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mGlu1 tonically regulates levels of calcium-permeable AMPA receptors in cultured nucleus accumbens neurons through retinoic acid signaling and protein translation

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Abstract

In several brain regions, ongoing metabotropic glutamate receptor 1 (mGlu1) transmission has been shown to tonically suppress synaptic levels of Ca^{2+}-permeable AMPA receptors (CP-AMPARs) while pharmacological activation of mGlu1 removes CP-AMPARs from these synapses. Consistent with this, we previously showed in nucleus accumbens (NAc) medium spiny neurons (MSNs) that reduced mGlu1 tone enables and mGlu1 positive allosteric modulation reverses the elevation of CP-AMPAR levels in the NAc that underlies enhanced cocaine craving in the 'incubation of craving' rat model of addiction. To better understand mGlu1/CP-AMPAR interactions, we used a NAc/prefrontal cortex co-culture system in which NAc MSNs express high CP-AMPAR levels, providing an *in vitro* model for NAc MSNs after the incubation of cocaine craving. The non-specific group I orthosteric agonist dihydroxyphenylglycine (10 min) decreased cell surface GluA1 but not GluA2, indicating CP-AMPAR internalization. This was prevented by mGlu1 (LY367385) or mGlu5 (MTEP) blockade. However, a selective role for mGlu1 emerged in studies of long-term antagonist treatment. Thus, LY367385 (24 h) increased surface GluA1 without affecting GluA2, whereas MTEP (24 h) had no effect. In hippocampal neurons, scaling up of CP-AMPARs can occur through a mechanism requiring retinoic acid (RA) signaling and new GluA1 synthesis. Consistent with this, the LY367385-induced increase in surface GluA1 was blocked by anisomycin (translation inhibitor) or 4-(diethylamino)-benzaldehyde (RA synthesis

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Author Contributions

JAL and MEW designed the study, with help from JMR, CTW, and MTS. JAL, JMR, AC, MTS, KYW, NCC and CTW performed experiments and analyzed data. JAL and MEW wrote the manuscript and prepared figures. MEW acquired funding for this project; JAL, CTW and MTS acquired fellowships to support their salary.

Competing Interests

The authors have no conflicts of interest.

Data Accessibility

All data are available from the corresponding author upon request.

inhibitor). Thus, mGlu1 transmission tonically suppresses cell surface CP-AMPAR levels, and decreasing mGlu1 tone increases surface CP-AMPARs via RA signaling and protein translation. These results identify a novel mechanism for homeostatic plasticity in NAc MSNs.

Keywords

GluA1; group I metabotropic glutamate receptors; homeostatic plasticity; primary culture; receptor trafficking

Introduction

The group I metabotropic glutamate receptors (mGluRs), mGlu1 and mGlu5, are predominantly postsynaptic receptors that couple to the Gq-like class of G-proteins and are important in modulating neurotransmission and plasticity through their linkages with multiple signaling pathways as well as NMDA receptors (Kano *et al.*, 2008). The consequences of group I mGluR transmission include multiple forms of long-term depression (LTD) (Luscher & Huber, 2010). In one type of LTD, observed when there is a significant contribution of GluA2-lacking Ca^{2+}-permeable AMPARs (CP-AMPARs) to synaptic transmission, mGlu1 stimulation elicits the removal of CP-AMPARs and their replacement by lower conductance GluA2-containing Ca^{2+}-impermeable AMPARs (CI-AMPARs). This has been demonstrated in cerebellar stellate cells [(Kelly *et al.*, 2009); see also: (Liu & Cull-Candy, 2000; Liu & Cull-Candy, 2002; 2005)], dopamine (DA) neurons of the ventral tegmental area (VTA) (Bellone & Luscher, 2005; 2006; Mameli *et al.*, 2007; Mameli *et al.*, 2009; Bellone *et al.*, 2011), and the lateral amygdala (Clem & Huganir, 2010).

In addition, we have demonstrated this mGlu1-LTD in medium spiny neurons (MSN) of the adult rat nucleus accumbens (NAc), in the context of our studies of the incubation of cocaine craving (McCutcheon *et al.*, 2011a; Loweth *et al.*, 2014; Scheyer *et al.*, 2015). Incubation refers to the progressive intensification of cue-induced drug craving that occurs over the first weeks of withdrawal from drug self-administration; elevated levels of craving then persist for months (Lu *et al.*, 2004; Pickens *et al.*, 2011; Wolf, 2016). This model is relevant to a human scenario in which heavy drug-taking is interrupted by a period of abstinence, e.g., due to hospitalization or incarceration. During abstinence, incubation of craving is proposed to increase vulnerability to relapse (Reichel & Bevins, 2009; Li *et al.*, 2016). Supporting the validity of the rodent model, incubation of craving has been observed in humans addicted to cocaine (Parvaz *et al.*, 2016) as well as other drugs of abuse (Bedi *et al.*, 2011; Wang *et al.*, 2013; Li *et al.*, 2015).

The NAc is critical for drug seeking because it serves as an interface between the cortical and limbic regions that initiate motivated behaviors and the motor regions that execute these behaviors (Sesack & Grace, 2010). In NAc MSNs of drug-naïve rats, excitatory synaptic transmission is dominated by CI-AMPARs consisting of GluA1 and GluA2, although there is a minority population of homomeric GluA1 CP-AMPARs (Conrad *et al.*, 2008; Reimers *et al.*, 2011). We and others showed that homomeric GluA1 CP-AMPARs accumulate in NAc synapses during incubation (Conrad *et al.*, 2008; Mameli *et al.*, 2009; McCutcheon *et al.*, 2011a; McCutcheon *et al.*, 2011b; Lee *et al.*, 2013; Purgianto *et al.*, 2013; Loweth *et al.*,

2014; Ma *et al.*, 2014; Terrier *et al.*, 2015) and that blocking, removing or preventing accumulation of these CP-AMPARs substantially reduces the 'incubated' component of cocaine craving (Conrad *et al.*, 2008; Lee *et al.*, 2013; Loweth *et al.*, 2014; Ma *et al.*, 2014; Wang *et al.*, 2018). We propose that CP-AMPAR accumulation strengthens glutamate synapses onto MSNs, enabling them to respond more strongly to glutamate released by cocaine cues and thereby leading to 'incubated' cue-induced cocaine seeking (Wolf, 2016).

Incubation of cocaine craving is also accompanied by alterations in group I mGluR function in NAc MSNs. The normally expressed form of group I mGluR-dependent synaptic depression (postsynaptically initiated by mGlu5 and presynaptically expressed via the CB1 receptor) is abolished, whereas robust mGlu1-dependent LTD, expressed postsynaptically via CP-AMPAR removal, is observed (McCutcheon *et al.*, 2011a; Scheyer et al., 2015). By targeting this mGlu1-LTD, either pharmacologically (Loweth *et al.*, 2014) or optogenetically (Lee *et al.*, 2013; Ma *et al.*, 2014), CP-AMPAR-mediated transmission in the NAc and cue-induced craving can be reduced. Furthermore, we showed that CP-AMPARs become stably elevated in the NAc core during cocaine incubation in part due to weakening of mGlu1-dependent mechanisms that normally limit the number of CP-AMPARs in NAc synapses; thus, restoring mGlu1 tone during early withdrawal, using an mGlu1 positive allosteric modulator (PAM), prevents CP-AMPAR accumulation and incubation during the period of PAM exposure (Loweth *et al.*, 2014). These data suggest that mGlu1 PAMs, given early in abstinence, might delay incubation of craving and thus create a window in which other interventions could be more successfully implemented. Interestingly, in a study of incubation of craving produced by a single cocaine self-administration session, a reduction in mGlu1 mRNA was found in NAc shell but not core (changes were also observed in other regions) and an enhancement of incubated cocaine seeking was observed after systemic mGlu1 blockade (Halbout *et al.*, 2014). Studies focused on VTA have also demonstrated negative modulation by mGlu1 of cocaine-induced plasticity and cocaine seeking (Bellone & Luscher, 2006; Mameli *et al.*, 2007; Mameli *et al.*, 2009; Bellone *et al.*, 2011; Yuan *et al.*, 2013).

