CaMKII modulates diacylglycerol lipase-α activity in the rat nucleus accumbens after incubation of cocaine craving

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3. Authors: Conor H. Murray1,2, Andrew D. Gaulden3, Alex B. Kawa2, Mike Milovanovic1, Aaron J. Caccamise1, Jonathan R. Funke2, Sachin Patel3 and Marina E. Wolf1,2*

1Department of Neuroscience, The Chicago Medical School at Rosalind Franklin University of Medicine and Science, North Chicago, IL USA (where the majority of this work was performed)
2Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR, USA (where some of this work was performed)
3Department of Psychiatry, Vanderbilt University, Nashville, TN


5. *Correspondence should be addressed to: Marina E. Wolf, Department of Behavioral Neuroscience, Oregon Health & Science University, 3181 SW Sam Jackson Park Rd, Portland, OR 97239, USA. Tel: 503-494-9797; Fax: (503) 494-6877; Email: wolfmar@ohsu.edu

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Present address of Dr. Conor H. Murray: Department of Psychiatry and Biobehavioral Sciences, University of California, Los Angeles, Los Angeles, CA

Present address of Aaron J. Caccamise: Department of Biomedical Sciences, Marquette University, Milwaukee, WI

Present address of Andrew D. Gaulden: Neuroscience Graduate Program, University of Cincinnati, Cincinnati, OH

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Abstract

Relapse is a major challenge to the treatment of substance use disorders. A progressive increase in cue-induced drug craving, termed incubation of craving, is observed after withdrawal from multiple drugs of abuse in humans and rodents. Incubation of cocaine craving involves strengthening of excitatory synapses onto nucleus accumbens (NAc) medium spiny neurons via postsynaptic accumulation of high-conductance Ca\(^2+\)-permeable AMPA receptors. This enhances reactivity to drug-associated cues and is required for the expression of incubation. Additionally, incubation of cocaine craving is associated with loss of the synaptic depression normally triggered by stimulation of metabotropic glutamate receptor 5 (mGlu5), leading to endocannabinoid production, and expressed presynaptically via cannabinoid receptor 1 activation. Previous studies have found alterations in mGlu5 and Homer proteins associated with the loss of this synaptic depression. Here we conducted co-immunoprecipitation studies to investigate associations of diacylglycerol lipase-\(\alpha\) (DGL), which catalyzes formation of the endocannabinoid 2-arachidonylglycerol (2-AG), with mGlu5 and Homer proteins. Although these interactions were unchanged in the NAc core at incubation-relevant withdrawal times, the association of DGL with total and phosphorylated Ca\(^2+\)/calmodulin-dependent protein kinase II\(\alpha\) (CaMKII\(\alpha\)) and CaMKII\(\beta\) was increased. This would be predicted, based on other studies, to inhibit DGL activity and therefore 2-AG production. This was confirmed by measuring DGL enzymatic activity. However, the magnitude of DGL inhibition did not correlate with the magnitude of incubation of craving for individual rats. These results suggest that CaMKII contributes to the loss of mGlu5-dependent synaptic depression after incubation, but the functional significance of this loss remains unclear.
Significance Statement

Cue-induced drug craving progressively intensifies or incubates after withdrawal from extended-access drug self-administration, augmenting relapse vulnerability. Incubation of cocaine craving in rats is accompanied by robust plasticity in the NAc including strengthening of excitatory synapses via postsynaptic AMPAR plasticity and the loss of endocannabinoid-dependent synaptic depression. Our results identify a novel mechanism that may account for this loss of synaptic depression, namely reduced activity of DGL, the enzyme that produces the endocannabinoid 2-AG, along with increased physical association of this enzyme with CaMKII, an association predicted to reduce DGL’s enzymatic activity. These findings expand our understanding of mechanisms underlying cocaine-induced alterations in endocannabinoid-dependent synaptic depression.
Introduction

Relapse is often triggered by exposure to drug-associated cues even after prolonged abstinence (O’Brien, 2005). During abstinence, cue-induced craving intensifies over time, a phenomenon termed incubation of craving. Incubation of cocaine craving has been well-documented in rats (Pickens et al., 2011) and also studied in humans (Parvaz et al., 2016), where cue-induced craving peaks between 1 and 6 months before declining by 1 year of abstinence. This trajectory is similar in rats, where craving is maximal after ~1 month, remains high for months, and then declines, although it remains significantly elevated, compared to withdrawal day 1, after 180 days (Lu et al., 2004).

Incubation involves adaptations in regions related to motivation and reward including the nucleus accumbens (NAc) (Li et al., 2015; Wolf, 2016; Wright and Dong, 2020). The NAc serves as an interface between cortical and limbic inputs and motor-related outputs, contributing to the execution of motivated behaviors (Meredith et al., 2008; Sesack and Grace, 2010). Medium spiny neurons (MSN), the principal cell type and projection neuron of the NAc, receive glutamatergic projections from cortical and limbic regions. Incubation of cocaine craving is associated with incorporation of high-conductance calcium-permeable AMPA receptors (CP-AMPARs) into excitatory synapses onto NAc core and shell MSNs (Wolf, 2016). In core, this begins after ~1 month of withdrawal (Wolf and Tseng, 2012). Once present, CP-AMPAR activation is required for expression of incubation (Conrad et al., 2008; Lee et al., 2013; Loweth et al., 2014; Ma et al., 2014). It is theoretically possible that expression of incubation also involves a reduction in mechanisms that normally constrain excitatory transmission in the NAc, such as long-term depression (LTD).

