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ARTICLE

Activation of the $mGlu_1$ metabotropic glutamate receptor has antipsychotic-like effects and is required for efficacy of M_4 muscarinic receptor allosteric modulators

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Abstract

Recent clinical and preclinical studies suggest that selective activators of the M_4 muscarinic acetylcholine receptor have potential as a novel treatment for schizophrenia. M_4 activation inhibits striatal dopamine release by mobilizing endocannabinoids, providing a mechanism for local effects on dopamine signaling in the striatum but not in extrastriatal areas. G protein-coupled receptors (GPCRs) typically induce endocannabinoid release through activation of $G\alpha_{q/11}$-type G proteins whereas M_4 transduction occurs through $G\alpha_{i/o}$-type G proteins. We now report that the ability of M_4 to inhibit dopamine release and induce antipsychotic-like effects in animal models is dependent on co-activation of the $G\alpha_{q/11}$-coupled $mGlu_1$ subtype of metabotropic glutamate (mGlu) receptor. This is especially interesting in light of recent findings that multiple loss of function single nucleotide polymorphisms (SNPs) in the human gene encoding $mGlu_1$ (*GRM1*) are associated with schizophrenia, and points to *GRM1*/$mGlu_1$ as a gene within the "druggable genome" that could be targeted for the treatment of schizophrenia. Herein, we report that potentiation of $mGlu_1$ signaling following thalamo-striatal stimulation is sufficient to inhibit striatal dopamine release, and that a novel $mGlu_1$ positive allosteric modulator (PAM) exerts robust antipsychotic-like effects through an endocannabinoid-dependent mechanism. However, unlike M_4, $mGlu_1$ does not directly inhibit dopamine D_1 receptor signaling and does not reduce motivational responding. Taken together, these findings highlight a novel mechanism of cross talk between $mGlu_1$ and M_4 and demonstrate that highly selective $mGlu_1$ PAMs may provide a novel strategy for the treatment of positive symptoms associated with schizophrenia.

Introduction

Currently approved antipsychotics are efficacious in treating positive symptoms of schizophrenia in many patients, however they offer little to no benefit for negative or cognitive symptoms, and are associated with a number of adverse effects [1, 2]. Thus, there is a critical need to develop fundamentally new approaches for treating schizophrenia that provide improved efficacy and induce fewer adverse effects than current medications.

In recent years, intense translational efforts suggest that highly selective positive allosteric modulators (PAMs) of the M_4 muscarinic acetylcholine receptor (mAChR) may provide a novel approach for the treatment of schizophrenia. Most notably, a four-week, double-blind, placebo-controlled outcome trial revealed that an M_1/M_4 subtype-preferring mAChR agonist, xanomeline, produced statistically significant improvements in total Positive and

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Negative Syndrome Scale (PANSS) in schizophrenia patients, as well as trends toward improvements in the PANSS positive and negative subscales, and specific domains of cognitive function [3]. Furthermore, xanomeline displays efficacy in animal models that predict clinical efficacy across these three symptom domains [4–6]. Interesting, the antipsychotic-like effects of xanomeline are absent in M_4 knockout mice [7] and mimicked by administration of highly selective M_4 PAMs [8–11], suggesting that some of the antipsychotic-like effects of xanomeline are mediated by M_4 activation. Finally, genetic studies reveal that single nucleotide polymorphisms (SNPs) of *CHRM4*, the gene encoding M_4, were associated with an increased risk of developing schizophrenia [12].

Hyperactivity in subcortical dopamine (DA) signaling contributes to the manifestation of positive symptoms in schizophrenia [13, 14] and imaging studies suggest that schizophrenia patients' display selective increases in DA release in the dorsal striatum, with decreases in extrastriatal DA release [15]. Interestingly, detailed preclinical, cellular, genetic, and optogenetic studies reveal that M_4 PAMs inhibit DA release in the dorsolateral striatum (DLS) by specific actions on M_4 in a subpopulation of spiny projection neurons (SPNs) that express D_1-DA receptors (D_1-SPNs) [11]. Activation of M_4 on D_1-SPNs induces an inhibition of DA release that is dependent upon both synthesis of the endocannabinoid (eCB) 2-arachinonoylglycerol (2-AG) and activation of CB_2 receptors located on neighboring DA terminals [11]. The mobilization of 2-AG by M_4 provides a novel mechanism that allows local inhibition of DA signaling in striatal regions that are most critical for positive symptoms of schizophrenia, without inhibiting DA in other regions where DA signaling is already impaired. However, despite this promising profile, activation of M_4 also directly inhibits D_1 signaling in D_1-SPNs through activation of $G\alpha_{i/o}$- type G proteins, which inhibits D_1 receptor-mediated activation of adenylyl cyclase [16]. Therefore, M_4 activation could excessively inhibit D_1 relative to D_2 DA receptor signaling, rather than maintaining balanced inhibition of both D_1 and D_2-dependent signaling pathways.

The finding that M_4 activation inhibits DA release through stimulation of 2-AG was somewhat surprising in light of studies showing that G protein-coupled receptors (GPCRs) typically induce eCB release by activation of $G\alpha_{q/11}$ and induction of intracellular calcium (Ca^{++}) [17, 18]. M_4 signals through $G\alpha_{i/o}$ and does not couple to $G\alpha_{q/11}$ or induce Ca^{++} mobilization in SPNs [11]. This raises the possibility that M_4-induced release of 2-AG and inhibition of DA release may require co-activation of another GPCR that activates $G\alpha_{q/11}$ and facilities Ca^{++} mobilization. If so, this $G\alpha_{q/11}$-coupled GPCR could provide a novel target that may be more proximal to eCB synthesis and inhibition of DA release, and may inhibit DA release without altering the balance between D_1 and D_2 signaling pathways.

Based on previous studies, the group I metabotropic glutamate (mGlu) receptors ($mGlu_1$ and Glu_5), are prime candidates as $G\alpha_{q/11}$-coupled GPCRs that may interact with M_4 and inhibit DA release through eCB signaling. Group I mGlu receptors are heavily expressed in striatal SPNs [19, 20], where they couple to $G\alpha_{q/11}$ and their signal transduction pathway induces Ca^{++} mobilization [21–23] and activates eCB signaling [24]. Furthermore, multiple loss of function SNPs in the human gene encoding $mGlu_1$ (*GRM1*) are associated with schizophrenia [25, 26], suggesting that $mGlu_1$ may play a key role in modulating schizophrenia-related circuitry. We now report a series of studies using ex vivo and in vivo cyclic voltammetry, along with genetic and optogenetic approaches, to show that activation of $mGlu_1$ is critical for M_4-induced reductions in DA release and antipsychotic-like effects in animal models. Furthermore, synaptic or agonist-induced activation of $mGlu_1$ is sufficient to inhibit DA release and a highly selective $mGlu_1$ PAM induces robust antipsychotic-like effects, which are dependent on eCB signaling. Interestingly, unlike M_4, $mGlu_1$ activation does not directly inhibit DA D_1 signaling and does not reduce motivational responding. Collectively, these findings provide strong evidence that $mGlu_1$ PAMs have robust antipsychotic efficacy and highlight the $mGlu_1$ receptor as an exciting novel target for the treatment of positive symptoms associated with schizophrenia.

Materials and methods

Animals

Adult, drug-naive, C57BL/6 J mice (Jackson Laboratory; 6–10 weeks old) and CB_2 knockout (KO) mice (Jackson Laboratory; 005786; 8–10 weeks old) were kept on a 12 h light/dark cycle and were tested during the light phase (lights on at 7:00 h). All experiments were approved by the Institutional Animal Care and Use Committee, Vanderbilt University. Detailed materials and methods are provided as Supplementary Information. All efforts were made to minimize the number of animals while maintaining statistical rigor.

Ex vivo fast scan cyclic voltammetry

Striatal DA release was measured as described previously [11] (See Supplemental Methods).

