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Co-Activation of Metabotropic Glutamate Receptor 3 and Beta-Adrenergic Receptors Modulates Cyclic-AMP and Long-Term Potentiation, and Disrupts Memory Reconsolidation

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Activation of β-adrenergic receptors (βARs) enhances both the induction of long-term potentiation (LTP) in hippocampal CA1 pyramidal cells and hippocampal-dependent cognitive function. Interestingly, previous studies reveal that coincident activation of group II metabotropic glutamate (mGlu) receptors with βARs in the hippocampal astrocytes induces a large increase in cyclic-AMP (cAMP) accumulation and release of adenosine. Adenosine then acts on A_1 adenosine receptors at neighboring excitatory Schaffer collateral terminals, which could counteract effects of activation of neuronal βARs on excitatory transmission. On the basis of this, we postulated that activation of the specific mGlu receptor subtype that mediates this response could inhibit βAR-mediated effects on hippocampal synaptic plasticity and cognitive function. Using novel mGlu receptor subtype-selective allosteric modulators along with knockout mice we now report that the effects of $mGlu_{2/3}$ agonists on βAR-mediated increases in cAMP accumulation are exclusively mediated by $mGlu_3$. Furthermore, $mGlu_3$ activation inhibits the ability of the βAR agonist isoproterenol to enhance hippocampal LTP, and this effect is absent in slices treated with either a glial toxin or an adenosine A_1 receptor antagonist. Finally, systemic administration of the $mGlu_{2/3}$ agonist LY379268 disrupted contextual fear memory in a manner similar to the effect of the βAR antagonist propranolol, and this effect was reversed by the $mGlu_3$-negative allosteric modulator VU0650786. Taken together, these data suggest that $mGlu_3$ can influence astrocytic signaling and modulate βAR-mediated effects on hippocampal synaptic plasticity and cognitive function.
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INTRODUCTION

Activation of noradrenergic projections from the locus coeruleus to the hippocampus and cortex enhances synaptic plasticity, aspects of cognition, and memory formation. Extensive studies suggest that noradrenergic regulation has a critical role in long-term retention of events that occur in novel environments or under conditions of mild stress. The majority of noradrenaline (NA) actions regulating hippocampal and cortical function are mediated through *β*-adrenergic receptors (*β*ARs). Interestingly, selective *β*AR agonists enhance induction of long-term potentiation (LTP) in hippocampal CA1 pyramidal cells and hippocampal-dependent cognitive function (O'Dell *et al*, 2015). Furthermore, hippocampal-dependent memory is impaired by reduced NA levels or *β*AR antagonists (Murchison *et al*, 2004; O'Dell *et al*, 2015).

A series of early studies revealed that *β*AR coupling to adenylyl cyclase activation in hippocampal and cortical astrocytes is highly regulated by group II metabotropic glutamate (mGlu) receptors ($mGlu_2$ and $mGlu_3$). Specifically, co-activation of *β*ARs and group II mGlu receptors in astrocytes results in a marked potentiation of *β*AR-induced cyclic-AMP (cAMP) accumulation (Moldrich *et al*, 2002; Winder and Conn, 1993, 1995; Winder *et al*, 1996). This large increase in astrocytic cAMP under coincident *β*AR and group II mGlu receptor activation has actions that could counteract the ability of neuronal *β*AR activation to enhance synaptic plasticity. Specifically, the large increase in cAMP induced by co-activation of group II mGlu receptors with *β*ARs leads to adenosine release from astrocytes and activation of presynaptic A_1 adenosine receptors on local excitatory SC terminals (Gereau *et al*, 1995; Winder *et al*, 1996). It has been hypothesized that this could serve as a mechanism to attenuate the physiological consequences of

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excessive excitatory transmission during periods of high noradrenergic activity, as would occur during periods of intense stress. However, A_1 adenosine receptor activation inhibits induction of LTP (de Mendonca and Ribeiro, 1994), and it is conceivable that activation of group II mGlu receptors could also reverse the ability of βARs to enhance induction of LTP. This is especially interesting in light of recent studies, suggesting that agonists and antagonists of group II mGlu receptors can, respectively, impair and enhance some forms of hippocampal-dependent cognitive function (Marek, 2010). On the basis of these studies, we postulated that the group II mGlu receptor subtype involved in this interaction with βARs could depress induction of hippocampal LTP under conditions in which βARs are also activated and could impair aspects of hippocampal-dependent learning that are sensitive to βAR activation.

We now report a series of studies using novel mGlu receptor subtype-specific allosteric modulators and knockout (KO) mice to show that the $mGlu_3$ receptor subtype is responsible for potentiating βAR-elicited cAMP responses in brain slices. Furthermore, $mGlu_3$ activation can block the enhancement of LTP by βARs by a mechanism that requires functional astrocytes and activation of A_1 adenosine receptors. Finally, we present evidence that $mGlu_3$ activation can disrupt contextual fear memories in a manner that is similar to the effect of the βAR antagonist propranolol.

MATERIALS AND METHODS

Materials and Chemicals

LY379268, propranolol, CCPA, and PQ69 were purchased from Tocris (Ellisville, Missouri). MNI-137 (Hemstapat *et al*, 2007), BINA (Galici *et al*, 2006), VU0469942 (Wenthur *et al*, 2013), and VU0650786 (Engers *et al*, 2015) were synthesized as previously described. Unless otherwise stated, all other reagents were purchased from Sigma-Aldrich (St Louis, Missouri).

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 NIH Guide for the Care and Use of Laboratory Animals. Male Sprague–Dawley (SD) rats (Harlan Labs, Indianapolis, IN) aged 4–6 weeks were used for cAMP, electrophysiology, and immunohistochemistry experiments. Male (age 32–38 days) ICR (CD1) mice (Harlan Labs) and $mGlu_2$ or $mGlu_3$ KO mice and their wild-type (WT) littermates (Linden *et al*, 2005; used by permission of Eli Lilly and bred by Taconic Farms, Cambridge City, IN) were also used in cAMP studies. Behavioral experiments were conducted with male SD rats (Harlan Labs) ordered to arrive weighing 250–275 g and allowed to acclimate in the animal facility for 4–5 days before use.

Rodent Tissue cAMP Accumulation

Agonist-induced increases in cAMP accumulation were measured in cross-chopped hippocampal or cortical slices prepared as previously outlined in detail (Gereau and Conn, 1994a). Slices were then incubated for 20 min with vehicle or $mGlu_{2/3}$ agonists (LY379268) ± the βAR agonist (isoproterenol (ISO)) before termination of the reaction and measurement of cAMP levels as previously described (Sheffler and Conn, 2008). Data were normalized to the percent vehicle cAMP response or the fold-over basal cAMP response where appropriate as indicated. Concentration-response curve data were fit using Prism 5.0 (GraphPad) to a four-parameter logistic equation to determine EC_{50} or IC_{50} values. Significance was determined using Prism 5.0, performing a one-way ANOVA followed by a Bonferonni post-test unless otherwise noted.

Hippocampal Slice Preparation for Electrophysiology

Rats were anesthetized with ketamine (100 mg/kg intraperitoneal (I.P.)) and xylazine (10 mg/kg I.P.) and perfused with 4 °C sucrose-based cutting buffer containing (in mM): 230 sucrose, 2.5 KCl, 10 MgCl, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 10 glucose, 26 $NaHCO_3$, and 0.5 sodium ascorbate. Horizontal hippocampal slices (400 μm) were prepared with a vibrating microtome (VT1200s, Leica), incubated 12–15 min at 32 °C in an N-methyl-D-glucamine-based recovery solution (Walker *et al*, 2015), and transferred to a holding chamber with artificial cerebrospinal fluid (aCSF) containing (in mM): 126 NaCl, 2.5 KCl, 1 MgCl, 2 $CaCl_2$, 1.25 NaH_2PO_4, 15 glucose, 26 $NaHCO_3$, and 5 sodium ascorbate. Recording aCSF was identical minus ascorbate.

