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$mGlu_5$ Positive Allosteric Modulators Facilitate Long-Term Potentiation via Disinhibition Mediated by $mGlu_5$-Endocannabinoid Signaling

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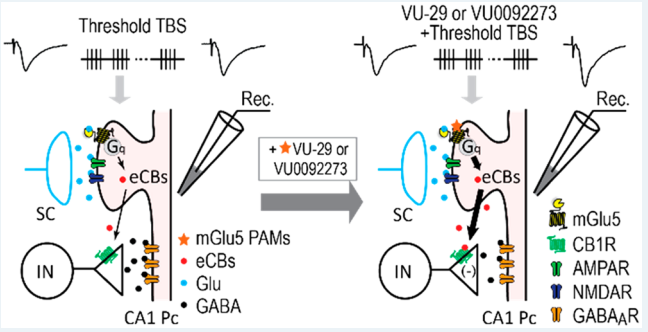
ABSTRACT: Metabotropic glutamate (mGlu) receptor type 5 ($mGlu_5$) positive allosteric modulators (PAMs) enhance hippocampal long-term potentiation (LTP) and have cognition-enhancing effects in animal models. These effects were initially thought to be mediated by potentiation of $mGlu_5$ modulation of *N*-methyl-D-aspartate receptor (NMDAR) currents. However, a biased $mGlu_5$ PAM that potentiates $G\alpha_q$-dependent $mGlu_5$ signaling, but not $mGlu_5$ 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 $mGlu_5$ PAMs. However, it is not clear whether the potentiation of NMDAR currents is critical for the ability of $mGlu_5$ PAMs to enhance hippocampal LTP. We now report the characterization of effects of two structurally distinct $mGlu_5$ PAMs, VU-29 and VU0092273, on NMDAR currents and hippocampal LTP. As with other $mGlu_5$ PAMs that do not display observable bias for potentiation of NMDAR currents, VU0092273 enhanced both $mGlu_5$ modulation of NMDAR currents and induction of LTP at the hippocampal Schaffer collateral (SC)-CA1 synapse. In contrast, VU-29 did not potentiate $mGlu_5$ 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 $mGlu_5$ PAMs to enhance LTP. Finally, both PAMs failed to enhance LTP in mice with the restricted genetic deletion of $mGlu_5$ in CA1 pyramidal cells. Taken together with previous findings, these results suggest that enhancement of LTP by $mGlu_5$ PAMs does not depend on $mGlu_5$ modulation of NMDAR currents but is mediated by a previously established mechanism in which $mGlu_5$ in CA1 pyramidal cells induces endocannabinoid release and CB1-dependent disinhibition.



KEYWORDS: *positive allosteric modulators, $mGlu_5$, hippocampus, long-term potentiation, endocannabinoid*

INTRODUCTION

The metabotropic glutamate (mGlu) receptor type 5 ($mGlu_5$) 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.[1,2] In recent years, $mGlu_5$ 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.[3−5] Multiple $mGlu_5$ 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 $mGlu_5$ PAMs enhance performance in the Morris water maze,[6] and reverse cognitive deficits in novel object recognition,[7] T-maze-based and operant-based set-shifting tasks,[8−10] and social novelty discrimination.[11] Furthermore, multiple $mGlu_5$ PAMs reverse amphetamine-induced hyperlocomotor activity in rats, a rodent model predictive of antipsychotic-like efficacy.[12−16] These results highlight the potential utility of $mGlu_5$ 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, $mGlu_5$ 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 $mGlu_5$ reduces LTP at Shaffer collateral (SC)-

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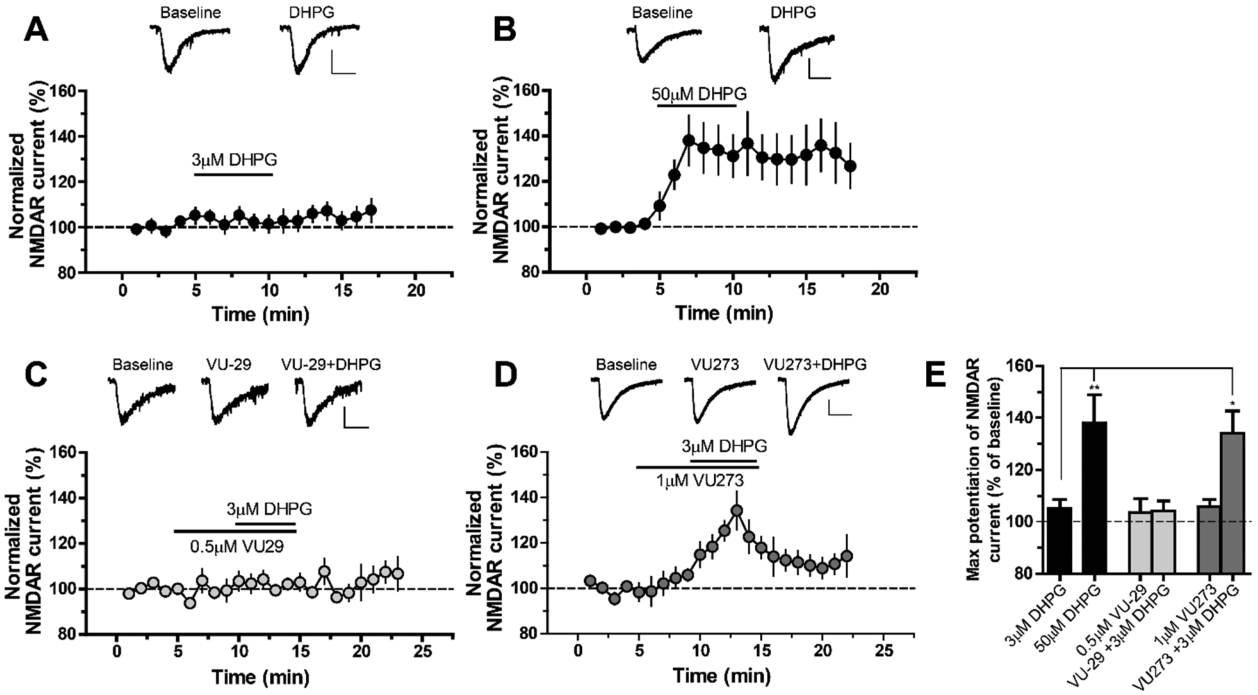