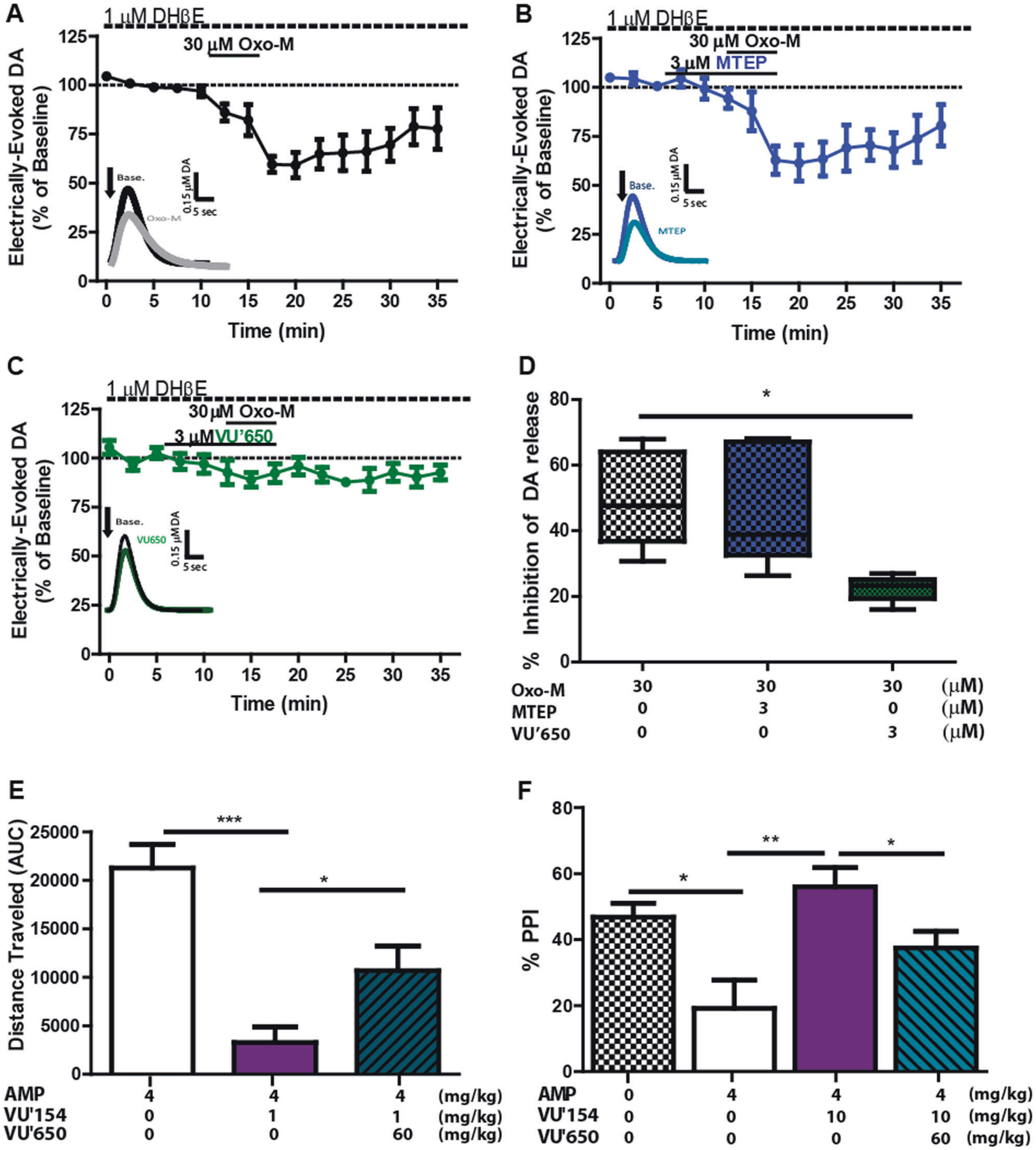


Fig. 1 Activation of mGlu$_1$ is Required for M$_4$-induced Reductions in Striatal DA Release and Subsequent Antipsychotic-like Effects. **a–c** Time courses of 30 μM Oxo-M-induced inhibition of electrically-evoked striatal DA release alone or in the presence of either 3 μM of the mGlu$_5$ negative allosteric modulator (NAM), MTEP, or the mGlu$_1$ NAM VU0469650 (VU'650). All time-course data are depicted as mean ± SEM. **d** Boxplot summaries depicting the percent inhibition of striatal DA release observed under different conditions at peak time points (n = 6–8; *significant from 30 μM Oxo-M; $p < 0.05$; one way ANOVA with Dunnett's multiple comparison posthoc test). **e** Total locomotor activity of area under the curve (AUC) in wildtype (WT) C57BL/6J mice following administration of 4 mg/kg amphetamine (AMP; s.c.), 1 mg/kg VU0467154 (VU'154; i.p.), and 60 mg/kg VU0469650 (VU'650; i.p; $n = 9–12$; *$p < 0.05$, significant difference from AMP/VEH condition; ***$p < 0.001$ significant difference from AMP/VEH condition; one-way ANOVA with Dunnett's multiple comparison posthoc test). Total locomotor counts are depicted as the mean ± SEM. **f** Averaged data showing percent prepulse inhibition (PPI) observed in WT mice following administration of vehicle (VEH; 10% Tween 80), 4 mg/kg amphetamine (AMP), 10 mg/kg VU0467154 (VU'154), and 60 mg/kg VU0469650 (VU'650; n = 15-20; *$p < 0.05$, significant difference from AMP/VU'154 condition; **$p < 0.01$, significant difference from AMP/VU'154 condition; one-way ANOVA with Dunnett's multiple comparison posthoc test). PPI data are depicted as the mean ± SEM

Whole-cell patch clamp

Slice preparation and recordings were conducted using procedures previously described [16] (See Supplemental Methods).

In vivo fast scan cyclic voltammetry

Mice were anesthetized and immobilized in a stereotaxic apparatus as described previously [27] (See Supplemental Methods). In sum, a twisted, bipolar, stimulating electrode was implanted into the medial forebrain bundle (MFB; mm from bregma, AP: -1.1, ML: ± 1.4; DV – 3.3) and a carbon fiber working electrode into the dorsal striatum (DLS; mm from bregma, AP: + 1.3; ML: ± 2.3, DV: -2.7). Biphasic current pulse (± 450 μA, 60 Hz, 4 ms pulse width) were applied for 2 s to the DA axons in the MFB to evoke DA release in the DLS following administration of vehicle (10% Tween 80, intraperitoneal injection (i.p.)) or the $mGlu_1$ PAM VU6004909 (60 mg/kg, i.p.). Sufficient time (i.e., five minutes) was allowed between stimulations for evoked responses to recover.

Microdialysis

Guide cannulae were implanted into the medial prefrontal cortex (mPFC) and were collected as described previously [10] (see Supplemental Methods). DA in dialysate samples were analyzed by the Vanderbilt University Neurochemistry Core using liquid chromatography (LC)-mass spectrometry. Only animals with accurate probe placement that showed three consecutive stable baseline values (within ≤ 20%) were included in the statistical analysis. Prior to analysis, samples (5 μL) were derivatized with benzoyl chloride [28]. LC was performed on a 2.0 × 50 mm, 1.7 μM particle Acquity BEH C18 column (Waters Corporation, Milford, MA, USA) using a Waters Acquity UPLC. Mobile phase A was 15% aqueous formic acid and mobile phase B was acetonitrile. Samples were separated by a gradient of 98–5% of mobile phase A over 11 min at a flow rate of 0.6 mL/min prior to delivery to a SCIEX 6500 + QTrap mass spectrometer (AB Sciex LLC, Framingham, MA, USA). Chromatograms were analyzed using MultiQuant 3.0.2 Software from SCIEX.

