold TBS alone, n = 12; Figure S5). As for the studies outlined above, the concentrations of PAMs used for these studies are based on previous studies in which these concentrations have been shown to have high efficacy and selectivity for mGlu5.

VU-29 and VU0092273 Do Not Potentiate LTP at SC-CA1 Synapses in Mice with mGlu5 Selectively Deleted in CA1 Pyramidal Cells. To directly test the hypothesis that the enhancement of LTP by the mGlu5 PAMs was mediated by mGlu5 activation in CA1 pyramidal cells, we generated mice in which mGlu5 was deleted from the CA1 pyramidal neurons (mGlu5-CA1-KO) by crossing mGluR5 loxP/loxP mice with transgenic mice expressing Cre recombinase under the control of CaMKIIa (CaMKIIa-Cre). The Cre recombinase-mediated deletion of mGlu5 in these mice has been shown to be complete in CA1 neurons at 8 weeks. Similar to WT mice, threshold TBS induced a slight potentiation of fEPSP slope measured at 50 min after TBS (118.5 ± 6.4% of baseline, n = 6) in 8–9 weeks old mGlu5-CA1-KO mice. In contrast to the WT mice, however, pretreatment of slices with VU-29 (0.1 μM) or VU0092273 (0.1 μM) was not able to enhance LTP induced by threshold TBS (117.8 ± 9.1% of baseline with VU-29, n = 6; or 117.1 ± 7.5% of baseline with VU0092273, n = 6; compared to 118.5 ± 6.4% of baseline with threshold TBS).
Suprathreshold TBS was still able to induce LTP at this synapse in mGlu5-CA1-KO mice. Together, these data suggest that mGlu5 in CA1 pyramidal cells is essential for mGlu5 PAM-induced facilitation of LTP at SC-CA1 synapses.

The mGlu5 PAM VU0092273 Enhances Trace Fear Conditioning in WT but Not in mGlu5-CA1-KO Mice. As mGlu5-eCB-CB1R signaling plays an important role in mGlu5 PAM enhancement of hippocampal LTP, we sought to determine if enhancement of LTP by these mGlu5 PAMs correlates with enhanced cognition. To test this, we evaluated temporal associative learning via trace fear conditioning as a specific hippocampal-dependent cognitive task that is critically dependent on mGlu5 signaling in the hippocampus. On day 1, administration of VU0092273 (10 mg/kg, i.p.) 30 min prior to trace conditioning in context A resulted in an enhancement of freezing behavior during the trace period of acquisition (Figure 7A). When memory retention was tested the following day in context B, WT mice previously treated with VU0092273 demonstrated significantly more freezing during 3 successive tones (Veh: 28.3 ± 4.1%; VU273: 47.8 ± 8.9%; p < 0.05, t test; Figure 7C), suggesting that a single systemic dose of the mGlu5 PAM VU0092273 is able to enhance the acquisition and expression of temporal associative fear learning. Conversely,
Figure 7. mGlu5 PAM VU0092273 enhances trace fear conditioning in WT mice but not in mGlu5-CA1-KO mice. Mice were trained with 3 CS (tone)-US (footshock) pairings in context A. Intertrial intervals (ITIs) were 240 s. CS and US were separated by a 30 s trace period. The amount of freezing to the 30 s trace is quantified for each pairing episode. (A) Trace fear conditioning of vehicle (black circles) and VU0092273 (gray circles; 10 mg/kg, i.p., 30 min prior to conditioning) treated WT mice (two-way repeated measures ANOVA, $F(3,54) = 10.33, p < 0.05$). (B) Tone test performed on subsequent day in context B. Animals were returned to a new context and were presented with three tones of 30 at 240 s intervals. Each point represents the total freezing during each of the 30 s tone presentations. (C) Quantification of total freezing during three successive tones (students $t$ test, $t(18) = 2.2, p < 0.05$). (D–F) Trace fear conditioning of vehicle (black squares) and VU0092273 (gray squares) treated mGlu5-CA1-KO mice. No significant differences in acquisition or tone were observed in trace fear conditioning or tone test in mGlu5-CA1-KO mice (two-way repeated measures ANOVA, $F(2,39) = 0.082, p > 0.05$).