Figure 1. Differential effects of VU-29 and VU0092273 on $mGlu_5$-mediated modulation of NMDAR currents in hippocampal CA1 pyramidal cells. (A,B) DHPG induces a concentration-dependent increase in NMDAR currents. Top: representative traces of NMDA-evoked currents in hippocampal CA1 pyramidal cells. Bottom: time courses of normalized NMDAR current amplitude before, during, and after application of DHPG (A. 3 μM and B. 50 μM, respectively). (C,D) Top: representative traces of NMDA-evoked currents. Bottom: time courses of normalized NMDAR current amplitude in baseline, during applications of an $mGlu_5$ PAM followed by a combination of the $mGlu_5$ PAM and DHPG (3 μM), and washout of the compounds. VU-29 (0.5 μM), a highly selective $mGlu_5$ PAM, does not potentiate NMDAR currents (C), whereas the $mGlu_5$ PAM VU0092273 (VU273, 1 μM) potentiates the effect of DHPG on NMDAR currents (D). (E) Bar graph summarizing the effects of DHPG, $mGlu_5$ PAMs alone and $mGlu_5$ PAMs in the presence of 3 μM DHPG on NMDAR currents (one-way ANOVA, $F(5, 39) = 6.15$, $p < 0.0005$, with Dunnett's post-test, $^{**}p < 0.01$; $^{*}p < 0.05$; $n = 6–7$). Calibration for sample traces: (A) 50 pA/3 s; (B) 30 pA/4 s; (C) 40 pA/4 s; (D) 50 pA/3 s.

CA1 synapses of the hippocampus in *ex vivo* slice preparations[17−19] and in freely moving rats.[20,21] Furthermore, the $mGlu_{1/5}$ agonist, DHPG, primes LTP induction[22,23] and multiple $mGlu_5$ PAMs can facilitate induction of LTP at SC-CA1 synapses.[6,16,24]

Multiple studies have revealed that activation of $mGlu_5$ can positively modulate NMDAR currents in multiple neuronal populations[25−28] and abundant evidence suggests that NMDAR signaling plays a critical role in hippocampal LTP and in regulating cognitive function.[29−31] Furthermore, NMDAR hypofunction can contribute to pathophysiological changes underlying cognitive disruption, schizophrenia, and other brain disorders.[32−34] These studies raise the possibility that $mGlu_5$ PAMs enhance synaptic plasticity and cognitive function by potentiation of $mGlu_5$-dependent regulation of NMDAR signaling. Despite this interesting connection, recent studies revealed that a novel $mGlu_5$ PAM, VU0049551, that selectively potentiates $G\alpha_q$-mediated calcium mobilization, but not $mGlu_5$ modulation of NMDAR currents, has robust antipsychotic-like and cognition-enhancing effects, suggesting that potentiation of $mGlu_5$ modulation of NMDAR currents is not critical for the *in vivo* efficacy of $mGlu_5$ PAMs.[16,35] However, it is not yet clear whether potentiation of NMDAR currents is required for effects of $mGlu_5$ PAMs on induction of LTP.

Previous studies have shown that $mGlu_5$ PAMs VU0092273[16,24] and VU-29,[6,36] which are structurally unrelated to one another and structurally unrelated to VU0409551, can both potentiate induction of LTP at SC-CA1 synapses. Furthermore, VU0092273 also potentiates $mGlu_5$ modulation of NMDAR currents in CA1 pyramidal cells.[16] However, we now report that VU-29 displays stimulus bias and does not potentiate $mGlu_5$ coupling to NMDAR currents in CA1 pyramidal cells. This suggests that the ability of $mGlu_5$ PAMs to enhance hippocampal LTP is not dependent on the ability of these compounds to potentiate $mGlu_5$-induced increases in NMDAR currents. Interestingly, recent studies suggest that, in addition to potentiating NMDAR currents, $mGlu_5$ 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.[37,38] This disinhibition provides another potential mechanism by which $mGlu_5$ 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 $mGlu_5$-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. $mGlu_5$ is a closely associated signaling partner with the NMDAR and its activation has been shown to potentiate NMDAR currents in hippocampal CA1 pyramidal cells.[16,27,28] Interestingly, we recently reported that some