Extracellular Field-Potential Recordings

Field excitatory postsynaptic potential (fEPSP) and LTP recordings were performed as previously described in detail (Noetzel *et al*, 2013). The adenosine A_1 receptor antagonist 8-cyclopentyltheophylline (CPT) was bath-applied for 10 min (200 nM; Schmitt *et al*, 2012) before beginning experiments. Sodium fluoroacetic acid (FAC) was bath-applied after a 5-min baseline period. We found that on average fEPSP slopes stabilized 15 min after FAC exposure. Slopes were normalized to the average from 20 to 30 min from the start of the recordings and drug applications began at 30 min. Data were analyzed as previously described (Walker *et al*, 2015). Statistical comparisons between drug conditions were made using unpaired *t*-tests, one-way or two-way ANOVA as appropriate followed by Tukey *post hoc* tests. See Supplementary Materials for NMDA current measurement methodology.

Imaging

See Supplementary Methods for immunohistochemistry details.

For RNAscope combined with immunohistochemistry experiments, slices were washed three times in 1× TBS after sectioning. To perform RNAscope with traditional immunohistochemistry, the manufacturer's directions (Advanced Cell Diagnostics (ACD)) were modified. After washing, sections were incubated in 95 °C pre-warmed ACD antigen retrieval buffer for 15 min, and then immediately washed three times in 1× TBS. After washing, sections were mounted to positively charged glass slides (ThermoFisher,

New York, NY) and dried overnight. Once dry, a hydrophobic barrier was placed around the section and dried for 1 h at 60 °C. The following steps were performed at 40 °C in an ACD HybEZ slide hybridization oven (Newark, CA). First, slides were incubated in ACD protease IV for 15 min and then washed 3× in ACD RNAscope wash buffer. Slides were then incubated with anti-ADRB2 and anti-GRM3 probes for 2 h and then washed 3× in ACD RNAscope wash buffer. After hybridization with probes, slides were hybridized with Amp 1-FL for 30 min, with Amp 2-FL for 15 min, with Amp 3-FL for 30 min, and then Amp 4 FL-A for 15 min, with three RNAscope wash buffer washes between each hybridization. After RNAscope staining was completed, slides were blocked in 10% normal donkey serum with 0.1% Triton X-100 in 1× TBS for 1 h at room temperature and then washed two times for 5 min in 1× TBS. After blocking, slides were incubated with 1 : 100 goat anti-GFAP (Abcam, Cambridge, UK) in 10% normal donkey serum for 24 h at 4 °C and then washed two times for 5 min in 1× TBS. After primary incubation, slides were incubated with 1 : 500 donkey anti-goat-Cy3 (Jackson Immuno-Research, West Grove, PA) for 2 h at room temperature, and then washed three times in 1× TBS. Slides were air-dried and mounted in pro-long gold-mounting media (Life Technologies, Grand Island, NY). Slides were imaged on a Zeiss LSM 510 inverted confocal microscope.

Contextual Fear Conditioning

Contextual fear-conditioning experiments were performed similar to previous reports (Taherian *et al*, 2014; see Supplementary Methods for details of behavioral training). We tested the effects of vehicle, propranolol (10 mg/kg), LY379268 (1–3 mg/kg), VU0650786 (56.6 mg/kg), CCPA (0.1–0.15 mg/kg), PQ69 (0.3–1.0 mg/kg), a mixture of LY379268 and VU0650786 or LY379268 and PQ69 on reconsolidation and/or consolidation. Propranolol, LY379268, CCPA, and PQ69 doses were based ranges in previously published studies showing efficacy *in vivo* (Concas *et al*, 1993; Lu *et al*, 2014; Pitsikas and Markou, 2014; Taherian *et al*, 2014). VU0650786 dosing was selected based on our extensive previous *in vivo* studies (Engers *et al*, 2015). All compounds for behavioral experiments were formulated with 10% Tween 80 in sterile water and the pH was adjusted to 7.4 with NaOH and injected at a volume of 2 ml/kg. Drug conditions were compared with a one-way ANOVA. *Post hoc* comparisons for significant omnibus tests were performed using a Dunnett's multiple comparison test *vs* vehicle.

RESULTS

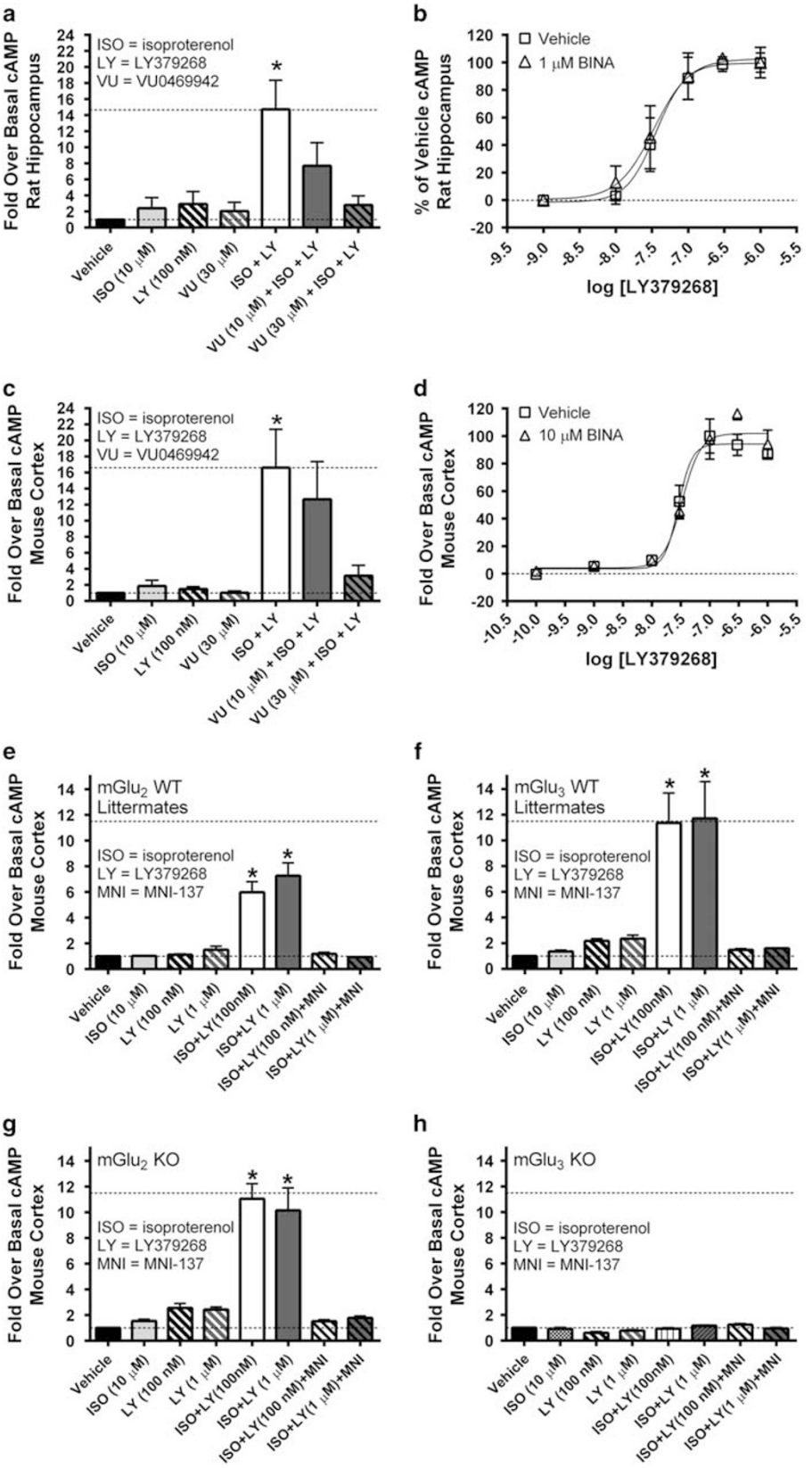
Group II mGlu Receptor-Induced Potentiation of βAR-Mediated cAMP Accumulation is Mediated by $mGlu_3$

We previously reported that coincident activation of group II mGlu receptors and βARs leads to robust increases in cAMP accumulation in a variety of rat brain regions including the cortex and hippocampus (Winder and Conn, 1993; Winder *et al*, 1996). However, the group II mGlu receptor subtype mediating this effect is unknown. In order to investigate the contribution of the individual group II mGlu receptor subtypes, we took advantage of two pharmacological tools that exhibit $mGlu_2$ and $mGlu_3$ subtype selectively. First, we confirmed that co-activation of βARs and group II mGlu receptors results in enhanced cAMP accumulation in brain slices. As shown in Figure 1a and c, application of the $mGlu_{2/3}$ agonist LY379268 (100 nM) significantly potentiates cAMP accumulation induced by the βAR agonist ISO (10 μM) in slices from both rat hippocampus ($F_{(7,16)} = 5.369$, $p < 0.05$; Bonferonni post-test $p < 0.05$) and mouse cortex ($F_{(7,16)} = 5.819$, $p < 0.05$; Bonferonni post-test $p < 0.05$) *vs* vehicle. When slices were pre-incubated with the $mGlu_3$-negative allosteric modulator (NAM) VU0469942 (10–30 μM), there was a concentration-dependent decrease in cAMP accumulation, whereby the response to LY379268 plus ISO was not statistically different from vehicle in the presence of VU0469942 ($p > 0.05$).

