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# mGlu5 function in the nucleus accumbens core during the incubation of methamphetamine craving

**Conor H. Murray**[a,b,1], **Daniel T. Christian**[a,2], **Mike Milovanovic**[a], **Jessica A. Loweth**[a,3], **Eun-Kyung Hwang**[b], **Aaron J. Caccamise**[a,4], **Jonathan R. Funke**[b], **Marina E. Wolf**[a,b,*]

[a]Department of Neuroscience, The Chicago Medical School at Rosalind Franklin University of Medicine and Science, 3333 Green Bay Road, North Chicago, IL 60064, USA (where the majority of this work was performed)

[b]Department of Behavioral Neuroscience, Oregon Health & Science University, 3181 SW Sam Jackson Park Rd, Portland, OR 97239, USA (where some of this work was performed)

## Abstract

Many studies have demonstrated that negative allosteric modulators (NAM) of metabotropic glutamate receptor 5 (mGlu5) reduce cocaine and methamphetamine seeking in extinction-reinstatement animal models of addiction. Less is known about effects of mGlu5 NAMs in abstinence models, particularly for methamphetamine. We used the incubation of drug craving model, in which cue-induced craving progressively intensifies after withdrawal from drug self-administration, to conduct the first studies of the following aspects of mGlu5 function in the rat nucleus accumbens (NAc) core during abstinence from methamphetamine self-administration: 1) functionality of the major form of synaptic depression in NAc medium spiny neurons, which is induced postsynaptically via mGlu5 and expressed presynaptically via cannabinoid type 1 receptors (CB1Rs), 2) mGlu5 surface expression and physical associations between mGlu5, Homer proteins, and diacylglycerol lipase-α, and 3) the effect of systemic and intra-NAc core administration of the mGlu5 NAM 3-((2-methyl-4-)ethynyl)pyridine (MTEP) on expression of

[*]**Correspondence to:** Marina E. Wolf, Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR, USA. Tel: 503-494-9797; Fax: (503) 494-6877; wolfmar@ohsu.edu.

[1]Present address: Dr. Conor H. Murray, Department of Psychiatry and Behavioral Neuroscience, University of Chicago, 5801 S Ellis Ave, Chicago, IL 60637, USA.

[2]Present address: Dr. Daniel T. Christian, Department of Physiology and Pharmacology, Des Moines University, 3200 Grand Ave, Des Moines, IA 50312, USA.

[3]Present address: Dr. Jessica A. Loweth, Department of Cell Biology and Neuroscience, Rowan University School of Osteopathic Medicine, 42 E Laurel Rd, Stratford, NJ 08084, USA.

[4]Present address: Aaron J. Caccamise, Department of Biomedical Sciences, Marquette University, 1250 W Wisconsin Ave, Milwaukee, WI 53233, USA.

CRediT author contribution statement

**Conor H. Murray**: Conceptualization, Investigation, Writing - original draft preparation. **Daniel T. Christian**: Investigation, Writing - review & editing. **Mike Milovanovic**: Investigation. **Jessica A. Loweth**: Investigation. **Eun-Kyung Hwang**: Investigation. **Aaron Caccamise**: Investigation. **Jonathan R. Funke**: Investigation. **Marina E. Wolf**: Conceptualization, Writing - review and editing, Project administration, Funding acquisition.

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incubated methamphetamine craving. We found that mGlu5/CB1R-dependent synaptic depression was lost during the rising phase of methamphetamine incubation but then recovered, in contrast to its persistent impairment during the plateau phase of incubation of cocaine craving. Furthermore, whereas the cocaine-induced impairment was accompanied by reduced mGlu5 levels and mGlu5-Homer associations, this was not the case for methamphetamine. Systemic MTEP reduced incubated methamphetamine seeking, but also reduced inactive hole nose-pokes and locomotion, while intra-NAc core MTEP had no significant effects. These findings provide the first insight into the role of mGlu5 in the incubation of methamphetamine craving and reveal differences from incubation of cocaine craving.

**Keywords**

incubation; methamphetamine; mGlu5; nucleus accumbens; rat; synaptic depression

## 1. Introduction

Methamphetamine is the most widely abused illicit drug in the world after cannabis (Brecht and Herbeck, 2014). The majority of individuals seeking treatment for methamphetamine use disorder relapse within the first year of abstinence (Brecht and Herbeck, 2014). There are no FDA-approved medications to help users of methamphetamine or other psychostimulants maintain abstinence. It is well-established that relapse vulnerability is linked to alterations in brain circuits that respond to drug-associated cues. This vulnerability can be studied using the 'incubation of craving' model wherein drug seeking in response to drug-associated cues progressively increases over the course of withdrawal from drug self-administration (Pickens et al., 2011). In rats, incubation of methamphetamine craving occurs during forced abstinence (Shepard et al., 2004; Scheyer et al., 2016; Adhikary et al., 2017) or voluntary abstinence, when rats are provided with mutually exclusive food rewards (Caprioli et al., 2015, 2017; Venniro et al., 2017) or faced with punishment following drug taking (Krasnova et al., 2014). Notably, incubation of methamphetamine craving also occurs in humans, with increases in cue-induced drug craving observed during the first 3 months of abstinence (Wang et al., 2013a).

Brain regions important for incubation of methamphetamine craving include the central nucleus of the amygdala (Li et al., 2015b; Venniro et al., 2017; Cates et al., 2018), the dorsal striatum (Li et al., 2015a, 2018a, 2018c; Caprioli et al., 2017), and the nucleus accumbens core (Scheyer et al., 2016; Rossi et al., 2020) (for review, see Altshuler et al., 2020). There is overlap with regions required for cocaine incubation (central nucleus: Lu et al., 2007, 2005; accumbens core: Conrad et al., 2008; Guillem et al., 2014) but also differences. For example, cocaine but not methamphetamine incubation appears to require the prefrontal cortex (Koya et al., 2009; Ma et al., 2014; Li et al., 2015b; Shin et al., 2017; see also Nicolas et al., 2016; Luis et al., 2017) and the nucleus accumbens shell (Lee et al., 2013; Ma et al., 2014; Rossi et al., 2020). One mechanism common to cocaine and methamphetamine incubation is strengthening of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) transmission in the nucleus accumbens (NAc) core. In drug-naïve or saline-treated animals, GluA2-containing $Ca^{2+}$-impermeable AMPARs are

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primarily responsible for excitatory transmission onto medium spiny neurons (MSNs), the major cell type and output neurons of the NAc (Kourrich et al., 2007; Conrad et al., 2008). However, after withdrawal from extended-access cocaine or methamphetamine self-administration, there is an accumulation of high conductance $Ca^{2+}$-permeable AMPA receptors (CP-AMPARs) in NAc core synapses; once this occurs, CP-AMPAR activation is required for the expression of cocaine (Conrad et al., 2008; Loweth et al., 2014) and methamphetamine (Scheyer et al., 2016) incubation. In summary, overlapping but distinct brain regions contribute to cocaine and methamphetamine incubation, and at least one underlying form of plasticity in the NAc – accumulation of CP-AMPARs – is held in common.

For cocaine, the incubation of craving is also associated with group I metabotropic glutamate receptor (mGluR) plasticity. In drug-naïve animals, the major form of long-term depression (LTD) observed in NAc medium spiny neurons (MSN) involves metabotropic glutamate receptor 5 (mGlu5) and cannabinoid receptor type 1 (CB1R) (Robbe et al., 2002; Zlebnik and Cheer, 2016; Araque et al., 2017; Augustin and Lovinger, 2018). Specifically, postsynaptic mGlu5 activation results in the generation of the endocannabinoid 2-arachidonoylglycerol (2-AG), which travels in a retrograde fashion to activate presynaptic CB1Rs thereby reducing glutamate release. The multiprotein mGlu5 complex that mediates 2-AG release is referred to as the 2-AG signalosome (Piomelli, 2014) and requires mGlu5 coupling through Homer scaffolding proteins to diacylglycerol lipase-α (DGL), the enzyme that produces 2-AG. Uncoupling of the signalosome is associated with abolished mGlu5/CB1R-dependent synaptic depression in the NAc (Jung et al., 2012). This synaptic depression is also lost in the NAc after various cocaine regimens (see Discussion) including incubation of cocaine craving (McCutcheon et al., 2011; Scheyer et al., 2018). However, mGlu1 activation, which does not affect excitatory synaptic transmission in drug-naïve MSN, elicits a postsynaptically-expressed LTD after incubation that is mediated by the removal of CP-AMPARs and results in decreased cocaine craving (McCutcheon et al., 2011; Lee et al., 2013; Loweth et al., 2014; Ma et al., 2014; Scheyer et al., 2018). This mGlu1-induced LTD has also been demonstrated in the NAc core after incubation of methamphetamine craving (Scheyer et al., 2016). In summary, cocaine and methamphetamine incubation have in common the emergence of mGlu1-LTD; this is accompanied by loss of mGlu5/CB1R-dependent synaptic depression after cocaine incubation, but whether the latter is impaired after methamphetamine incubation is unknown. Therefore, the first goal of this study was to evaluate mGlu5/CB1R-dependent synaptic depression and components of the 2AG signalosome after withdrawal from a methamphetamine self-administration regimen that produces incubation of craving.

Given that AMPAR transmission in the NAc is required for expression of incubated cocaine and methamphetamine craving, reducing excitatory transmission through mGlu5/CB1R-dependent LTD might be expected to decrease drug seeking. Contrary to this expectation, some evidence indicates that this LTD mediates reward (Novak et al., 2010; Bilbao et al., 2020) (see Discussion for more detail). Furthermore, many studies have found that reducing mGlu5 transmission through systemic or intra-NAc administration of negative allosteric modulators (NAM) such as 3-((2-methyl-4-thiazolyl)ethynyl)pyridine (MTEP) reduces reinstatement of cocaine seeking in extinction-reinstatement models (for

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reviews, see Caprioli et al., 2018; Mihov and Hasler, 2016; Olive, 2009) and context-induced cocaine seeking in abstinence models (Keck et al., 2014, 2013; Knackstedt et al., 2014; Knackstedt and Schwendt, 2016). Interestingly, mGlu5 NAMs reduce reinstatement of cocaine seeking under conditions in which mGlu5/CB1R-dependent LTD is impaired in the NAc, e.g., cocaine self-administration followed by extinction training (Knackstedt et al., 2010, 2014; Wang et al., 2013b). In summary, intra-NAc administration of mGlu5 NAMs reduces cocaine seeking in multiple addiction models, and results in the reinstatement model suggest that this effect is not attributable to prevention of mGlu5/CB1R-dependent LTD.

Far less is known about methamphetamine. Two studies reported systemic mGlu5 NAM-induced reductions in drug- and cue-primed reinstatement of methamphetamine seeking (Gass et al., 2009; Watterson et al., 2013), but there have been no studies in abstinence models. To fill this gap and provide further insight into the relationship between mGlu5/CB1R-dependent synaptic depression and incubation of methamphetamine craving, the second goal of this study was to determine if MTEP reduces cue-induced methamphetamine seeking before and after incubation. MTEP was selected because of vast preclinical findings with this compound (Mihov and Hasler, 2016), which have been replicated with newer compounds developed to avoid its off-target effects (Caprioli et al., 2018).