To study mechanisms underlying this mGlu1-dependent plasticity, we utilized postnatal day 1 (P1) NAc MSNs, co-cultured with prefrontal cortical (PFC) neurons to restore glutamate input (Sun *et al.*, 2008). MSNs in this co-culture system contain a significant population of CP-AMPARs, that is, as much as one-third of surface-expressed GluA1 may be present in GluA1 homomers (Sun & Wolf, 2009). They therefore provide a useful *in vitro* model for NAc MSNs after incubation of craving. Using these co-cultures, we assessed effects of acute mGlu1 or mGlu5 stimulation on cell surface GluA1 and GluA2 in NAc MSNs. We also determined if group I mGluR transmission exerts tonic control over NAc CP-AMPAR levels by investigating the effect of long-term (24–48 h) blockade of mGlu1 or mGlu5 on GluA1 and GluA2 surface expression. Our major finding is that a long-term decrease in mGlu1 tone resulted in increased surface expression of homomeric GluA1 CP-AMPARs through a mechanism requiring protein translation and retinoic acid (RA) signaling. These results are reminiscent of a previously described form of homeostatic plasticity in cultured hippocampal neurons, in which decreased excitatory synaptic transmission activates RA signaling which in turn increases dendritic translation of GluA1 leading to increased surface delivery of CP-AMPARs (Aoto *et al.*, 2008; Maghsoodi *et al.*, 2008; Poon & Chen, 2008;

Wang *et al.*, 2011; Chen *et al.*, 2014; Arendt *et al.*, 2015a). Our results identify a novel mechanism for homeostatic plasticity in NAc MSNs and may suggest new targets for therapeutic intervention.

Materials and Methods

Animals

As described previously (Sun *et al.*, 2008; Sun & Wolf, 2009; Reimers *et al.*, 2014; Werner *et al.*, 2017), postnatal day 1 (P1) offspring obtained from pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN, USA) were used to obtain NAc neurons, while P1 offspring from transgenic mice expressing enhanced cyan fluorescent protein (ECFP) [strain B6.129(ICR)-Tg(ACTB-ECFP)1Nagy/J; The Jackson Laboratory, Bar Harbor, ME, USA] were used to obtain PFC cells. The ECFP transgenic mouse line was maintained by mating homozygous ECFP male and female mice in house. All offspring express ECFP. Our experiments required approximately 60 rat pups and 80 mouse pups. All animal procedures were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of Rosalind Franklin University of Medicine and Science.

Primary neuronal cultures

NAc/PFC co-cultures were prepared as previously described (Sun *et al.*, 2008; Sun & Wolf, 2009; Reimers *et al.*, 2014; Werner *et al.*, 2017). Briefly, the PFC of P1 ECFP-expressing mice was dissociated with papain at 37°C and plated at a density of 30,000 cells/well onto coverslips coated with poly-D-lysine (100 μg/mL; Sigma Aldrich, St. Louis, MO, USA) in 24-well plates. Two to three days later, the NAc from P1 rats was dissociated with papain and plated at a density of 30,000 cells/well with the PFC cells. The NAc/PFC co-cultures were grown in Neurobasal medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2mM GlutaMAX, 0.5% Gentamicin and 2% B27 (Invitrogen). Half of the medium was replaced every 3–5 days. Cultures were used for experiments between weeks 2 and 3 after plating the NAc neurons.

Drug treatments

Reagents were obtained from Sigma Aldrich or Tocris (Minneapolis, MN). NAc/PFC co-cultures were either kept in control medium or in medium treated with drugs affecting mGlu1 or mGlu5 transmission: the nonselective group I mGluR agonist DHPG (50 μM), the mGlu1 antagonist LY367385 (100 μM), the mGlu5 antagonist MTEP (1 μM), the mGlu1 PAM Ro67–7476 (3 μM), or the mGlu5 PAM CDPPB (10 μM). We also used the translation inhibitor anisomycin (40 μM) and 4-(diethylamino)-benzaldehyde (DEAB; 10 μM), an inhibitor of retinal dehydrogenase (an enzyme in the RA synthesis pathway). Concentrations of mGlu drugs and anisomycin were selected based on our prior studies using the same NAc/PFC co-culture system (Sun *et al.*, 2008; Sun & Wolf, 2009; Reimers *et al.*, 2014; Stefanik *et al.*, 2018a) or NAc cultures (Mangiavacchi & Wolf, 2004). The selected concentration of DEAB has previously been used to block RA-dependent homeostatic plasticity in cultured hippocampal neurons (Aoto *et al.*, 2008; Soden & Chen, 2010).

Immunocytochemistry

For cell surface GluA1 and GluA2 double immunostaining, live neurons were incubated with polyclonal antibody to the extracellular N-terminal domain of GluA1 (1:10, PC246, aa 271–285; Millipore, Billerica, MA, USA) and monoclonal antibody to the extracellular N-terminal domain of GluA2 (1:20; MAB397, aa 175–430; Millipore) in NeuroBasal media (15 min, 37°C). Cells were then fixed with 4% paraformaldehyde, blocked with 5% donkey serum in phosphate-buffered saline for 1 h and incubated for 1 h with Cy3 conjugated donkey anti-rabbit secondary antibody (1:500; Jackson ImmunoResearch, West Grove, PA, USA) and Alexa 488 conjugated donkey anti-mouse secondary antibody (1:1000; Invitrogen) without permeabilization. All incubations following the primary antibody incubation were performed at room temperature (RT; ~21°C). In a prior study using the same antibodies (Sun & Wolf 2009), we performed control experiments that consisted of: (i) omitting the primary antibodies and applying the secondary antibodies alone and (ii) comparing double immunostaining with sequential single immunostaining with each antibody. These experiments confirmed that the fluorochromes used in the double-staining experiments did not introduce artifactual fluorescent labeling and there was no cross-reaction between ‘unmatched’ primary and secondary antibodies. Furthermore, immunoblotting studies have verified that a single band of the appropriate molecular weight is recognized by the GluA1 antibody (Petrovic *et al.*, 2017) and the GluA2 antibody (Millipore Product Information).

Image analysis

Images were analyzed as described previously (Sun *et al.*, 2008; Sun & Wolf, 2009; Reimers *et al.*, 2014; Werner *et al.*, 2017). NAc and PFC neurons were distinguished by fluorescence (Sun *et al.*, 2008) and NAc MSNs were identified based on previously defined morphological criteria (Shi & Rayport, 1994; Chao *et al.,* 2002; Sun *et al.,* 2008), namely a soma diameter of 10–15μm with two to four relatively closely projecting processes. Images of NAc MSNs adjacent to PFC pyramidal neurons, selected under phase contrast imaging to avoid experimenter bias based on the intensity of fluorescence staining, were acquired with a Nikon inverted microscope using a 40 x oil objective, an ORCA-ER digital camera and MetaMorph software (Universal Imaging, Downington, PA, USA). All experimental groups compared were from the same culture preparation and were processed simultaneously. Approximately 4–6 cells from at least 4 different wells for each group were analyzed. For each image, total AMPAR surface area in a fixed length (15 μm) of process, located at least one soma diameter away from the soma, was measured using a threshold set based on average background fluorescence in unstained areas. The soma was excluded from analysis because, in the intact brain, glutamate synapses from PFC onto MSNs occur exclusively on processes (Meredith & Totterdell, 1999), and nearly all GluA1 immunostaining is observed on processes, not on the soma (Chen *et al.,* 1998).