In order to evaluate the contribution of $mGlu_2$ to this biochemical response, we characterized the effects of the $mGlu_2$-selective positive allosteric modulator (PAM) BINA on the dose–response of a group II agonist-induced potentiation of βAR-mediated cAMP responses. As shown in Figure 1b, the $mGlu_{2/3}$ agonist LY379268 dose-dependently potentiated ISO-induced cAMP responses in rat hippocampal slices. However, pretreatment of the slices with 1 μM BINA does not alter the LY379268 dose–response (no difference in LY379268 potency or maximal response; $p > 0.05$). This concentration of BINA (1 μM) has near-maximal potentiating effects on $mGlu_2$-mediated responses in cell lines and in hippocampal slices (Galici *et al*, 2006). Nevertheless, we determined the effect of an even higher concentration of BINA (10 μM) on the dose–response of LY379268 for potentiation of ISO-induced cAMP responses in ICR (CD1) mouse cortical slices (Figure 1d). Similar to the effects in rat hippocampal slices, BINA does not potentiate the LY379268 dose–response in mouse cortical slices (no difference in LY379268 potency or maximal response; $p > 0.05$). Together, these studies suggest that $mGlu_3$ is likely the primary group II mGlu receptor subtype that potentiates βAR-mediated cAMP responses.

We next evaluated group II mGlu receptor-mediated potentiation of effects of βAR activation on cAMP accumulation in cortical slices prepared from $mGlu_2$ and $mGlu_3$ KO mice and their matched WT littermate pairs. As shown in Figure 1e and f, 100 nM and 1 μM LY379268 potentiated ISO-induced cAMP responses in cortical slices prepared from the WT littermates for either the $mGlu_2$ ($F_{(8,27)} = 32.39$, $p < 0.05$; Bonferonni post-test $p < 0.05$ *vs* vehicle) or $mGlu_3$ ($F_{(8,27)} = 10.15$, $p < 0.05$; Bonferroni post-test $p < 0.05$ *vs* vehicle) KO mice, and these effects are blocked by the $mGlu_{2/3}$ NAM MNI-137 ($p > 0.05$ *vs* vehicle). Furthermore, the group II mGlu receptor agonist potentiated βAR cAMP responses in cortical slices prepared from $mGlu_2$ KO mice (Figure 1g; $F_{(8,27)} = 10.15$, $p < 0.05$; Bonferroni post-test $p < 0.05$ *vs* vehicle). In contrast, LY379268 had no effect on ISO-induced increases in cAMP accumulation in slices from $mGlu_3$ KO mice (Figure 1h; $F_{(8,27)} = 39.25$, $p < 0.05$; Bonferroni post-test $p > 0.05$ for ISO +LY379268 *vs* vehicle). The absence of a cAMP potentiation response in $mGlu_3$ but not $mGlu_2$ KO mouse cortical slices is

consistent with the pharmacological studies and further confirms that mGlu$_3$ mediates potentiation of βAR-induced cAMP responses. It should be noted that there appears to be a blunted cAMP potentiation in WT littermates for the mGlu$_2$ KO mice. This effect was consistently observed in the process of performing these experiments.



Unfortunately, we do not currently have an explanation for this phenomenon.

$mGlu_3$ Activation Antagonizes the Enhancing Effects of βARs on Theta Burst-Induced LTP at the SC-CA1 Synapse

An important function of βAR activation in area CA1 is to enhance the ability of weak afferent stimulation patterns to induce LTP at the SC-CA1 synapse through actions on CA1 pyramidal cells (O'Dell *et al*, 2015). On the basis of previous findings that the large increase in cAMP accumulation induced by coincident activation of $mGlu_{2/3}$ and βARs on astrocytes leads to release of adenosine and activation of A_1 adenosine receptors on SC terminals (Gereau *et al*, 1995; Winder *et al*, 1996), we sought to determine whether group II mGlu receptors can inhibit the ability of βAR activation to enhance hippocampal LTP at the SC-CA1 synapse. We recorded extracellular fEPSPs at the SC-CA1 synapse in hippocampal slices prepared from rats. LTP was induced using two different theta burst stimulation (TBS) protocols (Figure 2a) to induce increasing levels of potentiation of excitatory transmission. As shown in Figure 2b, the weak TBS protocol results in an increased fEPSP slope ($148.3 \pm 8.3\%$) *vs* baseline ($n = 6$ slices). This LTP was submaximal as the more intense strong TBS protocol resulted in a greater increase in the fEPSP slope ($212.8 \pm 8.6\%$; $n = 5$). When slices were pretreated with 1 μM ISO (Gereau *et al*, 1995) for 10 min (Figure 2c), weak TBS resulted in a greater increase in fEPSP slope ($213.7 \pm 15.7\%$; $n = 5$) *vs* control slices that were not treated with ISO. Thus, as reported for other stimulation and slice preparation conditions, βAR activation enhances LTP in the presence of submaximal stimulation (O'Dell *et al*, 2015).

Next, we tested the hypothesis that activation of group II mGlu receptors would modulate the enhancement of weak TBS LTP by βARs by co-applying the $mGlu_{2/3}$ agonist LY379268 at the minimal concentration we observed in the cAMP experiments to produce maximum potentiation (100 nM) and ISO for 10 min before LTP induction (Figure 2d). Under these conditions, weak TBS resulted in less potentiation of the fEPSP slope ($147.1 \pm 6.0\%$; $n = 5$) *vs* ISO alone, suggesting that co-activating $mGlu_{2/3}$ receptors and βARs antagonized the enhancement produced by βARs alone. Because our cAMP experiments implicated a crucial role of $mGlu_3$, we repeated these experiments in the presence of an $mGlu_3$ NAM. When slices were pretreated with 30 μM VU0469942, a concentration that produced robust inhibition of cAMP accumulation, co-application of LY379268 no longer antagonized the effects of ISO after weak TBS ($195.8 \pm 15.7\%$; $n = 5$), suggesting that LY379268 is acting through $mGlu_3$ to antagonize the enhancement of LTP by βARs. Figure 2e summarizes the LTP experiments. Statistical analysis with a one-way ANOVA was significant ($F_{(5,29)} = 8.759$; $p < 0.001$). *Post hoc* analysis using the Tukey procedure showed that the strong TBS paradigm produces significantly greater LTP *vs* the weak TBS ($p < 0.05$). Weak TBS performed in the presence of ISO resulted in significantly greater potentiation than weak TBS alone ($p < 0.05$), and this potentiation by ISO was equivalent to that found in strong TBS experiments ($p > 0.05$). However, the ISO effect was significantly reduced when LY379268 was co-applied to slices ($p < 0.05$). While the $mGlu_3$ NAM VU0469942 had no effect on weak TBS on its own ($p > 0.05$), LY379268 did not antagonize the enhancement of weak TBS by ISO when applied in the presence of this compound ($p < 0.05$ *vs* weak TBS alone).

We also determined whether co-activation of $mGlu_3$ and βARs attenuates the induction of LTP by a strong TBS paradigm (Figure 2f and g). When strong TBS was applied in the presence of ISO, there was no enhancement of LTP *vs* control experiments ($F_{(2,14)} = 2.070$, $p > 0.05$; $n = 5$). Furthermore, co-application of ISO and LY379268 did not attenuate the effects of strong TBS in inducing LTP ($p > 0.05$; $n = 5$). We also ruled out that our results could be due to altered NMDA receptor signaling caused by the presence of residual ketamine anesthesia used for preparing brain slices. We performed experiments to measure currents in patch-clamped neurons in response to focally administered NMDA in brain slice prepared from animals anesthetized with ketamine and control animals that were not (Supplementary Figure S1). We found no difference in currents generated in response to the NMDA puff, suggesting that our results are not due to altered NMDA signaling.