when this assay was conducted with the mGlu5-CA1-KO mice, there was no effect of VU0092273 on acquisition of trace conditioning (Figure 7D) or tone-cued induced expression of fear (Veh: 38.7 ± 10.7%, VU273:35.4 ± 5.7%; $p = 0.78, t$ test; Figure 7F). Taken together, these experiments indicate that the mGlu5 PAM VU0092273 enhances cognition in the trace fear conditioning assay through potentiation of mGlu5 signaling in hippocampal CA1 pyramidal neurons.

**DISCUSSION**

The present studies demonstrate that two structurally distinct mGlu5 PAMs, VU-29 and VU0092273, have differential effects on mGlu5 modulation of NMDAR currents in CA1 pyramidal cells, but both are capable of facilitating induction of LTP at SC-CA1 synapses. These data suggest that the ability of mGlu5 PAMs to enhance hippocampal LTP is not dependent on potentiation of mGlu5 modulation of NMDAR currents. Furthermore, we identified a common mechanism underlying enhancement of LTP by the biased mGlu5 and nonbiased mGlu5 PAMs. Specifically, the PAMs potentiate mGlu5 receptors in CA1 pyramidal cells to stimulate production and release of eCBs, which, in turn, act on CB1Rs on neighboring interneuron terminals and suppress GABA release. This disinhibition could reduce inhibitory control on hippocampal CA1 pyramidal cells and subsequently facilitate LTP induction at SC-CA1 synapses.

Previous studies have shown that application of a group I mGlu agonist or low frequency stimulation (10 Hz) prior to TBS can facilitate or “prime” LTP induction at SC-CA1 synapses, and the same priming stimulation can also induce long-term depression (LTD) of inhibitory synaptic transmission (iLTD) in CA1 pyramidal cells. Both facilitation of LTP and induction of iLTD by the priming stimulation are diminished by group I mGlu receptor antagonists, mGlu5 NAMs, or CB1 receptor antagonists, and are also absent in CB1R-knockout mice or in transgenic mice in which mGlu5 is selectively ablated in CA1 pyramidal cells. In addition, the group I mGlu-CB1R mediated enhancement of LTP at this synapse can be prevented by GABA$_A$ receptor antagonists. Furthermore, the facilitation of LTP induced by mGlu5-mediated “priming” does not seem to involve direct modulation of NMDAR function in CA1 pyramidal cells, but rather depends on mGlu5 activation of CB1Rs, because the CB1 antagonist AM251 has been shown to have agonist effects on the GABA$_A$ receptor and are also absent in CB1R-knockout mice or in transgenic mice in which mGlu5 is selectively ablated in CA1 pyramidal cells. The data from these previous studies, combined with our present results, highlight the importance of mGlu5-induced disinhibition mediated by eCB signaling in the ability of mGlu5 PAMs to enhance LTP at SC-CA1 synapses.

There are multiple subtypes of GABAergic interneurons present in the CA1 region of the hippocampus. However, the eCB-mediated disinhibition is likely mediated by cholecystokinin-positive (CCK+) interneurons, because CB1Rs are mainly expressed in CCK+ interneurons and particularly found at the highest densities on the axon terminals and preterminal segments of these interneurons, but not in parvalbumin-positive (PV+) interneurons. The CCK+ interneurons provide substantial feed forward and feedback inhibition to CA1 pyramidal cells by targeting their perisomatic as well as dendritic regions. Considerable evidence indicates that eCBs acting on CB1Rs at CCK+ interneuron terminals suppress GABAergic transmission, which can subsequently reduce inhibitory control on hippocampal CA1 pyramidal cells and facilitate induction of LTP.