## 2. Methods

### 2.1. Subjects and surgery

All procedures were approved by the Rosalind Franklin University of Medicine and Science and the Oregon Health & Science University Institutional Animal Care and Use Committees in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. Male Sprague-Dawley rats (Envigo, Indianapolis, IN or South Kent, WA) weighing 275-300 g were housed 3/cage under a reverse 12-hour light/dark cycle with food and water available ad libitum. One week after arrival, rats were implanted with a jugular catheter under ketamine/xylazine anesthesia and thereafter were single-housed. Catheters were flushed daily with Cefazolin (100 mg/ml in sterile 0.9% saline; 0.15 ml; Webster Veterinary Supply, Devens, MA) for the week prior to self-administration to prevent infection and maintain catheter patency. A total of 152 rats were used in these studies.

### 2.2. Drug self-administration

Methamphetamine was obtained from the NIDA Controlled Substances Program (RTI, Research Triangle, NC), and dissolved in 0.9% saline. Rats self-administered methamphetamine (0.1 mg/kg; 0.065 ml/infusion) or saline (0.065 ml/infusion) during 10 sessions (6 hours/day) conducted over 11 or 12 days with 1 or 2 days off in a chamber equipped with two nose-poke holes. Active hole nose-pokes resulted in intravenous delivery of the drug paired with a 20-second light cue (white light illuminating the active hole) on a fixed-ratio 1 schedule. Each infusion was followed by a 20-second time-out period. Nose poking in the inactive hole had no consequences. After 10 days of training, rats underwent 1-48 days of withdrawal in home cages and were handled weekly. This regimen produces incubation of cue-induced craving that is maximal after ~1 week of withdrawal and persists at least through withdrawal day (WD) 45 (Scheyer et al., 2016), consistent with results from

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similar regimens (Shepard et al., 2004; Adhikary et al., 2017). Statistical analyses comparing self-administration data for methamphetamine groups (and where appropriate, saline groups) within each Experiment are described in the main text. Statistical analyses comparing methamphetamine groups from different Experiments, and comparing methamphetamine groups (combined) to saline groups (combined), is provided in Supplementary Table 1. As detailed there, using infusions over the last 3 days of training as a measure of acquisition, no difference between the methamphetamine groups in different Experiments was found; furthermore, the overall average methamphetamine infusions were significantly greater than overall saline infusions. Versions of behavioral data panels from main text figures showing individual data points are provided in Supplementary Fig. 1.

### 2.3. Intracranial cannulation

Rats destined for intra-NAc drug infusions during cue-induced seeking tests (see description of individual Experiments below) underwent jugular catheter implantation as described above (Section 2.1) and guide cannula implantation during the same surgery. Guide cannula were aimed bilaterally at the NAc core (anteroposterior: 1.4; mediolateral: 2.2; dorsoventral: −7.2) at a 6-degree angle. After one week of recovery, drug self-administration training proceeded as described above (Section 2.2).

### 2.4. Cue-induced seeking tests

After discontinuing methamphetamine self-administration, some rats (see description of individual Experiments below) underwent cue-induced seeking tests (30 min) in early and/or late withdrawal, during which responding in the previously active hole delivered the light cue but no drug. Responding under these conditions is our operational measure of cue-induced methamphetamine craving. During the tests, photobeams in the operant boxes enabled measurement of locomotor activity.

### 2.5. Biotinylation and co-immunoprecipitation

For all biochemical studies, rats were decapitated and bilateral NAc core was rapidly dissected from two 1 mm slices prepared with a brain matrix (ASI Instruments; Warren, MI) using 1.5 mm biopsy punches (Thermo Fisher Scientific, Waltham, MA). As previously described in detail (Ferrario et al., 2011; Loweth et al., 2014; Murray et al., 2019), the freshly dissected tissue was quickly chopped into smaller pieces using a scalpel and then incubated with 1mM membrane impermeant biotinylating reagent sulfo-NHS-S-S-Biotin (Thermo Scientific, Rockford, IL) for 30 min before stopping the reaction. Tissue was lysed, sonicated, and aliquots stored at −80°C. Some aliquots were destined for use in immunoprecipitation experiments (see below); this portion of the biotinylated starting material was reserved without any further processing. Other aliquots were further processed to separate biotinylated surface-expressed proteins bound to NeutrAvidin beads (bound fraction) from the non-biotinylated (unbound) material, and the bound fraction was used to measure surface-expressed mGlu5. The presence of the small biotin molecule (~0.24 kD) on the extracellular portion of surface-expressed proteins is extremely unlikely to affect interactions of their intracellular portion with binding partners, and using the same starting material for both types of experiments (biotinylation and immunoprecipitation) increases our ability to compare the results. We have used this approach previously (e.g.,

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Loweth et al., 2014; Murray et al., 2019). In the present experiments, either mGlu5 was immunoprecipitated to assess its association with Homer1bc and Homer2 (WD3, WD21 and WD48) or DGL was immunoprecipitated to assess its association with CaMKII (WD3) (see description of individual Experiments below). Briefly, 3 μg of mGlu5 antibody (AB5675, Millipore Sigma, Burlington, MA) or DGL-α antibody (a generous gift from Dr. Ken Mackie, Indiana University, Bloomington) was incubated overnight (4°C) with protein A/G agarose slurry. The resulting antibody-coated beads were incubated overnight (4°C) with 75-100 μg of tissue. The material bound to the antibody-coated beads was isolated by centrifugation (two rounds of immunoprecipitation were performed to maximize recovery). The combined bound material was suspended in 2X Laemmli sample buffer with 1X XT Reducing Agent (Bio-Rad, Hercules, CA) in a volume equal to the input tissue volume. Samples were heated to 100°C for 3 minutes and stored at −20°C. For immunoprecipitation experiments measuring associations between Homer proteins and mGlu5, the following control studies were performed and published previously (Loweth et al., 2014). First, after immunoprecipitation of NAc tissue with mGlu5 antibody, immunoblotting with the same antibody verified that virtually all of the mGlu5 dimer present in the starting material (input) is recovered in the bound material and not the unbound material. Then, we confirmed that the signal observed in the bound material is not due to non-specific binding because, after immunoprecipitation with IgG (control condition), mGlu5 protein is detected in the unbound material rather than the bound material. For DGL immunoprecipitation, we conducted preliminary studies in homogenates from drug-naïve rats. First, using DGL-α antibody and DGL blocking peptide kindly provided by Dr. Ken Mackie from Indiana University, we showed that preabsorption of the antibody with the blocking peptide (5 μg/ml overnight at 4°C) largely eliminated the ~100 kDa DGL band detected without antibody preabsorption. After DGL pull-down, we confirmed a robust ~100 kDa band in the bound material with a much weaker signal in the unbound material.

### 2.6. Immunoblotting

Samples were run on 4-12% Bis-Tris gels (Bio-Rad) and transferred to PVDF membranes as previously described (Conrad et al., 2008; Loweth et al., 2014). Primary antibodies used were: DGL-α (1:750; a generous gift from Dr. Ken Mackie, Indiana University, Bloomington), p-Ser (1:120, sc-81514, Santa Cruz Biotechnology, Dallas, TX), mGlu5 (1:10,000, AB5675, Millipore Sigma, Burlington, MA), CaMKII (1:1,000, 3362, Cell Signaling Technology, Hillsboro, OR), p-Thr286 CaMKII (1:1,000, p1005-286, Phospho Solutions, Aurora, CO), Homer1b/c (1:200, sc-55463, Santa Cruz Biotechnology, Dallas, TX), and Homer2 (1:500, H00009455-B01P, Abnova, Taipei, Taiwan). GAPDH was used as a loading control when immunoblotting total tissue homogenates; for immunoblotting of surface-expressed (biotinylated) fractions, we have not identified a surface-expressed protein that we are confident using as a control and so we load carefully based on protein assay data. The mGlu5 dimer band (~260 kDa) was analyzed because it represents the functional pool of these receptors (Jingami et al., 2003). Secondary antibodies to rabbit or mouse IgG light chain were used (211-032-171 or 115-035-174, Jackson ImmunoResearch Laboratories, West Grove, PA) when immunoblotting immunoprecipitated tissue to prevent interference with the Homer band (~45 kDa) by the heavy chain (~50 kDa). Visualization was achieved by chemiluminescence (ECL; GE Healthcare, Piscataway, NJ). Immunoblots were analyzed

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with TotalLab (Life Sciences Analysis Essentials) as described previously (Conrad et al., 2008; Ferrario et al., 2011; Loweth et al., 2014). Data were excluded only if imperfections in the gel or blot interfered with analysis. Representative bands are shown in main text figures and full immunoblots are shown in Supplementary Fig. 2.

### 2.7. Electrophysiology

All electrophysiological procedures were adapted from those previously described (Conrad et al., 2008; McCutcheon et al., 2011; Scheyer et al., 2016, 2018). Briefly, rats were anesthetized with chloral hydrate (400–600 mg/kg, i.p.) and the brains were rapidly removed. Coronal slices at the level of the NAc (350 μm) were cut with a vibrating microtome in ice-cold sucrose-based cutting solution and then transferred to warm (32-34°C) artificial cerebral spinal fluid (aCSF) containing (in mM): 122.5 NaCl, 20 glucose, 25 $NaHCO_3$, 2.5 KCl, 0.5 $CaCl_2$, 3 $MgCl_2$, 1.0 $NaH_2PO4$, 1.0 ascorbic acid. All recordings were conducted at least 1 h after slicing. All solutions were constantly oxygenated (95% O2/5% $CO_2$). Whole cell patch clamp recordings were conducted in room temperature aCSF with $CaCl_2$ increased to 2.5 mM and $MgCl_2$ reduced to 1.0 mM. Both picrotoxin (0.1 mM) and APV ((2R)-amino-5-phosphonopentanoate) (0.05 mM) were added into the recording aCSF to pharmacologically isolate AMPAR transmission. Recordings were conducted using patch pipettes (6–8 MΩ) filled with a Cs-based/spermine-containing internal solution (in mM): 140 CsCl, 10 HEPES, 2 $MgCl_2$, 5 NaATP, 0.6 NaGTP, 2 QX-314, 0.1 spermine. A bipolar tungsten stimulating electrode (FHC, Inc., Bowdoin, ME) placed ~200 μm from the recording site was used to elicit excitatory postsynaptic currents (EPSC) in MSN. Only neurons that exhibited stable baseline synaptic responses (<15% variability, 15 min) were included. Bath application of the group I mGluR agonist 3,5-dihydroxyphenylglycine (DHPG; 50 μM) in the presence of the mGlu1 antagonist LY367385 (50 μM) determined the contribution of mGlu5 to DHPG-mediated synaptic depression in MSNs.

### 2.8. Statistical Analyses

Data are expressed as mean ± SEM. Drug effects were compared using one-way and two-way ANOVAs or student's t-tests, as detailed in Results for each Experiment. Differences between groups were considered statistically significant when $p<0.05$.

### 2.9. Experiments

**Experiment 1.—**Rats self-administered saline ($n = 5$) or methamphetamine ($n = 15$) as described in Section 2.2. After different periods of withdrawal ranging from 1-38 days, rats were killed and NAc core MSN recorded as described in Section 2.7.