$mGlu_3$ Modulation of βAR-Induced Enhancement of LTP is Inhibited by the A_1 Adenosine Receptor Antagonist CPT and FAC

Previous work demonstrated that co-activation of βARs and group II mGlu receptors in the hippocampus induces release of adenosine and activation of A_1 adenosine receptors on presynaptic SC terminals (Gereau and Conn, 1994a). To determine whether $mGlu_3$ activation modulates βAR effects on LTP through this mechanism, we performed a series of experiments in the presence of 200 nM (Schmitt *et al*, 2012) of the A_1 adenosine receptor antagonist CPT. Similar to control experiments, there was a differential effect of

Figure 1 Pharmacological and genetic studies indicate that $mGlu_3$ is the group II mGlu subtype that mediates potentiated cyclic-AMP (cAMP) accumulation. Rat hippocampal slice (a and b) or mouse cortical slice (c–h) cAMP assays were performed in the absence (Vehicle) or presence of either 10 μM isoproterenol (ISO), 100 nM LY379268 (LY), 30 μM VU0469942 (VU), ISO+LY, ISO+LY+10 μM VU, or ISO+LY+30 μM VU. Data are normalized to the fold-over basal cAMP response and represent the mean of at least three independent experiments performed in at least triplicate with error bars representing SEM. *$p < 0.05$ compared with all other conditions except for ISO+LY+10 μM VU. Concentration response curves (CRCs) of LY379268 were performed in rat hippocampal slice (b) or mouse cortical slice (d). cAMP assays in the presence of 10 μM ISO and in the absence or presence of 1 μM (b) or 10 μM (d) BINA. For studies utilizing $mGlu_2$ and $mGlu_3$ knockout mice and their wild-type littermates, mouse cortical slice cAMP assays were performed in the absence (Vehicle) or presence of either 10 μM ISO, 100 nM, or 1 μM LY379268 (LY) as indicated, and ISO+LY, or ISO+LY+1 μM MNI-137 (MNI). (e and f) Represent data that were obtained from experiments in WT littermates and data from $mGlu_2$ and $mGlu_3$ KO mice are presented in g and h, respectively. Data are normalized to the fold-over basal cAMP response and represent the mean of four independent experiments performed blinded to the genotype in quadruplicate with error bars representing SEM. *$p < 0.05$ compared with all other conditions except for either ISO+LY (100 nM) or ISO+LY (1 μM) where applicable.

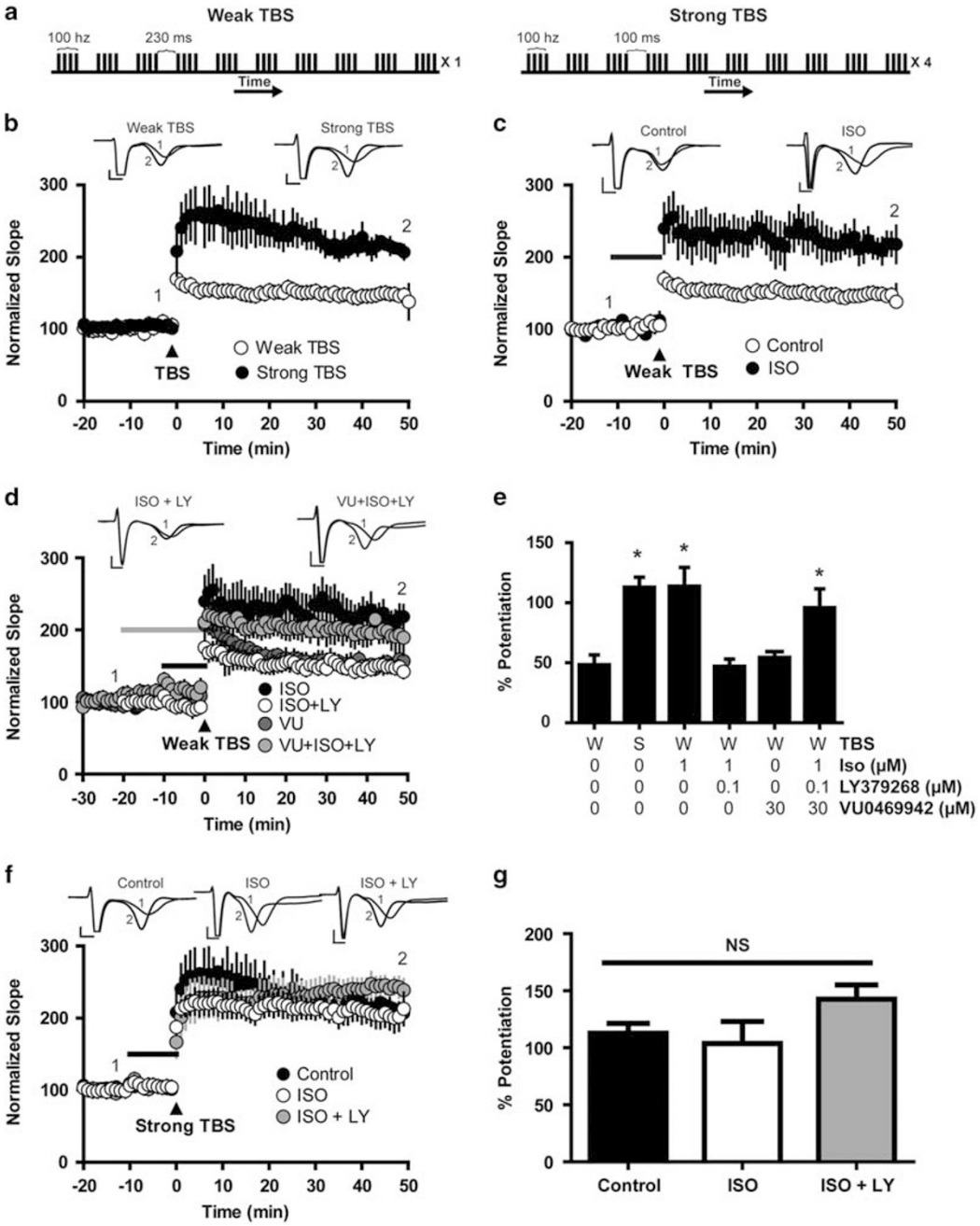


Figure 2 mGlu$_3$ activation blocks the long-term potentiation (LTP) enhancing effects of βARs. (a) Schematic of weak and strong theta burst stimulation (TBS) protocols. (b) Average time course of normalized field excitatory postsynaptic potential (fEPSP) slopes recorded from the SC-CA1 synapse in rat hippocampal slices. Strong TBS induced a larger potentiation of fEPSPs compared with weak TBS measured 50 min post stimulation. (c) Bath application of ISO (1 μM) enhances the effects of weak TBS. Black horizontal bar represents drug add. (d) When ISO and LY379268 (100 nM) are co-applied, the enhancing effects of ISO were antagonized relative to ISO-alone experiments. This blockade was reversed when slices were pretreated with the mGlu$_3$ NAM VU0469942 (30 μM). The gray horizontal bar represents the NAM drug addition and the black horizontal bar represents ISO ± LY379268. (e) Summary data for experiments in (b–d). Bars represent mean ± SEM. *$p < 0.05$ *vs* control, 1 μM ISO+LY379268, and 30 μM VU0469942. (f and g) ISO does not enhance and ISO+LY379268 does not attenuate the effects of strong TBS. Insets for b–d and f are representative fEPSP traces for the various experimental conditions measured during baseline (1) and 50 min post stimulation (2). Calibration: 1 mV vertical and 1 ms horizontal for all traces.

stimulation intensity on fEPSP slope with strong TBS ($n = 4$) producing significantly greater potentiation than weak TBS ($n = 3$; Figure 3a and b; two-way ANOVA main effect of stimulation $F_{(1,11)} = 14.3$; $p < 0.05$). ISO also was able to significantly enhance the effects of weak TBS in the presence of CPT (Figure 3c and d; 166.1+8.8% *vs* 127.1 ± 12.3%; $F_{(2,15)} = 6.505$, $p < 0.05$; Tukey post-test *vs* no drug $p < 0.05$; $n = 5$). However, activation of mGlu$_3$ with LY379268 ($n = 4$) no longer antagonized the enhancement of weak TBS by ISO in presence of CPT and also resulted in a significant LTP enhancement *vs* control slices (181.1 ± 8.1% baseline slope; $p < 0.05$ *vs* no drug). It should be noted that CPT appears to

blunt the potentiating effects of ISO on weak TBS *vs* experiments performed in the absence of CPT (compare Figure 2e and Figure 3d). In fact, it appears that both the levels of LTP in the control (no drug) and ISO conditions are equivalently reduced. However, there is still a clear differentiation between the control (no drug) and ISO