Statistical Analysis

Data were not normally distributed and therefore were analyzed with a Kruskal-Wallis one-way ANOVA on ranks. When a significant group effect was found, *post hoc* comparisons were performed using a Dunn’s test. The criterion for significance was set at $P < 0.05$ (*n*,

number of cells). Data were analyzed and graphed with SigmaPlot and GraphPad Prism software.

Results

Acute group I mGluR activation decreases cell surface GluA1 levels in cultured NAc MSNs

Electrophysiological studies in several cell types, including NAc MSNs of rats that have undergone incubation of cocaine craving, have shown that mGlu1 stimulation produces a form of LTD in which CP-AMPARs are removed from synapses and replaced by lower conductance CI-AMPARs (see Introduction). Here we directly assessed the effects of increasing or decreasing group I mGluR transmission on AMPAR trafficking in NAc MSNs co-cultured with PFC neurons. The co-cultures were subjected to a media change (control group) or incubated for 10 or 20 min with the nonselective group I mGluR agonist DHPG (50 μM), followed by live-cell labeling with antibodies directed against the extracellular N-terminal regions of GluA1 and GluA2 (Fig. 1A & B). Our analysis was restricted to MSNs of the NAc, which were identified based on morphology and absence of the cyan fluorescence that serves as a marker for PFC neurons in the co-cultures (see Materials and Methods). Analysis by Kruskal-Wallis one-way ANOVA on ranks revealed a significant difference between the three treatment groups (Control, DHPG 10 min, DHPG 20 min) in levels of cell surface GluA1 ($H=10.97$, $P=0.004$). Dunn's *post hoc* comparisons revealed that, compared to the control group, incubation with DHPG for both 10 and 20 min produced a significant decrease in surface GluA1 levels ($Q=3.21$, $P=0.003$; $Q=2.41$, $P=0.032$, respectively). Interestingly, the decrease in GluA1 surface expression was not as pronounced after 20 min of DHPG treatment (60±15% of control) compared to the 10 min time-point (46±12% of control) (Fig. 1C, Top left). No group differences were observed for GluA2 ($H=0.44$, $P=0.801$), but there was a trend towards increased GluA2 surface expression after 20 min incubation with DHPG (141±28% of control) relative to the 10 min time-point (117±22% of control) (Fig. 1C, Bottom left). Scatter plots show variability within each treatment group (Fig. 1C, Right). Together, these results suggest that DHPG elicits removal of homomeric GluA1 CP-AMPARs that is followed by insertion of CI-AMPARs containing GluA1 and GluA2.

Acute DHPG-induced decreases in GluA1 surface levels are mediated by both mGlu1 and mGlu5 receptors

In order to determine which receptor subtype (mGlu1 or mGlu5) was mediating the effect of DHPG on AMPAR trafficking, co-cultures were treated for 10 min with media, DHPG (50 μM), DHPG and the mGlu1 antagonist LY367385 (100 μM), or DHPG and the mGlu5 antagonist MTEP (1 μM) (Fig. 2A). Analysis by Kruskal-Wallis one-way ANOVA on ranks revealed a significant difference between the four treatment groups (Control, DHPG, DHPG + LY367385, DHPG + MTEP) in levels of cell surface GluA1 ($H=12.83$, $P=0.005$). Dunn's *post hoc* comparisons revealed that, compared to the control group, incubation with DHPG for 10 minutes produced a significant decrease in surface GluA1 levels ($Q=2.99$, $P=0.008$) that was prevented by both LY367385 ($Q=0.18$, $P=1.00$) and MTEP ($Q=0.42$, $P=1.00$) (Fig. 2A, Top left). No changes in GluA2 levels were observed across the four treatment groups ($H=0.37$, $P=0.95$) (Fig. 2A, Bottom left). Scatter plots show individual variability within

each treatment group (Fig. 2A, Right). Thus, in contrast to our findings in the incubation model, where CP-AMPAR removal is mediated exclusively by mGlu1 (McCutcheon et al., 2011; Loweth et al., 2014), blocking either mGlu5 or mGlu1 prevented the acute DHPG-induced decrease in GluA1 surface expression.

Next, we compared results obtained with DHPG to those produced by selectively enhancing mGlu1 or mGlu5 transmission by incubating co-cultures for 10 min with the mGlu1 PAM Ro67–7476 (3 μM) or the mGlu5 PAM CDPPB (10 μM) (Fig. 2B). Analysis by Kruskal-Wallis one-way ANOVA on ranks revealed a significant difference between the four treatment groups (Control, DHPG, Ro67–7476, CDPPB) in levels of cell surface GluA1 ($H=23.52$, $P<0.001$). Dunn's *post hoc* comparisons revealed that, compared to the control group, incubation with DHPG for 10 minutes produced a significant decrease in surface GluA1 levels ($Q=3.40$, $P=0.002$) that was also observed following incubation with Ro67–7476 ($Q=4.68$, $P<0.001$) and CDPPB ($Q=2.97$, $P=0.009$) (Fig. 2B, Top left). No changes in GluA2 levels were observed across the four treatment groups ($H=1.93$, $P=0.59$) (Fig. 2B, Bottom left). Scatter plots show variability within each treatment group (Fig. 2B, Right). These results show that acute treatment (10 min) with either an mGlu1 or mGlu5 PAM produced a decrease in GluA1 surface expression similar to that observed following DHPG application, with no change in GluA2 levels, indicating internalization of CP-AMPARs.

Long-term inhibition of mGlu1 but not mGlu5 transmission increases GluA1 surface expression in cultured NAc MSNs

In our studies of the intact NAc during incubation of craving, we found that a reduction in mGlu1 transmission during withdrawal preceded and enabled the accumulation of CP-AMPARs (Loweth et al., 2014). To assess whether altering group I mGluR tone *in vitro* affects CP-AMPAR surface expression, NAc/PFC co-cultures were treated for 24 or 48 h with control media, the mGlu1 antagonist LY367385 (100 μM) or the mGlu5 antagonist MTEP (1 μM) (Fig. 3). Kruskal-Wallis one-way ANOVA on ranks revealed a significant difference between the five treatment groups (control, 24 h LY367385, 48 h LY367385, 24 h MTEP, 48 h MTEP) in surface levels of GluA1 ($H=15.07$, $P=0.005$). Dunn's *post hoc* comparisons revealed a significant increase in GluA1 levels compared to the control group following both 24 h ($Q=2.98$, $P=0.012$) and 48 h ($Q=2.90$, $P=0.015$) treatment with LY367385, while treatment with MTEP for 24 h ($Q=0.69$, $P=1.00$) or 48 h ($Q=0.58$, $P=1.00$) had no effect (Fig. 3, Top left). No change in GluA2 levels was observed between treatment groups ($H=1.55$, $P=0.82$) (Fig. 3, Bottom left). Scatter plots show variability within each treatment group (Fig. 3, Right). These results show that long-term inhibition of mGlu1 transmission leads to an increase in CP-AMPARs on the cell surface of NAc MSNs, reminiscent of what we have observed during incubation of cocaine craving.