**Experiment 2.—**Three cohorts of rats self-administered saline or methamphetamine as described in Section 2.2. The cohorts were killed on WD3 (saline, $n = 12$; methamphetamine, $n = 12$), WD21 (saline, $n = 12$; methamphetamine, $n = 11$), and WD48 (saline, $n = 11$; methamphetamine, $n = 10$). Biochemical procedures are described in detail in Sections 2.5 and 2.6. Briefly, NAc tissue (primarily core) was biotinylated and then lysed. Some aliquots of this lysed homogenate were used without further processing to measure total protein levels by immunoblotting. Results for total mGlu5 protein are presented here;

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previously, identical aliquots from the same animals were used to measure total mGlu1, Homer1b/c and Homer2 protein levels (Murray et al., 2019). Other aliquots were used without further processing as starting material for immunoprecipitation experiments in which mGlu5 was immunoprecipitated and the amount of Homer1b/c or Homer 2 pulled down with mGlu5 was analyzed by immunoblotting. Finally, some aliquots were further processed via avidin-biotin pull-down to isolate surface-expressed proteins for measurement of surface mGlu5 by immunoblotting; previously, identical aliquots from the same animals were previously used to measure surface-expressed mGlu1 (Murray et al., 2019).

**Experiment 3.—**Rats self-administered saline ($n = 11$) or methamphetamine ($n = 12$) as described in Section 2.2 and were killed on WD1. Using biochemical procedures described in Sections 2.5 and 2.6, NAc tissue (primarily core) was lysed and used without further processing to measure total protein levels of DGL and as starting material for immunoprecipitation experiments in which DGL was immunoprecipitated and the amount of mGlu5, Homer1b/c or Homer 2 pulled down with DGL was determined by immunoblotting. The only difference between processing of this tissue and processing of tissue in all other Experiments is that we used a lysis buffer containing 1% Triton and 0.5% deoxycholate instead of our standard buffer (Ferrario et al., 2011); both yielded similar results.

**Experiment 4.—**This experiment began with the WD3 NAc tissue prepared as part of Experiment 2. This biotinylated, lysed tissue was used without further processing to measure phosphorylated and total levels of CaMKIIα and CaMKIIβ, and as starting material for immunoprecipitation experiments in which DGL was immunoprecipitated and the amount of pan or phosphorylated CaMKIIα and CaMKIIβ pulled down with DGL was determined by immunoblotting.

**Experiment 5.—**A total of 24 rats self-administered methamphetamine (Section 2.2) and were then divided into two cohorts that did not differ in infusions, active hole nose poke responding or inactive hole nose poke responding during training (see Results). On WD21, one cohort received the mGlu5 NAM MTEP (Tocris Bioscience, Bristol, UK) (3 mg/kg, i.p.; $n = 12$) and the other cohort received vehicle (0.9% Sodium Chloride Injection, USP; $n = 12$) 10 min before a 30 min cue-induced seeking test (Section 2.4). To determine effects of a lower dose of MTEP (1 mg/kg), these same animals were subsequently retested on WD33 using a counter-balanced design (cohort previously treated with intra-NAc MTEP now received vehicle).

**Experiment 6 (A and B).—**For Experiment 6A, rats ($n = 8$) implanted with a guide cannula aimed at the NAc core (Section 2.3) self-administered methamphetamine (Section 2.2) and then received a cue-induced seeking test (Section 2.4) on WD1 to establish baseline craving. They were subsequently divided into two cohorts that did not differ in infusions, active hole nose poke responding, or inactive hole nose poke responding during training and likewise did not differ in active hole or inactive hole responding on WD1 (see Results). On WD21, one cohort received intra-NAc core MTEP ($n = 3$; 1 μg/μl dissolved in 0.9% Sodium Chloride Injection, USP; 0.5 μl/min for 2 min, for a total of 1 μg per side) and the other cohort received vehicle ($n = 5$) 10 min prior to placement in the operant chamber

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for a 30 min cue-induced seeking test. On WD25, the cohort that received intra-NAc MTEP now received vehicle and vice versa. In Experiment 6B, an additional cohort of rats ($n = 9$) was used to examine the effect of intra-NAc MTEP on cue-induced cocaine seeking in early withdrawal. These rats underwent guide cannula implantation (Section 2.4) and methamphetamine self-administration (Section 2.2) and were subsequently divided into two cohorts that did not differ in the average number of infusions, active hole nose poke responding, or inactive nose poke hole responding over training (see Results). Using a counterbalanced design, on WD1, one cohort ($n = 5$) received intra-NAc MTEP (1 μg per side) and the second ($n = 4$) received vehicle 10 min prior to placement in the operant chamber for a 30 min cue-induced seeking test. On WD4, the cohort that previously received intra-NAc MTEP now received vehicle, and vice versa. The interval between tests was 1 day shorter for these rats (compared to Experiment 6A) because we wanted to test prior to incubation and incubation develops within 7 days after discontinuing methamphetamine self-administration. After experiments were concluded, histological analysis was conducted to confirm guide cannula placements above the NAc core. Only those rats with accurate placements were used for the analysis and in presentation of behavioral data.

# 3. Results

## 3.1. Experiment 1: Early withdrawal from methamphetamine self-administration is associated with a loss of mGlu5/CB1R-dependent synaptic depression

We have shown previously that DHPG-induced mGlu5/CB1R-dependent synaptic depression (hereafter referred to as DHPG-induced synaptic depression) is abolished in late withdrawal (>WD35) from a cocaine self-administration regimen leading to incubation of craving (McCutcheon et al., 2011; Scheyer et al., 2018). To determine if similar plasticity accompanies methamphetamine incubation, we used rats that self-administered saline (control condition) or methamphetamine (10 sessions of 6 hours/day) (Fig. 1A). Statistical analysis of infusions, which included the between-subjects factor Group (saline, methamphetamine) and the within-session factor Training Day (1–10), showed a significant effect of Group ($F_{1,18} = 20.25$, $p < 0.0005$), a non-significant effect of Training Day ($F_{9,162} = 0.79$, $p = 0.62$), and a significant interaction between the two factors ($F_{9,162} = 4.02$, $p < 0.0005$). Statistical analysis of active and inactive hole nose-pokes showed a significant effect of Group, with methamphetamine active hole responses significantly higher than methamphetamine inactive or saline active or inactive hole responses (one-way ANOVA: $F_{3,36} = 15.52$, $p < 0.0005$; LSD post-hoc tests, all $p < 0.0005$).

Rats were randomly selected for whole-cell patch-clamp recordings of NAc core MSNs at different withdrawal times. Previous work has shown that the incubation of methamphetamine craving in rats is relatively rapid (compared to cocaine), reaching maximal levels after roughly a week of withdrawal and then remaining stably elevated through at least WD51 (Shepard et al., 2004; Scheyer et al., 2016; Adhikary et al., 2017). Therefore, in this study, our experiments were designed to assess time-points on the rising phase of incubation and then combine later withdrawal times when incubation is stably expressed (i.e., recordings conducted between WD15-WD38). As in our prior cocaine studies (McCutcheon et al., 2011; Loweth et al., 2014; Scheyer et al., 2018), we did

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not verify incubation in rats destined for electrophysiology. However, methamphetamine intake in these rats was very similar to cohorts shown to express incubation in prior studies (Scheyer et al., 2016; Murray et al., 2019) and they did not differ in acquisition of methamphetamine self-administration (infusions over last 3 days of training) compared to methamphetamine groups in other Experiments (Supplementary Table 1).

We first applied DHPG (50 μM, 10 min) and measured evoked $EPSC_{-70mV}$ amplitudes in MSN recorded from saline rats (7 cells/5 rats) or MSN recorded from methamphetamine rats between WD1 and WD10 (8 cells/4 rats). To assess the final magnitude of DHPG-induced synaptic depression, we used a standard analysis (e.g., McCutcheon et al., 2011) in which the mean $EPSC_{-70mV}$ amplitude during the last 3 min of DHPG application (gray shading in Fig. 1B) is compared to the pre-DHPG baseline for each cell, and the percent reduction versus baseline is calculated for each cell (Fig. 1C). Within-group analysis revealed a significant DHPG-induced depression of $EPSC_{-70mV}$ in MSNs from saline animals ($t_6$ = 4.46, $p = 0.004$), as in prior studies (McCutcheon et al., 2011; Scheyer et al., 2018), but not in MSNs from the methamphetamine animals ($t_7 = -0.54$, $p = 0.61$) (Fig. 1C). Between-group analysis of the percent reduction achieved in cells from saline and methamphetamine groups also indicated a significant difference (unpaired t-test, $^{**}p < 0.01$). To extend this analysis, we performed two-way repeated measures ANOVA on 5 baseline time-points and the last 3 post-DHPG time-points. This revealed significant effects of Group ($F_{1,13} = 20.26$, $p = 0.001$) and Time ($F_{7,91} = 8.60$, $p < 0.0001$), as well as a significant interaction ($F_{7,91}$ = 9.33, $p < 0.0001$). Overall, these results indicate loss of DHPG-induced synaptic depression in early withdrawal from methamphetamine self-administration.

Given that DHPG is a non-specific group I mGluR agonist, we pharmacologically isolated mGlu5-dependent effects by applying DHPG in the presence of the mGlu1 antagonist LY367385 (50μM). Using this approach, we compared MSNs recorded from methamphetamine rats at the beginning and towards the end of the rising phase of incubation (WD1-2 and WD7, respectively) and at later times when incubation is stably expressed (WD15-38) (Fig. 1D). We found a significant DHPG-induced depression of $EPSC_{-70mV}$ (last 3 min), relative to the pre-DHPG baseline, in MSNs recorded on WD15-38 but not MSNs recorded at earlier withdrawal times. This was revealed using either within-group paired t-tests (WD1-2: $t_6 = 1.98$, $p = 0.10$; WD7: $t_9 = 0.86$, $p = 0.41$; WD15-38; $t_5 = 4.1$, $p$ = 0.01) or one-way ANOVA ($F_{2,20} = 4.01$, $p = 0.03$; LSD post-hoc tests: WD15-38 differed from WD1-2, $p = 0.041$, and WD7, $p = 0.01$) (Fig. 1E). Consistent with these results, two-way repeated measures ANOVA on 5 baseline time-points and the last 3 post-DHPG time-points revealed significant effects of WD ($F_{2,20} = 4.00$, $p = 0.04$) and Time ($F_{7,140}$ = 6.30, $p < 0.0001$) as well as a significant interaction ($F_{14,140} = 2.14$, $p = 0.01$).