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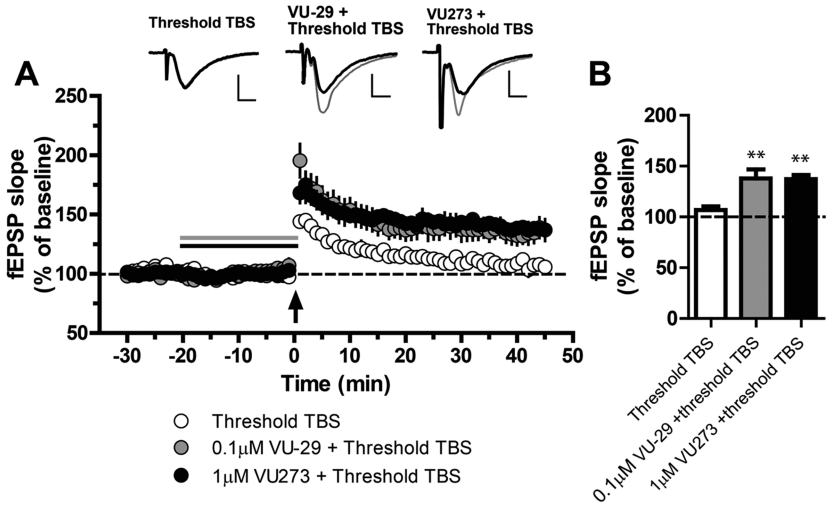

Figure 2. $mGlu_5$ PAMs VU-29 and VU0092273 enhance LTP induction at SC-CA1 synapses in rats. (A) Time courses of normalized fEPSP slope before and after threshold theta burst stimulation (TBS) (open symbols), threshold TBS in the presence of 0.1 μM VU-29 (gray symbols), and threshold TBS in the presence of 1 μM VU0092273 (VU273, black symbols). Horizontal gray and black lines indicate the duration of bath application of VU-29 and VU273, respectively. Arrow indicates the time at which threshold TBS was applied. Averaged sample traces on top: black, baseline; gray, 45 min after threshold TBS. Calibration for all sample traces: 0.4 mV/5 ms. (B) Bar graph summarizing the normalized fEPSP slope measured 45 min after TBS. Bath application of VU-29 or VU0092273, followed by threshold TBS, resulted in significantly greater LTP compared to that with threshold TBS alone (one-way ANOVA, $F(2, 34) = 9.005$, $p < 0.001$, with Dunnett's post-test, $^{**}p < 0.01$; $n = 11-13$).

$mGlu_5$ PAMs can induce stimulus bias in $mGlu_5$ signaling and potentiate $mGlu_5$-induced calcium mobilization and ERK1/2 phosphorylation without potentiating the effect of $mGlu_5$ activation on NMDAR currents in CA1 pyramidal cells.[16] To determine whether $mGlu_5$-dependent potentiation of NMDAR currents is critical for the ability of $mGlu_5$ PAMs to enhance hippocampal LTP, we examined the effects of a structurally unique $mGlu_5$ PAM, VU-29, that has been shown to potentiate hippocampal LTP,[6] on $mGlu_5$-dependent modulation of NMDAR currents. VU-29 has an EC50 of 9 nM at $mGlu_5$ in cell line Ca^{2+} 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, $mGlu_1$, $mGlu_2$, or $mGlu_4$.[36] Very close analogues of VU-29 have been evaluated at all eight mGlu receptor subtypes and are highly selective for $mGlu_5$.[39] In agreement with previous studies, whole cell voltage clamp recordings revealed that the orthosteric $mGlu_{1/5}$ agonist DHPG induced a concentration-dependent enhancement of NMDAR-mediated inward currents in CA1 pyramidal cells evoked by local application of NMDA through a patch pipette positioned in the dendritic field near the recorded cell (105.1 ± 3.6% of baseline in 3 μM DHPG, $n = 7$; 138.1 ± 10.9% of baseline in 50 μM DHPG, $n = 7$; Figure 1A,B,E). Interestingly, application of VU-29 had no effect on NMDAR currents and did not potentiate the effect of 3 μM DHPG on NMDAR currents in CA1 pyramidal cells (103.7 ± 5.2% and 104.2 ± 3.9% of baseline in 0.5 μM VU-29 alone and in combination of 0.5 μM VU-29 and 3 μM DHPG, respectively, Repeated measures ANOVA, $F(2,17) = 0.5809$, $p > 0.5$; Figure 1C,E). In contrast, a bath application of another structurally distinct $mGlu_5$ PAM VU0092273 (1 μM) had no significant effect on NMDA-evoked currents when applied alone but potentiated the effect of 3 μM DHPG on NMDAR-mediated currents (106.0 ± 2.5% and 134.3 ± 8.3% of baseline in VU0092273 alone and combination of VU0092273 and DHPG, respectively; repeated measures ANOVA, $F(2,20) = 14.87$, $p < 0.001$; with Dunnett's post-test, $p > 0.05$ when comparing VU0092273 vs baseline, $p < 0.0001$ when comparing VU0092273 + DHPG vs baseline; Figure 1D,E), which is consistent with our previous studies.[16] VU0092273 is a highly selective $mGlu_5$ PAM, which did not potentiate responses at any of the other seven mGlu receptor subtypes at concentrations up to 10 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 $mGlu_5$ PAMs on both NMDA-induced responses and NMDAR EPSCs. These previous studies showed similar effects of PAMs on the two responses.[16]

$mGlu_5$ 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, $n = 11$), whereas pretreatment 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, $^{**}p < 0.01$; Figure 2). As noted, application of the $mGlu_5$ PAM VU-29 or VU0092273 alone had no effect on fEPSP slope, which is consistent with our previous results.[6,16] Importantly, 0.1 μM VU-29 was sufficient to potentiate threshold LTP, whereas this compound did not potentiate NMDAR currents at 5 fold higher concentrations.