Behavioral tests

Tests for locomotor activity [29], prepulse inhibition (PPI) of the acoustic startle reflex [30], motivated behavior [31], rotarod and TreadScan were conducted, as previously described and outlined in supplemental materials. Randomizations were performed for counter-balanced behavioral assays by alternating mice based on mouse number. Rotarod and TreadScan measures were measured and recorded by a blinded investigator. All other behavioral data was collected by MedAssociates software.

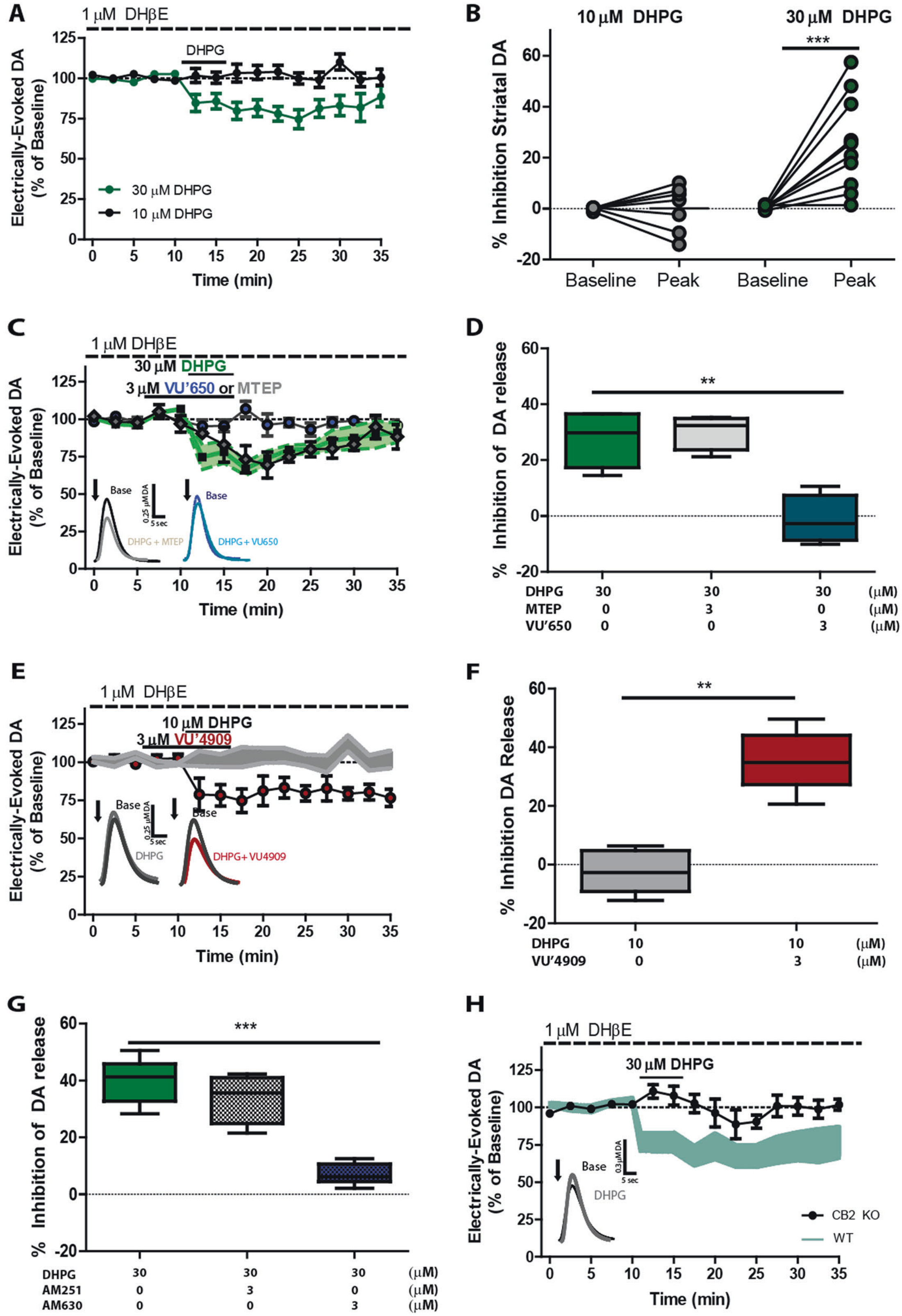
Statistics

When appropriate, data are represented as the mean ± SEM. Statistical analyses were performed using two-tailed Student's t-test, one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison post hoc test and two-way ANOVA followed by Bonferroni's post hoc test, as described in the figure legends. Samples sizes are indicated in figure legends. A value of $p < 0.05$ was considered as statistically significant. All comparisons met the assumptions of the test used, including similar variance between groups being compared. Post hoc power analyses ensured a sufficient number of slices and mice were used.

Results

$mGlu_1$ activation is required for M_4-induced reductions in striatal DA release and antipsychotic-like effects

Due to the localization of group I mGlu receptors in D_1-SPNs [19, 20, 32] and their well-defined role in modulating SPNs via eCB production [33, 34], we tested the hypothesis that M_4-induced reductions in DA release require co-activation of group I mGlu receptors. The effect of the muscarinic acetylcholine receptor (mAChR) agonist oxotremorine-M (Oxo-M) on electrically-evoked DA release in striatal slices was assessed using fast-scan cyclic voltammetry (FSCV) in the absence or presence of selective negative allosteric modulators (NAMs) of individual group I mGlu receptor subtypes. All studies were performed in the presence of the nicotinic acetylcholine receptor antagonist dihydro-β-erythroidine hydrobromide (DHβE, 1 μM). Consistent with previous studies [11], 30 μM Oxo-M induced a sustained M_4-mediated inhibition of DA release (Fig. 1a, d). This effect of Oxo-M on DA release was not blocked by the selective $mGlu_5$ NAM, MTEP (3 μM) (Fig. 1b, d), but was significantly attenuated by application of an $mGlu_1$ NAM VU0469650 (3 μM) (46.7% ± 9.02 in the absence and 21.37% ± 3.14 in the presence of VU0469650; one-way ANOVA, $p < 0.05$; Fig. 1c, d), suggesting that the M_4-mediated reduction in DA release is dependent upon $mGlu_1$ activation. Consistent with this, VU0469650 also blocked the inhibition of DA release observed following application of the M_4 PAM VU0467154 (rat M_4 $EC_{50} = 17.7$ nM, inactive at rat $M_{1,2,3,5}$) [9] with a submaximal concentration of Oxo-M (10 μM) (52% ± 6.07 in the absence and 9.13% ±



2.72 in the presence of VU0469650; students *t*-test, $p < 0.01$; Supplementary Fig. 1A, B).