### 3.2. Experiment 2: Loss of mGlu5/CB1R-dependent synaptic depression is not associated with impaired assembly of the 2-AG signalosome

In cocaine-incubated animals, the loss of DHPG-induced synaptic depression in late withdrawal is associated with postsynaptic reductions in surface mGlu5 and in mGlu5 association with Homer scaffolding proteins (Loweth et al., 2014; see Discussion). To determine if similar alterations occur during early withdrawal from methamphetamine,

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we conducted biochemical analyses of mGlu5, Homer proteins and DGL-α, components of the signaling complex known to mediate mGlu5/CB1R-dependent synaptic depression (Piomelli, 2014). We utilized NAc core tissue collected from three cohorts of rats killed on either WD3, WD21, or WD48. WD3 and WD21 represent timepoints during incubation of methamphetamine craving with impaired and intact DHPG-induced synaptic depression, respectively, while WD48 represents a timepoint when incubation of cocaine craving is maximal and rats display loss of DHPG-induced synaptic depression (McCutcheon et al., 2011; Scheyer et al., 2018). Statistical analysis of infusions over the 10 days of self-administration training (Fig. 2A) using two-way ANOVA revealed a significant effect of Group (saline/methamphetamine; WD3: $F_{1,22} = 90.90$, $p < 0.0001$; WD21: $F_{1,21} = 68.45$, $p < 0.0001$; WD48: $F_{1,19} = 29.98$, $p < 0.0001$), Training Day (WD3: $F_{9,198} = 2.25$, $p = 0.02$; WD48: $F_{9,171} = 2.58$, $p = 0.01$; but not WD21: $F_{9,189} = 1.84$, $p = 0.06$) and a significant Group x Training Day interaction (WD3: $F_{9,198} = 18.83$, $p < 0.0001$; WD21: $F_{9,189} = 6.52$ $p < 0.0001$; WD48: $F_{9,171} = 6.73$ $p < 0.0001$) for each of the three different WD cohorts. Most importantly, methamphetamine rats assigned to these cohorts did not differ in the average number of infusions obtained during self-administration training ($F_{2,30} = 1.56$, $p = 0.23$), nor were there differences in average active hole ($F_{2,30} = 1.64$, $p = 0.21$) or inactive hole ($F_{2,30} = 3.10$, $p = 0.06$) nose poke responding over the 10 days of self-administration training.

We began by measuring cell surface expression of mGlu5 using biotinylation. Both cell surface and total protein levels of mGlu5 were unchanged across timepoints in methamphetamine compared to saline groups as determined by two-way ANOVA (Fig. 2B,C; Surface mGlu5: Group: $F_{1,62} = 0.97$, $p = 0.33$; WD: $F_{2,62} = 1.26$, $p = 0.29$; Group x WD: $F_{2,62} = 1.26$, $p = 0.29$. Total mGlu5: Group: $F_{1,61} = 2.20$, $p = 0.14$; WD: $F_{2,61} = 1.83$, $p = 0.17$; Group x WD: $F_{2,61} = 2.04$, $p = 0.14$). Next, we assessed Homer1b/c and Homer2, selected because their expression in the NAc is implicated in vulnerability to methamphetamine reward and reinforcement (see Discussion). Using homogenates from the same NAc samples used for studying mGlu5 (WD3, WD21, WD48), we evaluated mGlu5-Homer associations by immunoprecipitating mGlu5 and measuring bound Homer levels and found no differences between saline and methamphetamine groups (Fig. 2D,E; mGlu5-Homer1b/c: Group: $F_{1,60} = 0.03$, $p = 0.87$; WD: $F_{2,60} = 0.51$, $p = 0.60$; Group x WD: $F_{2,60} = 0.17$, $p = 0.84$. mGlu5-Homer2: Group: $F_{1,61} = 1.41$, $p = 0.24$; WD: $F_{2,61} = 0.48$, $p = 0.62$; Group x WD: $F_{2,61} = 0.48$, $p = 0.62$). We have previously published data obtained using aliquots of the same homogenates which showed no changes in total Homer1b/c or Homer2 protein levels (Murray et al., 2019).

### 3.2 Experiment 3: Associations of DGL with mGlu5 and Homer2 are unchanged in early withdrawal

In addition to mGlu5 and Homer scaffolding proteins, the 2-AG signalosome requires DGL-α enzymatic activity to produce 2-AG from the hydrolysis of DAG following mGlu5 stimulation and PLCβ activation (Piomelli, 2014). Furthermore, DGL associates with Homer scaffolding proteins and this association is required for membrane-bound DGL activity and colocalization with mGlu5 (Jung et al., 2007). To study these interactions, we generated a new cohort of rats that self-administered saline or methamphetamine (Fig. 3A). Statistical analysis of infusions over the 10 days of training using two-way repeated

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measures ANOVA revealed a significant effect of Group (saline/methamphetamine; $F_{1,21} = 59.26$, $p < 0.0001$), a non-significant effect of Training Day ($F_{9,189} = 1.65$, $p = 0.10$) and a significant Group x Training Day interaction ($F_{9,189} = 19.88$, $p < 0.0001$). Using NAc core homogenates obtained from these rats on WD1, when mGlu5/CB1R-dependent synaptic depression is abolished (Fig. 1), we assessed total DGL-α levels. Surprisingly, DGL levels were significantly increased in methamphetamine tissue ($t_{20} = 2.87$, $p = 0.009$), possibly representing compensation related to loss of synaptic depression. Then, using the same homogenates, we assessed DGL associations with Homer and mGlu5 by immunoprecipitating DGL and measuring bound Homer protein and bound mGlu5 (DGL does not bind directly to mGlu5, so we are presumably detecting an indirect association via intermediate Homer interactions). DGL associations with Homer2 and mGlu5 were unaffected (DGL-Homer2: $t_{21} = 0.64$, $p = 0.53$; DGL-mGlu5: $t_{21} = -0.36$, $p = 0.72$) (Fig. 3C), further indicating that the 2-AG signalosome is intact after methamphetamine withdrawal. Homer1b/c was not detected in our DGL immune complexes in NAc core after saline or methamphetamine self-administration (data not shown), suggesting that this Homer protein scaffolds mGlu5 to binding partners other than DGL.

### 3.4. Experiment 4: Loss of DHPG-induced synaptic depression is not associated with increased interactions between CaMKII and DGL or with CaMKII activation

CaMKIIα and CaMKIIβ bind DGL, with activated CaMKIIα phosphorylating DGL and inhibiting 2-AG signaling (Shonesy et al., 2013). To determine whether CaMKII/DGL interactions are affected during early withdrawal from methamphetamine self-administration, when DHPG-induced synaptic depression is lost, we utilized WD3 NAc core tissue (from rats shown in Fig. 2) to immunoprecipitate DGL and measure bound CaMKII. Saline and methamphetamine groups did not differ in associations between DGL and either total or phosphorylated (Thr286) CaMKIIα and CaMKIIβ (DGL-CaMKIIα: ($t_{15} = 0.76$, $p = 0.56$); DGL-pCaMKIIα ($t_{14} = 0.07$, $p = 0.94$); DGL-CaMKIIβ ($t_{14} = 0.38$, $p = 0.71$); DGL-pCaMKIIβ ($t_{15} = 1.00$, $p = 0.533$) (Fig. 4A). However, while not significant, there were trends toward an increased ratio of phosphorylated to total CaMKIIβ (saline vs methamphetamine: $t_{22} = 1.88$, $p = 0.07$) and to a lesser extent for CaMKIIα (saline vs methamphetamine: $t_{22} = 1.18$, $p = 0.25$) in starting material (homogenate) in the methamphetamine group compared to saline controls (Fig. 4B). We tried to determine if DGL was differentially phosphorylated in methamphetamine vs saline groups by immunoblotting the DGL antibody-immunoprecipitated tissue with antibody recognizing phosphorylated serine residues, but we could not detect a clear band in the molecular weight range of DGL that was suitable for quantification. Overall, these data suggest that the loss of DHPG-induced synaptic depression in early methamphetamine withdrawal is not attributable to increased associations between DGL and CaMKII.

### 3.5. Experiments 5 and 6. Effect of negative allosteric modulation of mGlu5 on incubation of methamphetamine craving

Many studies using different animal models indicate that mGlu5 NAMs reduce relapse-like behavior (see Introduction), but whether their efficacy extends to the incubation of methamphetamine craving is unknown. In Experiment 5, we tested this using systemic administration of the mGlu5 NAM MTEP. Rats ($n = 24$) self-administered

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methamphetamine as described above (Fig. 5A) and then were divided into two cohorts that did not differ in the average number of infusions obtained during self-administration training ($F_{1,22}$ = 2.11, $p$ = 0.16), or in average active hole ($F_{1,22}$ = 2.65, $p$ = 0.12) or inactive hole ($F_{1,22}$ = 2.05, $p$ = 0.17) nose poke responding over the 10 days of self-administration training. On WD21, a time when incubation of methamphetamine craving has plateaued, one cohort received the mGlu5 NAM MTEP (3 mg/kg, i.p.; $n$ = 12) and the other cohort received vehicle ($n$ = 12) 10 min before a 30 min cue-induced seeking test. At this 3 mg/kg dose, previously reported to reduce cue-induced reinstatement of methamphetamine seeking to extinction levels in an extinction-reinstatement model (Gass et al., 2009), responding in the previously drug-paired active hole was reduced dramatically ($t_{22}$ = 8.69, $p$ < 0.0001), but this was accompanied by nonspecific behavioral depression, since responding in the inactive hole ($t_{22}$ = 4.01, $p$ = 0.0006) and locomotor activity during the seeking test ($t_{22}$ = 2.86, $p$ = 0.009) were also robustly decreased (Fig. 5B). These same animals were subsequently retested on WD33 in a counter-balanced design (the cohort previously treated with MTEP now received vehicle) using a lower dose (1 mg/kg) that also reduced cue-induced reinstatement to extinction levels (Gass et al., 2009). Again, significantly decreased responding was observed in both active ($t_{22}$ = 4.67, $p$ = 0.002) and inactive holes ($t_{22}$ = 2.54, $p$ = 0.018), and there was a significant reduction in locomotion ($t_{22}$ = 3.10, $p$ = 0.005) (Fig. 5C). We note that reinstatement studies using these MTEP doses have sometimes reported non-significant but appreciable reductions in inactive hole or lever responding (see Discussion).

In Experiment 6, we turned to intra-NAc administration of MTEP, which has been tested against cocaine seeking (see below and also Discussion) but not methamphetamine seeking. In Experiment 6A, rats ($n$ = 8) implanted with guide cannula targeting the NAc core underwent methamphetamine self-administration (Fig. 6A) and then received a cue-induced seeking test on WD1 (active hole nose-pokes: 18.5 ± 1.4; inactive hole nose-pokes: 6.8 ± 1.0; locomotor activity measured via photobeams in operant box: 237.5 ± 45.0; mean ± SEM; data not shown in figures). They were subsequently divided into two cohorts that did not differ in the average number of infusions ($t_6$ = 0.14, $p$ = 0.89), active hole nose poke responding ($t_6$ = −0.20, $p$ = 0.85) or inactive hole nose poke responding ($t_6$ = 1.45, $p$ = 0.28) over the 10 days of self-administration training (Fig. 6A), and also did not differ in active hole nose-pokes ($t_6$ = −0.65, $p$ = 0.54) or inactive hole nose-pokes ($t_6$ = 1.32, $p$ = 0.24) on WD1. Additional seeking tests were performed in the same rats after incubation of craving. Using a counter-balanced design with cue-induced seeking tests on WD21 and WD25, rats received intra-NAc core infusion of vehicle or MTEP (1 μg per side) 10 min prior to placement in the operant chamber for a 30 min cue-induced seeking test. We selected this dose because when administered into the core it blocked cue-induced reinstatement of cocaine seeking (Wang et al., 2013b; Knackstedt et al., 2014) and context-induced cocaine seeking during abstinence (Knackstedt et al., 2014). In contrast to these results, we found that intra-NAc core MTEP infusion produced only a trend toward reduction of active hole responding during WD21 or WD25 tests without affecting responding on the inactive hole or locomotor activity during the test (Fig. 6B). In Experiment 6B, an additional cohort of rats ($n$ = 9) was used to examine the effect of intra-NAc MTEP on cue-induced cocaine seeking in early withdrawal. These rats underwent methamphetamine self-administration

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(Fig. 6D) and were subsequently divided into two cohorts that did not differ in the average number of infusions ($t_7 = -0.76$, $p = 0.47$), active hole nose poke responding ($t_7 = -0.94$, $p = 0.38$) or inactive hole nose poke responding ($t_7 = -0.53$, $p = 0.61$) over the 10 days of self-administration training. Using a counterbalanced design in which one cohort received MTEP on WD1 and vehicle on WD4, while the other received the opposite treatments, we found no significant effects of intra-NAc core MTEP infusion on cue-induced cocaine seeking in early withdrawal (Fig. 6E). Histological analyses of cannula placements for these rats are depicted in Fig. 6C, F.