VU-29 and VU0092273 Inhibit eIPSCs in CA1 Pyramidal Cells. The finding that VU-29 can enhance hippocampal LTP without potentiating $mGlu_5$ effects on NMDAR currents raises the important question of the mechanism by which $mGlu_5$ PAMs enhance hippocampal LTP. It is well established that induction of LTP at SC-CA1 synapses can be regulated by $GABA_A$ receptor-mediated

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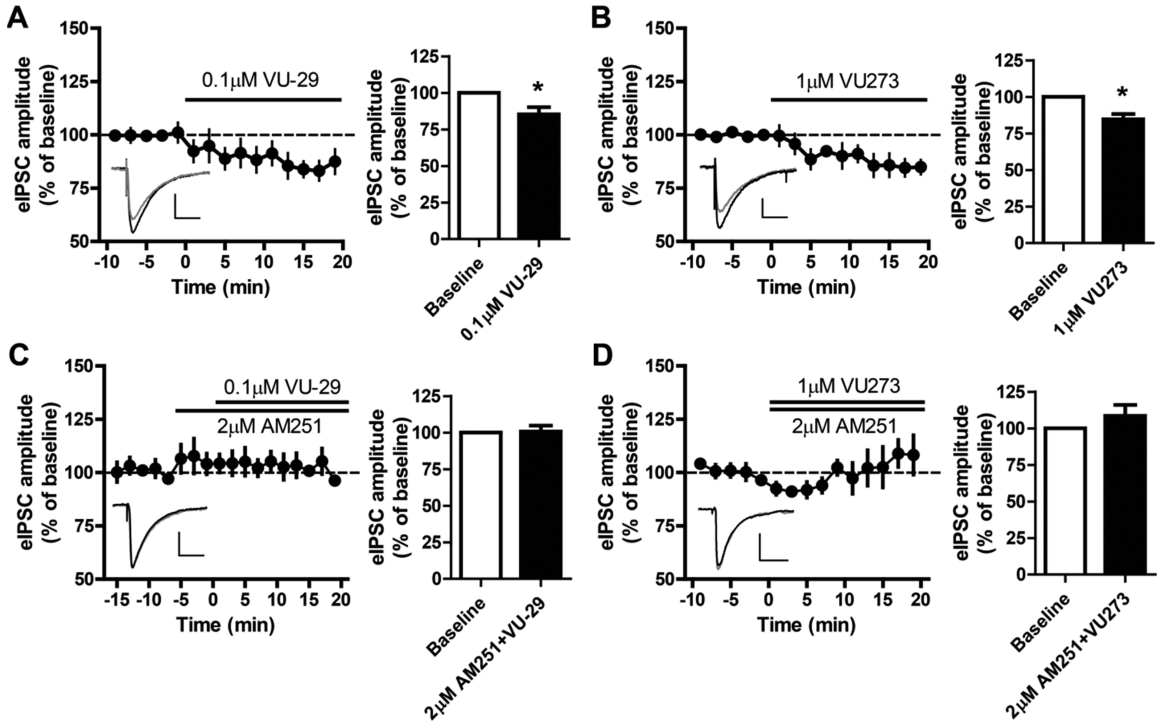

Figure 3. $mGlu_5$ PAMs VU-29 and VU0092273 inhibit evoked IPSCs (eIPSCs) in CA1 pyramidal cells via activation of CB1 receptors. (A,B) Bath application of VU-29 (0.1 μM, A) or VU0092273 (1 μM, B) inhibits IPSCs in CA1 pyramidal cells evoked by a stimulating electrode placed in the stratum radiatum of 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). Right panels in panels A and B: bar graphs summarizing the effects 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.

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 $mGlu_5$ 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 $mGlu_5$ PAMs, are able to suppress inhibitory synaptic transmission in CA1 pyramidal cells, and this effect is mediated by CB1R activation.

Involvement of CB1R Signaling in the $mGlu_5$ 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 $mGlu_5$ PAM-induced inhibition of evoked IPSCs (Figure 3), these results support the notion that enhancement of LTP by both biased $mGlu_5$ PAM VU-29 and an $mGlu_5$ PAM that does not display observable bias for calcium mobilization relative to potentiation of NMDAR currents, VU0092273, shares a common mechanism involving $mGlu_5$-eCB-CB1R signaling.

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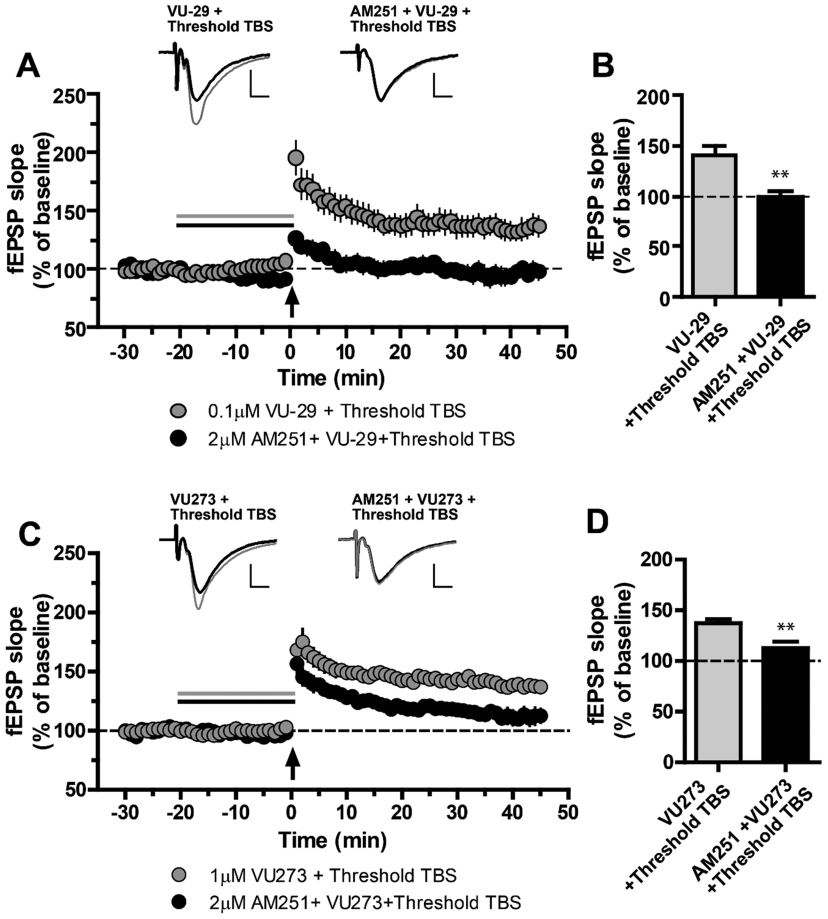