It has been shown that mGlu5 receptors are highly expressed at the perisynaptic region of dendritic spines in CA1 pyramidal cells. Interestingly, the same area of the spine also encompasses the molecular machinery that synthesizes eCBs, particularly diacylglycerol lipase $\alpha$ (DGL$\alpha$) that is involved in the synthesis of a major eCB in the brain, 2-arachidonoyl-glycerol (2-AG). In addition, the CB1R enriched terminals of CCK+ interneurons target both somatic and dendritic regions of CA1 pyramidal cells. These provide the anatomical and molecular basis for mGlu5-induced enhancement of LTP via disinhibition mediated by eCB signaling. Of note, CB1 antagonist AM251 has been shown to have agonist activity at an orphan G-protein coupled receptor GPR55. Interestingly, activation of GPR55 receptors has recently been
shown to enhance LTP at SC-CA1 synapses, while our current studies showed that AM251 blocked the mGlu5 PAM-induced enhancement of LTP. These results suggest that the inhibitory effect of 2 μM AM251 on mGlu5 PAM-mediated enhancement of LTP observed in the present studies is primarily due to blocking the CB1 receptors.

Our previous studies show that VU0092273, but not VU-29, displays allosteric agonist activity in cell lines that overexpress mGlu5. However, we did not observe any effects of VU0092273 or VU-29 on the baseline fEPSP slope at SC-CA1 synapses (Figure 2), indicating that both mGlu5 PAMs have no agonist activity in this native tissue response, particularly in the CA1 region of the hippocampus. These results suggest that data obtained from cell line assays may not be predictive of physiological responses in native tissue or related behaviors in vivo. In the present studies, both VU-29 and VU0092273 suppress evoked IPSCs in CA1 pyramidal cells (Figure 3A,B), an effect that likely results from potentiation of endogenous glutamate action on mGlu5 receptors on CA1 pyramidal cells, which induces production of eCBs that act on CB1Rs at GABAergic axon terminals and reduce GABA release. Our results showing that the CB1R antagonist, AM251, blocks the mGlu5 PAM-induced suppression of IPSCs (Figure 3C,D) are consistent with this notion.

In addition to mGlu5-induced disinhibition, we cannot rule out the possibility of other mechanisms that are involved in the enhancement of LTP by mGlu5-activated eCB signaling. For example, it has been shown that eCB release from CA1 pyramidal cells can activate CB1Rs on astrocytes and induce release of glutamatergic neurotransmitter glutamate, which, in turn, has been shown to elicit slow inward currents in CA1 pyramidal cells by acting on NMDARs. The depolarization of CA1 pyramidal cells induced by this reciprocal astrocyte-neuron communication could potentially lower the threshold of LTP induction and contribute to the mGlu5-eCB induced enhancement of LTP at this synapse. It remains to be determined if this glial mechanism is also involved in addition to the disinhibition mediated by interneurons.

Previous studies have shown that mGlu5 in hippocampal CA1 pyramidal cells plays a critical role in trace fear conditioning, a hippocampal-dependent learning and memory task that has a temporal processing component. In the present studies, we showed that the mGlu5 PAM VU0092273 is able to enhance trace fear conditioning, and this effect is absent in transgenic mice in which mGlu5 receptors have been selectively ablated in CA1 pyramidal cells. Along with the ex vivo electrophysiological data that VU0092273 enhances hippocampal LTP in WT mice but not in CA1-mGlu5-KO mice, the results from these behavioral studies suggest that the enhancement of hippocampal temporal processing by mGlu5 PAMs could be through their actions on mGlu5 receptors in CA1 pyramidal cells. This mGlu5 enhancement of trace fear conditioning is likely mediated by eCB signaling because increasing 2-AG signaling facilitates trace fear learning in both WT and CA1-mGlu5-KO mice. It would be more informative if we could compare the effect of VU-29 on the same behavioral paradigm, analogous to that of VU0092273. Unfortunately, VU-29 does not possess a favorable pharmacokinetic profile compatible with in vivo studies. In the future, the identification of centrally penetrant mGlu5 PAMs that display similar stimulus bias to VU-29 may allow for this hypothesis to be tested.