Long-term inhibition of mGlu1 upregulates surface CP-AMPARs in cultured NAc MSNs through a mechanism requiring protein translation and RA synthesis

To determine if the increase in surface GluA1 required new protein synthesis, we incubated co-cultures with the translation inhibitor anisomycin (40 μM) with or without LY367385 for 24 h (Fig. 4A). Analysis by Kruskal-Wallis one-way ANOVA on ranks revealed a significant difference between the four treatment groups (control, LY367385, LY367385 + anisomycin,

anisomycin) in surface levels of GluA1 (H=14.31, P=0.003). Dunn's test *post hoc* comparisons revealed that, compared with the control group, 24 h treatment with LY367385 significantly elevated surface expression of GluA1 (Q=2.83, P=0.014) and that co-treatment with anisomycin completely prevented this increase (Q=0.40, P=1.00), while 24 h treatment with anisomycin alone had no effect on GluA1 levels (Q=0.55, P=1.00) (Fig. 4A, Top left). In contrast, no significant differences in GluA2 levels were observed between the four treatment groups (H=2.04, P=0.56), although GluA2 levels following 24 h of treatment with anisomycin alone did trend downward (68±16% of control group) (Fig. 4A, Bottom left). Scatter plots show variability within each treatment group (Fig. 4A, Right). These results demonstrate that protein translation is required for the increase in cell surface GluA1 elicited by LY367385.

We wondered if LY367385 might be upregulating surface GluA1 through the RA-dependent homeostatic cascade described previously in hippocampal neurons (Chen *et al.*, 2014) (see Introduction and Discussion). To test this, we incubated co-cultures for 24 h with LY367385 in the absence or presence of DEAB (10 μM), an inhibitor of retinal dehydrogenase, an enzyme in the RA synthesis pathway (Fig. 4B). Analysis by Kruskal-Wallis one-way ANOVA on ranks revealed a significant difference between the four treatment groups (control, LY367385, LY367385 + DEAB, DEAB) in surface levels of GluA1 (H=16.68, P<0.001). Dunn's test *post hoc* comparisons revealed that, compared to the control group, 24 h LY367385 treatment produced a significant increase in surface GluA1 levels (Q=2.89, P=0.023) that was blocked in the LY367385 + DEAB group (Q=0.91, P>0.05), while 24 h treatment with DEAB alone had no effect (Q=0.03, P=1.00) (Fig. 4B, Top left). In contrast, no significant group differences were observed in GluA2 surface levels (H=3.12, P=0.37) (Fig. 4B, Bottom left). Scatter plots show variability within each treatment group (Fig. 4B, Right). Overall, the results shown in Fig. 4 demonstrate that long-term mGlu1 blockade upregulates surface GluA1 through RA signaling and protein synthesis.

DISCUSSION

Overview of mGlu1 modulation and drug craving

Compounds that negatively or positively modulate group I mGluRs have been the focus of intense interest due to their potential to tune glutamate transmission up or down in disease states. In animal models of drug addiction, most attention has focused on mGlu5, with less attention paid to mGlu1 (Olive, 2009). As described in the next section, rodent studies from our lab and others suggest that mGlu1 PAMs, through negative regulation of CP-AMPAR levels, might reduce craving and relapse if given to recovering drug users during abstinence. As discussed elsewhere, mGlu1 PAMs may also alleviate cognitive deficits associated with cocaine use (Olive, 2010). It is therefore important to better understand the cellular mechanism by which mGlu1 regulates CP-AMPAR levels in the NAc. Here we studied this in NAc neurons that were co-cultured with PFC neurons to restore excitatory synaptic inputs (Sun *et al.*, 2008). This line of investigation is timely given the recent development of improved mGlu1 PAMs based on indications in other brain disorders (Garcia-Barrantes *et al.*, 2015).

mGlu1 PAMs and the incubation of cocaine craving

Studies in multiple brain regions have shown that, when CP-AMPARs are present in excitatory synapses, activation of mGlu1 produces a postsynaptically expressed LTD that is mediated through CP-AMPAR internalization (Bellone & Luscher, 2005; 2006; Mameli *et al.*, 2007; Kelly *et al.*, 2009; Mameli *et al.*, 2009; Clem & Huganir, 2010; Loweth *et al.*, 2013). This regulatory mechanism can be demonstrated in slice recordings from NAc MSNs of rodents who have undergone incubation of craving ((McCutcheon *et al.*, 2011a; Scheyer et al., 2015) and targeted *in vivo*, using systemically active mGlu1 PAMs (Loweth et al., 2014) or optogenetic LTD (Lee *et al.*, 2013; Ma *et al.*, 2014), to remove CP-AMPARs from NAc synapses and thereby reduce cue-induced cocaine craving. More recently, we found that mGlu1 PAMs can similarly reduce cue-induced craving by removing CP-AMPARs from NAc core synapses of rats that have undergone incubation of methamphetamine craving (Scheyer *et al.*, 2016). These findings suggest that mGlu1 PAMs might minimize the risk of craving and relapse if taken by abstinent drug users prior to entering an environment associated with prior cocaine use. There is presently no prophylactic treatment of this kind available. A different potential therapeutic context is suggested by the finding that decreased mGlu1 surface expression in the NAc precedes the accumulation of CP-AMPARs during cocaine withdrawal, and restoring mGlu1 tone during this early withdrawal period (through repeated mGlu1 PAM injections), delays the onset of CP-AMPAR accumulation and incubation, although these processes resume 2–3 days after mGlu1 PAM injections are discontinued (Loweth *et al.*, 2014). Thus, mGlu1 PAMs, given early in abstinence, may delay the onset of incubation, allowing time for other interventions to be implemented. Studies in other brain regions also indicate that ongoing mGlu1 transmission exerts a tonic suppressive effect on CP-AMPAR levels (Kelly *et al.*, 2009; Mameli *et al.*, 2009; Bellone *et al.*, 2011).

Acute group I mGluR stimulation leads to exchange of CI-AMPARs for CP-AMPARs

Using the nonselective agonist DHPG, we found that group I mGluR activation leads to a rapid reduction in surface expression of CP-AMPARs on NAc MSNs in NAc/PFC co-cultures and a slightly delayed trend towards an increase in surface expression of GluA2-containing AMPARs (Fig. 1). Slice recordings have previously provided evidence that mGlu1-induced CP-AMPAR internalization is associated with insertion of CI-AMPARs in NAc MSNs (McCutcheon *et al.*, 2011a; Scheyer *et al.*, 2015), as well as VTA DA neurons (Bellone & Luscher, 2005; 2006; Mameli *et al.*, 2007) and cerebellar stellate cells (Liu & Cull-Candy, 2000; Kelly *et al.*, 2009). This “swap” leads to LTD because a high conductance CP-AMPAR is exchanged for a lower-conductance CI-AMPAR. Interestingly, in oligodendrocyte precursor cells, group I mGluR stimulation leads to the opposite effect, namely insertion of CP-AMPARs (Zonouzi *et al.*, 2011).

By directly visualizing GluA1 and GluA2 trafficking during mGlu1-LTD, our present results add to the literature on mGlu1-LTD in neurons by showing that CI-AMPAR insertion may proceed more slowly than CP-AMPAR internalization, since we detected CP-AMPAR removal at both 10 and 20 min time-points after DHPG application whereas the trend towards CI-AMPAR insertion was most pronounced at the 20-min time-point (Fig. 1). The different timing suggests some mechanistic independence. Indeed, while CP-AMPAR

removal and CI-AMPAR insertion are induced through a common PKC-dependent signaling cascade and CP-AMPAR activation is required in order for CI-AMPARs to be inserted (McCutcheon *et al.*, 2011a), we found that the two halves of the exchange can be dissociated: thus, disrupting the association of CI-AMPARs with the trafficking protein PICK1 did not affect DHPG-induced CP-AMPAR internalization but prevented the accompanying insertion of CI-AMPARs (Scheyer *et al.*, 2015). In contrast, in the VTA, PICK1 prevents DHPG-induced LTD and no difference in the total number of synaptic AMPARs was detected during DHPG-induced LTD in VTA DA neurons, indicating immediate replacement of CP-AMPARs with CI-AMPARs (Mameli *et al.*, 2007). Interpretation of PICK1 experiments in cerebellar stellate cells (Liu & Cull-Candy, 2005) is more complex, because PICK1 potentially interacts with both GluA2 and GluA3 (Hanley, 2008) and therefore with both the GluA2-containing CI-AMPARs and the homomeric GluA3 CP-AMPARs found in these neurons (Petralia *et al.*, 1997; Liu & Cull-Candy, 2002).