M$_4$ PAMs have robust efficacy in multiple animal models of antipsychotic activity, including an ability to attenuate amphetamine-induced hyperlocomotion (AHL) and disruption of pre-pulse inhibition (PPI) [8–10], and these effects are thought to be mediated, at least in part, by inhibition of DA release. Thus, we examined the ability of

◀ **Fig. 2** $mGlu_1$-mediates DHPG induced Reductions in Striatal DA Release and Requires CB_2 Receptor Activation. **a** Time course of a concentration response curve of 10 and 30 μM DHPG, a group I mGlu receptor agonist, in the presence of the nAChR antagonist DhβE on electrically evoked striatal DA release. **b** Before and after graphs of peak effects of either 10 or 30 μM DHPG on DA release compared to baseline (BL) conditions ($n = 6$–8; ***$p < 0.001$ significant difference from BL; students *t*-test). **c** Time courses of 30 μM DHPG in the presence of either 3 μM of the $mGlu_5$ NAM, MTEP, or the $mGlu_1$ NAM VU0469650 (VU'650). **d** Boxplot summaries of peak effects of 30 μM DHPG following blockade of $mGlu_5$ or $mGlu_1$ receptors ($n =$ 6–10; **$p < 0.01$, significant difference from 30 μM DHPG; one-way ANOVA with Dunnett's multiple comparison posthoc test). **e** Time course of 10 μM DHPG in the presence of 3 μM VU6004909 (VU'4909). **f** Boxplot summaries of peak effects of 10 μM DHPG alone or in combination with 3 μM $mGlu_1$ PAM VU6004909 (VU'4909; $n = 6$–8; **$p < 0.01$, significant difference from 10 μM DHPG; students *t*-test). **g** Boxplot summaries of peak effects of 30 μM DHPG alone or in the presence of 3 μM AM251, CB_1 antagonist, or 3 μM AM630, CB_2 antagonist, on striatal DA release (*n = 6–10*; ***$p <$ 0.001, significant difference from 30 μM DHPG; one-way ANOVA with Dunnett's multiple comparison posthoc test). **h** Time courses of 30 μM DHPG in wildtype (WT; blue) or CB_2 knockout mice (KO; black)

$mGlu_1$ blockade to blunt the efficacy of M_4 PAMs on AHL and PPI. Consistent with previous findings [9–11], VU0467154 (1 mg/kg, intraperitoneal (i.p.)), reversed AHL (Fig. 1e; one-way ANOVA, $p < 0.001$) and VU0467154 (10 mg/kg) attenuated amphetamine-induced deficits in PPI (Fig. 1f; one-way ANOVA, $p < 0.01$). Interestingly, the effects of the M_4 PAM on AHL (one-way ANOVA, $p <$ 0.05; Fig. 1e) and PPI (one-way ANOVA, $p < 0.01$; Fig. 1f) were attenuated by prior administration of the $mGlu_1$ NAM, VU0469650 (60 mg/kg). In both AHL and PPI, all treatment conditions had similar baseline activity counts (data not shown) and acoustic startle responses were not altered by dosing with amphetamine, VU0467154 or VU0469650 alone or in combination (data not shown). Finally, the effects of M_1/M_4 preferring agonist xanomeline on AHL were also partially attenuated by prior administration of the $mGlu_1$ NAM, VU0469650 (60 mg/kg) (one-way ANOVA, $p < 0.05$; Supplemental Fig. 1C, D). Taken together, these findings suggest that $mGlu_1$ is a critical modulator of the antipsychotic-like effects of M_4 activation.

Activation of $mGlu_1$ reduces striatal DA release via activation of CB_2 cannabinoid receptors

Previous studies suggest that a non-selective agonist of group I mGlu receptors can inhibit striatal DA release [35]. This raises the possibility that $mGlu_1$ may not only be required for M_4 PAMs to inhibit DA release, but may be capable of inducing this response in the absence M_4 agonists. We now report that application of 30 μM DHPG, a group I mGlu receptor agonist, induces a robust inhibition of DA release compared to baseline (25.415% ± 5.856 and −2.576% ± 1.106, respectively; students *t*-test, $p <$ 0.001; Fig. 2a, b) and this response was completely blocked by the $mGlu_1$ NAM VU0469650 (3 μM) but not by the $mGlu_5$ NAM MTEP (3 μM) (Fig. 2c, d; one-way ANOVA, $p < 0.01$). These results suggest that DHPG-induced reductions in striatal DA release are mediated by $mGlu_1$ activation. To further evaluate the potential role of $mGlu_1$ on DA release, we determined the effects of two selective $mGlu_1$ PAMs, Ro-07-11401 [36] and VU6004909 (rat $mGlu_1$ $EC_{50} = 31$ nM, inactive at $mGlu_{2,3,5,7,8}$; $EC_{50} >$ 10 μM at $mGlu_4$) [37], on a subthreshold concentration of DHPG (10 μM). In contrast to 30 μM DHPG, application of 10 μM DHPG alone did not produce a significant inhibition in striatal DA release (students *t*-test, $p > 0.05$; Fig. 2a, b). However, this concentration of DHPG induced a robust inhibition of DA release when co-applied with either 3 μM Ro-07-11401 (12.180 ± 3.229; students *t*-test, $p < 0.01$; Supplemental Fig. 2A, B) or 3 μM VU6004909 (22.748 ± 7.519; students *t*-test, $p < 0.01$; Fig. 2e, f). Collectively, these results suggest that activation of $mGlu_1$ inhibits stimulus-induced DA release in the striatum.

Activation of $mGlu_1$ generates diacylglycerol, which is converted to 2-AG by the Ca^{++}-dependent enzyme diacylglycerol lipase (DAGL) [38, 39], raising the possibility that $mGlu_1$-mediated reductions in DA release are similar to M_4-mediated effects in that they are mediated by eCB mobilization and activation of CB_2 receptors [11]. Consistent with this, inhibition of CB_2 receptors via the CB_2 antagonist AM630 (3 μM) significantly blocked DHPG-induced inhibition of DA release (one-way ANOVA, $p <$ 0.001, Fig. 2g; Supplemental Fig. 2C). Furthermore, the effects of DHPG were absent in slices from CB_2 knockout (KO) mice (Fig. 2h; Supplemental Fig. 2D), confirming a critical role for the CB_2 receptor in mediating this response. In contrast to the CB_2 receptor antagonist, bath application of 3 μM AM251, a CB_1 antagonist, did not significantly attenuate 30 μM DHPG-induced reductions in striatal DA release (one-way ANOVA, $p > 0.05$, Fig. 2g; Supplemental Fig. 2C).

$mGlu_1$ activation following stimulation of thalamostriatal afferents attenuates striatal DA release

The striatum receives extensive glutamatergic innervation from the cortex [40] and several thalamic nuclei [41]. Both afferent inputs have been reported to modulate striatal DA release [35, 42, 43], however, it is critical to determine whether activation of $mGlu_1$ by these glutamatergic inputs inhibits DA release, and whether this response is specific to either cortico-striatal or thalamo-striatal synapses. To test the hypothesis that endogenous glutamate acts on $mGlu_1$ to inhibit DA release, we selectively activated corticostriatal or