Table 1

| Ca2+ assay in cell line (EC50 nM) | NMDAR current in CA1 | LTP at SC-CA1 synapse | LTD at SC-CA1 synapse |
|----------------------------------|----------------------|-----------------------|----------------------|
| VU-29                            | (9)                  | −d                    | +e/df               | +e                  |
| VU0092273                        | (35)                 | +e/df                 | +e/df               | +b                  |
| VU0409551                        | (235)                | −c                    | −c                  | +e                  |

**present studies. Ayala et al, 2009.**

In summary, despite mGlu5 being a close signaling partner of NMDARs and mGlu5 modulation of NMDAR function being postulated as a potential mechanism underlying mGlu5 PAM-induced enhancement of hippocampal LTP, the present studies provide evidence that enhancement of LTP at SC-CA1 synapses by mGlu5 PAMs does not require potentiation of mGlu5 modulation of NMDARs. Instead, our data, along with previous reports, suggest that mGlu5 PAMs potentiate LTP by a mechanism that involves GABAergic disinhibition mediated by endocannabinoid signaling. This might provide a cellular and subcellular basis for mGlu5 PAM-induced enhancement of some learning and memory tasks that require the temporal coding function of the hippocampus. However, mGlu5 PAMs can enhance multiple aspects of cognitive function and may act by other mechanisms to regulate circuits involved in other cognitive tasks. In recent years, a range of mGlu5 PAMs that have distinct physiological and behavioral profiles have been identified. Interestingly, the structurally distinct mGlu5 PAMs, VU0092273, VU0409551, and CDPPB (an analogue of VU-29), all have cognition-enhancing and/or antipsychotic-like effects in animal models. These data suggest that mGlu5 PAM-induced pro-cognitive and antipsychotic-like effects are likely to involve multiple mechanisms. The availability of a range of novel mGlu5 allosteric modulators (Table 1) that have distinct modes of efficacy in regulating mGlu5 function provides a valuable set of tools that can be used to shed light on the roles of specific signaling modalities in specific physiological and behavioral responses modulated by mGlu5. Development of biased mGlu5 PAMs, or biased allosteric ligands targeting GPCRs in general, represents a novel avenue for drug development for treatment of neurological and neuropsychiatric disorders. Developing biased ligands that favor one signaling pathway over the others might provide more selectivity in modulation of specific brain circuits or neuronal population at cellular and subcellular levels that are associated with the particular disorder with less adverse effects. In the case of mGlu5 PAMs, development of biased ligands that facilitate synaptic plasticity and/or enhance cognition but not potentiate NMDAR currents might be preferable, in light of possible neurotoxicity associated with increased NMDAR currents by nonbiased mGlu5 PAMs.

**METHODS**

**Animals.** The present studies used male Sprague–Dawley (SD) rats (3–8 weeks old), and both male and female C57BL/6 mice (7–9 weeks old). Conditional mGlu5 knockout (KO) mice with restricted deletion of mGlu5 in hippocampal CA1 pyramidal cells were generated by crossing mGluR5loxP/loxP mice with transgenic mice expressing Cre recombinase under the control of the regulatory region of CaMKIIa (CaMKII-Cre; Jackson Labo-
atory, stock no. 005359). Animals were kept under a 12 h light/dark cycle with lights on from 6 AM to 6 PM and were used for experiments during the light phase unless stated otherwise. All experimental procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee and followed the guidelines set forth by the Guide for the Care and Use of Laboratory Animals.

