Metabotropic glutamate receptor 3 activation is required for long-term depression in medial prefrontal cortex and fear extinction

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Clinical studies have revealed that genetic variations in metabotropic glutamate receptor 3 ($mGlu_3$) affect performance on cognitive tasks dependent upon the prefrontal cortex (PFC) and may be linked to psychiatric conditions such as schizophrenia, bipolar disorder, and addiction. We have performed a series of studies aimed at understanding how $mGlu_3$ influences PFC function and cognitive behaviors. In the present study, we found that activation of $mGlu_3$ can induce long-term depression in the mouse medial PFC (mPFC) in vitro. Furthermore, in vivo administration of a selective $mGlu_3$ negative allosteric modulator impaired learning in the mPFC-dependent fear extinction task. The results of these studies implicate $mGlu_3$ as a major regulator of PFC function and cognition. Additionally, potentiators of $mGlu_3$ may be useful in alleviating prefrontal impairments associated with several CNS disorders.

GRM3 | medial prefrontal cortex | fear extinction | long-term depression | group II mGlu receptors

Metabotropic glutamate receptor 3 ($mGlu_3$) has become of increasing clinical interest due to its genetic association with psychiatric conditions. For example, several studies have identified single-nucleotide polymorphisms (SNPs) in *GRM3*, the human gene encoding $mGlu_3$, that are associated with poor performance on cognitive tests that are dependent on function of the prefrontal cortex (PFC) and hippocampus (1, 2). Additionally, these SNPs have also been associated with variations in functional magnetic resonance imaging (fMRI) indexes of prefrontal cortical activity during working memory tasks (1, 3). Moreover, converging lines of evidence indicate that *GRM3* represents a major locus associated with schizophrenia (1, 2, 4), bipolar disorder (5, 6), and substance abuse disorders (6–8). Because $mGlu_3$ is densely expressed in PFC (9), a brain region implicated as a site of pathology in these disorders (10–12), this genetic evidence has led to an increased interest in determining the role of $mGlu_3$ in regulating PFC function and behavior.

Previous studies have revealed that pharmacological activation of group II mGlu receptors ($mGlu_2$ and $mGlu_3$) results in long-term depression (LTD) of excitatory transmission in layer V of the rat medial prefrontal cortex (mPFC) (13–16). Although it is not known whether induction of LTD in the mPFC is mediated by $mGlu_2$ or $mGlu_3$, previous studies suggest that presynaptically localized $mGlu_2$ is typically responsible for inhibition of synaptic transmission by group II mGlu receptor agonists at other synapses (17–23). However, evidence suggests that induction of LTD in the mPFC is dependent upon activation of a postsynaptic group II mGlu receptor (15, 16), suggesting that this response is mechanistically distinct from presynaptic effects of group II mGlu receptor agonists on transmission at other synapses. Unfortunately, a lack of pharmacological agents that can selectively antagonize $mGlu_3$ or $mGlu_2$ has impaired progress in this area. To allow us to begin studies aimed at understanding the role of $mGlu_3$ in regulation of mPFC function, we developed a series of negative allosteric modulators (NAMs) that are highly selective for $mGlu_3$ and are suitable for in vivo use (24). In addition, we now report characterization of a highly selective $mGlu_2$ NAM. We used these compounds, along with $mGlu_2$ and $mGlu_3$ knockout (KO) mice, to evaluate the respective roles of $mGlu_2$ and $mGlu_3$ in acute regulation of synaptic transmission and induction of LTD in mPFC. Interestingly, we found that $mGlu_2$ is involved in acute inhibition of synaptic transmission in the mPFC, but that induction of LTD at this synapse by group II mGlu receptor agonists is mediated exclusively by $mGlu_3$. Furthermore, we found that $mGlu_3$ NAMs impair extinction of conditioned fear, a behavioral task that is critically dependent upon the integrity of the mPFC. These data suggest that $mGlu_3$ plays an essential role in the regulation of a specific form of synaptic plasticity in the mPFC that could be important for forms of cognitive function that require depression of excitatory inputs to mPFC and are thought to be disrupted in patients suffering from a range of CNS disorders.

Results

NMDA Receptor-Independent LTD with a Postsynaptic Component Is Induced by Pharmacological Activation of Group II mGlu Receptors in mPFC. We recorded field excitatory postsynaptic potentials (fEPSPs) from layer V in response to stimulation of layer II/III in

Significance

Recent genetic studies suggest that variations in the gene encoding metabotropic glutamate receptor 3 ($mGlu_3$) can influence aspects of cognitive function that involve the prefrontal cortex (PFC). Furthermore, mutations in this gene may predispose individuals to developing psychiatric disorders in which altered function of the PFC has been implicated. However, little is known about the precise roles of $mGlu_3$ in regulating the function of the PFC. In the present study, we took advantage of newly identified molecular probes to show that $mGlu_3$ can strongly influence synaptic plasticity within the PFC and that blockade of this receptor impairs specific learning abilities in mice. These results suggest that $mGlu_3$ may be a therapeutic target for cognitive dysfunction in mental disorders.