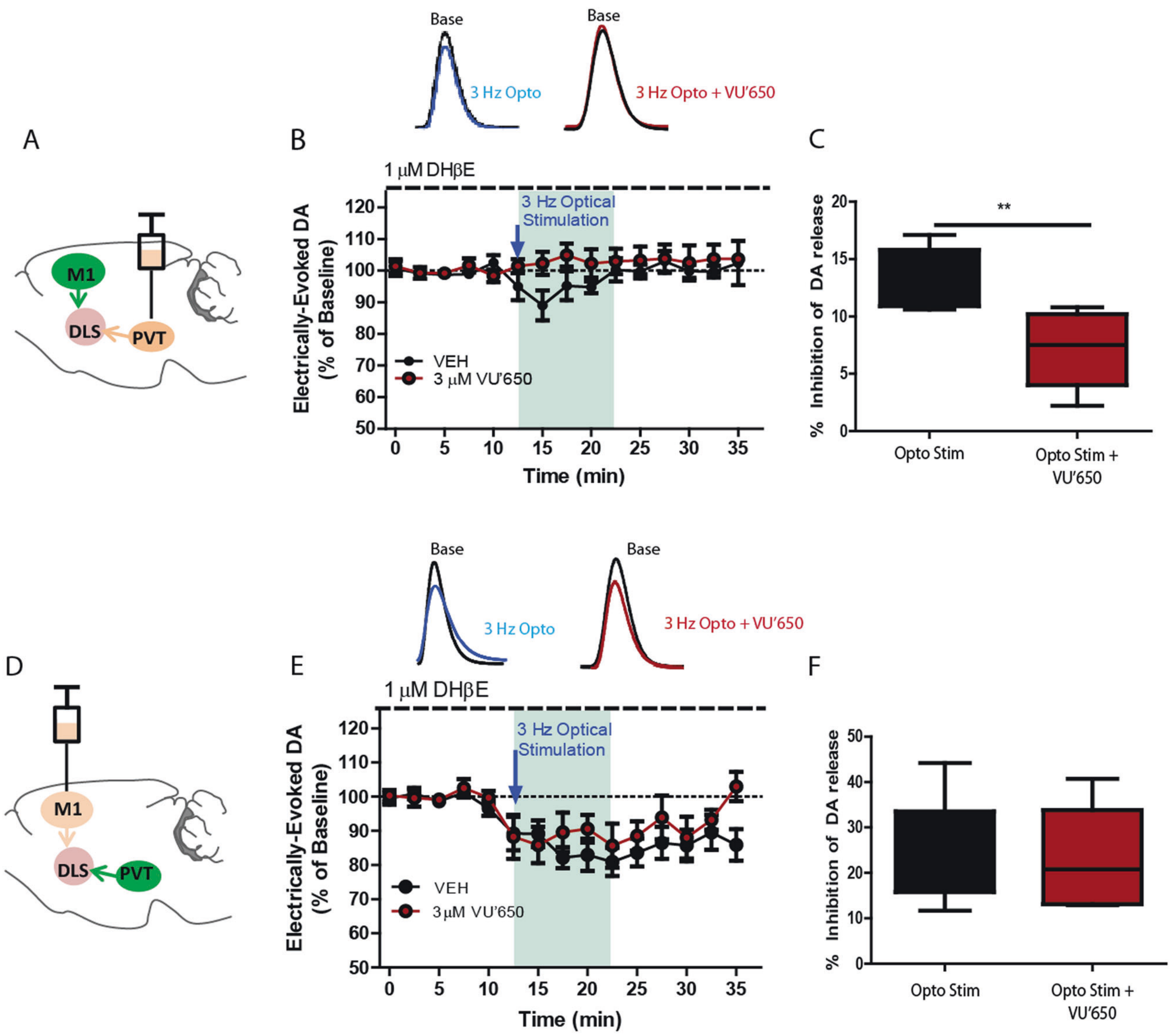


Fig. 3 Activation of mGlu$_1$ following Stimulation of Thalamostriatal Afferents modulates Striatal DA Release. **a** Schematic of viral injection strategy to target glutamatergic afferents from the paraventricular nucleus (PVT) of the thalamus to the dorsal lateral striatum (DLS). **b** Time course depicting the effect of 3 Hz optical stimulation of thalamic inputs (black) on electrically-evoked striatal DA release, an effect that is blocked following application of the mGlu$_1$ NAM VU0469650 (VU'650; red). **c** Boxplot summary depicting the percent inhibition of DA release at the peak time point following stimulation of thalamostriatal afferents ($n = 12$–16, $^{**}p < 0.01$, significant difference from peak inhibition of optical stimulation; students t-test). **d** Schematic of viral injection strategy to target glutamatergic afferents from the motor cortex (M1) to the dorsal lateral striatum (DLS). **e** Time course depicting the effect of 3 Hz optical stimulation of cortical inputs to the striatum (black) on electrically-evoked striatal DA release, an effect that is not blocked following application of the mGlu$_1$ NAM VU0469650 (VU'650; red). **f** Boxplot summary depicting the percent inhibition of DA release at the peak time point following stimulation corticostriatal afferents ($n = 12$–16; not significantly different, students t-test $p > 0.05$)

thalamostriatal axons in the striatum by virally expressing ChR2-eYFP in the motor cortex (M1) or the paraventricular nucleus of the thalamus (PVT; Fig. 3a, d). In coronal sections containing ChR2-eYFP expression, we recorded electrically evoked DA in the presence of 1 μM DHβE prior to, during, and following optical stimulation (3 Hz, 12.5 min). Stimulation of thalamostriatal afferents produced a 15% inhibition in striatal DA release (Fig. 3b, c), which returned to baseline after terminating optical stimulation. The mGlu$_1$ NAM VU0469650 (3 μM) attenuated thalamostriatal induced reductions in DA release (students t-test; $p < 0.01$; Fig. 3b, c), whereas antagonists of ionotropic glutamate (iGluRs) and mGlu$_5$ receptors did not inhibit this response (Supplemental Fig. 3A, B). Interestingly, selective activation of corticostriatal afferents produced a 27% sustained inhibition in striatal DA release, but this response was not inhibited by the mGlu$_1$ NAM VU0469650 (Fig. 3e, f). The reduction in striatal DA release observed following corticostriatal stimulation was eliminated by incubation with a cocktail of antagonists for other glutamate receptors (Supplemental Fig. 3C, D). Taken together, these findings suggest that $mGlu_1$ inhibits striatal DA release in an input-specific manner and regulates DA release by glutamate from thalamostriatal but not corticostriatal afferents.

The mGlu1 PAM, VU6004909, reduces dorsolateral striatal DA release in vivo and displays antipsychotic efficacy

The finding that $mGlu_1$ activation inhibits striatal DA release raises the possibility that selective $mGlu_1$ PAMs could reduce DA release in vivo and have antipsychotic-like effects. To determine whether a selective $mGlu_1$ PAM [37] reduces striatal DA release, we examined the effects of systemic administration of VU6004909 using FSCV in isoflurane anesthetized mice. DA release was monitored in the dorsolateral striatum (DLS) following electrical stimulation of the medial forebrain bundle (MFB) in animals treated with either vehicle (10% Tween 80) or 60 mg/kg VU6004909 (Fig. 4a–c). Compared to vehicle, VU6004909 significantly reduced striatal DA release 40 min after administration (Fig. 4b), which corresponds to the T_{max} for this compound [37]. The averaged peak effect, observed 60 minutes after administration, was statistically significant from vehicle-control conditions (students *t*-test, $p < 0.01$; Fig. 4c). These results confirm our ex vivo voltammetry studies and indicate that administration of the $mGlu_1$ PAM can reduce striatal DA release in vivo.

Next, we assessed the ability of VU6004909 to exert antipsychotic-like activity in rodent models that are dependent on increased DA transmission and are known to be responsive to antipsychotic agents and M_4 PAMs. Consistent with previous studies [11, 44], amphetamine (4 mg/kg, s.c.) induced a robust decrease in PPI (one-way ANOVA, $p < 0.001$), an effect that was dose-dependently reversed by pretreatment with 30 mg/kg ($p < 0.05$) or 60 mg/kg VU6004909 (one-way ANOVA, $p < 0.01$; Fig. 4d). The highest dose of VU6004909 (60 mg/kg, i.p.) alone had no effect on PPI compared to vehicle (1409.42 ± 102.21 and 1164.19 ± 81.16, respectively; Supplemental Fig. 4A).

Amphetamine (4 mg/kg, s.c.) also produced a significant increase in locomotor activity, and this effect was attenuated by administration of 60 mg/kg VU6004909 (students *t*-test, $p < 0.01$; Fig. 4e; Supplemental Fig. 4B). Acute treatment with VU6004909 (60 mg/kg) alone induced a slight, but statistically significant reduction in total spontaneous locomotor activity compared to vehicle-treated animals (students *t*-test, $p < 0.05$; Supplemental Fig. 5A, B) but was without effect on cerebellum-dependent motor tasks, such as the rotarod and TreadScan (Supplemental Fig. 5C, D–F, respectively). Interestingly, the ability of VU6004909 to attenuate amphetamine disruptions in PPI (one-way ANOVA, $p < 0.05$; Supplemental Fig. 6C) and AHL (one-way ANOVA, $p < 0.05$; Supplemental Fig. 6A, B) were absent following systemic administration of a CB_2 antagonist (AM630). Administration of AM630 alone did not affect basal levels of locomotion or disrupt the startle response (Supplemental Fig. 6A, D, respectively). Together, these findings demonstrate that VU6004909 exerts antipsychotic-like effects, and these effects are dependent on CB_2 activation.