While the counter-balanced design used in Figs. 5 and 6 can have the advantage of reducing variability, a potential concern is extinction between tests. However, our data do not indicate that this is occurring. For the systemic MTEP experiment (Fig. 5B,C), if extinction was occurring between tests, the rats that received vehicle on their second test day (WD33; white bar in Fig. 5C left) should have shown significantly lower active hole responding compared to the cohort that received vehicle on their first test day (WD21; white bar in Fig. 5B left), but this was not the case (t-test between vehicle tests, $p = 0.187$). Also potentially relevant to our counter-balanced design, MTEP was found to inhibit subsequent extinction learning (evaluated on four consecutive days following a context-induced seeking test), but this inhibitory effect was observed after its infusion into the dorsal striatum but not the NAc core (Knackstedt et al., 2014). To rigorously evaluate whether this is occurring under our conditions, we would need to conduct multiple extinction tests after MTEP exposure, as done by Knackstedt and colleagues.

## 4. Discussion

Long-lasting adaptations in glutamate transmission within the reward circuitry are a cardinal feature of the enduring vulnerability to relapse captured in animal models of substance use disorders (D'Souza, 2015; Lüscher, 2016; Scofield et al., 2016; Wolf, 2016). While many studies have addressed the role of mGlu5 in reinstatement of psychostimulant seeking after extinction training, less is known about mGlu5's role in drug craving during forced abstinence, and no studies have examined this for methamphetamine. Here we conducted the first studies of mGlu5/CB1R-dependent synaptic depression, surface and total mGlu5 levels, and associations between mGlu5, Homer and DGL proteins in the NAc core during incubation of methamphetamine craving. We also performed the first studies on the effect of systemic and intra-NAc core administration of an mGlu5 NAM (MTEP) on cue-induced methamphetamine craving during incubation.

### 4.1. Synaptic plasticity in NAc core after incubation of cocaine versus methamphetamine craving

We previously showed that incubation of cocaine craving is associated with increased synaptic levels of homomeric GluA1 CP-AMPARs in the NAc core, the stimulation of which is required for expression of incubated craving (Conrad et al., 2008). This is accompanied by emergence of an mGlu1-mediated LTD that is expressed via CP-AMPAR internalization (McCutcheon et al., 2011; Scheyer et al., 2018). As expected from the mediating role of CP-AMPARs, this LTD reduces incubated cocaine craving (Lee et

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al., 2013; Loweth et al., 2014; Ma et al., 2014). Interestingly, mGlu1 levels decline in the NAc core during cocaine withdrawal (although a sufficient number remain for their pharmacological stimulation to induce LTD) and this decline contributes to CP-AMPAR accumulation (Loweth et al., 2014).

CP-AMPARs also accumulate in NAc core synapses during incubation of methamphetamine craving and their stimulation is a requirement for its expression (Scheyer et al., 2016). This CP-AMPAR accumulation is associated with increased GluA1 translation (Murray et al., 2019), as shown previously for cocaine (Stefanik et al., 2018). Furthermore, mGlu1-LTD expressed via CP-AMPAR removal is present after incubation of methamphetamine craving and can be targeted to reduce incubation (Scheyer et al., 2016). However, mGlu1 levels do not decrease in NAc core after methamphetamine withdrawal (Murray et al., 2019). mGlu1-Homer associations are not altered during incubation of either cocaine or methamphetamine craving (Loweth et al., 2014; Murray et al., 2019).

Incubation of cocaine craving also involves plasticity related to mGlu5, as the DHPG-induced synaptic depression that is apparent in MSN from drug-naïve rats is lost in MSN recorded after WD35 from extended-access cocaine self-administration (McCutcheon et al., 2011; Scheyer et al., 2018); earlier withdrawal times have not been not assessed. Here we extended this work to methamphetamine, showing that DHPG-induced synaptic depression in the NAc core is lost during the first 7-10 days of abstinence from extended-access methamphetamine self-administration. This period corresponds to the rising phase of incubation of methamphetamine craving (Scheyer et al., 2016; Adhikary et al., 2017). At later withdrawal times, this synaptic depression recovered. This contrasts with incubation of cocaine craving, where loss of the synaptic depression is a persistent feature of the plateau phase of incubation (see above).

In other cocaine paradigms, electrically-evoked mGlu5-dependent LTD in NAc core was impaired after extinction but unaltered after 3 weeks of abstinence from short-access cocaine self-administration (Moussawi et al., 2009; Knackstedt et al., 2010). Electrically-evoked and DHPG-induced mGlu5/CB1R-dependent LTD were impaired in NAc core 24 h after a single i.p. cocaine injection (Fourgeaud et al., 2004), while impairment of both DHPG-induced/mGlu5-dependent and NMDAR-dependent LTD was observed in NAc shell but not core after repeated i.p. cocaine and 14 days of abstinence (Huang et al., 2011, 2015). Using a pairing protocol previously shown to elicit NMDAR-dependent LTD, LTD was reduced in core and shell 1 day after discontinuing extended-access cocaine self-administration but only in shell after 21 days (Martin et al., 2006), and rats that progressively develop behavioral hallmarks of addiction during long-term cocaine self-administration show abolished NMDAR-dependent LTD in NAc core 24 h after the last drug exposure (Kasanetz et al., 2010). Thus, the subregion-specificity and withdrawal-dependence of cocaine's actions seems to depend on the drug regimen and the type of LTD assessed. Whether this is true for methamphetamine remains to be determined.

### 4.2. Potential mechanisms underlying loss of DHPG-induced synaptic depression

Loss of DHPG-induced synaptic depression following cocaine exposure appears to be attributable to postsynaptic alterations in the mGlu5 signaling complex. Thus,

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impaired synaptic depression was associated with reduced mGlu5 levels following acute noncontingent cocaine (Fourgeaud et al., 2004), chronic noncontingent cocaine (Huang et al., 2011), and incubation of cocaine craving (in NAc core; McCutcheon et al., 2011; Loweth et al., 2014). Two of these studies also tested CB1R function and found no impairment (Fourgeaud et al., 2004; McCutcheon et al., 2011). Altered coupling to Homer scaffolding proteins may also contribute, although divergence is apparent between cocaine regimens, as acute cocaine increased Homer levels (Fourgeaud et al., 2004), while incubation of cocaine craving did not alter protein levels of Homer1b/c or Homer2 although associations between mGlu5 and these Homer proteins were reduced (Loweth et al., 2014). Interestingly, in NAc core, injection of a peptide interfering with mGlu5-Homer interactions prevented cocaine- and cue-induced reinstatement (Wang et al., 2013b). Finally, in the NAc of rats that have undergone incubation of cocaine craving, CaMKII phosphorylation is increased (Ferrario et al., 2011) and there is an increased association between phosphorylated CaMKII and DGL along with reduced DGL enzymatic activity (C.M. Murray, A.D. Gaulden, S. Patel, M.E. Wolf, unpublished observations). Studies in dorsal striatum have found that CaMKII phosphorylation of DGL inhibits its activity and, conversely, that 2-AG dependent depolarization-induced suppression of excitation is augmented by pharmacological inhibition of CaMKII (Shonesy et al., 2013). Thus, our preliminary findings suggest that CaMKII-mediated negative regulation of DGL may contribute to loss of DHPG-induced synaptic depression after incubation of cocaine craving.

Surprisingly, none of the mechanisms described above appear to hold for incubation of methamphetamine craving. Thus, in the present study we observed no changes in mGlu5 surface or total expression in NAc core and no changes in the association between mGlu5 and Homer scaffolding proteins at any timepoint (WD3, WD21, WD48). A previously published study that used the same tissue found no change in Homer1b/c and Homer2 total protein levels (Murray et al., 2019). The present study further found that the association between DGL and CaMKII was also unchanged (WD1). Interestingly, however, we observed an increase in DGL protein levels on WD1 after methamphetamine self-administration. We speculate that this reflects a compensatory adaptation to lost DHPG-induced synaptic depression at this withdrawal time. Given that DGL's binding partners include key synaptic anchoring proteins such as PSD95, PSD93, Shank3, SAPAP2, and SAPAP3 (Shonesy et al., 2013) and that these proteins regulate activity of glutamate receptors including mGlu5 (e.g., Ade et al., 2016; Scheefhals et al., 2019), this could have numerous consequences for synaptic function, but more information about methamphetamine's effect on binding partners would be necessary to construct a specific hypothesis. Overall, the results described above suggest that impaired DHPG-induced synaptic depression during the rising phase of methamphetamine incubation is not due to uncoupling of the mGlu5 signaling complex or reduced DGL activity secondary to CaMKII activation, although the latter possibility cannot be completely ruled out as we detected a trend towards a small increase in CaMKII phosphorylation on WD3 (see Results). Activated CaMKII can also reduce mGlu5-mediated $Ca^{2+}$ mobilization (Marks et al., 2018). The role of CaMKII, as well other pathways regulating DGL activity (e.g.,Shonesy et al., 2020), could be investigated in future studies.

A number of other possible explanations for impaired DHPG-induced synaptic depression should be noted. First, it may be attributable to functionally significant reductions in mGlu5

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levels or its interactions with Homer proteins that are not detectable with the present methods. Second, there may be a change in the regulation of presynaptic glutamate release. This would be consistent with a study showing elevated baseline and methamphetamine challenge-evoked glutamate levels in the NAc of rats during withdrawal from long-term intravenous methamphetamine self-administration (Lominac et al., 2012). Dysregulation of glutamate release could be secondary to altered CB1R expression or function, possibilities which should be investigated in the future. Third, our results do not address mGlu5 signaling outside of its ability to elicit synaptic depression. For instance, mGlu5 stimulation has been shown to increase NAc MSN excitability (D'Ascenzo et al., 2009) and increase phosphorylation of AMPAR subunits in dorsal striatum (Ahn and Choe, 2009; Dell'Anno et al., 2013). There is also evidence for presynaptic mGlu5 receptors in rat striatum (Rodrigues et al., 2005). Fourth, regulator of G-protein signaling 4 (RGS4) is implicated in psychostimulant action through negatively modulating mGlu5 signaling (Schwendt et al., 2012) and serves as a point of convergence for signaling pathways modulating striatal mGlu5 signaling and endocannabinoid-mediated LTD (Shen et al., 2015). Thus, alterations in RGS4 could contribute to our observed results. Finally, studies on cocaine and mGlu5 implicate reduced coupling of mGlu5 to G proteins (Hao et al., 2010) and altered mGlu5-dependent phosphorylation of ERK and CREB (Hoffmann et al., 2017).