In the NAc of rats that have undergone incubation of cocaine craving, slice recordings have demonstrated that DHPG-induced internalization of CP-AMPARs is mediated by mGlu1; in fact, stimulation of mGlu5, while leading to presynaptically expressed LTD in MSNs of control rats, produces little effect in MSNs after incubation of cocaine craving (McCutcheon *et al.*, 2011a; Loweth *et al.*, 2014). In contrast to these findings in NAc tissue from adult rats, we found that blockade of either mGlu1 or mGlu5 inhibited DHPG-induced internalization of CP-AMPARs in co-cultured NAc MSNs (Fig. 2). The next section considers how to reconcile this with subsequent experiments indicating selective regulation of CP-AMPARs by long-term mGlu1 blockade.

Long-term decreases in mGlu1 but not mGlu5 tone regulate CP-AMPAR expression

As discussed above, a decrease in NAc mGlu1 surface expression precedes CP-AMPAR accumulation during cocaine withdrawal, and enhancing mGlu1 transmission during this critical withdrawal period prevents CP-AMPAR accumulation and incubation of cocaine craving, while inhibiting mGlu1 transmission during the same period accelerates CP-AMPAR accumulation (Loweth *et al.*, 2014). Consistent with these *in vivo* findings, here we found that long-term (24–48 h) incubation with an mGlu1 antagonist (LY367385) increased GluA1 but not GluA2 surface expression in co-cultured NAc neurons. Identical treatment with an mGlu5 antagonist (MTEP) did not significantly alter GluA1 or GluA2 surface expression (Fig. 3).

As discussed in more detail in the next section, the LY367385-induced increase in surface GluA1 was blocked by the translation inhibitor anisomycin (Fig. 4). We had previously hypothesized that decreased mGluR1 surface expression observed in the NAc during cocaine withdrawal enabled CP-AMPAR accumulation by reducing mGlu1-dependent LTD. The present findings raise the possibility that reduced mGlu1 levels enable CP-AMPAR accumulation through a different mechanism, namely an increase in GluA1 translation, or that both mechanisms contribute to CP-AMPAR accumulation during cocaine withdrawal. A caveat is that we have shown that the LY367385-induced increase in surface GluA1 depends on new translation but we have not specifically demonstrated an increase in GluA1 translation. These studies are underway.

How can GluA1 upregulation after long-term mGlu1 but not mGlu5 blockade (Fig. 3) be reconciled with our acute DHPG and PAM studies, in which the effect of these drugs could be blocked by either an mGlu1 or mGlu5 antagonist (Fig. 2)? The acute data suggest that both mGlu1 and mGlu5 are capable of coupling to signaling cascades that trigger internalization of CP-AMPARs, and that exogenous agonists or PAMs can access both mGlu1 and mGlu5 to accomplish CP-AMPAR internalization. However, studies in which mGlu antagonists were applied alone indicate that endogenous glutamate is mainly accessing mGlu1 receptors to elicit CP-AMPAR internalization. This could be explained if mGlu1 is in closer proximity to glutamate release sites.

While this may be the case in our co-culture system, electron microscopy studies of mGlu1 and mGlu5 in the adult rat NAc paint a more complex picture (Mitrano & Smith, 2007; Mitrano *et al.*, 2008; Mitrano *et al.*, 2010). The first study by Mitrano, Young and colleagues showed that, for both receptors, the plasma membrane-bound pools are >80% extrasynaptic (i.e., not associated with synapses), with some perisynaptic labeling (within a 20-nm range of the edges of postsynaptic specializations) and only a very small degree of synaptic labeling (in contact with the main body of postsynaptic specializations) (Mitrano & Smith, 2007). A follow-up study assessed the ultrastructural relationships between specific glutamatergic afferents (cortex, thalamus and amygdala) and mGlu1- or mGlu5-containing neurons in the rat NAc (Mitrano *et al.*, 2010). Although the general subcellular distribution of mGlu1 and mGlu5 in relation to these inputs was similar to that described in the earlier study (mainly extrasynaptic with some perisynaptic and very low synaptic labeling), it was found that glutamate afferents innervated mGlu5-labeled spines more frequently than mGlu1-labeled spines and, most relevant to our results, that the prevalence of perisynaptic labeling tended to be higher for mGlu5 than mGlu1 and extrasynaptic mGlu5 tended to be closer to the edges of asymmetric synapses than mGlu1 (Mitrano *et al.*, 2010). The latter findings are not consistent with preferential activation of mGlu1 in the adult rat NAc in response to glutamate transmission or spillover.

On the other hand, mGlu1 and mGlu5 colocalize to a substantial degree in NAc dendrites (~30%) and spines (50–55%) and yet, as in other brain regions that show such colocalization, it is clear that mGlu1 and mGlu5 can couple to different effectors and mediate distinct cellular effects (see discussion in (Mitrano & Smith, 2007)). For example, in the NAc of drug-naïve rats, mGlu5 but not mGlu1 stimulation leads to presynaptically expressed CB1R-dependent LTD, whereas after incubation of cocaine craving, mGlu1 but not mGlu5 stimulation leads to CP-AMPAR internalization (McCutcheon *et al.*, 2011a). Resolving the relationship between localization and function of mGlu1 versus mGlu5 is an important challenge for future studies. A complicating factor is the existence of a substantial intracellular pool of these receptors in the adult rat NAc (Mitrano & Smith, 2007) combined with evidence that mGlu5 and other GPCRs can signal from intracellular compartments (Jong *et al.*, 2018). Furthermore, group I mGluRs are expressed by astrocytes (Hovelsø *et al.*, 2012) and we cannot exclude a role for this mGluR population in our observed effects.

Role of RA-dependent protein translation in CP-AMPAR upregulation after long-term mGlu1 blockade

Synaptic scaling is a form of homeostatic plasticity that adjusts synaptic strength to compensate for changes in neuronal activity. For example, prolonged inactivity leads to strengthening of synapses (scaling up) via mechanisms including postsynaptic AMPAR insertion (Turrigiano, 2012). In one type of scaling up, decreased synaptic activity leads to local GluA1 synthesis and synaptic insertion of homomeric GluA1 CP-AMPARs (Ju *et al.*, 2004; Sutton *et al.*, 2006). As described in the Introduction, work from the Chen lab has identified RA as a link between decreased synaptic activity and the resultant increase in homomeric GluA1 receptors (Aoto *et al.*, 2008; Chen *et al.*, 2014). Under normal conditions in hippocampal neurons, ongoing synaptic transmission maintains dendritic Ca^{2+} at levels sufficient to repress RA synthesis. Inhibition of synaptic activity leads to a reduction in Ca^{2+} levels and a resultant reduction in activity of the Ca^{2+}-dependent phosphatase calcineurin; this disinhibits RA synthesis (Wang *et al.*, 2011; Arendt *et al.*, 2015b). RA increases GluA1 translation through a novel non-genomic mechanism involving accumulation of retinoic acid receptor alpha in dendritic RNA granules, where GluA1 synthesis is subsequently activated (Maghsoodi *et al.*, 2008).