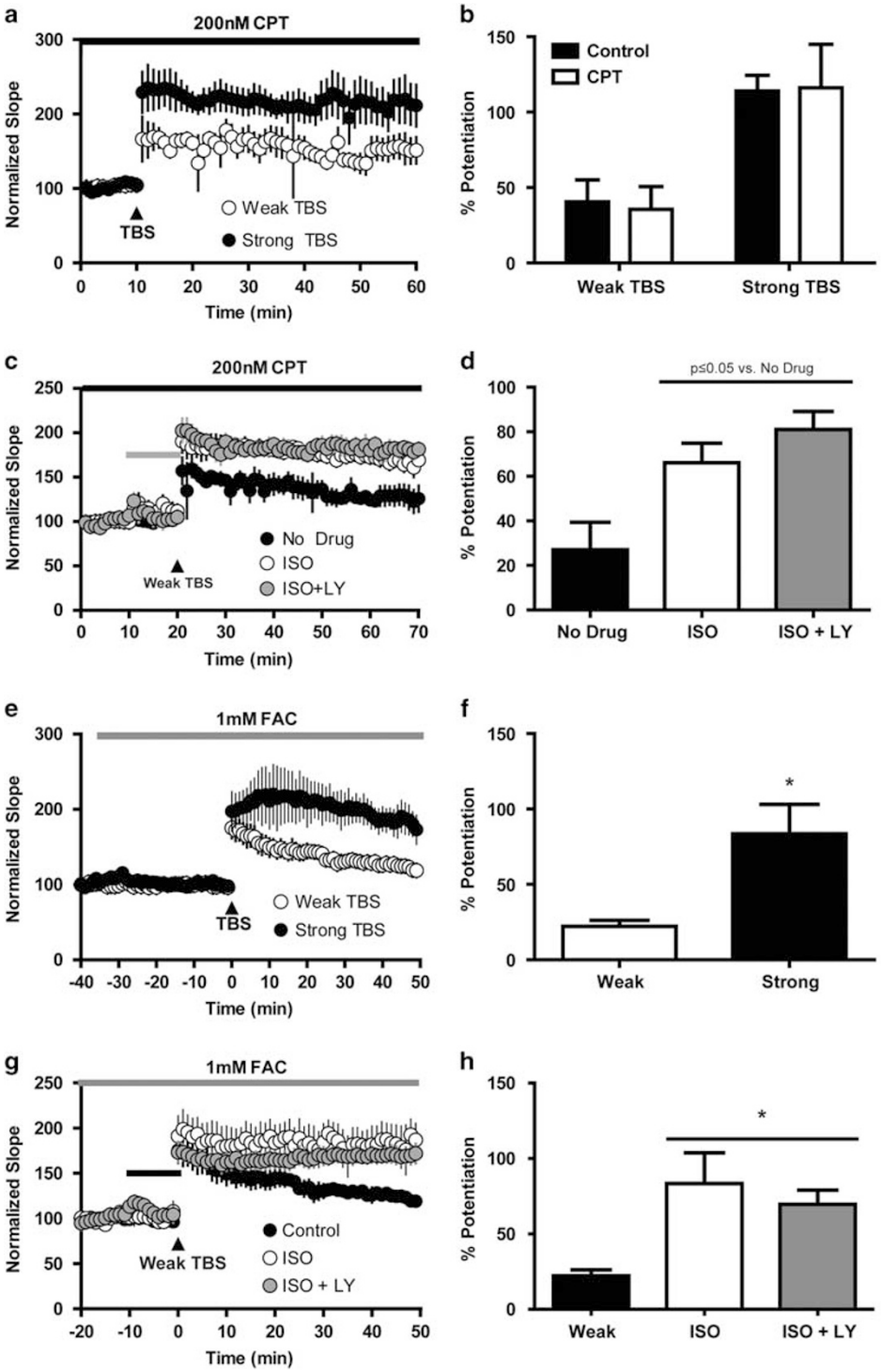


Figure 3 CPT, an A_1 adenosine antagonist, and fluoroacetic acid (FAC) occlude $mGlu_3$-mediated blockade of βAR-enhanced LTP. (a) Differential potentiating effects of weak and strong TBS in the presence of the A_1 adenosine receptor antagonist CPT (200 nM). (b) Quantification of the effects of weak and strong TBS from a. Weak and strong TBS results in similar levels of LTP when compared with control experiments presented in Figure 3. (c and d) Relative to control, ISO (1 μM) applied in the presence of CPT enhances the effects of weak TBS, but ISO+LY379268 (100 nM) no longer attenuates this enhancement. (e) Time course of normalized fEPSP slopes recorded in the presence of the glial-specific toxin FAC before and after TBS stimulation. Weak and strong TBS produce differential levels of LTP measured 50 min post stimulation in the presence of FAC. (f) Quantification of data presented in e represented as the mean ± SEM. (g) Relative to control experiments, ISO (1 μM) and ISO+LY379268 (100 nM) enhance the effects of weak TBS. (h) Quantification of data presented in g represented as the mean ± SEM $^*p < 0.05$ compared with weak TBS alone.

conditions; thus, we are confident that there would still be a signal window to observe antagonizing effects of LY379268. Apart from the presence of CPT, the conditions for these experiments were identical and were performed in parallel, often on the same day in slices from the same animals. It is possible that this blunting effect is due to off-target effects of CPT, such as activity toward phosphodiesterases. Despite this caveat, results of these experiments indicate that modulation of LTP by $mGlu_3$ requires activation of A_1 adenosine receptors.

Results from anatomical studies indicate that $mGlu_3$ is primarily localized on glial cells and not neurons in area CA1 of the hippocampus (Ohishi *et al*, 1993). In addition, previous work suggests that the large increases in cAMP accumulation and adenosine release induced by co-activation of group II mGlu receptors and βARs are mediated by actions on receptors on astrocytes and can be blocked by selective astrocytic metabolic toxins (Winder *et al*, 1996). Thus, we hypothesized that $mGlu_3$ modulation of enhancement of LTP by βAR activation is also dependent upon an astrocytic mechanism. To test this hypothesis, we utilized the metabolic toxin FAC that selectively inhibits astrocyte function. FAC (1 mM; Zhang *et al*, 2003) was bath-applied after a 5-min drug-free baseline period (Figure 3e). Consistent with our initial LTP experiments, strong TBS ($n=3$) resulted in significantly greater potentiation of the fEPSP slope *vs* the weak TBS ($n=5$; Figure 3e and f; $F_{(2,13)}=7.573$; $p<0.05$; Tukey *post hoc* test $p<0.05$). Furthermore, in the presence of FAC, ISO was able to enhance the effects of weak TBS (Figure 3g and h), resulting in greater LTP *vs* control slices ($p<0.05$; $n=4$). Slices co-treated with ISO and LY379268 also resulted in enhanced weak TBS-induced LTP *vs* control ($p<0.05$; $n=5$), but there was no significant difference in TBS-induced LTP *vs* ISO alone experiments ($p>0.05$). Taken together, these data suggest that βAR activation enhances weak TBS-induced LTP through a neuronal mechanism, but the effect of $mGlu_3$ activation on βAR-mediated potentiation of LTP may require astrocytic signaling.

$mGlu_3$ and βAR are Co-Expressed in Hippocampal Astrocytes

Our biochemical and electrophysiological studies indicate that $mGlu_3$ and βARs act in astrocytes to modulate synaptic plasticity. This mechanism would require $mGlu_3$ and βARs to be expressed together in individual astrocytes within the hippocampus. Although there is evidence in the literature that $mGlu_3$ (Bradley and Challiss, 2012) and βAR (Laureys *et al*, 2010) are expressed in astrocytes, to our knowledge coexpression of these receptors in hippocampal astrocytes has not been evaluated. Thus, we first performed co-localization experiments where we labeled for the astrocyte-specific protein GFAP, β2AR, and $mGlu_3$. As shown in Figure 4, $\times 100$ images reveal a neuropil-like distribution of both β2AR and $mGlu_3$ and that a portion of the immunoreactivity for these receptors is colocalized with GFAP. While the neuropil staining for both $mGlu_3$ and β2AR likely includes substantial neuronal staining, colocalization of these receptors in GFAP-immunopositive elements is consistent with astrocytic expression. High-magnification examination ($\times$200 images, $\times 100$ optics with $\times 2$ digital zoom) indicates that $mGlu_3$ and β2AR appear to be expressed within the same astrocyte. Areas with clusters of vesicle-like structures that co-label for β2ARs and $mGlu_3$ within GFAP+ structures are indicated by arrowheads. Importantly, the $mGlu_3$ antibody appears to be specific as staining was absent in the presence of an $mGlu_3$-blocking peptide.