### 4.3. What is the functional consequence of the impaired synaptic depression observed in early methamphetamine withdrawal?

CB1R activation attenuates excitatory synaptic transmission onto NAc MSNs (Robbe et al., 2002; Lovinger, 2008) and excitatory synaptic transmission in the NAc is required for expression of incubated methamphetamine seeking (Scheyer et al., 2016). This could suggest that mGlu5/CB1R-dependent synaptic depression opposes methamphetamine seeking and that its loss in early withdrawal is promoting methamphetamine seeking. Alternatively, this synaptic epression could promote reward seeking. Supporting this, mice with constitutive mGlu5 knockdown in D1R-expressing MSN do not express mGlu5/CB1R-LTD in these MSN or exhibit cue-induced reinstatement (to cocaine, ethanol, or saccharin), while elevation of CB1R levels using a MAGL inhibitor restored LTD and reinstatement of saccharin seeking (Novak et al., 2010; Bilbao et al., 2020). Furthermore, an mGlu5- and CB1R-mediated reduction in extracellular glutamate levels in the NAc was found to enable cocaine-primed (but not cue-induced) reinstatement (Li et al., 2018b). This latter finding may be related to work showing that reduced extracellular glutamate levels promote relapse-like behavior by reducing activation of presynaptic glutamate autoreceptors that normally oppose such behavior (Kalivas, 2009). Other studies link mGlu5-dependent reward seeking to AMPAR internalization, i.e., a postsynaptically expressed LTD (Schmidt et al., 2013; Benneyworth et al., 2019).

In the case of incubation of methamphetamine craving, our results do not support either positive or negative regulation by mGlu5/CB1R-dependent synaptic depression. First, we show that this synaptic depression is absent throughout the rising phase of incubation (WD1-10) and then recovers during a period (WD15-38) when incubation is still maintained (time-course of incubation based on Shepard et al., 2004; Scheyer et al., 2016; Adhikary et al., 2017). Thus, there is no relationship between the magnitude of methamphetamine

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seeking and the integrity of the synaptic depression. Second, while MTEP prevents DHPG-induced synaptic depression in NAc neurons (McCutcheon et al., 2011), intra-NAc MTEP did not prevent expression of methamphetamine incubation at a withdrawal time (WD21/WD25) when this synaptic depression was intact. Along the same lines, for cocaine, mGlu5 NAMs reduce reinstatement of cocaine seeking under conditions when mGlu5/CB1R-LTD is impaired in the NAc, e.g., cocaine self-administration followed by extinction training (Knackstedt et al., 2010, 2014; Wang et al., 2013b).

The relationship between mGlu1/CB1R-dependent LTD and incubated methamphetamine seeking could be further tested by infusing drugs into the NAc core that inhibit or promote endocannabinoid signaling. However, CB1Rs in the striatum are present on GABAergic terminals as well as glutamate terminals, and cocaine affects CB1R regulation of GABA transmission (Centonze et al., 2007). CB1Rs also contribute to the modulation of striatal dopamine release (Mateo et al., 2017). These varied roles of the CB1R could underlie the complexity of results from studies that targeted the endocannabinoid system to influence psychostimulant seeking. For example, both elevating endocannabinoid levels (Nawata et al., 2019) and reduction of CB1R transmission via the inverse agonist rimonabant (Anggadiredja et al., 2004) or a NAM (Jing et al., 2014) reduced cue-induced reinstatement of methamphetamine seeking.

### 4.4 mGlu5-Homer interactions across models of methamphetamine reward

Our study is the first to measure mGlu5 and its interaction with Homer proteins in the NAc after intravenous methamphetamine self-administration. Non-contingent methamphetamine did not alter mGlu5 in the NAc (Herrold et al., 2011, 2013). Complex results have been obtained in studies of genetic (high and low methamphetamine-drinking selectively bred mouse lines) and idiopathic (inbred C57BL/6J mice exhibiting spontaneously divergent place-conditioning phenotypes) variance in methamphetamine preference and intake. High methamphetamine drinking mice exhibited greater mGlu5 and Homer2 (but not Homer1b/c) levels in NAc core and shell compared to low drinking mice, and in C57BL/6J mice conditioned place preference scores correlated with higher mGlu5, mGlu1 and Homer2 levels in NAc core whereas only the CPP-Homer2 correlation was significant in shell (Szumlinski et al., 2017). Interestingly, Homer2b knockdown in shell and core of C57BL/6J mice generally reduced and enhanced methamphetamine reward/reinforcement, respectively (Szumlinski et al., 2017; Brown et al., 2020). However, constitutive Homer2 knockout mice exhibited an enhancement of methamphetamine reward/reinforcement that was not normalized by Homer2b over-expression in either core or shell, suggesting an important locus for Homer2 effects outside the NAc. (Szumlinski et al., 2017; Brown et al., 2020). These results are somewhat consistent with the present results showing no effect of intra-NAc MTEP on expression of incubated methamphetamine craving (see Section 4.4), and point to the potential importance of mGlu5 and Homer adaptations observed in other regions, including prefrontal cortex (Lominac et al., 2016), perirhinal cortex (Reichel et al., 2011; Peters et al., 2015) and dorsal striatum (Knackstedt et al., 2014).

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### 4.5. mGlu5 negative allosteric modulation and cue-induced methamphetamine seeking

To determine if mGlu5 NAMs reduce incubated methamphetamine seeking, we began with systemic administration of MTEP (1 and 3 mg/kg, i.p.). As reviewed previously, these doses produce 50-100% occupancy of mGlu5 and block numerous addiction-related behaviors (cocaine, ethanol and nicotine) while generally showing much lower efficacy when food is the reinforcer (Mihov and Hasler, 2016). Most relevant to our study, this MTEP dose range has been found to decrease cue-induced reinstatement of cocaine seeking (e.g., Kumaresan et al., 2009; Martin-Fardon et al., 2009) and context-induced cocaine seeking after ~3 weeks of abstinence (Knackstedt and Schwendt, 2016). In a study where incubation of context-induced cocaine seeking was demonstrated, there was only a trend towards reduction after 1 mg/kg MTEP, whereas a robust effect was observed after 10 mg/kg MTEP but inactive lever responding was also affected (Keck et al., 2014). Incubated context-induced cocaine seeking was also inhibited by other mGlu5 NAMs (Keck et al., 2013, 2014). In the single study of MTEP and methamphetamine, 1 and 3 mg/kg MTEP inhibited cue-and drug-induced reinstatement (Gass et al., 2009). A different mGlu5 NAM had similar effects (Watterson et al., 2013). In our study, the first to explore MTEP's effect on methamphetamine seeking after incubation, both 1 and 3 mg/kg MTEP robustly inhibited the expression of incubation but also significantly reduced inactive hole responding and locomotor activity, indicative of nonspecific behavioral depression (see below).

Due to nonspecific effects observed after systemic MTEP, we went on to perform the first study of intra-NAc MTEP on methamphetamine seeking, using a dose (1 ug/side) that, when delivered into the NAc core, blocked cue-induced cocaine reinstatement (Wang et al., 2013b; Knackstedt et al., 2014; but see Bäckström and Hyytiä, 2007) and context-induced seeking during abstinence (Knackstedt et al., 2014). We found no effect on expression of incubated methamphetamine seeking, inactive hole responding, or locomotor activity - both during early withdrawal when DHPG-induced synaptic depression is impaired (WD1 and WD4) and in late withdrawal when it has recovered to normal levels (WD21 and WD25). These results suggest that the inhibitory effects of systemic MTEP on incubated methamphetamine craving, as well as its inhibitory effects on general behavioral activity (as indicated by inactive hole responding and locomotor activity measures), are mediated outside the NAc.

It is possible that effects of systemic MTEP on incubated methamphetamine craving are secondary to its inhibition of general behavioral activity; possible brain sites for activity reduction are discussed in the next paragraph. Alternatively, systemic MTEP may reduce seeking apart from nonspecific behavioral suppression, which raises the question of where it acts in the brain to reduce seeking. This is difficult to address, as no analogous studies (i.e., studies of the effect of MTEP infusion on methamphetamine seeking during abstinence from methamphetamine self-administration) have been conducted in other brain regions. While various types of methamphetamine exposure have been shown to alter mGlu5 signaling in other brain regions, the directionality of the effects does not suggest these regions as potential sites where MTEP may be acting in our experiments. Thus, mGlu5 levels in perirhinal cortex are reduced after abstinence from extended-access methamphetamine self-administration, correlating with cognitive impairments that are ameliorated by positive allosteric modulation of mGlu5 (Reichel et al., 2011; Peters et al., 2015). Likewise, results

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from mouse models suggest an inverse relationship between mGlu5 or Homer2a/b levels and methamphetamine preference (Lominac et al., 2016). Future studies could explore MTEP effects in regions other than the NAc that are implicated in incubation of methamphetamine craving (CeA and dorsal striatum; see Introduction).

Regarding the generalized behavioral depression we observed following systemic MTEP (above), this is at odds with many prior studies although in some of these studies substantial albeit non-significant reductions (50-83% of vehicle) in inactive lever presses were reported (Mihov and Hasler, 2016). It is possible that neurobiological effects of prolonged abstinence from extended-access methamphetamine self-administration (a condition that did not apply in any studies included in the cited review) may, through adaptations in mGlu5 signaling, enhance the ability of MTEP to produce these nonspecific behavioral effects. Focusing specifically on the MTEP-induced reduction in locomotor activity, it is notable that we measured locomotion in the operant chamber during a seeking test, whereas other studies have assessed MTEP effects on locomotion in drug-naïve rats in a novel environment where reductions are less likely to be evident (e.g., Bäckström and Hyytiä, 2007; Martin-Fardon et al., 2009). One might speculate that the drug taking environment could be capturing context-induced sensitization that is sensitive to MTEP (Vezina and Leyton, 2009). When attempting to integrate the present results with studies on the effects of these MTEP doses on responding for natural rewards, it should be noted that such effects appear to depend on the nature of the test (e.g., Martin-Fardon et al., 2009; Keck et al., 2014 versus Gass et al., 2009). Furthermore, while studies of natural rewards can provide an important control for studies of methamphetamine, and are important in their own right, effects of MTEP in such studies do not account for potential interactions between prior methamphetamine exposure and mGlu5 signaling. As to where MTEP might be acting in the brain to reduce locomotor activity, this effect has been observed after infusion of *a* high concentration of MTEP (10 μg/μl) into NAc shell (Gass and Oliver, 2009) and by infusion of a different mGlu5 NAM into ventral striatum or primary motor cortex (Guimaraes et al., 2015).

Please note that for brevity Section 4.5 has focused on intra-NAc core MTEP and cue-induced psychostimulant seeking, but cocaine-primed reinstatement was reduced by intra-NAc infusion of mGlu5 NAMs (core: Schmidt et al., 2015; shell: Kumaresan et al., 2009; Schmidt et al., 2013) and potentiated by DHPG (core: Schmidt et al., 2015; shell: Schmidt et al., 2013) or an mGlu5-selective agonist (core: Wang et al., 2013b) (cue-induced reinstatement was not evaluated in these experiments).