Here we show that RA and protein translation are similarly required for increased CP-AMPARs in cultured NAc neurons after 24 h of mGlu1 blockade (Fig. 4). Although RA-dependent homeostatic plasticity was demonstrated in hippocampal neurons after blockade of ionotropic glutamate receptors or voltage-gated Ca^{2+} channels (Chen *et al.*, 2014), mGlu1 activation is known to increase postsynaptic Ca^{2+} levels (Kano *et al.*, 2008; Luscher & Huber, 2010). Therefore, it is not surprising that decreasing mGlu1 signaling in NAc neurons would trigger increased RA signaling. Future studies will confirm the relationship between postsynaptic Ca^{2+} levels and RA synthesis in NAc neurons, and determine if calcineurin is also the sensor for RA-dependent homeostatic plasticity in these neurons. Ultimately our goal is to determine if this mechanism is responsible for CP-AMPAR upregulation during cocaine withdrawal. Electrophysiological studies and measurements of blood flow in rodents suggest reduced activity in the NAc and in brain regions sending excitatory inputs to the NAc following cocaine self-administration (Hammer *et al.*, 1993; Macey *et al.*, 2004; Sun & Rebec, 2006; Gozzi *et al.*, 2011; Wolf, 2016). We propose that this leads to a reduction in Ca^{2+} levels in NAc MSNs that through RA synthesis results in upregulation of CP-AMPARs and heightened cocaine craving.

It is important to appreciate the diversity of mechanisms that can underlie homeostatic plasticity (see (Chen *et al.*, 2014)). For example, in the same NAc/PFC co-culture system, we have previously demonstrated that blocking excitatory synaptic transmission with tetrodotoxin or ionotropic glutamate receptor antagonists (24–72 h) leads to AMPAR scaling up in NAc MSNs that required protein translation and was occluded by inhibition of the ubiquitin-proteasome system; however, in this form of scaling, it is GluA1A2 CI-AMPARs that upregulate (Sun & Wolf, 2009). It is possible that mGlu1 preferentially regulates Ca^{2+} pools in NAc MSNs that are linked to GluA1 translation. Supporting potential selectivity for GluA1, we have failed to detect an effect of mGlu1 blockade on overall basal protein

translation in either cultured NAc neurons (Stefanik *et al.*, 2018a) or NAc neurons of the intact rat (Stefanik *et al.*, 2018b).

Conclusions

The present results in cultured NAc MSNs, together with prior slice recordings in MSNs from the NAc of rats that have undergone incubation of cocaine craving, suggest that acute activation of mGlu1 triggers internalization of CP-AMPARs that is coupled, albeit with a slight delay, to insertion of CI-AMPARs. In cultured MSNs, but not the intact NAc, acute mGlu5 stimulation can also elicit CP-AMPAR internalization; on the other hand, our results indicate that ongoing mGlu1 transmission, but not mGlu5 transmission, maintained by endogenous glutamate levels exerts a tonic inhibitory influence on surface CP-AMPAR levels through a distinct mechanism, namely by suppressing protein translation. Thus, when mGlu1 tone is interrupted for 24 h, surface homomeric GluA1 receptors increase through a mechanism dependent on RA synthesis and new protein translation. This identifies a novel form of homeostatic plasticity in NAc neurons and may explain how reduced mGlu1 tone during cocaine withdrawal leads to accumulation of the CP-AMPARs that underlie persistent increases in cocaine craving.

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Abbreviations

AMPAR	AMPA receptor
CI-AMPAR	calcium-impermeable AMPA receptor
CP-AMPAR	calcium-permeable AMPA receptor
DHPG	dihydroxyphenylglycine
ECFP	enhanced cyan fluorescent protein
GluA1, 2 and 3	glutamate receptor types 1, 2 and 3
LTD	long-term depression
MSN	medium spiny neuron
NAc	nucleus accumbens
P	postnatal day
PAM	positive allosteric modulator
PFC	prefrontal cortex

RA	retinoic acid
RT	room temperature

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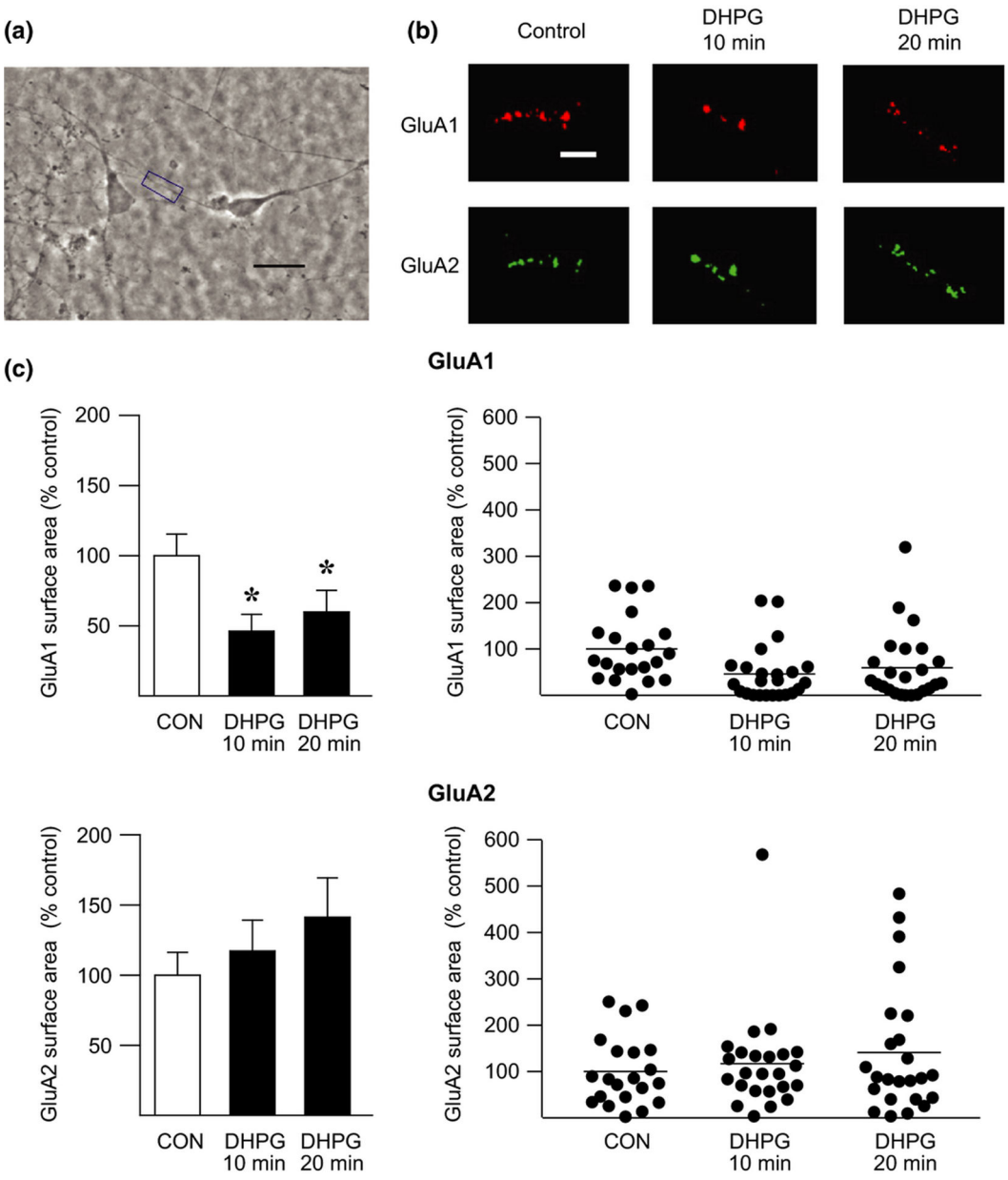

Figure 1.