Because of the dense neuropil-like staining of β2ARs and $mGlu_3$ throughout the hippocampus, we sought to increase confidence in coexpression of β2ARs and $mGlu_3$ in astrocytes through the use of RNAscope-based staining methods. In order to determine whether β2AR and $mGlu_3$ RNA were coexpressed in the same astrocytes, we developed a hybrid method that used traditional immunohistochemical (IHC) to label GFAP+ astrocytes with RNAscope technology to label β2AR and $mGlu_3$. Again, both β2AR and $mGlu_3$ were found throughout the hippocampus. However, $\times 200$ images that allow for the inspection of single astrocytes clearly revealed that both β2AR (arrows) and $mGlu_3$ (filled-in arrows) RNAs could be found within the same GFAP+ structures. Again, negative controls (probes for non-mammalian genes) were nearly completely blank. Taken together, our traditional IHC and RNAscope provide strong evidence that both β2AR and $mGlu_3$ are found within the same astrocyte, giving anatomical verification that our proposed mechanism is possible.

Blockade of βAR and Activation of $mGlu_3$ Have Similar Effects on Contextual Fear Conditioning

When taken together with previous studies of $mGlu_3$ and βAR signaling, our biochemical and electrophysiological studies suggest that astrocytic $mGlu_3$ and βARs cooperate to produce unique signaling that results in functional antagonism of the ability of neuronal βARs to enhance LTP in the hippocampus. Therefore, we reasoned that activation of $mGlu_3$ could inhibit forms of hippocampal-dependent learning that are dependent on βAR activation in a manner similar to βAR antagonists. To test this hypothesis, we trained rats in a contextual fear memory reconsolidation task (Figure 5a). As previously reported (Taherian *et al*, 2014), propranolol-treated animals exhibited significantly less freezing (Figure 5b; $29.6\pm4.6\%$) *vs* vehicle-treated rats ($56.1\pm8.3\%$; $F_{(5,73)}=3347$, $p<0.05$, Dunnett's post-test *vs* vehicle $p<0.05$), indicative of a disruption of memory reconsolidation. Furthermore, the $mGlu_{2/3}$ agonist LY379268 dose-dependently decreased freezing to context, reaching significance at a dose of 3 mg/kg ($19.8\pm6.1\%$; $p<0.05$ *vs* vehicle). When LY379268 was co-administered with the $mGlu_3$ NAM, VU0650786, there was no longer a significant reduction in freezing *vs* vehicle-treated rats ($36.1\pm5.0\%$; $p>0.05$). While the $mGlu_3$ NAM did not fully reverse the LY379268 effect, this partial reversal is consistent with our hypothesis that activation of $mGlu_3$ can disrupt contextual fear memory reconsolidation. In addition to having an important role in reconsolidation of fear memory, previous studies also suggest that βARs have an important role in consolidation of some forms of fear memory and that propranolol disrupts fear memory consolidation (Gao *et al*, 2016). Thus, we also determined the effect of the group II mGlu receptor agonist on contextual fear memory consolidation (Supplementary Figure S2). Rats treated with propranolol (10 mg/kg) or LY3792628 (3 mg/kg) after fear

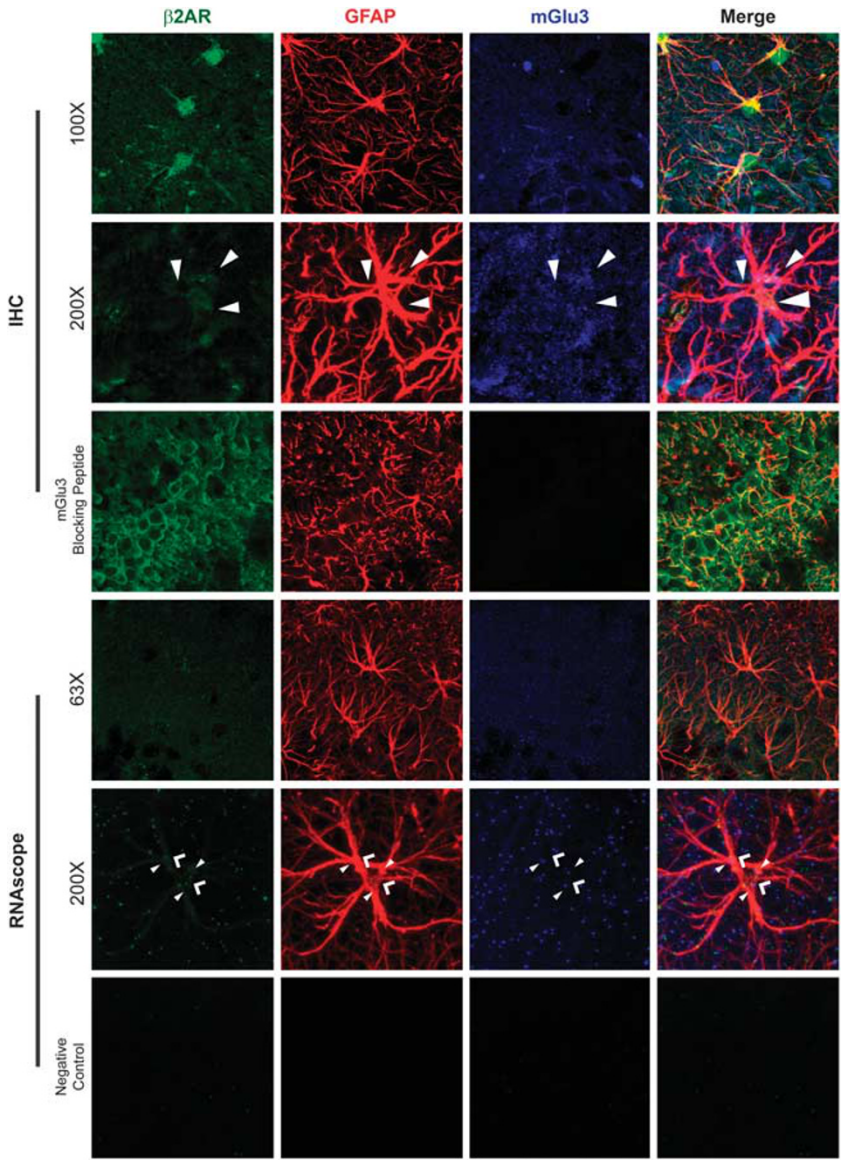


Figure 4 mGlu$_3$ and β2 adrenergic receptors (β2ARs) can co-localize to astrocytes. For immunohistochemical (IHC) analysis, × 100 images indicate several instances of β2ARs (green) and mGlu$_3$ (blue) within the cell bodies and processes of astrocytes demonstrated by GFAP staining (red). Additional β2AR and mGlu$_3$ staining appears in a neuropil-like pattern, implicating expression in diverse cell types. × 200 images (×100 objective with a × 2 digital zoom) implicate that β2ARs (green) and mGlu$_3$ (blue) can be found in vesicular-like structures throughout astrocytes (red), which are marked by filled-in arrowheads. Inclusion of an mGlu$_3$ antibody-blocking peptide within the staining reaction abolishes the visualization of mGlu$_3$. RNAscope analysis of β2AR and mGlu$_3$ also reveals expression throughout the hippocampus. High-magnification × 200 images reveal that β2AR (arrowheads) and mGlu$_3$ (filled arrowheads) can be found within GFAP+ structures. Negative control probes yield nearly completely clean images. Images are representative of sections taken from at least three animals.

conditioning exhibited significantly less contextual freezing 24 h later ($49.6 \pm 5.3\%$ and $42.9 \pm 6.0\%$, respectively, *vs* vehicle-treated animals ($69.8 \pm 5.1\%$; $F_{(5,71)} = 3.3.893$, $p < 0.05$, Dunnett's post-test *vs* vehicle $p < 0.05$). When the LY379268 (3 mg/kg) was co-administered with the mGlu$_3$ NAM VU0650786 (56.6 mg/kg), there was no longer a significant reduction in freezing *vs* vehicle-treated rats ($64.5 \pm 5.4\%$; $p > 0.05$).