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the prelimbic (PL) subregion of mPFC in ICR(CD1) mice. Application of the selective $mGlu_{2/3}$ agonist LY379268 (30–100 nM for 10 min) produced a concentration-dependent, transient inhibition of the fEPSP slope (Fig. 1 *A* and *B*). Strong pharmacological activation of $mGlu_{2/3}$ with 100 nM LY379268 produced LTD of fEPSPs up to 60 min after drug washout (40.3 ± 3.0% depression from baseline, n = 7; Fig. 1 *B* and *F*). Analysis of paired-pulse ratios (PPR) (25-ms interstimulus interval) throughout the experiment showed a phasic PPR increase corresponding to the peak of the transient inhibition, which then returned to baseline levels 60 min later when LTD was observed (Fig. 1*C*). This suggests that, although the initial fEPSP inhibition may have a presynaptic component, the observed LTD was not simply due to a long-term decrease in the neurotransmitter release probability. When experiments were performed in the presence of the *N*-methyl-D-aspartate (NMDA) receptor antagonist AP5 (50 μM), LTD was still observed (38.1 ± 6.3%, n = 5; Fig. 1 *D* and *F*). Conversely, when LY379268 was applied in the presence of group II antagonist LY341495 (500 nM), both the transient inhibition and induction of LTD were blocked (12.3 ± 4.6%, n = 6; Fig. 1 *E* and *F*), confirming this effect was solely due to actions at group II mGlu receptors. Thus, selective pharmacological activation of group II mGlu receptors produces an NMDA receptor-independent form of LTD of fEPSPs that is expressed postsynaptically.

Induction of Group II mGlu LTD Requires Activation of $mGlu_3$. Next we sought to evaluate the contribution of the $mGlu_2$ and $mGlu_3$ subtypes to this form of LTD. We took advantage of two $mGlu_3$-selective NAMs, VU0469942 and VU0477950, that we recently reported and characterized (24). When slices were pretreated with the $mGlu_3$-selective NAM, VU0469942 (10 μM), the agonist LY379268 caused a large transient depression, but the slope returned to near baseline levels during the 60-min drug washout (12.3 ± 4.6%, n = 6, Fig. 2*A*). Thus, the $mGlu_3$ NAM blocked the ability of LY379268 to induce LTD, but did not inhibit the acute inhibition of synaptic transmission. VU0477950 is a deuterated structural analog of VU0469942, which provides improved pharmacokinetics in terms of clearance relative to the nondeuterated compound (24). When experiments were repeated in the presence of VU0477950, a similar profile emerged (Fig. 2*B*).

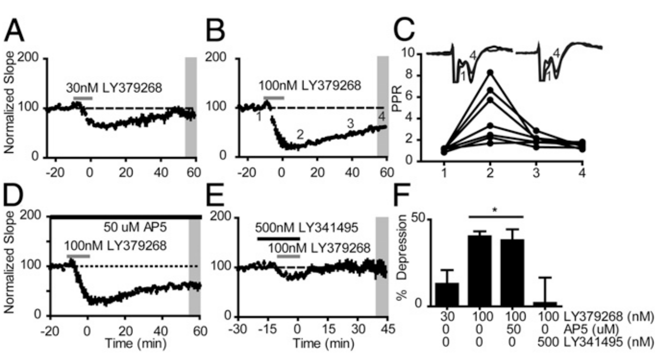

Fig. 1. Group II mGlu LTD in mouse mPFC. (*A* and *B*) Average time course of fEPSP slopes recorded from layer V mPFC. Application of LY379268 at 30 nM (n = 6) and 100 nM (n = 7) transiently decreased the slope, but induced LTD only at 100 nM. (*C*) Paired-pulse ratio analysis for fEPSPs recorded from all slices in *B*. *x*-axis labels correspond to the time-points *Inset* in *B*. *Insets* show sample paired-pulse fEPSP traces from baseline (*1*) and 60 min after drug washout (*4*). (*D*) LTD induced by LY379268 was not altered by the NMDA receptor antagonist AP5 (n = 5). (*E*) LTD was blocked by a selective $mGlu_{2/3}$ orthosteric antagonist LY341495 (n = 6). (*F*) Quantification of LTD measured 55–60 min after drug washout (average of shaded region in *A*, *B*, *D*, and *E*). * indicates P < 0.05 Tukey posttest vs. 30 nM and 500 nM LY341495. Data are expressed as mean ± SEM.