Acute DHPG application reduces surface GluA1 levels. (A) Representative phase contrast image of NAc/PFC co-cultures. The blue rectangle shows the fixed length of process (15 μm) on a medium spiny neuron (MSN) used to analyze surface GluA1 and GluA2 levels. Scale bar = 20 μm. (B) Enlarged representative fluorescent images of surface GluA1 (red) and GluA2 (green) on MSNs in NAc/PFC co-cultures under control conditions (CON; media) or after treatment with DHPG (50 μM, 10 or 20 min). Scale bar = 5 μm. (C) Left: Quantification of surface GluA1 (Top) and GluA2 (Bottom) levels following treatments described in (A). Acute application of DHPG for 10 minutes significantly decreased surface GluA1 but not GluA2, indicating internalization of CP-AMPARs. After 20 minutes of DHPG treatment, the decrease in GluA1 surface expression was not as pronounced and there

was a trend towards increased GluA2 surface expression relative to the 10 min time-point, suggesting insertion of CI-AMPARs containing GluA1 and GluA2. Data are shown as % control (mean ± SEM). *p<0.05 versus control. Right: Scatter plots for GluA1 (top) and GluA2 (bottom) showing individual data points for all cells in each treatment group. Data are shown as % control and lines represent each group mean. n=21–24 cells/group.

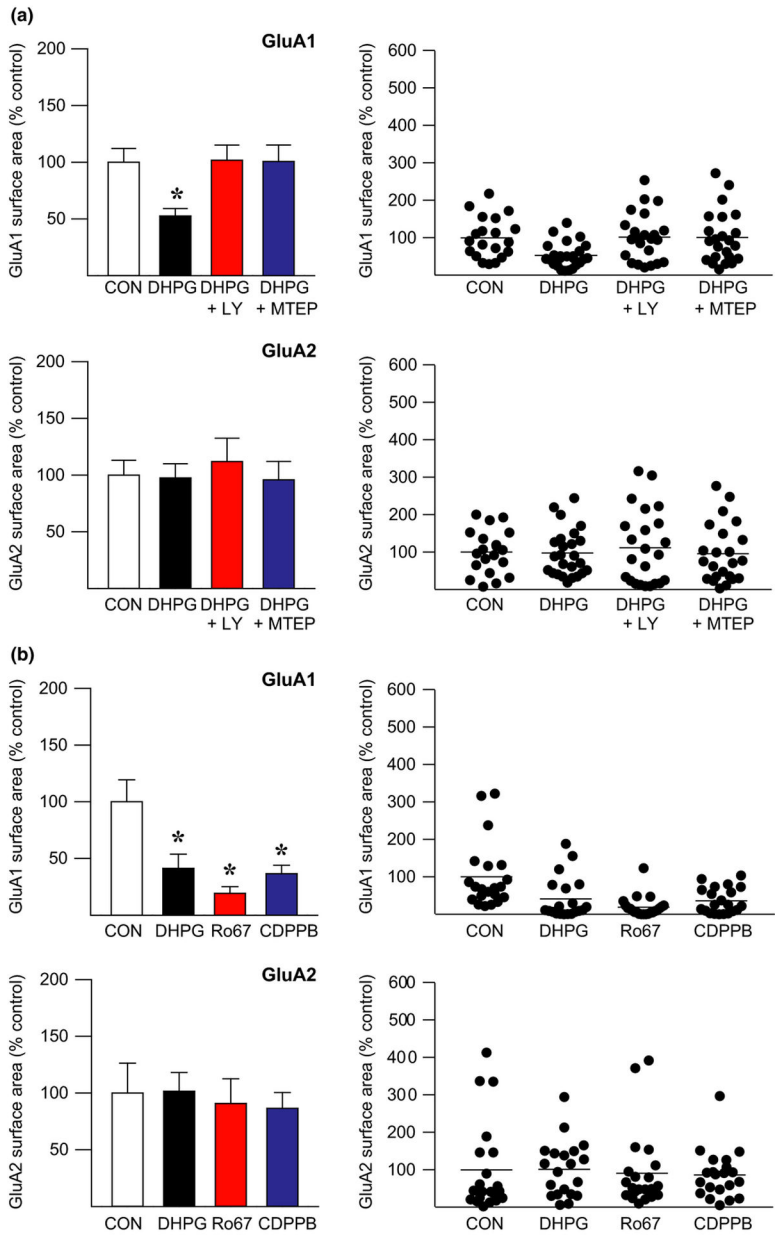

Figure 2.

The DHPG-induced reduction in surface GluA1 is mediated by mGlu1 and mGlu5 and mimicked by mGlu1 or mGlu5 PAMs. (A) Left: Quantification of surface GluA1 (Top left) and GluA2 (Bottom left) levels following a 10 minute treatment with media (control, CON), the group I mGluR agonist DHPG (50 μM), DHPG plus the mGlu1 antagonist LY367385 (LY, 100 μM), or DHPG plus the mGlu5 antagonist MTEP (1 μM) (antagonists added 5 min prior to DHPG). Blocking either mGlu5 or mGlu1 prevented the acute DHPG-induced decrease in GluA1 surface expression while having no effect on GluA2 surface expression. Data are shown as % control (mean ± SEM). *$p<0.05$ versus control. Right: Scatter plots for GluA1 (top) and GluA2 (bottom) showing individual data points for all cells in each treatment group. Data are shown as % control and lines represent each group mean. n=20–25

cells/group. (B) Left: Quantification of surface GluA1 (Top left) and GluA2 (Bottom left) levels following a 10 minute treatment with media (control), the group I mGluR agonist DHPG (50 μM), the mGlu1 positive allosteric modulator (PAM) Ro67–7476 (Ro67, 3 μM), or the mGlu5 PAM CDPPB (10 μM). Consistent with the antagonist study, both mGlu1 and mGlu5 PAMs produced a decrease in GluA1 surface expression similar to that observed following DHPG application, with no change in GluA2 levels, indicating internalization of CP-AMPARs. Data are shown as % control (mean ± SEM). *$p<0.05$ versus control. Right: Scatter plots for GluA1 (top) and GluA2 (bottom) showing individual data points for all cells in each treatment group. Data are shown as % control and lines represent each group mean. n=20–22 cells/group.