$mGlu_1$ does not directly inhibit signaling at the DA D_1 receptor and does not impair motivation

In addition to inhibiting DA release, we recently reported that M_4 PAMs directly inhibit D_1-mediated increases in GABA-mediated synaptic responses in terminals of D_1-SPNs in the substantia nigra pars reticulata (SNr), through $G\alpha_{i/o}$ –dependent inhibition of cAMP formation [16]. Based on these findings, M_4 could induce an excessive inhibition of D_1 relative to D_2 signaling. D_1 plays a criticla role in regulating motor function, cognition, and motivation [16, 45–48], therefore a mechanism that maintains balance of D_1/D_2 signaling may be preferable for the treatment of schizophrenia [49–51]. Since $mGlu_1$ primarily couples to $G\alpha_{q/11}$ rather than $G\alpha_{i/o}$, we would not expect $mGlu_1$ PAMs to directly inhibit D_1 signaling in SNr SPN terminals. To test this hypothesis, we performed whole-cell patch clamp recordings from GABAergic cells of the SNr and assessed the effects of an $mGlu_1$ PAM on miniature inhibitory post synaptic currents (mIPSCs). As previously reported [16], the D_1 agonist SKF82958 (10 µM) induced a leftward shift in mIPSC cumulative probability plots with a ~40% increase in mIPSC frequency compared to baseline (Supplemental Fig. 7A–C). In contrast to effects of M_4 PAMs [16], pretreatment with the $mGlu_1$ PAM VU6004909 (3 or 10 µM) did not attenuate the effect of SKF82958 on mIPSCs (Supplemental Fig. 7A–C), suggesting that $mGlu_1$ activation does not inhibit effects of D_1 agonists on mIPSC frequency. Furthermore, in contrast to M_4 PAMs [16], VU6004909 did not attenuate SKF82958 (1 mg/kg, i.p.) induced increases in locomotor activity (Supplemental Fig. 7D, E) (students *t*-test, $p > 0.05$). Taken together, these results suggest that $mGlu_1$ PAMs do not directly inhibit effects of direct-acting D_1-receptor agonists.

Since reductions in DA D_1 signaling have been implicated in reduced motivation [48, 52, 53], we determined the effects of the $mGlu_1$ PAM (VU6004909), M_4 PAM (VU0467154), and the typical antipsychotic haloperidol on motivational responding in a traditional progressive ratio (PR) schedule [31], where rodents need to nose poke for a 30% Strawberry Ensure solution (Fig. 5a). We assessed

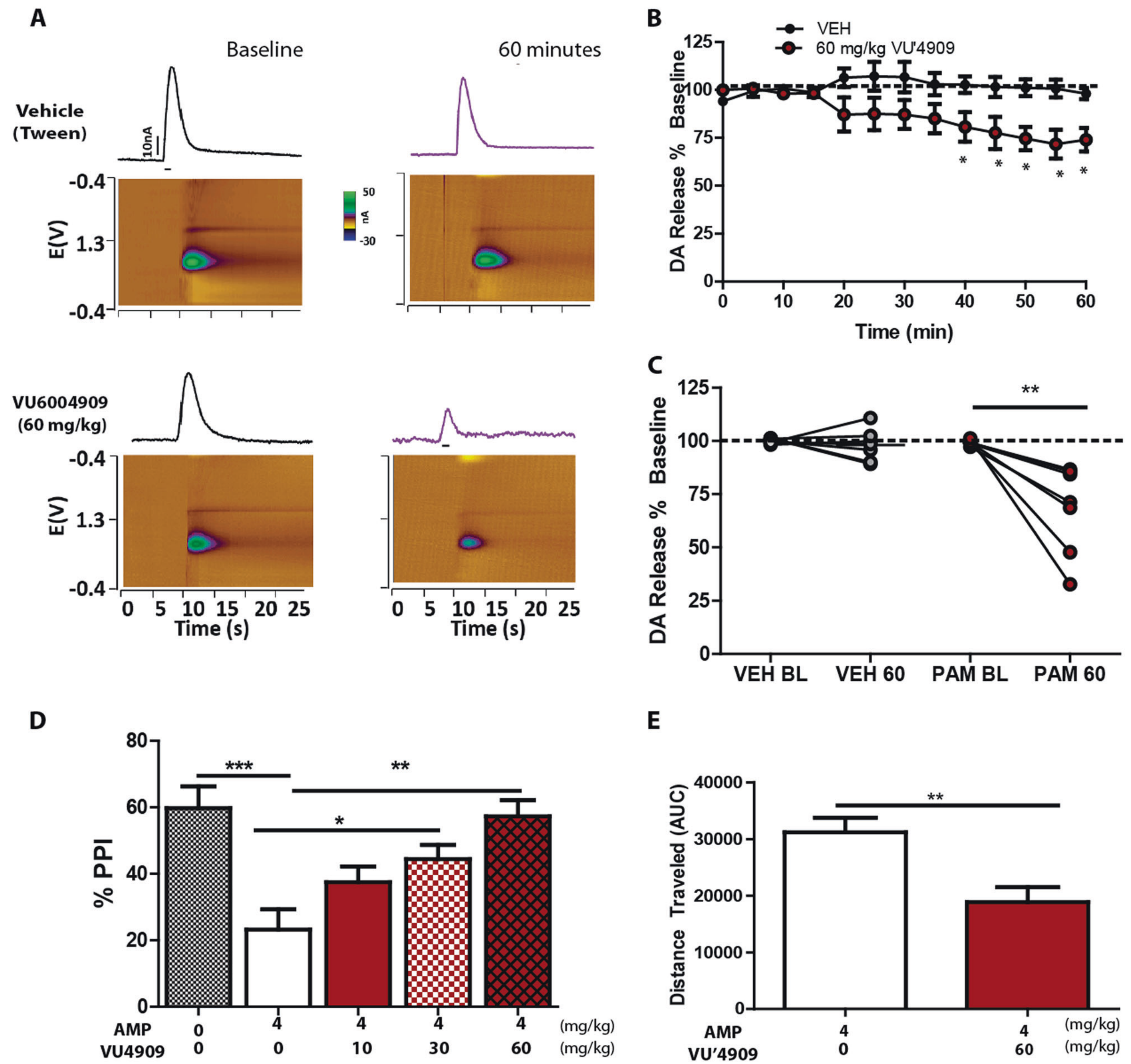


Fig. 4 The Novel $mGlu_1$ PAM VU6004909 Reduces Striatal DA Release in vivo and Displays Antipsychotic Efficacy. **a** Color plots from a representative vehicle (10% Tween 80) and 60 mg/kg VU6004909 (VU'4909) treated animal; potential is on the y-axis plotted against time on the *x*-axis and the current response is represented in false color. **b** Time course of striatal DA release following i.p. administration of VEH or PAM ($n = 6$–8; $^*p < 0.05$, significant difference between VEH and PAM at the time sampled; two-way factorial ANOVA with posthoc sample *t*-test). All time-course data are depicted as mean ± SEM. **c** Averaged DA inhibition at baseline (BL) and 60 minutes post administration ($n = 6$–8; $^{**}p < 0.01$, significant difference between PAM BL and 60 minutes after administration; students *t*-test). **d** Averaged data showing percent prepulse inhibition (PPI) observed in wildtype (WT) mice following administration of vehicle (VEH; 10% Tween 80), 4 mg/kg amphetamine (AMP; s.c.), and various doses of VU6004909 (VU'4909; 10, 30, 60 mg/kg; i.p.) plus AMP ($n = 15$–20; $^*p < 0.05$, significant difference from AMP/VEH condition; $^{**}p < 0.01$, significant difference from AMP/VEH condition; $^{***}p < 0.001$, significant difference from AMP/VEH condition one-way ANOVA with Dunnett's multiple comparison posthoc test). **e** Total locomotor activity of area under the curve (AUC) in wildtype (WT) mice following administration of 4 mg/kg amphetamine (AMP) and 60 mg/kg VU6004909 (VU'4909; $n = 14$; $^{**}p < 0.01$, significant difference from AMP/VEH condition; students *t*-test)

motivation as total number of pokes and highest ratio achieved. Consistent with prior reports [54], administration of haloperidol (0.2 mg/kg, i.p.), a dose that possesses antipsychotic efficacy following amphetamine challenges (data not shown) significantly decreased total pokes and highest ratio achieved (repeated measures ANOVA, $p < 0.001$; Fig. 5b, c). While a low dose of the M_4 PAM VU0467154 (0.3 mg/kg) did not have effects on responding in this model, a higher dose (3.0 mg/kg), significantly reduced total pokes and highest ratio achieved compared to vehicle