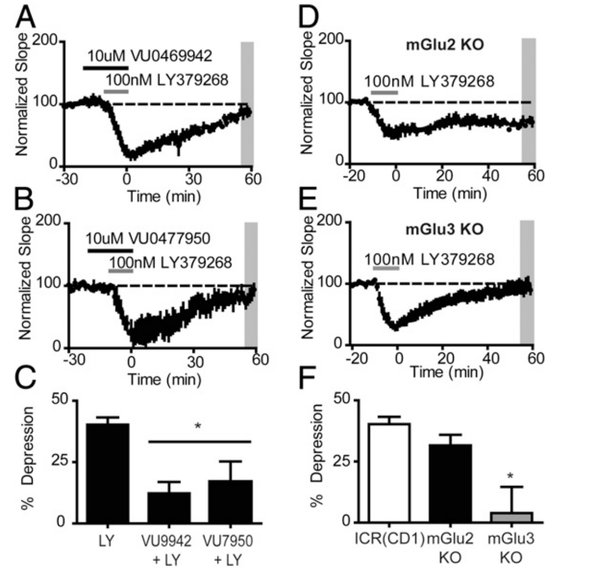

Fig. 2. $mGlu_3$ activation is required for the induction of LTD by a group II agonist. (*A* and *B*) Preincubating slices with the $mGlu_3$ NAMs VU0469942 (*A*, n = 6) and VU0477950 (*B*, n = 4) does not affect the transient inhibition of the fEPSP slope, but blocks LTD induced by LY379268. (*C*) Quantification of the effects of $mGlu_3$ NAMs on LTD measured 55–60 min after drug washout (average of shaded region in *A* and *B*). * indicates P < 0.05 Tukey posttest vs. LY379268. (*D* and *E*) LY379268 induces LTD in mPFC slices from $mGlu_2$ (n = 6) but not $mGlu_3$ KO mice (n = 9). (*F*) Quantification of LTD in $mGlu_2$ KO, $mGlu_3$ KO, and the background strain ICR(CD1) (average of shaded region in *D* and *E*). * indicates P < 0.05 Tukey posttest vs. $mGlu_2$ and ICR(CD1) mice. Data are expressed as mean ± SEM.

LY379268 caused a transient depression of the fEPSP slope, which then returned to baseline levels by the end of the experiment (16.9 ± 8.4%, n = 4). Compared with LY379268 alone (Fig. 2*C*), both VU0469942 and VU0477950 significantly decreased the magnitude of LTD measured 55–60 min after washout (P < 0.05; one-way ANOVA with a Tukey posttest).

To further evaluate the role of the individual group II mGlu receptor subtypes, we compared LTD induced by LY379268 in $mGlu_2$ and $mGlu_3$ KO mice (Fig. 2 *D* and *E*). In $mGlu_2$ KO mice agonist application induced a lasting depression of fEPSPs, indicative of LTD measured 60 min after drug washout (31.6 ± 4.3%, n = 5). In contrast, when LY379268 was applied to slices from $mGlu_3$ KO mice, a transient depression was observed, but LTD was absent when assessed 60 min after washout of the agonist (4.0 ± 10.7%, n = 9). Compared with LTD measured in the ICR(CD1) background strain and $mGlu_2$ KO mice (Fig. 2*F*), the magnitude of LTD was significantly smaller in $mGlu_3$ KO mice (P < 0.05; Tukey posttest). Moreover, there was no difference between the magnitude of LTD observed in wild-type (WT) and $mGlu_2$ KO mice (P > 0.05).

To further test the contribution of $mGlu_2$ activation to this LTD response, we synthesized and characterized the reported $mGlu_2$-selective NAM MRK-8-29 (25, 26). The synthesis of MRK-8-29 was completed in seven steps from commercially available 4,7-dichloroquinoline, using a modified version of the originally reported route (*SI Materials and Methods*). We then used a calcium-fluorescence assay to generate concentration-response curves for MRK-8-29 in stable cell lines expressing rat $mGlu_2$ and rat $mGlu_3$ receptors to assess the selectivity of the compound (*SI Materials and Methods*). Although MRK-8-29 did not alter the response of rat $mGlu_3$ cells to an EC_{80} concentration of glutamate, it fully inhibited rat $mGlu_2$ responses with an IC_{50} value of 146 nM (Fig. 3*A*). Furthermore, increasing concentrations of MRK-8-29 induced a progressive depression of

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the maximal efficacy of rat $mGlu_2$ in response to glutamate, indicating an allosteric mechanism of inhibition (Fig. 3*B*). When LY379268 (100 nM) was applied to slices in the presence of MRK-8-29 (10 μM), there was a rapid and lasting depression of the fEPSP slope that was still evidenced 60 min after agonist washout, indicative of induction of LTD (33.7 ± 9.8%; $n = 4$; Fig. 3*C*). Compared with LY379268 alone, MRK-8-29 did not significantly affect the magnitude of LTD measured 60 min after agonist washout (Fig. 3*D*; $P > 0.05$; unpaired *t* test). Although the transient inhibition induced by LY379268 appeared to be reduced by MRK-8-29 relative to control and VU0469942 (Fig. S1*A*), this effect was not statistically significant ($P > 0.05$; one-way ANOVA). Likewise, the transient depression appeared to be attenuated in $mGlu_2$ KO mice relative to WT and $mGlu_3$ KO mice (Fig. S1*B*), and this effect also did not reach statistical significance ($P > 0.05$; one-way ANOVA). Taken together, these data indicate that whereas activation of $mGlu_2$ plays an important role in the transient depression of synaptic transmission at this synapse, $mGlu_3$ activation is required for the induction of group II mGlu LTD.