Ex Vivo Electrophysiology. Extracellular Field Potential Recordings. Horizontal hippocampal slices (400 μm) from SD rats (5–7 weeks old) (Charles River, Wilmington, MA), or coronal hippocampal slices from both male and female mice (The Jackson Laboratory; or bred in house) were prepared as previously described (Ayala et al., 2009; Noetzel et al., 2012). In brief, after being anesthetized with isoflurane, animals were decapitated, and the brains were quickly removed and submerged into ice-cold cutting solution either containing (in mM) 110 sucrose, 60 NaCl, 3 KCl, 1.25 NaH2PO4, 25 NaHCO3, 5 d-glucose, 0.6 (+)-sodium-L-ascorbate, 0.5 CaCl2, and 7 MgCl2 or (in mM) 220 glucose, 2.5 KCl, 8 MgSO4, 0.5 CaCl2, 1.25 NaH2PO4, 26 NaHCO3, and 10 d-glucose. The cutting solution was continuously bubbled with 95% O2/5% CO2. Slices (400 μm) were made using a CompuCresstome (Precisionary Instruments, Greenville, North Carolina), or a Leica VT1200S microtome (Leica Microsystems Inc.). Slices containing the hippocampus were incubated at 32°C for 90 min in oxygenated artificial cerebrospinal fluid (ACSF; in mM): 126 NaCl, 2.5 KCl, 2.0 CaCl2, 1.0 MgSO4, 1.25 NaH2PO4, 26 NaHCO3, and 10 d-glucose. The addition of 12 mM N-acetyl-d-cysteine (pH adjusted to 7.3–7.4 with NaHCO3 and osmolality to 300–310 mOsm with deionized water), and then maintained at room temperature afterward until transferred to a recording chamber. The slice was continuously superfused (1.5–2 mL/min) with oxygenated ACSF at 30–31°C. A concentric bipolar stimulating electrode was placed in the stratum radiatum near the CA3-CA1 border to stimulate the Schaffer collaterals. Recording electrodes were pulled using a Narishige puller (model PP-830; Narishige International USA, East Meadow, NY) or a Flaming/Brown micropipette puller (Sutter Instrument Company, Novato, CA), and had a resistance of 3–5 MΩ when filled with ACSF. Field potential recordings were acquired using a MultiClamp 700B amplifier (Molecular Devices, Union City, CA) and pClamp 10 software (Molecular Devices). A stimulus at intensity that produced about ~50% of the maximum fEPSP slope was set before each experiment for baseline recordings (0.2 ms duration, 0.05 Hz) using a constant current stimulus isolator (DS3, Digitimer North America, Ft. Lauderdale, FL). mGlu5 compounds were diluted to the appropriate concentrations in dimethyl sulfoxide (0.1% final) in ACSF and applied to the bath for 10–20 min. Threshold LTP was induced by one train of theta burst stimulation (TBS; nine bursts of four pulses at 100 Hz, 230 ms interburst interval).

Whole-Cell Voltage Clamp Recordings. Horizontal hippocampal slices (300 μm) were prepared from male SD rats (3–5 weeks old) (Charles River, Wilmington, MA). The procedure of slice preparation was similar to that described in the previous section. Whole-cell recordings were made from visually identified hippocampal CA1 pyramidal neuron soma under an Olympus BX50WI upright microscope (Olympus, Lake Success). A low-power objective (4×) was used to identify the CA1 region of the hippocampus, and a 40× water immersion objective coupled with oblique illumination and a video system was used to visualize individual pyramidal cells. Patch pipettes were prepared from borosilicate glass (World Precision Instrument, Sarasota, FL) using a Narishige puller (model PP-830; Narishige International USA, East Meadow, NY) or a Flaming/Brown micropipette puller (Sutter Instrument Company, Novato, CA). In experiments examining the effects of mGlu5 PAMs on NMDA induced currents in CA1 pyramidal cells, patch pipettes were filled with the intracellular solution containing (in mM) 130 Cs-MeSO3, 5 NaCl, 10 TEA, 5 QX-314, 10 HEPES, 0.2 ETGTA, 0.4 Mg-ATP-Mg, and 0.4 GTP-Na; the pH was adjusted to ~7.3 with CsOH and osmolality to ~290 mOsm with deionized water. NMDA receptor mediated currents were recorded at ~60 to ~65 mV and evoked by pressure ejection of 0.5–1 mM NMDA onto the dendritic field near the soma of the recorded CA1 pyramidal cell every 30 s through a patch pipette using a Picospritzer II (General Valve, Fairfield, NJ). The experiment was carried out in the presence of tetrodotoxin (1 μM) to block voltage-gated sodium channels. In experiments examining the effects of mGlu5 PAMs on GABAergic synaptic transmission in CA1 pyramidal cells, the patch pipette was filled with the following intracellular solution (in mM): 70 CsMeSO3, 60 CsCl, 1 MgCl2, 10 HEPES, 0.2 ETGTA, 2 ATP-Mg, 0.3 GTP-Na, 10 phosphocreatine, 10 TEA, and 5 QX-314. The pH was adjusted to ~7.3 and osmolality to ~295 mOsm. IPSCs were recorded from CA1 pyramidal cells at ~70 mV and evoked at 0.05 Hz with a concentric bipolar-stimulating electrode placed in the stratum radiatum of the CA1 region approximately 100 μm from the recorded cell. The experiment was carried out in the presence of CNQX (20 μM) and AP-5 (50 μM) to block ionotropic glutamate receptor-mediated transmission. All drugs were bath applied.