In our electrophysiology experiments, the LTP-antagonizing effects of mGlu$_3$ activation were dependent upon A$_1$ adenosine receptor signaling. We next performed a series of experiments to test the hypothesis that disruption of contextual fear memory reconsolidation by mGlu$_3$ is similarly dependent upon A$_1$ receptors. First, we tested the hypothesis that A$_1$ receptor activation could disrupt reconsolidation. Similar to the effects we observed with propranolol and LY379268, the selective A$_1$ agonist CCPA dose-dependently (0.1–0.15 mg/kg) decreased freezing during the context test (Figure 5c) reaching significance at the highest dose tested ($26.8 \pm 5.8\%$; $F_{(2,31)} = 3.351$, $p < 0.05$; Dunnett's post-test $p < 0.05$ *vs* vehicle). To test the hypothesis that the behavioral effects of mGlu$_3$ activation by LY379268 were dependent upon A$_1$ receptors, we used the selective antagonist PQ69. When administered alone, we found no

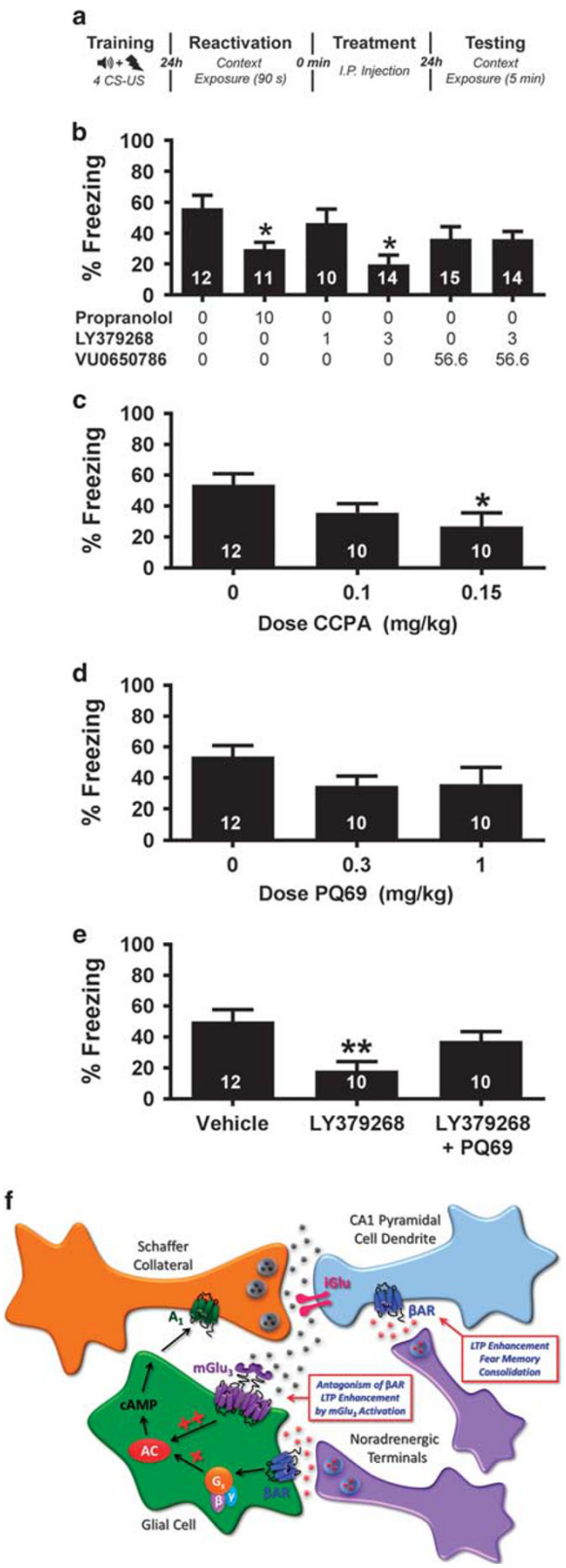


Figure 5 Activation of mGlu$_3$ disrupts reconsolidation of contextual fear memory. (a) Schematic of the behavioral testing protocol. (b) Propranolol and LY379268 disrupt reconsolidation of contextual fear memory as demonstrated by decreased freezing during 5 min context exposure. The mGlu$_3$ NAM VU0650786 reverses the disruption induced by LY379268. (c) The A$_1$ receptor agonist CCPA dose-dependently disrupts reconsolidation. (d and e) The A$_1$ antagonist PQ69 had no effect alone, but blocks the disruption induced by LY379268. (f) A model for mGlu$_3$-mediated glial–neuronal communication at the SC-CA1 hippocampal synapse. When glial mGlu$_3$ is coincidently activated with β-adrenergic receptors (βARs), the βAR-mediated production of cAMP by stimulation of adenylate cyclase (AC) is greatly potentiated. cAMP metabolites then activate A$_1$ adenosine receptors presynaptically expressed on the Schaffer Collateral (SC) terminals, resulting in a depression of neurotransmission at the SC-CA1 synapse in the hippocampus. This glial–neuronal signaling also results in antagonism of βAR agonist-mediated potentiation of weak TBS-induced LTP. Quantification of contextual fear memory on testing day presented as mean ± SEM of the percentage of time freezing during the 5-min context exposure. Inset numbers in bars indicate the number of animals in each condition. *$p \leqslant 0.05$ and **$p < 0.01$ relative to vehicle-treated animals.

effect of PQ69 (0.3–1 mg/kg) on freezing during the context test (Figure 5d; $F_{(2,31)} = 1.720$ $p > 0.05$). However, when co-administered with LY37268 (3 mg/kg), PQ69 (1 mg/kg) blocked the disruption effects of LY37268 in reducing freezing behavior *vs* vehicle (Figure 5e; $F_{(2,35)} = 6.024$, $p < 0.05$; Dunnett's post-test *vs* vehicle $p > 0.05$). Taken together, this is consistent with the hypothesis that mGlu$_3$ is acting through A$_1$ receptors in order to disrupt contextual fear memory reconsolidation.

DISCUSSION

The present work has identified a role of mGlu$_3$ in hippocampal synaptic plasticity and modulation of fear memory. As previously reported, we found that activation of group II mGlu receptors potentiates cAMP formation induced by agonists of the G$_s$-coupled *β*AR (Moldrich *et al*, 2002; Winder and Conn, 1993, 1995; Winder *et al*, 1996). Our results suggest that this effect is mediated by mGlu$_3$ as we found that cAMP potentiation is antagonized by the selective mGlu$_3$ NAM VU0469942 and cannot be potentiated by the mGlu$_2$ PAM BINA. Furthermore, this effect occurs in mGlu$_2$ but not in mGlu$_3$ KO mice. Although it is counterintuitive for a G$_{i/o}$-coupled GPCR to stimulate the production of cAMP, the effects of G$_{i/o}$-coupled GPCR activation on adenylate cyclase (AC) are dependent on the form of AC that is expressed in a particular cell type. AC type II is activated by the *βγ* subunits associated with G$_{i/o}$, allowing a G$_{i/o}$-mediated potentiation of a G$_s$-coupled GPCR's (such as *β*ARs) activation of AC (Tang and Gilman, 1991). Type II AC (but not Type IV) has been shown to be enriched by transcriptome analysis in astroglia (Cahoy *et al*, 2008; Orre *et al*, 2014). In addition, an RNA-Seq transcriptome and splicing database of glia, neurons, and vascular cells (Zhang *et al*, 2014) in the cerebral cortex showed that type II AC is present in astrocytes, whereas Type IV AC is not present.