Figure 4. Involvement of CB1R signaling in $mGlu_5$ PAM-induced enhancement of LTP at SC-CA1 synapses. (A) VU-29 (0.1 μM) enhances LTP induced by threshold TBS (gray symbols), but fails to enhance LTP when coapplied with CB1R antagonist AM251 (2 μM, black symbols). (B) Bar graph summarizing the effects of VU-29 alone and VU-29 coapplied with AM251 on threshold TBS-induced LTP measured at 45 min after threshold TBS (**p < 0.01, Mann−Whitney test, n = 6−11). (C) VU0092273 (VU273, 1 μM) enhances LTP induced by threshold TBS (gray symbols), but fails to enhance LTP when coapplied with CB1R antagonist AM251 (2 μM, black symbols). (D) Bar graph summarizing the effects of VU0092273 alone and VU0092273 coapplied with AM251 on threshold TBS-induced LTP measured at 45 min after threshold TBS (**p < 0.01, Mann−Whitney test, n = 8−13). Averaged sample traces: black, baseline; gray, 45 min after threshold TBS. Calibration bars for all sample traces: 0.3 mV/5 ms.

VU-29 and VU0092273 Enhance LTP at SC-CA1 Synapses in Mice. $mGlu_5$ is expressed in pyramidal cells, inhibitory interneurons, and astrocytes in the CA1 region of the hippocampus.[1,43−45] Previous studies have demonstrated that $mGlu_5$ 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.[38] Thus, if this is the mechanism by which $mGlu_5$ PAMs act, the effect of $mGlu_5$ PAMs on LTP should be absent in hippocampal slices from mice in which $mGlu_5$ is selectively deleted from pyramidal cells. Before determining if enhancement of LTP by the $mGlu_5$ PAMs is mediated by $mGlu_5$ in CA1 pyramidal cells using conditional $mGlu_5$ KO mice, we repeated the $mGlu_5$ PAM facilitation of LTP experiment in wild-type (WT) mice to confirm that mice have responses to the $mGlu_5$ 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 5). 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 be have high efficacy and selectivity for $mGlu_5$.

VU-29 and VU0092273 Do Not Potentiate LTP at SC-CA1 Synapses in Mice with $mGlu_5$ Selectively Deleted in CA1 Pyramidal Cells. To directly test the hypothesis that the enhancement of LTP by the $mGlu_5$ PAMs was mediated by $mGlu_5$ activation in CA1 pyramidal cells, we generated mice in which $mGlu_5$ was deleted from the CA1 pyramidal neurons ($mGlu_5$-CA1-KO) by crossing $mGluR5^{loxP/loxP}$ mice[46] with transgenic mice expressing *Cre* recombinase under the control of CaMKIIa (CaMKIIa-Cre). The Cre recombinase-mediated deletion of $mGlu_5$ in these mice has been shown to be complete in CA1 neurons at 8 weeks.[38] 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 $mGlu_5$-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

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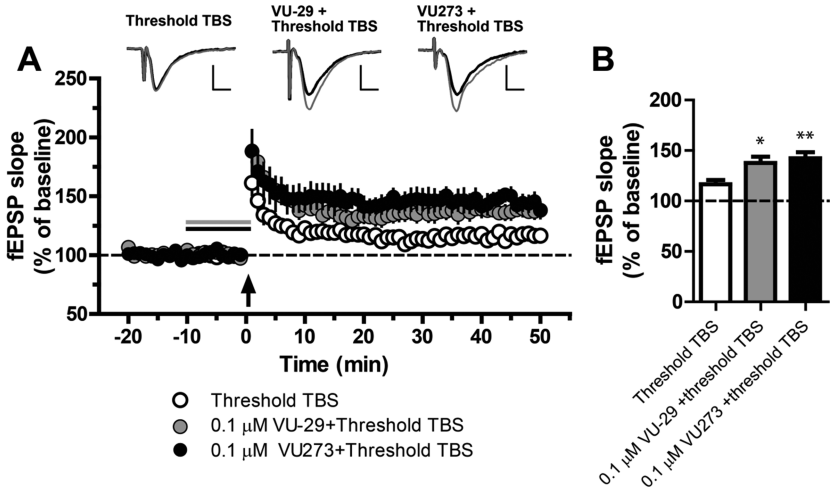