Trace Fear Conditioning. The design for the trace fear conditioning experiments was modified from previous studies. On day 1, mice were placed in a sound-attenuating conditioning chamber with a shock grid (Med Associates, St. Albans, VT) with white walls in the presence of 1 mL of 10% vanilla odor cue (context A). Mice were acclimated for 60 s before the presentation of CS-trace-US. The conditioned stimulus (CS) used was a 15 s tone (85 db, 3000 Hz), and the unconditioned stimulus (US) was a 0.5 mA footshock for 1 s. The tone and footshock were separated by a precise time interval (trace, 30 s). Intertrial intervals (ITIs) were 240 s. A total of 3 CS-trace-US pairings were used for the conditioning phase. The memory test for trace fear conditioning was conducted 24 h after training in a novel chamber with black insert walls, in the presence of 1 mL of 10% almond odor cue (context B). Mice were presented with 3, 30 s tones, each separated by 240 s. VU0092273 was diluted in vehicle (10% Tween80) and formulated at 0.01 mL/g body weight. VU0092273 and vehicle were administered intraperitoneally (i.p.) 30 min prior to the trace conditioning session only. Chambers were cleaned with 70% ethanol between each set of mice. Freezing behavior was scored by video software (VideoFreeze, MedAssociates) and confirmed by a scorer blinded to treatment conditions. Freezing was considered as lack of all movement except for respiration.

Data Analysis. Data were analyzed using Clampfit 10 (Molecular Devices), Excel (Microsoft), and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA), and were presented as mean ± SEM and statistically analyzed with one-way ANOVA with Dunnett’s post hoc test, Mann–Whitney test, or Wilcoxon matched-pairs signed rank test. Behavioral experiments were analyzed with two-way ANOVA repeated measures.
with Bonferroni post hoc test and student’s t test. Statistical significance was set at \( P < 0.05 \).

### AUTHOR INFORMATION

**Corresponding Author**
*E-mail: jeff.conn@vanderbilt.edu.*

**ORCID**
Zixiu Xiang: 0000-0002-1678-209X
Ayan Ghoshal: 0000-0003-3247-8706
Craig W. Lindsley: 0000-0003-0168-1445

### Present Address

*A.G.: Stanley Center for Psychiatric Research, Broad Institute, Cambridge, MA 02142.*

### Author Contributions

Z.X., X.L., J.M., B.S., A.G., R.G.G., C.M.N., and P.J.C. designed, performed, or provided input to the studies. C.W.L. contributed reagents and other resources. Z.X., B.S., and P.J.C. wrote the manuscript. All the authors edited and reviewed the manuscript.

### Funding

This work was supported by a grant from the National Institute of Mental Health: MH062646 (P.J.C.). J.M. was supported by Canadian Institutes for Health Research DFS146189 and the Vanderbilt International Scholars Program.

### Notes

The authors declare no competing financial interest.

### ACKNOWLEDGMENTS

We thank Ms. Weimin Peng for help with breeding of the conditioned mGlu_5 KO mice.

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