Finally, to confirm that the actions of the $mGlu_3$ NAM VU0469942 require activity at $mGlu_3$, we evaluated the effects of this compound in slices prepared from $mGlu_2$ and $mGlu_3$ KO mice (Fig. S2 *A*–*C*). In control slices from both $mGlu_2$ and $mGlu_3$ KO mice, the agonist LY379268 induced a transient depression of fEPSPs (43.5 ± 9.3%, $n = 6$, and 66.4 ± 5.9%, $n = 9$, respectively). However, when LY379268 was applied in the presence of VU0469942, inhibition was almost completely attenuated in $mGlu_2$ KO slices (11.3 ± 7.9%, $n = 5$), which express only $mGlu_3$. In contrast, the $mGlu_3$-selective NAM VU0469942 did not antagonize the effect of LY379268 in slices from $mGlu_3$ KO mice (67.2 ± 1.9%, $n = 3$), in which case the agonist would be acting only on the $mGlu_2$ subtype. These data confirm that the actions of VU0469942 require the expression of $mGlu_3$ and are therefore mediated by selective inhibition of this receptor.

Postsynaptic $mGlu_3$ Mediates Group II mGlu Agonist-Induced Ca^{2+} Elevations in Layer V Pyramidal Neurons in mPFC. Previous reports and our analysis of PPRs suggest that group II LTD in mPFC is expressed postsynaptically. Furthermore, LTD is dependent upon intracellular Ca^{2+} mobilization induced by activation of group II mGlu receptors in layer V pyramidal cells (16). Based on our findings that LTD is dependent upon $mGlu_3$ activation, we tested the hypothesis that Ca^{2+} signaling is downstream of $mGlu_3$ by monitoring Ca^{2+} in individual layer V pyramidal neurons (Fig. 4 *A*–*C*). Cells were loaded with the Ca^{2+}-sensitive dye, Fluo-4, through a patch pipette; experiments were performed in the presence of tetrodotoxin (TTX) (1 μM) to isolate postsynaptic receptor actions. In control experiments, when group II mGlu receptors were activated with LY379268 (100 nM for 10 min) there was an increase in fluorescence intensity relative to baseline (0.47 ± 0.11 ΔF/F peak), indicating an elevation in intracellular Ca^{2+}. However, when these experiments were performed in the presence of the $mGlu_3$ NAM, VU0469942 (10 μM), there was a significant reduction in the change in fluorescence (0.21 ± 0.024 ΔF/F peak; $P < 0.05$; unpaired *t* test). This is consistent with the hypothesis that group II agonists induce intracellular Ca^{2+} signals through activation of $mGlu_3$ in layer V pyramidal neurons. Furthermore, these data suggest that postsynaptic $mGlu_3$ is the critical site of action for induction of group II mGlu LTD.

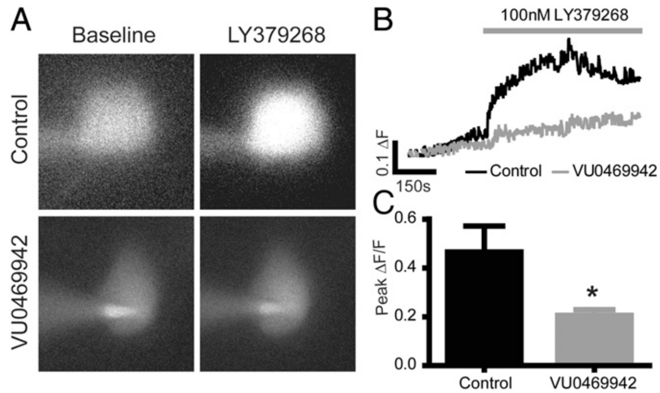

Fig. 4. Activation of postsynaptic $mGlu_3$ increases intracellular Ca^{2+} in layer V pyramidal neurons. (*A*) Representative images demonstrating fluorescence levels during baseline (*Left*) and in the presence of LY379268 (*Right*). *Top* images are from a control experiment and *Bottom* images are from an experiment performed in the presence of the $mGlu_3$ NAM VU0469942. (*B*) Time course of fluorescence measurements for the experiments shown in *A*. (*C*) Quantification and summary of Ca^{2+} imaging experiments ($n = 7$ per group). * indicates $P < 0.05$ unpaired *t* test vs. control. Data are expressed as mean ± SEM.