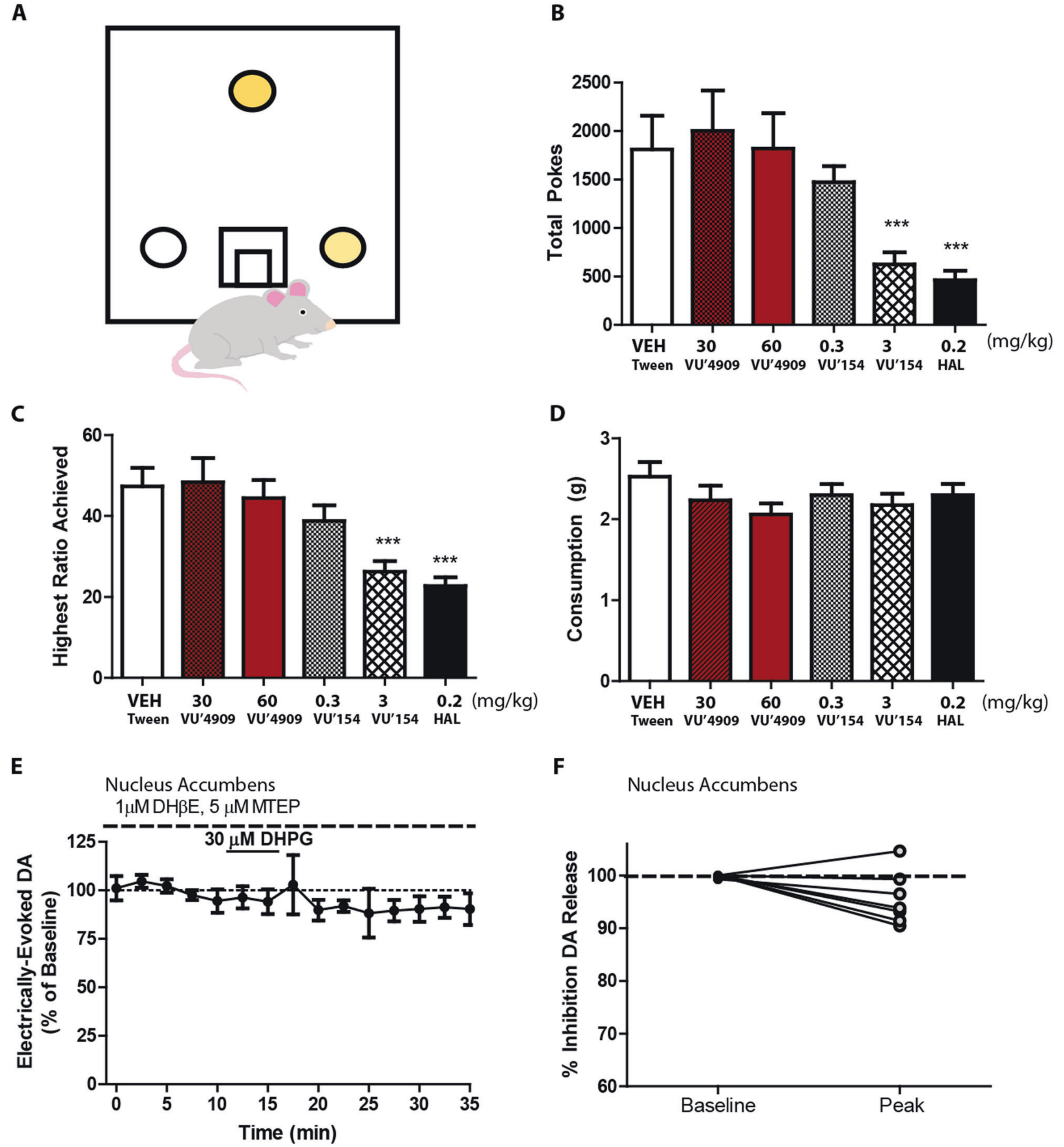


Fig. 5 Effects of $mGlu_1$ Activation on Progressive Ratio Performance and Ventral Striatal DA Release. **a** Schematic of the progressive ratio (PR) assay, where mice are required to nose poke for 30% Ensure reward. Mean (±SEM) number of total nose pokes (**b**) and highest ratio achieved (**c**). **d** Total amount of ensure consumed in a free feeding session (in grams; $^{**}p < 0.001$, significant difference from VEH conditions; repeated measures ANOVA with Dunnett's multiple comparison posthoc test; $n = 12$–24). **e** Time course of 30 μM DHPG in the presence of 5 μM MTEP, $mGlu_5$ NAM, and 1 μM DhβE on ventral striatal DA release. **f** Averaged ventral striatal DA release at baseline (BL) and following application of MTEP and DhβE to isolate $mGlu_1$ specific effects ($n = 8$; students t-test $p > 0.05$)

(repeated measures ANOVA, $p < 0.001$). These reductions in motivation are not due to an appetite suppressant effect (repeated measures ANOVA, $p > 0.05$; Fig. 5d) or reduction in spontaneous locomotion (students t-test, $p > 0.05$; data not shown), but rather the work requirements of the task. Interestingly 30 and 60 mg/kg VU6004909, doses that attenuate deficits in AHL and PPI, did not affect motivation (Fig. 5b, c).

Reductions in operant responding have been correlated with reduced levels of DA in the ventral striatum [55] and with the manifestation of negative symptoms, including motivational deficits [53, 56]; therefore, we assessed $mGlu_1$ activation on ventral striatum DA release. To test this, we performed ex vivo FSCV and isolated $mGlu_1$ via bath application of the nACh antagonist DhβE (1 μM) and the $mGlu_5$ NAM MTEP (5 μM), and recorded changes in DA release in the nucleus accumbens (NAc) following application of 30 μM DHPG. Interestingly, in contrast to its effects in DLS, activation of $mGlu_1$ does not produce a significant change in NAc DA release compared to baseline (Fig. 5e, f; paired students *t*- test, $p > 0.05$). Furthermore, since some negative symptoms and cognitive disturbances in schizophrenia patients are associated with aberrant frontal lobe function [57, 58] and reduced DA levels in the prefrontal cortex (PFC) [59], we examined DA levels following $mGlu_1$ activation in the PFC through both ex vivo FSCV (Supplemental Fig. 8A) and microdialysis. Our FSCV data demonstrates that activation of $mGlu_1$ produced a slight rise, although not statistically significant from baseline, in PFC DA release (Supplemental Fig. 8B; students *t*-test, $p > 0.05$). Moreover, data collected from microdialysis, with probes implanted in the infralimbic or prelimibic PFC (Supplemental Fig. 8C), revealed that similar to ex vivo DA recordings, systemic administration of the $mGlu_1$ PAM VU6004909 did not produce a significant change compared to vehicle-control nor a significant sample × treatment interaction (two-way ANOVA, $p > 0.05$) in PFC DA levels (Supplemental Fig. 8D). However, there was a significant effect of time (two-way ANOVA, $p < 0.05$), suggesting that DA in both treatment groups increased relative to baseline levels. Taken together, these findings suggest that by it's self, activation of $mGlu_1$ does not affect extracellular DA in the PFC.