Figure 5. VU-29 and VU0092273 enhance LTP induction at SC-CA1 synapses in mice. (A) Time courses of normalized fEPSP slope before and after threshold TBS (open symbols), or threshold TBS in the presence of 0.1 μM VU-29 (gray symbols) and threshold TBS in the presence of 0.1 μM VU0092273 (VU273, black symbols). Horizontal lines indicate the duration of the bath application of VU-29 (gray) and VU0092273 (black), respectively. Arrow indicates the time at which threshold TBS was applied. (Insets on top) Average sample traces in different conditions as indicated: black traces, baseline; gray traces, 50 min after threshold TBS. Calibration bars for all sample traces, 0.4 mV/5 ms. (B) Bar graph summarizing the normalized fEPSP slope measured 50 min after threshold TBS. Threshold TBS in the presence of VU-29 or VU0092273 resulted in a significantly greater increase in fEPSP slope measured 50 min after the stimulation, compared to that after threshold TBS alone (one-way ANOVA, $F(2,25) = 7.461$, $p < 0.005$, with Dunnett's post-test, $^*p < 0.05$, $^{**}p < 0.005$).

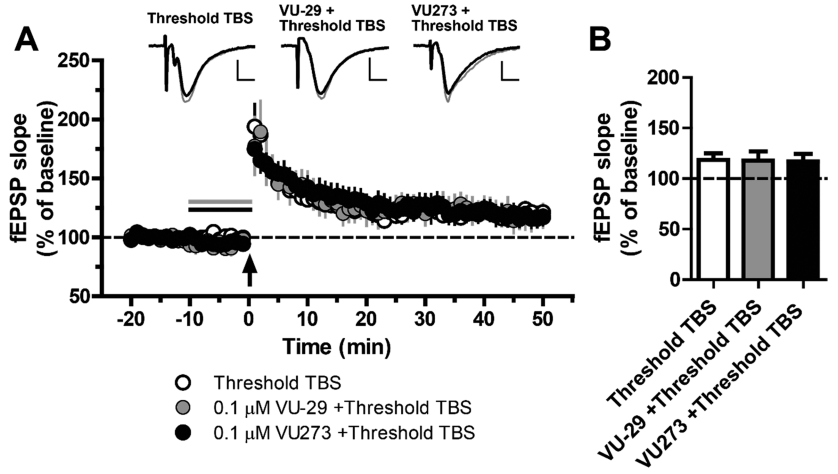

Figure 6. VU-29 and VU0092273 are not able to enhance LTP at SC-CA1 synapses in mice with restricted deletion of mGlu5 in CA1 pyramidal cells. (A) Time courses of normalized fEPSP slope before and after threshold TBS alone (open symbols), or threshold TBS in the presence of 0.1 μM VU-29 (gray symbols) and threshold TBS in the presence of 0.1 μM VU0092273 (black symbols). Horizontal lines indicate the duration of the bath application of VU-29 (gray) and VU0092273 (black), respectively. Arrow indicates the time at which threshold TBS was applied. (Insets on top) Average sample traces in different condition as indicated: llack traces, baseline; gray traces, 50 min after threshold TBS. Calibration bars for sample traces: 0.2 mV/5 ms (left), 0.3 mV/5 ms (middle), 0.3 mV/6 ms (right). (B) Bar graph summarizing the normalized fEPSP slope measured 50 min after threshold TBS. Bath application of VU-29 or VU0092273 had no significant effect on threshold TBS-induced LTP measured 50 min after the stimulation, compared to that after threshold TBS alone (one-way ANOVA, $F(2,17) = 0.0085$, $p > 0.05$).

alone, $n = 6$; $p > 0.5$, Figure 6). It is worth noting that suprathreshold TBS was still able to induce LTP at this synapse in $mGlu_5$-CA1-KO mice.[38] Together, these data suggest that $mGlu_5$ in CA1 pyramidal cells is essential for $mGlu_5$ PAM-induced facilitation of LTP at SC-CA1 synapses.

The $mGlu_5$ PAM VU0092273 Enhances Trace Fear Conditioning in WT but Not in $mGlu_5$-CA1-KO Mice. As $mGlu_5$-eCB-CB1R signaling plays an important role in $mGlu_5$ PAM enhancement of hippocampal LTP, we sought to determine if enhancement of LTP by these $mGlu_5$ 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 $mGlu_5$ signaling in the hippocampus.[38] 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 $mGlu_5$ PAM VU0092273 is able to enhance the acquisition and expression of temporal associative fear learning. Conversely,

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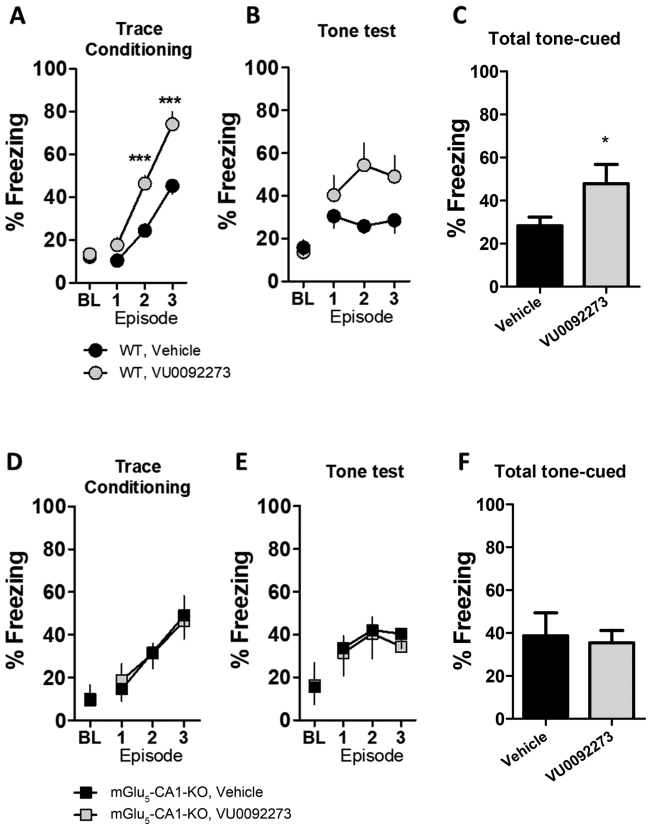