The $mGlu_3$ NAM VU0477950 Impairs Fear Extinction Learning. We next sought to investigate how $mGlu_3$ is able to modulate fear extinction, a behavior that is dependent upon the integrity of the mPFC (27). On day 1, drug naive mice were conditioned by pairing a tone conditioned stimulus (CS) with a mild foot-shock unconditioned stimulus (US). After seven CS–US presentations, there was a significant increase in the amount of time spent freezing during the CS presentation across trials for all subjects ($P < 0.0001$), indicating all mice were conditioned to associate the tone with the foot shock (Fig. 5*A*). Twenty-four hours later, mice received an injection of vehicle or the $mGlu_3$ NAM VU0477950 (3–100 mg/kg i.p.). Thirty minutes after injection, mice were placed in a new context and received 20 CS-alone presentations to evaluate initial cue memory and subsequent extinction learning (Fig. 5*B*). No effect of VU0477950 on initial cue memory (Fig. 4*B* and Fig. S3*A*) was observed as all mice had equivalent levels of freezing during the first block of CS-alone trials ($P > 0.05$). During subsequent CS presentations, vehicle-treated mice decreased freezing to an asymptotic level, a pattern of behavior consistent with extinction learning. However, in mice treated with the $mGlu_3$ NAM, there was a dose-dependent impairment in extinction learning ($P < 0.05$, block × dose interaction). Specifically, mice treated with a 30-mg/kg or a

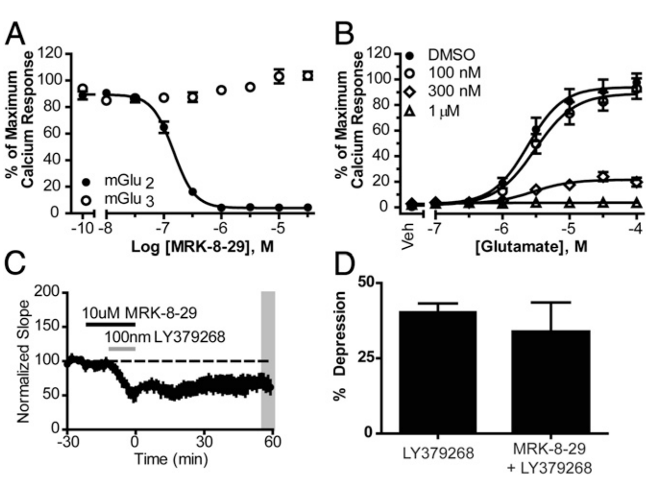

Fig. 3. MRK-8-29 is a selective $mGlu_2$ NAM. (*A*) Concentration-response curves for MRK-8-29 in the presence of an EC_{80} for glutamate at rat $mGlu_2$ and rat $mGlu_3$ receptors. (*B*) Progressive fold shift for MRK-8-29 at rat $mGlu_2$ receptors. (*C*) Preincubation of slices with MRK-8-29 ($n = 5$) does not affect LTD induced by LY379268. (*D*) Quantification of the effects of MRK-8-29 on LTD at 55–60 min after drug washout (average of shaded region in *C*). Data are expressed as mean ± SEM.

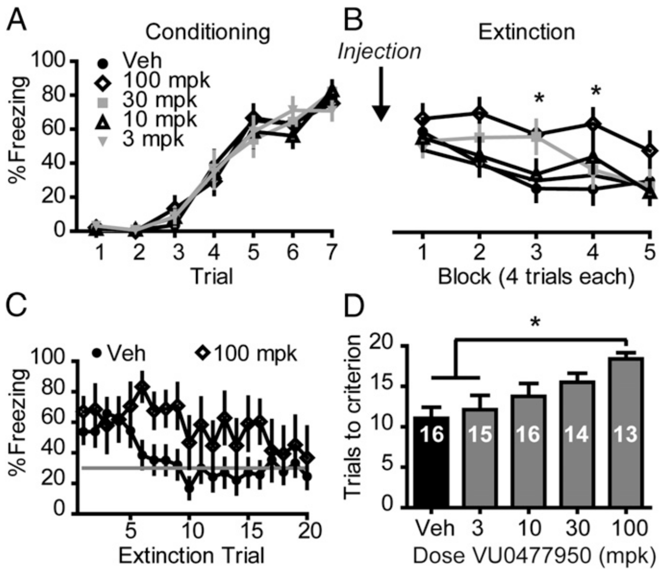

Fig. 5. An $mGlu_3$ NAM impairs fear extinction learning in mice. (*A*) On day 1, mice were fear conditioned with 7 paired CS–US trials. No drug was administered on the conditioning day. (*B*) On day 2, mice received an i.p. injection of vehicle or the $mGlu_3$ NAM VU0477950 (3–100 mg/kg) and then were trained on fear extinction with 20 CS-alone trials. Data are presented as mean freezing across 4 consecutive trials. * indicates $P < 0.05$, 100-mg/kg Bonferroni posttest vs. vehicle-treated mice. (*C*) Extinction learning criterion was established by examining asymptotic learning (shaded horizontal line) across all 20 CS-alone trials in vehicle-treated animals. Freezing behavior of mice treated with 100 mg/kg is plotted for comparison. (*D*) Quantification of the learning impairment induced by VU0477950 as measured by trials to criterion. Numbers within the individual bars indicate number of mice within the respective group. * indicates $P < 0.05$ Tukey posttest vs. vehicle and 3-mg/kg–treated mice. Data are expressed as mean ± SEM.