## 5. Conclusions

mGlu5/CB1R-dependent synaptic depression in NAc core MSNs was lost during the rising phase of methamphetamine incubation but then recovered, in contrast to its persistent loss during the plateau phase of incubation of cocaine craving. Furthermore, whereas the cocaine-induced loss was accompanied by adaptations related to mGlu5 and Homer proteins, this was not the case for methamphetamine. Finally, behavioral experiments do not rule out a contribution of mGlu5 to the expression of incubated methamphetamine seeking but the locus is unlikely to be the NAc. Overall, adaptations in mGlu5 signaling in the NAc

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core appear modest compared to those observed after cocaine and are not clearly linked to regulation of the strength of cue-induced methamphetamine craving during abstinence.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Highlights

- Cue-induced methamphetamine craving incubates during forced abstinence
- mGlu5-induced synaptic depression is transiently lost in NAc core during abstinence
- Surface mGlu5, mGlu5-Homer interactions and DGL-CaMKII interactions are unaltered
- Systemic mGlu5 NAM reduces incubated seeking alongside general behavioral reduction
- Intra-NAc core mGlu5 NAM does not affect incubated methamphetamine seeking

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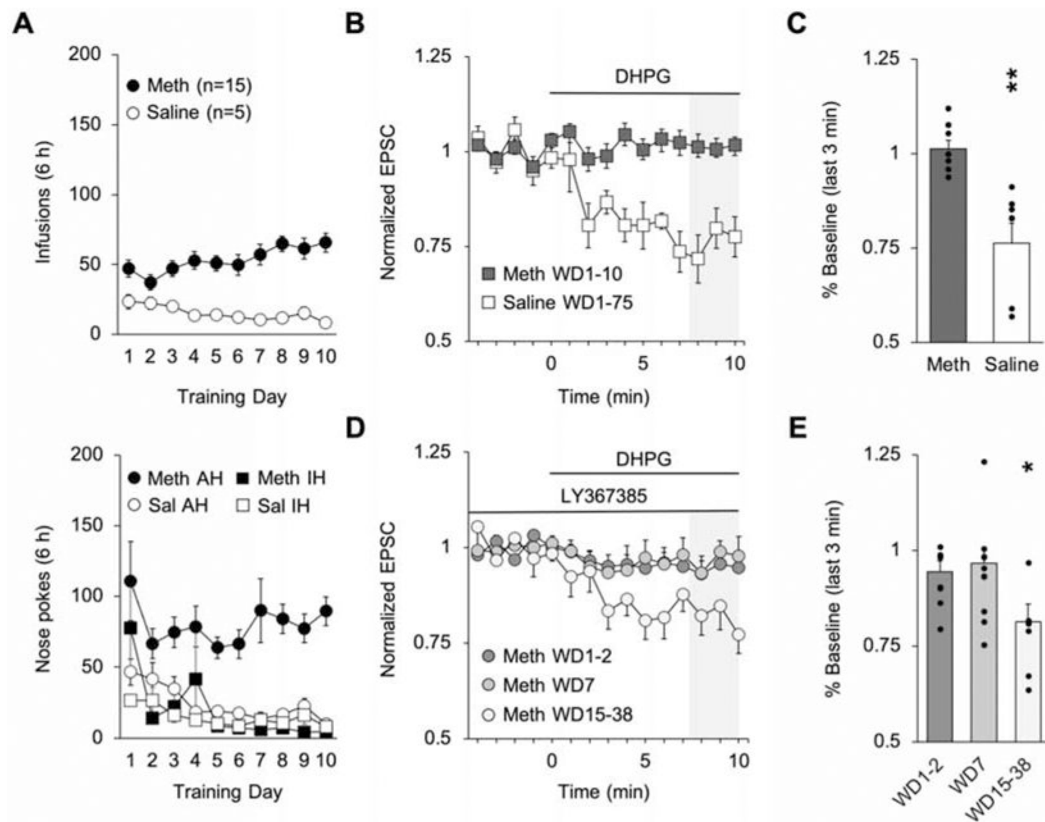


**Figure 1.**

DHPG-induced synaptic depression is impaired in the NAc core of rats during early withdrawal from extended-access methamphetamine self-administration. (**A**) Self-administration (SA) training data. AH, active hole; IH inactive hole. See Supplementary Fig. 1 for versions of this and all subsequent behavioral graphs modified to show individual data points. (**B**) DHPG-induced attenuation of $EPSC_{-70mV}$ in NAc MSN occurs in saline (Sal) controls ($n = 7$ cells/5 rats), but is absent after 1-10 days of withdrawal from methamphetamine (Meth) self-administration ($n = 8$ cells/4 rats). (**C**) The mean $EPSC_{-70mV}$ amplitude during the last 3 min of DHPG application (gray shaded area in panel **B**) expressed as a fraction of the pre-DHPG baseline for cells in each group ($*p < 0.01$, unpaired t-test). (**D**) In the presence of the mGlu1 antagonist LY36785 (50 μM), DHPG-induced attenuation of $EPSC_{-70mV}$ in NAc MSN was not detected in early withdrawal from Meth self-administration (WD1-2, $n = 7$ cells/4 rats; WD7, $n = 10$ cells/4 rats; WD, withdrawal day) but was detected in MSN from rats recorded between WD15 and WD38 ($n = 6$ cells/5 rats). (**E**) The mean $EPSC_{-70mV}$ amplitude during the last 3 min of DHPG application (gray shaded area in panel **D**) expressed as a fraction of the pre-DHPG baseline for cells in each group ($*p < 0.05$, WD15-38 versus WD1-2 and WD7; one-way ANOVA followed by LSD post-hoc test). Please see Results section for additional statistical analyses.

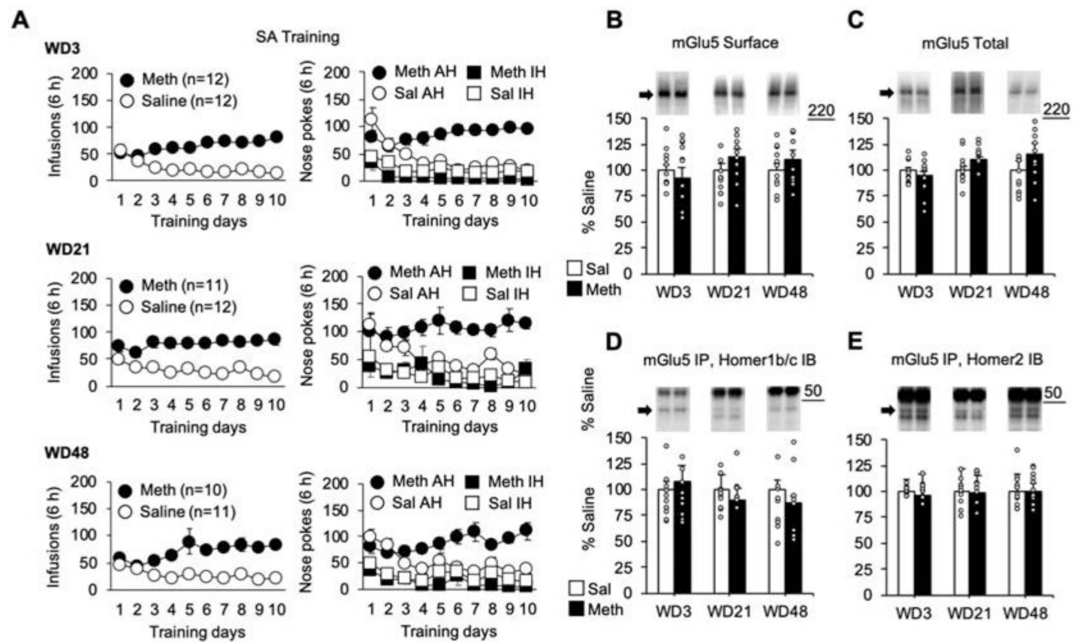


**Figure 2.**

Withdrawal from extended-access methamphetamine self-administration does not alter expression of mGlu5 or its association with Homer scaffolding proteins in the NAc core. (**A**) Self-administration (SA) training data, replotted from a previous paper that used tissue from the same rats (Murray et al., 2019). AH, active hole; IH, inactive hole. (**B, C**) Cell surface and total protein levels of mGlu5 at three time-points after discontinuing self-administration of saline (Sal; control condition) or methamphetamine (Meth): withdrawal day (WD) 3, WD21 and WD48. Data are expressed as percent control at each time-point ($n$ = 10-12 rats/group; mean ± SEM; two-way ANOVA, N.S.). (**D, E**) Co-immunoprecipitation experiments assessing the physical association between mGlu5 and Homer scaffolding proteins, expressed as percent of control ($n$ = 10-12 rats/group; mean ± SEM; two-way ANOVA, N.S.). Representative blots (cropped) are shown for a Sal rat (left) and a Meth rat (right) for each experimental group. For this and all subsequent figures, full blots are shown in Supplementary Fig. 2. Arrows indicate bands analyzed and lines indicate location of molecular weight marker. Please note that differences in optical density of bands between experiments conducted on different WDs reflect differences in exposure time. IB, immunoblot; IP, immunoprecipitation.



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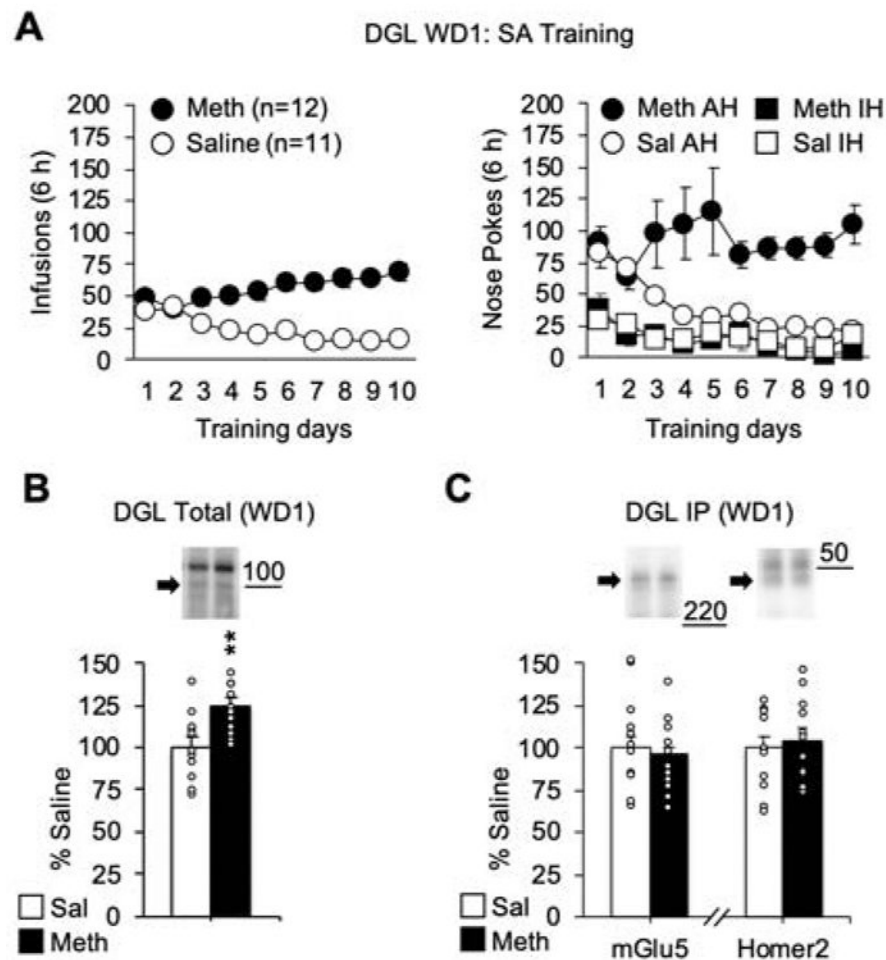


**Figure 3.**

DGL associations with mGlu5 and Homer2 in the NAc core are unaltered on withdrawal day 1 from methamphetamine self-administration. (**A**) Self-administration (SA) training data. AH, active hole; IH inactive hole. (**B**) Total protein levels of DGL expressed as percent of saline (Sal) control ($n$ = 11-12 rats/group; mean ± SEM; unpaired t-test, $^{**}p < 0.01$ vs. saline). (**C**) Co-immunoprecipitation experiments assessing the physical associations between DGL and both mGlu5 and Homer2, expressed as percent control ($n$ = 11-12 rats/group; mean ± SEM; unpaired t-tests, N.S.). Representative blots (cropped) are shown for a Sal rat (left) and a methamphetamine (Meth) rat (right) for each experimental group. Arrows indicate bands analyzed and lines indicate location of molecular weight marker. Please note that differences in optical density of bands between experiments conducted on different WDs reflect differences in exposure time. IB, immunoblot; IP, immunoprecipitation.