To evaluate the electrophysiological consequences of mGlu$_3$-*β*AR synergistic signaling, we evaluated LTP at the SC-CA1 synapse in rat hippocampal slices. At this synapse, activation of *β*ARs can enhance excitatory synaptic transmission and synaptic plasticity (Chavez-Noriega and Stevens, 1994; Gereau and Conn, 1994b) and can facilitate the induction of LTP when low-frequency stimulation paradigms are performed in the presence of a *β*AR agonist

(O'Dell *et al*, 2015). We found that the βAR agonist ISO enhances induction of LTP by a weak TBS protocol. When βARs and $mGlu_{2/3}$ were co-activated with ISO and LY379268, the coincident activation of group II mGlu receptors reversed the ISO-induced enhancement of LTP. Our data suggest that the ability of the group II mGlu receptor agonist to reverse ISO effects on LTP is completely blocked by the $mGlu_3$-selective NAM VU0469942, suggesting that this effect is mediated by $mGlu_3$. In addition, the effect of the group II mGlu receptor agonists requires A_1 adenosine receptors and functional astrocytes as LY379268 no longer antagonized the effects of ISO in the presence of the A_1 antagonist CPT or the glial toxin FAC. Moreover, the results of our anatomical studies showed that βARs and $mGlu_3$ colocalize in hippocampal astrocytes. Overall, this is consistent with previous reports that co-activating group II mGlu receptors and βARs on hippocampal astrocytes leads to release of adenosine, which can then modulate synaptic transmission at neighboring SC synapses (Gereau and Conn, 1994b; Winder *et al*, 1996). Previous studies have shown that group II mGlus cooperates with other Gs-coupled receptors to potentiate cAMP in a similar manner, including A_2 adenosine receptors (Winder and Conn, 1993). It is therefore conceivable that astrocyte-derived adenosine may have a dual function by acting on astrocytic A_2 receptors to perpetuate cAMP accumulation and neuronal A_1 receptors to suppress synaptic transmission.

Multiple studies suggest that the ability of βAR activation to enhance LTP is likely to have an important role in facilitating memory processes (Murchison *et al*, 2004; O'Dell *et al*, 2015), and that this can be important for memory formation in novel environments. However, it is also conceivable that during periods of intense stress when noradrenergic activity is high, this mechanism may contribute to formation of pathological memories, as would be the case in post-traumatic stress disorder (PTSD). Recent work indicates that memories undergo a reconsolidation process after retrieval, during which they are labile and susceptible to manipulation (Otis *et al*, 2015). βARs have a critical role in this process and the antagonist propranolol can disrupt reconsolidation of fearful memories in both rodents and humans (Otis *et al*, 2015; Taherian *et al*, 2014). In fact, pharmacological disruption of memory reconsolidation may serve a therapeutic approach for PTSD (Otis *et al*, 2015). In the present study, we found that the $mGlu_{2/3}$ agonist LY379268 disrupted contextual fear memory reconsolidation in rats in a manner similar to propranolol. This disruption was reversed by the selective $mGlu_3$ NAM VU0650786, suggesting a critical role of this receptor in this effect. The fact that VU0650786 did not produce a full reversal is likely because of the fact that the potency of the NAM is relatively weak compared with that of LY379268 as an agonist. Alternatively, this could indicate that $mGlu_2$ may also contribute to this response and should be explored in future studies. Consistent with our electrophysiology studies, the effects of LY379268 were dependent upon A_1 receptors as LY379268 no longer disrupted reconsolidation in the presence the A_1 antagonist PQ69. Also of note, animals that received VU0650786 alone appeared to have reduced freezing relative to controls. While this effect was not statistically significant, one hypothesis for this reduction is that $mGlu_3$ activation is required for the reconsolidation process. However, it would be expected that activation of $mGlu_3$ by LY379268 would enhance reconsolidation and freezing during the context or at the very least result in equivalent freezing relative to controls. Because LY379268 caused a clear reduction in freezing, this does not appear to be the case.

We also observed that propranolol and LY379268 also disrupt fear memory consolidation, and the effects of LY379268 were blocked by the $mGlu_3$ NAM VU0650786. This is especially interesting in light of a recent study demonstrating that astrocytic βARs modulate consolidation of fear memory using the inhibitory avoidance task (Gao *et al*, 2016). Taken together with the current study, these results suggest that the effects of activation of astrocytic βARs on hippocampal-dependent memory consolidation are likely to be dependent on the physiological state and can be reduced by coincident activation of astrocytic $mGlu_3$. In future studies it will be important to determine the behavioral or physiological context in which these receptors are co-activated. It will also be important to determine the role astrocytes have in $mGlu_3$-induced disruption of memory reconsolidation. Unfortunately, very few tools exist to selectively manipulate astrocytic receptors *in vivo*. Because the progenitor cells for many neuronal populations are radial glia, including cortical and hippocampal pyramidal cells (Casper and McCarthy, 2006), genetic manipulations of receptors selectively in astrocytes are difficult (Casper *et al*, 2007). Even using inducible strategies may not result in astrocyte-specific effects, as recently demonstrated for the dnSNARE mouse (Fujita *et al*, 2014). Overall, combined with our recent report that activation of $mGlu_3$ is required for fear extinction learning (Walker *et al*, 2015), the results of our behavioral experiments indicate that potentiators of this receptor may be a promising therapeutic strategy for the treatment of PTSD.

Coincident activation of $mGlu_3$ and βARs could also serve as a protective feedback mechanism to prevent excitotoxic injury during instances of prolonged stress. The activation of group II mGlu receptors has long been known to have neuroprotective activity both *in vitro* (Battaglia *et al*, 1998; Bruno *et al*, 1995; Bruno *et al*, 1994) and *in vivo* (Kingston *et al*, 1999a; Kingston *et al*, 1999b). Previous studies have shown that $mGlu_{2/3}$ agonist-induced neuroprotection in mixed cultures of cortical neurons and astrocytes occurs via a glial–neuronal mechanism requiring astrocytic $mGlu_3$, and that neuronal $mGlu_2$ activation may actually be harmful to neurons exposed to toxic insults (Corti *et al*, 2007). Importantly, the neuroprotective effects of group II mGlu receptor activation absolutely require $mGlu_3$ as they are absent when mixed cultures of cortical neurons and astrocytes contain astrocytes prepared from $mGlu_3$ KO mice (Caraci *et al*, 2011; Corti *et al*, 2007).

The astrocytic activation of $mGlu_3$ leads to a number of neuroprotective signaling cascades including the stimulation of the mitogen-activated protein kinase and phosphoinositide 3-kinase pathways (Ciccarelli *et al*, 2007; Corti *et al*, 2007). The activation of these pathways then leads to the increased production and release of transforming growth factor-β (TGF-β) from glia (Bruno *et al*, 1998), which in turn acts to protect neurons against excitotoxicity (Bruno *et al*, 1998; Bruno *et al*, 1997). In addition, these studies have also been recently extended to show a role for glial $mGlu_3$

activation in mediating neuroprotection from β-amyloid (Aβ) toxicity through a TGF-β mechanism (Caraci *et al*, 2011), suggesting that mGlu$_3$ activation may be a viable therapeutic approach for the treatment of Alzheimer's disease. Activation of mGlu$_3$ in astrocytes also leads to the release of a number of other important factors for neuronal survival, including nerve growth factor, S-100β protein (Ciccarelli *et al*, 1999), brain-derived neurotrophic factor (Di Liberto *et al*, 2010), and glial-derived neurotrophic factor (Battaglia *et al*, 2009), providing additional non-TGF-β mechanisms for mGlu$_3$-mediated neuroprotection. The role of mGlu$_3$ in the form of glial–neuronal communication outlined here may provide another distinct mechanism of neuroprotection driven by astroglial mGlu$_3$. Thus, the current studies also support the development of mGlu$_3$ activators as a novel therapeutic approach for neuroprotection.

In conclusion, the results of the present studies provide a conceptual framework in which mGlu$_3$ and βARs interact to influence cAMP signaling, synaptic plasticity, and potential behavior (Figure 5f). Noradrenergic afferents can modulate cellular excitability and potentiate LTP through neuronal βARs. Co-activation of mGlu$_3$ and βARs located on astrocytes will produce large cAMP accumulation in these cells and release of cAMP metabolites into the perisynaptic space. These metabolites then activate presynaptic A$_1$ adenosine receptors to antagonize the LTP-enhancing effects of βAR agonists. Systemically administered agonists or potentiators of mGlu$_3$ could interact with endogenous NA through this mechanism to modulate behavior such as fear memory reconsolidation and afford neuroprotection against cellular damage from excess glutamatergic activity. However, this could also result in the formation of pathological associations, as is observed in cases of PTSD. Consistent with this, there are reports that compounds that act as βAR antagonists are efficacious in both preclinical models of PTSD as well in the clinic (Otis *et al*, 2015).

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)