100-mg/kg dose maintained high levels of freezing through blocks 3 and 4, which reached significance in the 100-mg/kg group relative to vehicle-treated animals (Bonferroni posttests; $P < 0.05$). We quantified the impact of VU0477950 on extinction learning by analyzing the number of trials required to reach criterion. The learning criterion was established by examining the performance of vehicle-treated animals across all extinction trials and determining asymptotic performance (Fig. 5*C*). On average, vehicle-treated mice achieved criterion learning of 30% freezing in ∼11 trials (Fig. 5*D*). Consistent with analysis of freezing across blocks of trials, there was a dose-dependent increase in the number of trials required to reach criterion in mice treated with VU0477950. Moreover, mice treated with the 100-mg/kg dose of the compound showed a significant increase in the number of trials to reach criterion compared with mice in the vehicle and 3-mg/kg groups ($P < 0.05$; Tukey posttest). Interestingly, there appeared to be no impairment in memory retrieval for extinction in animals treated with 30 mg/kg or 100 mg/kg relative to vehicle-treated animals when measured 24 h later in a second extinction session (Fig. S3 *B* and *C*). Furthermore, increased levels of freezing could not simply be attributed to general motor suppression (Fig. S3*D*) or an anxiogenic effect induced by VU0477950 (Fig. S4 *A*–*D*), as demonstrated by no change in open field activity.

Discussion

We have demonstrated that $mGlu_3$ plays a critical role in the regulation of mPFC neuroplasticity and is required for a specific learned behavior that is dependent upon the integrity of this brain region. In agreement with reports from rat brain slices (13–16), we found that strong pharmacological activation of group II mGlu receptors results in LTD of fEPSPs recorded in layer V mPFC. The initial transient depression of the fEPSP slope was accompanied by a robust increase in the PPR, suggesting a presynaptic modulation of neurotransmitter release, which is a well-known function of group II mGlu receptors, and especially $mGlu_2$ (28). One hour after the maximal transient depression, when we quantified LTD, the PPR had returned to baseline levels, suggesting the expression of lasting depression of synaptic transmission was likely mediated by a postsynaptic mechanism. This is consistent with previous studies showing postsynaptic actions of group II mGlu receptor agonists in mPFC pyramidal cells (15, 16) and with our finding that activation of $mGlu_3$ induces calcium transients in these cells. Furthermore, under our experimental conditions this LTD does not require activation of NMDA receptors, as the magnitude was unaffected by an NMDA receptor antagonist.

To delineate the roles the $mGlu_2$ and $mGlu_3$ receptor subtypes, we used newly identified $mGlu_2$ or $mGlu_3$ NAMs, as well as $mGlu_2$ and $mGlu_3$ receptor KO mice. Although the selective $mGlu_2$ NAM MRK-8-29 slightly reduced the magnitude of the transient depression, it did not prevent the induction of LTD. In contrast, $mGlu_3$ NAMs VU0469942 and VU0477950 completely blocked induction of LTD by the group II mGlu receptor agonist, but were without effect on the transient depression of fEPSPs. Similarly, LTD was observed in slices prepared from $mGlu_2$, but not $mGlu_3$, KO mice, whereas acute depression of synaptic transmission was intact in slices from $mGlu_3$ KO mice. Taken together, these results provide strong evidence that activation of $mGlu_2$ can induce transient depression of synaptic transmission in mPFC neurons, a response to $mGlu_2$ activation that has been established at multiple other synapses (19–23). However, although $mGlu_2$ can regulate transmission at this synapse, these data also reveal that activation of $mGlu_3$ is required for the induction of LTD in mPFC pyramidal cells. In contrast to presynaptic effects of $mGlu_2$ receptor activation at this and other synapses, our data suggest an important role for postsynaptically localized $mGlu_3$ in induction of LTD. Also, similar to $mGlu_5$-mediated LTD in the hippocampus, it is likely that maintenance of LTD at this synapse is mediated by postsynaptic mechanisms. Thus, in agreement with previous studies (16), we found that a selective $mGlu_{2/3}$ agonist increases intracellular Ca^{2+} in layer V pyramidal cells and now show that this response is mediated by $mGlu_3$. This effect is likely due to direct actions of the group II agonist on the postsynaptic neuron, as the experiments were performed in the presence of TTX. Although the exact mechanism by which $mGlu_3$, a $G_{i/o}$ coupled receptor, induces intracellular Ca^{2+} increases is unknown, similar effects of group II mGlu receptor agonists on intracellular Ca^{2+} are observed in hippocampal CA3 pyramidal cell and interneuron populations, and these responses are thought to be mediated by activation of $mGlu_3$ (29). In addition, there are examples of other $G_{i/o}$ coupled receptors inducing intracellular Ca^{2+} elevations (30). Overall, the results from our electrophysiology and imaging studies provide strong support for a critical role of postsynaptic $mGlu_3$ signaling in the induction of LTD in mPFC.