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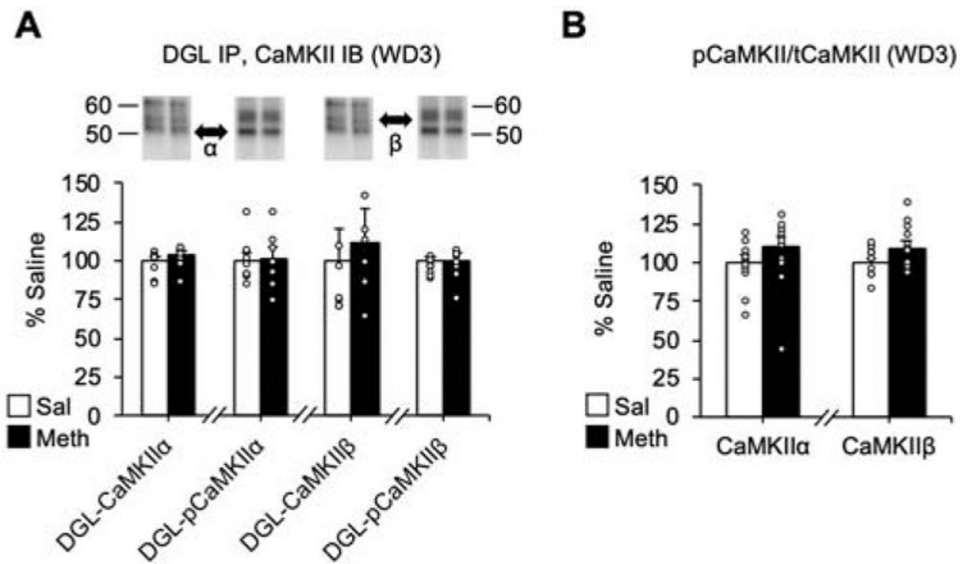


**Figure 4.**

CaMKII activation and associations with DGL in the NAc core are unchanged on withdrawal day 3 from methamphetamine self-administration. (**A**) Co-immunoprecipitation experiments assessing the physical associations between DGL and CaMKII (α and β isoforms). DGL-immunoprecipitated material was immunoblotted with antibodies detecting phosphorylated CaMKII (Thr286) and total CaMKII. Data are expressed as percent of saline (Sal) controls ($n = 12$ rats/group; mean ± SEM; unpaired t-tests, N.S.). (**B**) Ratio of phosphorylated/total CaMKII levels from saline (Sal) control and methamphetamine (Meth) rats, expressed as percent of Sal group ($n = 12$ rats/group; mean ± SEM; unpaired t-tests, N.S.). Representative blots (cropped) are shown for a Sal rat (left) and a Meth rat (right). Arrows indicate bands analyzed and lines indicate location of molecular weight marker. IB, immunoblot; IP, immunoprecipitation.

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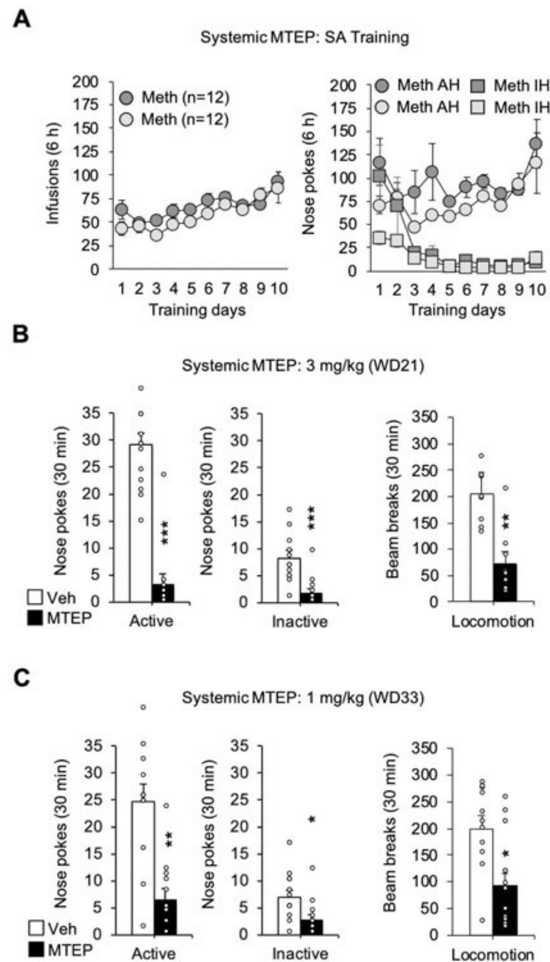


**Figure 5.**

Effect of systemic administration of the mGlu5 negative allosteric modulator MTEP on the expression of incubation of cue-induced methamphetamine craving. Rats self-administered methamphetamine (Meth; 6 h/day x 10 days; AH, active hole; IH, inactive hole) (**A**), and then received cue-induced seeking tests (30 min) during which active hole nose-pokes led to presentation of a 20-second light cue previously paired with each Meth infusion. One cohort of rats received vehicle (Veh; $n = 12$) or MTEP (3 mg/kg, i.p.; $n = 12$) 10 min before a seeking test on WD21 (**B**). This same cohort was retested with 1 mg/kg MTEP on withdrawal day (WD) 33 after interchanging MTEP and vehicle groups (**C**). Both doses of MTEP reduced AH and IH responses, as well as locomotor activity during the seeking test (unpaired t-tests, Veh vs. MTEP on WD21 and Veh vs. MTEP on WD33: *$p < 0.05$, **$p < 0.01$, ***$p < 0.0005$ vs. Veh).

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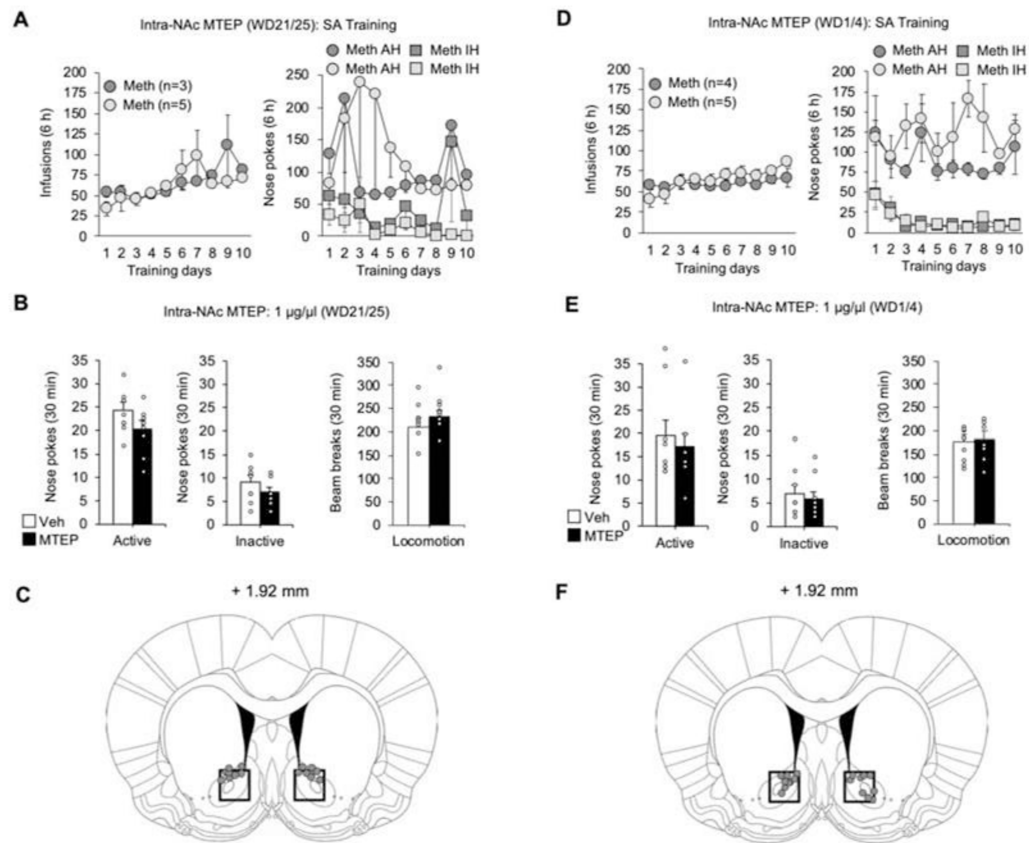


**Figure 6.**

Effect of intra-NAc core infusions of MTEP on expression of incubation of cue-induced methamphetamine craving. (**A**) Self-administration (SA) training data for rats shown in panel **B**. AH, active hole; IH inactive hole. These data and data in other panels are presented as mean ± SEM. (**B**) MTEP (1 µg/side) or Veh was infused bilaterally into the NAc core 10 min before a 30-min seeking test using a counterbalanced design in which rats that received vehicle on WD21 received MTEP on WD25, and vice versa ($n = 8$ rats; paired t-test, $p = 0.148$ MTEP vs. Veh). (**C**) Diagrams illustrate the location of cannula tips where injections were made into the NAc core. Coronal sections are adapted from (Paxinos and Watson, 2013); numbers indicate distance from bregma in the anteroposterior plane. (**D**) Self-administration (SA) training data for rats shown in panel **E**. (**E**) Using the same counterbalanced design in early withdrawal (WD1 and WD4), intra-NAc MTEP (1 µg/side) had no effect on AH or IH nose-pokes or locomotor activity ($n = 9$ rats; paired t-test, $p = 0.140$ MTEP vs. Veh). (**F**) Location of cannula tips for rats in panel E (see legend to panel **C** for details).