Figure 7. $mGlu_5$ PAM VU0092273 enhances trace fear conditioning in WT mice but not in $mGlu_5$-CA1-KO mice. Mice were trained with 3 CS (tone)-US (footshock) pairings in context A. Intertrial intervals (ITIs) were 240 s. CS and US were separated by a 30 s trace period. The amount of freezing to the 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 $mGlu_5$-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 $mGlu_5$ PAM VU0092273 enhances cognition in the trace fear conditioning assay through potentiation of $mGlu_5$ signaling in hippocampal CA1 pyramidal neurons.

DISCUSSION

The present studies demonstrate that two structurally distinct $mGlu_5$ PAMs, VU-29 and VU0092273, have differential effects on $mGlu_5$ 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 $mGlu_5$ PAMs to enhance hippocampal LTP is not dependent on potentiation of $mGlu_5$ modulation of NMDAR currents. Furthermore, we identified a common mechanism underlying enhancement of LTP by the biased $mGlu_5$ and nonbiased $mGlu_5$ PAMs. Specifically, the PAMs potentiate $mGlu_5$ 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,[22,37,38] and the same priming stimulation can also induce long-term depression (LTD) of inhibitory synaptic transmission (iLTD) in CA1 pyramidal cells;[37,38,47] Both facilitation of LTP and induction of iLTD by the priming stimulation are diminished by group I mGlu receptor antagonists, $mGlu_5$ NAMs, or CB1 receptor antagonists,[37,38] and are also absent in CB1R-knockout mice[37] or in transgenic mice in which $mGlu_5$ is selectively ablated in CA1 pyramidal cells.[38] In addition, the group I mGlu-CB1R mediated enhancement of LTP at this synapse can be prevented by $GABA_A$ receptor antagonists.[37] Furthermore, the facilitation of LTP induced by $mGlu_5$-mediated "priming" does not seem to involve direct modulation of NMDAR function in CA1 pyramidal cells, but rather depends on $mGlu_5$ activation of the $G\alpha_q$ and PLC signaling pathway.[37] The same signaling pathway is also involved in iLTD in the CA1 region of the hippocampus.[48] The data from these previous studies, combined with our present results, highlight the importance of $mGlu_5$-induced disinhibition mediated by eCB signaling in the ability of $mGlu_5$ PAMs to enhance LTP at SC-CA1 synapses.

There are multiple subtypes of GABAergic interneurons present in the CA1 region of the hippocampus.[49−51] However, the eCB-mediated disinhibition is likely mediated by cholecystokinin-positive (CCK+) interneurons, because CB1Rs are mainly expressed in CCK+ interneurons[52,53] and particularly found at the highest densities on the axon terminals and preterminal segments of these interneurons,[54] but not in parvalbumin-positive (PV+) interneurons.[52,53] The CCK+ interneurons provide substantial feed forward and feedback inhibition to CA1 pyramidal cells by targeting their perisomatic as well as dendritic regions.[54−56] Considerable evidence indicates that eCBs acting on CB1Rs at CCK+ interneuron terminals suppress GABAergic transmission,[57,58] which can subsequently reduce inhibitory control on hippocampal CA1 pyramidal cells and facilitate induction of LTP.

It has been shown that $mGlu_5$ receptors are highly expressed at the perisynaptic region of dendritic spines in CA1 pyramidal cells.[59] Interestingly, the same area of the spine also encompasses the molecular machinery that synthesizes eCBs, particularly diacylglycerol lipase α (DGLα) that is involved in the synthesis of a major eCB in the brain, 2-arachidonoylglycerol (2-AG).[60,61] In addition, the CB1R enriched terminals of CCK+ interneurons target both somatic and dendritic regions of CA1 pyramidal cells.[54] These provide the anatomical and molecular basis for $mGlu_5$-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.[62] Interestingly, activation of GPR55 receptors has recently been

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shown to enhance LTP at SC-CA1 synapses,[63] while our current studies showed that AM251 blocked the $mGlu_5$ PAM induced enhancement of LTP. These results suggest that the inhibitory effect of 2 μM AM251 on $mGlu_5$ PAM mediated enhancement of LTP observed in the present studies is primarily due to blocking the CB1 receptors.

Our previous studies show that VU0092273,[15] but not VU-29,[36] displays allosteric agonist activity in cell lines that overexpress $mGlu_5$. 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 $mGlu_5$ 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 $mGlu_5$ 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 $mGlu_5$ PAM-induced suppression of IPSCs (Figure 3C,D) are consistent with this notion.