The finding that activation of $mGlu_3$ is required for induction of a form of synaptic plasticity in the mPFC is especially important in light of extensive studies demonstrating a central role of the mPFC in multiple domains of cognitive function and previous genetic studies implicating $mGlu_3$ in aspects of cognitive function that require integrity of this cortical region. Based on this, it is possible that $mGlu_3$-mediated LTD in the mPFC could be important for some aspects of mPFC-dependent cognition. Interestingly, previous studies suggest that intact functioning of the mPFC and especially regulation of excitatory inputs to the PFC from paralimbic regions (27, 31, 32) are central for fear extinction learning. Thus, our finding that the selective $mGlu_3$ NAM VU0477950 induced a dose-dependent increase in the number of trials required to extinguish fear responses is consistent with a possible role of $mGlu_3$ in this specific form of prefrontal cortical-dependent cognitive function. The highest dose of the $mGlu_3$ NAM tested nearly doubled the number of trials needed to reach the extinction criterion. Furthermore, some animals failed to reach criterion after the maximum number of cues given, suggesting a major role for $mGlu_3$ in the process of acquisition of extinction learning. Interestingly, there was no difference in the amount of freezing during the initial cue presentations, signifying that the $mGlu_3$ NAM did not create a heightened fear state, which is corroborated by our data indicating that the $mGlu_3$ NAM was not anxiogenic in an open-field assay. Furthermore, there was no difference in retrieval of the extinction memory assessed 24 h posttraining, despite the robust learning delay.

Importantly, the doses of VU0477950 used for these studies were based on extensive pharmacokinetic studies that revealed that these doses lead to free brain concentrations that are estimated to be in the range of those required to inhibit $mGlu_3$ but well below concentrations tested for selectivity against other mGlu receptor subtypes. The relatively high doses required to achieve these concentrations in the CNS and for behavioral efficacy are related to the rapid clearance and high plasma protein binding of VU0477950, which limit the amount of free drug available to bind to the target (24). Estimates of unbound brain concentrations are based on precise measures of total brain concentrations, which are then corrected for in vitro measures of plasma and brain homogenate binding and do not provide a definitive measure of the actual concentration achieved at the receptor site. Thus, it is impossible to directly estimate the level of receptor occupancy achieved in the CNS with the doses used. However, it is also possible that in vivo efficacy requires high $mGlu_3$ receptor occupancy, a property that has been reported for $mGlu_1$ and $mGlu_5$ NAMs (33, 34). In contrast, mGlu receptor positive allosteric modulators (PAMs) can produce full efficacy with relatively low occupancy in the CNS because of the contributions of both affinity and cooperativity to PAM potency at a receptor (35, 36). Unfortunately, there are currently no selective $mGlu_3$ radioligands that would allow us to measure receptor occupancy of VU0477950. In the future, development of radiolabeled compounds that can be used with positron emission tomography (PET) and in vivo radioligand binding studies will be crucial for assessing the level of $mGlu_3$ occupancy and blockade required for different behavioral responses to $mGlu_3$ NAMs.

The fear extinction circuit is composed of reciprocal interactions between the mPFC subregions and the amygdala and hippocampus (27, 31, 32, 37). In vivo recordings have demonstrated that activity within the PL cortex, the region of the mPFC where we studied LTD, is correlated with freezing behavior during fear extinction (32, 38–40). During states of high fear and freezing, neurons within the PL will display robust firing in response to fear cues, such as a tone CS. Recent work has demonstrated that this pattern of activity likely reflects excitatory drive from the amygdala to the PL subregion of mPFC (31, 41). Moreover, PL neurons provide reinforcing feedback by sending robust projections to the amygdala, which may help drive freezing behavior. Furthermore, as the animals successfully extinguish fear responding, CS-induced firing of mPFC/PL neurons diminishes (38–40). Our data raise the intriguing possibility that this lasting depression of CS-induced firing in this mPFC subregion is an $mGlu_3$-dependent process and could reflect a lasting depression of transmission from amygdala afferents. However, at present, it is not known whether the $mGlu_3$-dependent LTD established here reflects depression of transmission at this specific synapse. Group II mGlu-mediated LTD of cortical inputs to the amygdala has been reported (42). Thus, it is also possible that the $mGlu_3$ NAM could be acting within the amygdala to prolong freezing and impair extinction learning. However, activation of either $mGlu_2$ or $mGlu_3$ alone appears to be sufficient for the induction of LTD at this synapse (42); therefore selective attenuation of $mGlu_3$ signaling is likely to have a minimal physiological effect. Future studies using complementary in vivo and in vitro methods making use of the newly available tools will help provide more mechanistic information about the role of $mGlu_3$ in this form of mPFC-dependent cognitive function.