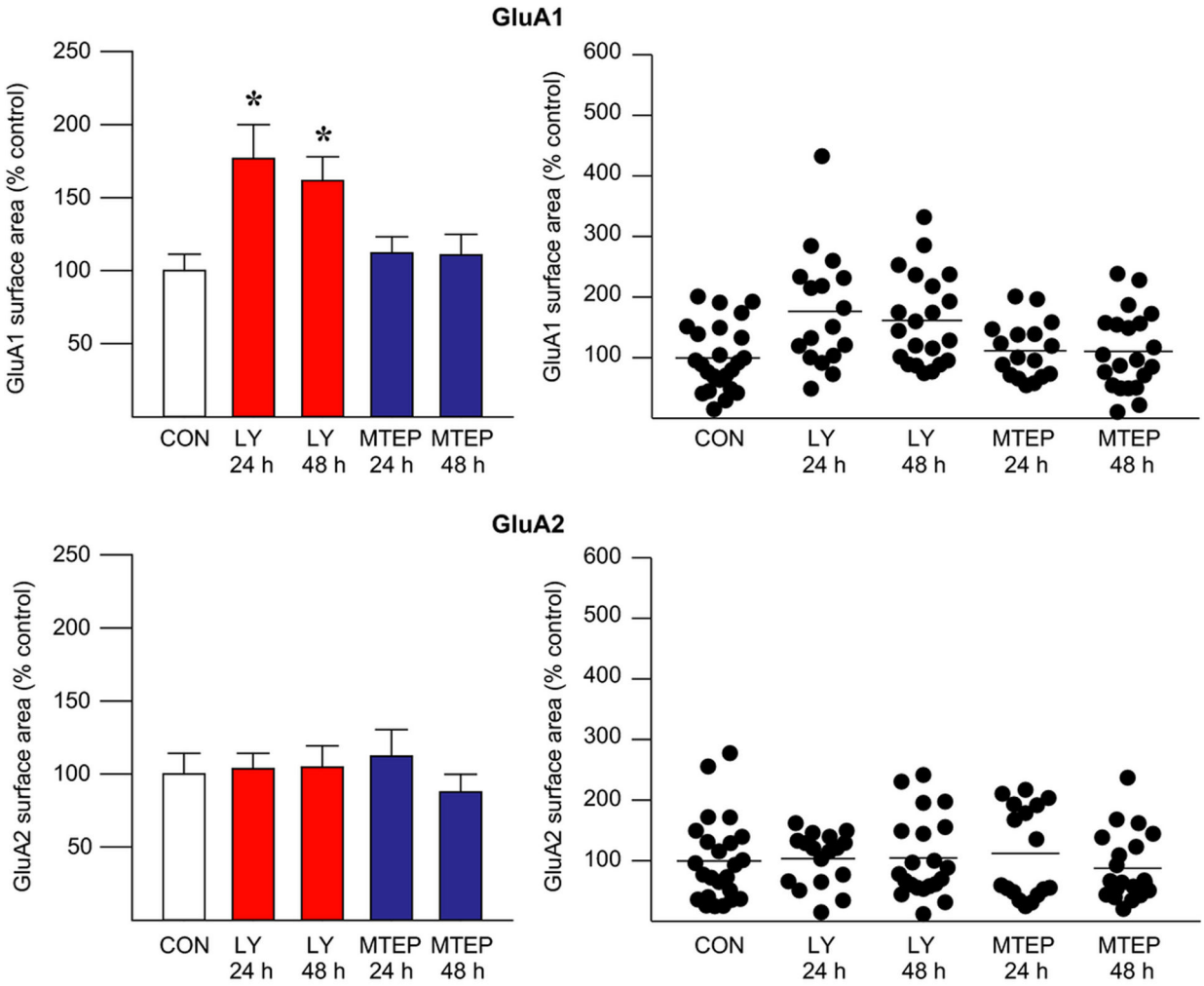

Figure 3.

Long-term reduction in mGlu1 transmission, but not mGlu5 transmission, leads to upregulation of surface GluA1. Left: Quantification of surface GluA1 (Top) and GluA2 (Bottom) levels following long-term treatment (24–48 h) with media (control, CON), the mGlu1 antagonist LY367385 (LY, 100 μM) or the mGlu5 antagonist MTEP (1 μM). Both 24 and 48 h incubation with the mGlu1 antagonist LY367385 (but not the mGlu5 antagonist MTEP) increased GluA1 (left) but not GluA2 (right) surface levels in cultured NAc neurons, suggesting that long-term inhibition of mGlu1 transmission leads to scaling up of CP-AMPARs. Data are expressed as % control (mean ± SEM). *$p<0.05$ versus control. Right: Scatter plots for GluA1 (top) and GluA2 (bottom) showing individual data points for all cells in each treatment group. Data are shown as % control and lines represent each group mean. n=17–24 cells/group.

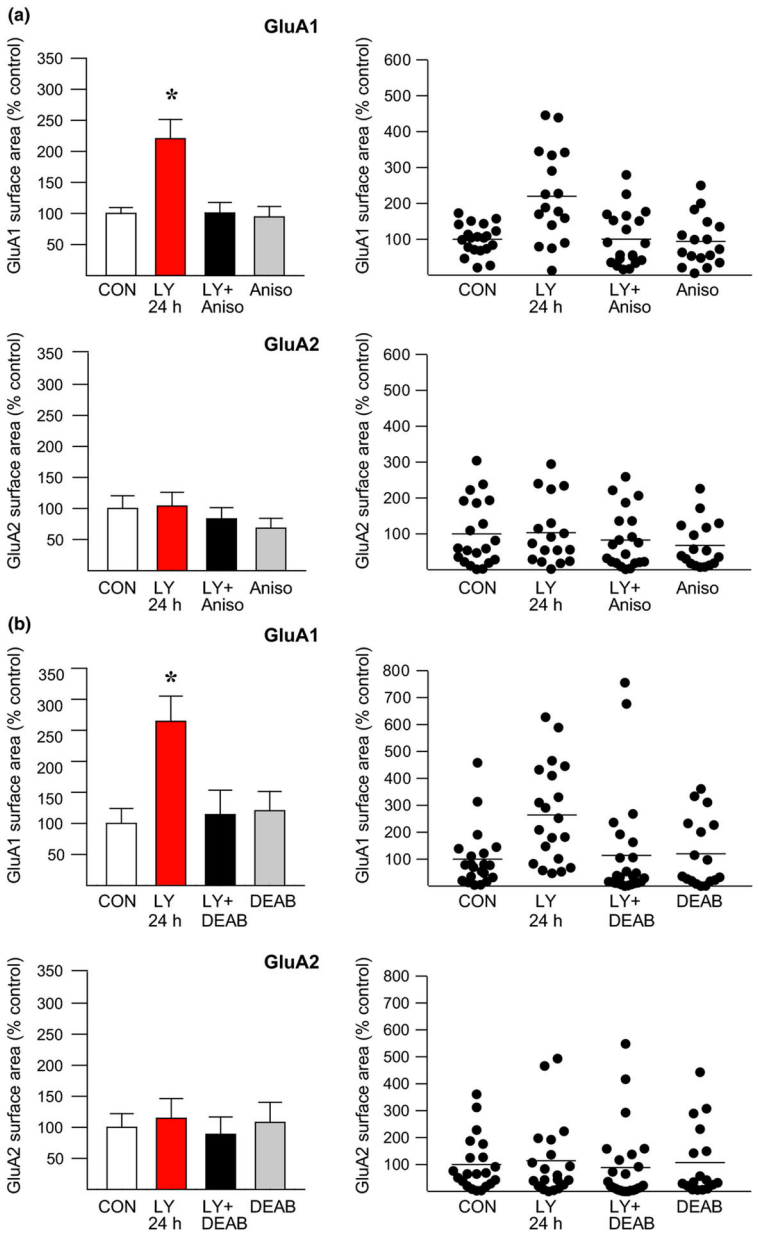

Figure 4.

Upregulation of surface GluA1 after long-term blockade of mGlu1 requires protein translation and retinoic acid signaling. Cultures were treated for 24 h with the mGlu1 antagonist LY367385 (LY, 100 μM) in the presence of the protein synthesis inhibitor anisomycin (Aniso, 40μM) (A) or the inhibitor of retinoic acid synthesis DEAB (10 μM) (B). Controls (CON) were treated with media. Inhibiting either protein synthesis or the production of retinoic acid completely blocked the LY-induced increase in surface GluA1 expression. Left: Data are expressed as % control (mean ± SEM). *$p<0.05$ versus control. Right: Scatter plots for GluA1 and GluA2 showing individual data points for all cells in each

treatment group. Data are shown as % control and lines represent each group mean. n=17–20 cells/group (A) and n=17–25 cells/group (B).