In addition to $mGlu_5$-induced disinhibition, we cannot rule out the possibility of other mechanisms that are involved in the enhancement of LTP by $mGlu_5$-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 gliotransmitter glutamate,[64] which, in turn, has been shown to elicit slow inward currents in CA1 pyramidal cells by acting on NMDARs.[64−67] 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 $mGlu_5$-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 $mGlu_5$ 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.[38] In the present studies, we showed that the $mGlu_5$ PAM VU0092273 is able to enhance trace fear conditioning, and this effect is absent in transgenic mice in which $mGlu_5$ 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-$mGlu_5$-KO mice, the results from these behavioral studies suggest that the enhancement of hippocampal temporal processing by $mGlu_5$ PAMs could be through their actions on $mGlu_5$ receptors in CA1 pyramidal cells. This $mGlu_5$ 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-$mGlu_5$-KO mice.[38] 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 $mGlu_5$ PAMs that display similar stimulus bias to VU-29 may allow for this hypothesis to be tested.

In summary, despite $mGlu_5$ being a close signaling partner of NMDARs and $mGlu_5$ modulation of NMDAR function being postulated as a potential mechanism underlying $mGlu_5$ PAM-induced enhancement of hippocampal LTP, the present studies provide evidence that enhancement of LTP at SC-CA1 synapses by $mGlu_5$ PAMs does not require potentiation of $mGlu_5$ modulation of NMDARs. Instead, our data, along with previous reports, suggest that $mGlu_5$ PAMs potentiate LTP by a mechanism that involves GABAergic disinhibition mediated by endocannabinoid signaling. This might provide a cellular and subcellular basis for $mGlu_5$ PAM-induced enhancement of some learning and memory tasks that require the temporal coding function of the hippocampus. However, $mGlu_5$ 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 $mGlu_5$ PAMs that have distinct physiological and behavioral profiles have been identified. Interestingly, the structurally distinct $mGlu_5$ PAMs, VU0092273, VU0409551, and CDPPB (an analogue of VU-29), all have cognition-enhancing and/or antipsychotic-like effects in animal models.[6,12,14,16,68] These data suggest that $mGlu_5$ PAM-induced pro-cognitive and antipsychotic-like effects are likely to involve multiple mechanisms. The availability of a range of novel $mGlu_5$ allosteric modulators (Table 1) that have distinct modes of efficacy in regulating $mGlu_5$ 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 $mGlu_5$. Development of biased $mGlu_5$ 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 $mGlu_5$ 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 $mGlu_5$ PAMs.

Table 1

	Ca^{2+} assay in cell line (EC_{50}, nM)	NMDAR current in CA1 pyramidal cells	LTP at SC-CA1 synapse	LTD at SC-CA1 synapse
VU-29	+(9)[a]	−[d]	+[d,e]	+[e]
VU0092273	+(35)[b]	+[c,d]	+[c,d]	+[b]
VU0409551	+(235)[c]	−[c]	−[c]	+[c]

[a]Chen et al., 2007.[36] [b]Noetzel et al., 2012.[14] [c]Rook et al., 2015.[16] [d]Present studies. [e]Ayala et al, 2009.[6] (+) Potentiation; (−) no effect.

METHODS

Animals. The present studies used male Sprague−Dawley (SD) rats (3−8 weeks old), and both male and female C57BL/J6 mice (7−9 weeks old). Conditional $mGlu_5$ knockout (KO) mice with restricted deletion of $mGlu_5$ in hippocampal CA1 pyramidal cells were generated by crossing $mGluR5^{loxP/loxP}$ mice (Jackson Laboratory, stock no. 028626) with transgenic mice expressing Cre recombinase under the control of the regulatory region of CaMKIIa (CaMKII-Cre; Jackson Labo-

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ratory, 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 NaH_2PO_4, 25 $NaHCO_3$, 5 D-glucose, 0.6 (+)-sodium-L-ascorbate, 0.5 $CaCl_2$, and 7 $MgCl_2$; or (in mM) 220 glucose, 2.5 KCl, 8 $MgSO_4$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 D-glucose. The cutting solution was continuously bubbled with 95% O_2/5% CO_2. Slices (400 μm) were made using a Compresstome (Precisionary Instruments, Greenville, North Carolina), or a Leica VT1200S microtome (Leica Microsystems Inc.). Slices containing the hippocampus were incubated at 32 °C for 30 min in oxygenated artificial cerebrospinal fluid (ACSF; in mM): 126 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 1.0 $MgSO_4$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 D-glucose) with the addition of 12 mM *N*-acetyl-L-cysteine (pH adjusted to 7.3−7.4 with *N*-methyl-D-glucamine and osmolality to 300−310 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). $mGlu_5$ 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 $mGlu_5$ PAMs on NMDA induced currents in CA1 pyramidal cells, patch pipettes were filled with the intracellular solution containing (in mM) 130 Cs-$MeSO_3$, 5 NaCl, 10 TEA, 5 QX-314, 10 HEPES, 0.2 EGTA, 4 Mg-ATP-Mg, and 0.4 GTP-Na; the pH was adjusted to ∼7.3 with CsOH and osmolarity 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 $mGlu_5$ PAMs on GABAergic synaptic transmission in CA1 pyramidal cells, the patch pipette was filled with the following intracellular solution (in mM): 70 $CsMeSO_3$, 60 CsCl, 1 $MgCl_2$, 10 HEPES, 0.2 EGTA, 2 ATP-Mg, 0.3 GTP-Na, 10 phosphocreatine, 10 TEA, and 5 QX-314. The pH was adjusted to ∼7.3 and osmolarity 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.[38] 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

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with Bonferroni post hoc test and student's t test. Statistical significance was set at $P < 0.05$.

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Notes

The authors declare no competing financial interest.

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