In summary, our data help define the role of $mGlu_3$ in the regulation of prefrontal cortical function. Together with recent reports of working memory deficits in $mGlu_3$ KO mice (43, 44), the effects of $mGlu_3$ NAMs suggest that $mGlu_3$ plays an important role in certain PFC-dependent behaviors. Additionally, allelic variations in *GRM3*, the human gene encoding $mGlu_3$, have been reported to affect prefrontal activity and cognitive performance in healthy human subjects (45) and several studies have found associations between mutations in *GRM3* and psychiatric disorders (1, 2, 4, 6–8). If these mutations are found to lead to a loss of $mGlu_3$ function, this would suggest that selective positive allosteric modulators of $mGlu_3$ may represent a novel therapeutic strategy for enhancing prefrontal function in patients.

Materials and Methods

Animals. All animal studies were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (46). Male ICR (CD1) (Harlan Laboratories) mice were used in electrophysiology (4–8 wk old) and behavioral studies (6–7 wk old). Male $mGlu_2$ and $mGlu_3$ KO mice (6–12 wk old; gift from Eli Lilly and Company) were also used for electrophysiology studies.

Electrophysiology. Coronal slices through the mPFC (300–400 μm) were prepared from ICR(CD1), $mGlu_2$, and $mGlu_3$ knockout mice with a vibrating microtome (VT1200s; Leica). After anesthesia with a mixture of ketamine and xylazine (100 mg/kg and 10 mg/kg i.p.), mice were perfused with a 4 °C sucrose-based cutting buffer containing 230 mM sucrose, 2.5 mM KCl, 10 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 10 mM glucose, 26 mM $NaHCO_3$, and 0.5 mM sodium ascorbate. Brain slices were then incubated at 32 °C for 12–15 min in an *N*-methyl-D-glucamine (NMDG)-based recovery solution as previously described (47) and then transferred to a holding chamber with artificial cerebrospinal fluid (aCSF) containing 126 mM NaCl, 2.5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 10 mM glucose, 26 mM $NaHCO_3$, 5 mM sodium ascorbate, and 12 mM *N*-acetylcystine. Recording aCSF was identical aside from the exclusion of sodium ascorbate and *N*-acetylcystine. fEPSPs were recorded from layer V of PL, using a pulled-glass pipette (3–5 MΩ), and evoked by electrical stimulation of layer II/III (0.05 Hz), using a concentric bipolar electrode. Three consecutive fEPSP slopes were averaged and then normalized to the mean baseline slope before drug application. LTD was measured as the average slope across the last 5 min of the recording session.

For calcium (Ca^{2+}) imaging experiments, individual neurons in layer V of mPFC exhibiting properties of pyramidal cells were loaded with the indicator dye Fluo-4, Pentapotassium Salt, cell impermeant (Life Technologies), through a glass patch pipette as previously described (48). Detailed information for the Ca^{2+} imaging experiments can be found in *SI Materials and Methods*.

Behavioral Studies. Behavioral studies were conducted on wild-type (WT) male ICR(CD1) mice. Before behavioral experiments, all animals were habituated to handling, transportation procedures, and injections for 2 consecutive days. Mice were fear conditioned with seven pairings of a tone CS (3.5 kHz, 80 dB, 30 s) with mild foot-shock US (2 s, 0.6 mA). Mice were returned to their home cages. Twenty-four hours after fear conditioning, CS retrieval and extinction learning were assessed with 20 CS-alone trials (5-s intertrial interval). To limit the effects of contextual conditioning, mice were fear conditioned in a round-walled, metal bar-floored chamber that was scented with 10% (vol/vol) vanilla extract odor and housed in a room with white ceiling lights. Extinction training occurred in a square-walled, solid-floored chamber that was scented with 10% (vol/vol) peppermint and housed in a room with red ceiling lights. Mice were dosed with vehicle or $mGlu_3$ NAM VU0477950 (3–100 mg/kg) via i.p. injection 30 min before extinction training. Freezing behavior defined as the absence of movement other than respiration was used to measure fear and was quantified by computer video analysis software (Video Freeze; Med Associates). For locomotion experiments, mice were injected with vehicle or VU0477950 (100 mg/kg) i.p. and placed in an open field chamber equipped with infrared beams (Med Associates) to monitor motor activity for 1 h.

Please see *SI Materials and Methods* for information on drugs, statistical analysis, chemistry methodology, and details of the cell line Ca^{2+} mobilization assay.

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