Discussion

Several clinical and preclinical studies suggest that dysfunction at glutamatergic synapses may play a critical role in the pathophysiological changes that underlie schizophrenia [60–63]. However, specific changes in glutamate signaling that contribute to symptoms in different subpopulations of schizophrenia patients are not well understood. Interestingly, recent genetic studies identified multiple nonsynonymous SNPs in the human gene encoding $mGlu_1$ (*GRM1*) that are associated with schizophrenia [26, 64]. Furthermore, we reported that these mutations lead to deficits in $mGlu_1$ signaling [25, 26], raising the possibility that disrupted signaling of $mGlu_1$ could contribute to the symptoms of schizophrenia in some patients. However, the roles of $mGlu_1$ in brain circuits that are disrupted in schizophrenia patients are not understood.

Here, we show that activation of $mGlu_1$ through application of exogenous agonists or selective stimulation of thalamostriatal afferents induces a robust inhibition of DA release in the dorsolateral striatum and that selective $mGlu_1$ PAMs exert antipsychotic-like effects in rodent models. These findings are consistent with previous studies showing that mGlu receptor agonists reduce striatal DA release [34], $mGlu_1$ KO mice have altered locomotor responses to amphetamine [65] and with anatomical studies suggesting that $mGlu_1$ is preferentially expressed at thalamostriatal synapses [66]. Our data are especially interesting in light of a large body of studies suggesting that striatal dopaminergic hyperactivity is associated with psychotic symptoms in schizophrenic patients [15, 67, 68] and that excessive striatal DA predicts treatment response to current antipsychotics [69]. Furthermore, we have previously shown that $mGlu_1$ PAMs reverse deficits in $mGlu_1$ signaling observed with schizophrenia-associated mutations [25]. Taken together, these data raise the possibility that selective $mGlu_1$ PAMs may provide a novel approach to treatment of positive symptoms both in a broad schizophrenia patient population, as well as in schizophrenia patients with *GRM1* mutations.

Interestingly, our studies also suggest that $mGlu_1$ activation is required for M_4 PAM-induced inhibition of DA release and antipsychotic-like effects. M_4 PAMs have received increasing attention as a novel approach to treatment of schizophrenia, and clinical studies suggest that mAChR agonists have efficacy in schizophrenia patients [3]. Thus, the present findings provide a mechanistic link between $mGlu_1$ PAMs, and two clinically validated targets (muscarinic agonists and DA receptor antagonists). However, in contrast to available antipsychotic agents, the present results and previous studies [11] suggest that $mGlu_1$ and M_4 PAMs reduce DA signaling through local release of an eCB from striatal SPNs and activation of CB_2 receptors on neighboring DA terminals. Interestingly, tonic eCB signaling does not appear to play a key role in DA release; however, when eCBs are mobilized by an $mGlu_1$ PAM a CB_2 antagonist can block these effects. These local effects are interesting in the light of recent clinical imaging studies suggesting that the symptoms in schizophrenia patients are associated with selective increases in striatal DA signaling while extrastriatal regions display hypo-dopaminergic function [70, 71]. Thus, $mGlu_1$ and M_4 PAMs may provide a mechanism for selective inhibition of DA release in striatal regions that are important for antipsychotic efficacy, without further disruptions in extrastriatal DA signaling.

While these studies suggest that the effects of M_4 PAMs on DA release require activation of $mGlu_1$, we have also found that these targets have important differences. Most notably, M_4 PAMs also directly inhibit D_1 signaling in D_1-SPN terminals in the SNr and this effect likely regulates M_4-dependent actions on locomotor activity [16]. In contrast, we found that $mGlu_1$ activation does not inhibit D_1/cAMP-mediated increases in transmission at GABAergic synapses of striatal D_1-SPNs and that $mGlu_1$ PAMs do not suppress D_1 agonist-induced increases in locomotor activity. While the relative importance of these different actions of M_4 PAMs is not entirely clear, the finding that $mGlu_1$ PAMs do not inhibit D_1 signaling through actions that are independent of DA release could provide therapeutic benefits over traditional antipsychotics and M_4 PAMs. Most notably, current antipsychotics [56, 72], as well as D_1 antagonists [48] reduce motivation, as well as ventral striatal and PFC DA release. Consistent with this, we found that therapeutically relevant doses of a typical antipsychotic, haloperidol, or an M_4 PAM significantly reduce motivational responding in a progressive ratio operant paradigm. Interestingly, low doses of M_4 PAMs do not impair motivated behavior but do possess antipsychotic-efficacy, suggesting that it is possible to provide efficacy for the positive symptom domain without inducing or worsening negative symptoms. In contrast, efficacious doses of an $mGlu_1$ PAM did not impair motivated performance or alter DA release in the ventral striatum. It is possible that differential effects of these agents on D_1 signaling and distinct actions in other brain regions, such as the PFC or ventral striatum, contribute to their different effects on motivational responding. The mesolimbic DA system and interconnected forebrain regions are a critical component of brain circuitry that regulate behavioral activation and motivated behavior [73, 74] and it will be important in the future to fully evaluate the effects of $mGlu_1$ and M_4 PAMs as compared to currently available antipsychotics in regions that may be important for motivated behavior.

In addition to modulating motivated behavior, DA release in the striatum and PFC are heavily implicated in the manifestation of negative symptoms in schizophrenia. Accordingly, future studies are needed to explore the efficacy of $mGlu_1$ PAMs on the negative symptoms, such as preclinical models of NMDA hypofunction, which are thought to recapitulate all symptom clusters. Previous studies have shown that M_4 PAMs display robust efficacy following challenge with an NMDA antagonist, such as MK801 [9]. Therefore, it will be of interest to compare the effects of $mGlu_1$ PAMs in these preclinical models to those observed following M_4 administration.

In conclusion, we present a series of studies that build upon extensive preclinical mechanistic and drug discovery efforts, as well as human genetics, imaging, and clinical intervention studies, that raise the possibility, that $mGlu_1$ PAMs may provide a novel approach for the treatment of the positive symptoms of schizophrenia. Future studies are needed to evaluate the therapeutic potential of $mGlu_1$ PAMs on negative and cognitive symptom domains. It will be important to develop a more complete understanding of the actions of $mGlu_1$ PAMs in disease relevant models as well as the impact of specific *GRM1* mutations on identified circuits that have been implicated in schizophrenia.

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Author contributions: SEY and PJC conceived the studies and wrote the manuscript. SEY, DJF, DPC, MSM, CKJ, JFC, and MB designed experiments. SEY, DPC, MSM, JG, ALB, HPC, and MB conducted experiments and analyzed the data. CWL and PMBG provided pharmacological tools utilized in this study.

Competing interests CWL and PJC are inventors on patents that protect different classes of metabotropic glutamate allosteric modulators. CWL has been funded by the NIH, Johnson and Johnson, Bristol-Myers Squibb, AstraZeneca, Michael J. Fox Foundation, as well as Seaside Therapeutics. He has consulted for AbbVie and received compensation. PJC has been funded by NIH, AstraZeneca, Bristol-Myers Squibb, Michael J. Fox Foundation, Dystonia Medical Research Foundation, CHDI Foundation, and Thome Memorial Foundation. Over the past three years he has served on the Scientific Advisory Boards for Michael J. Fox Foundation, Stanley Center for Psychiatric Research Broad Institute, Karuna Pharmaceuticals, Lieber Institute for Brain Development, Clinical Mechanism and Proof of Concept Consortium, and Neurobiology Foundation for Schizophrenia and Bipolar Disorder. SEY, DJF, DPC, MSM, JG, PMGB, HPC, MB, ALB, MEJ, JFC, and CKJ declare no potential conflicts of interest.

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