A well-characterized form of LTD in the NAc is triggered by metabotropic glutamate receptor 5 (mGlu5), leading to generation of endocannabinoids (eCB) that act as retrograde messengers to stimulate presynaptic cannabinoid receptor 1 (CB1), resulting in reduction of glutamate release from excitatory afferents in the NAc (Robbe et al., 2001; Robbe et al., 2002;
The best characterized eCB ligands are anandamide (AEA) and 2-arachidonylglycerol (2-AG) (Ohno-Shosaku and Kano, 2014). Although some evidence implicates AEA in mediating eCB-LTD in dorsal striatum (Ronesi et al., 2004; Ade and Lovinger, 2007) and both presynaptic CB1R- and postsynaptic TRPV1-dependent LTD in NAc (Grueter et al., 2010), we focused on 2-AG because its contribution to eCB-LTD in the NAc is linked to regulation of motivated behavior (Novak et al., 2010; Bilbao et al., 2020; Folkes et al., 2020). Formation of 2-AG at excitatory synapses in the NAc is mediated by the 2-AG signalosome – a postsynaptic multiprotein complex comprised of mGlu5, enzymes involved in 2-AG production, and Homer scaffolding proteins (Piomelli, 2014). Stimulation of mGlu5 activates phospholipase-C which cleaves plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP2) to form 1,2-diacylglycerol (DAG), a second messenger that is hydrolyzed by DAG lipase-α (DGL) to yield 2-AG, which acts on presynaptic CB1Rs (Jung et al., 2007).

NAc synaptic depression, evoked electrically or via pharmacological activation of mGlu5, is abolished 1 day after acute non-contingent cocaine (Fourgeaud et al., 2004; Grueter et al., 2010), 14 days after repeated non-contingent cocaine injections (Huang et al., 2011), and after >35 days of withdrawal from extended-access cocaine self-administration (McCutcheon et al., 2011; Scheyer et al., 2014). Neither acute cocaine (Fourgeaud et al., 2004) nor incubation of craving (McCutcheon et al., 2011) impairs CB1R function, implying a postsynaptic locus. Indeed, alterations in the 2-AG signalosome have been observed after regimens leading to loss of this synaptic depression, namely a reduction in mGlu5 surface expression (Fourgeaud et al., 2004; Huang et al., 2011; Loweth et al., 2014) and altered Homer levels (Fourgeaud et al., 2004) or Homer-mGlu5 associations (Loweth et al., 2014). However, no studies have assessed the enzymatic activity of DGL or its associations within the 2-AG signalosome after cocaine exposure.
Here, we used co-immunoprecipitation to assess associations between DGL and other components of the signalosome and also assayed enzymatic activity of DGL in NAc core. Compared to saline controls, associations between total and phosphorylated Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) and DGL were increased at incubation-relevant withdrawal times. This was accompanied by decreased DGL activity, as predicted by studies demonstrating that CaMKII phosphorylation reduces DGL activity (Shonesy et al., 2013; Park et al., 2017). However, our results fall short of demonstrating that loss of this synaptic depression contributes to incubated craving. In fact, other findings argue that this synaptic depression promotes reward seeking (Novak et al., 2010; Bilbao et al., 2020; Mitra et al., 2021).

**Materials and methods**

**Subjects and Surgery**

All animal procedures were performed in accordance with the [Author University] animal care committee's regulations and the US Public Health Service 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 (80/10 mg/kg, i.p.). Prior to surgery, rats received an injection of the analgesic Banamine (flunixin meglumine, 2.5 mg/kg, s.c.) to minimize post-operative discomfort. Thereafter, rats were single-housed for 7 days before beginning self-administration training. Catheters were flushed daily with Cefazolin (100 mg/ml in sterile 0.9% saline; 0.15 ml) during this period to prevent infection and maintain catheter patency.

**Drug Self-Administration and Cue-Induced Seeking Tests**

Cocaine was obtained from NIDA and dissolved in 0.9% saline. Rats self-administered cocaine (0.5 mg/kg/infusion in a 100 μl/kg volume over 3 sec) or saline, during 10 consecutive sessions.
(6 hours/day starting at Zeitgeber Time ZT15), 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 forced abstinence in home cages and were handled weekly until being used for experiments on or after withdrawal day (WD) 40. This regimen reliably results in incubation of cocaine craving that is robustly expressed during this withdrawal period (Conrad et al., 2008; Loweth et al., 2014). Therefore, we did not assess incubation of craving in rats used some of our biochemical studies. However, two cohorts of rats, used for DGL activity assays, underwent cue-induced seeking tests after the same cocaine self-administration regimen to verify incubation of craving. During these seeking tests, responses in the previously active hole delivered the light cue but no drug. Responding under these conditions is our operational measure of cue-induced cocaine craving. One cohort underwent seeking tests on WD1 and WD40, and the other on WD1 and WD42.

Co-immunoprecipitation

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 a 1.5 mm biopsy punch (Thermo Fisher Scientific, Waltham, MA). NAc punches were homogenized in 500 μl of lysis buffer consisting of 25 mM HEPES pH 7.4, 500 mM NaCl, 2 mM EDTA, 20 mM NaF, 1 mM PMSF, 0.1% NP-40 (v/v), 1 mM NaOV, 1X protease inhibitor cocktail Set 1 (Millipore), 1% Triton and 0.5% deoxycholate. 3 μg of DGL-α antibody (a generous gift from Dr. Ken Mackie, Indiana University, Bloomington), validated in previous studies (Katona et al., 2006; Keimpema et al., 2010), was incubated overnight at 4°C with protein A/G agarose slurry. The pellet containing antibody-coated beads was then incubated overnight at 4°C with 100 μg of NAc core tissue homogenate. The agarose-bound antibody was pelleted by centrifugation to isolate the bound
fraction (two rounds of immunoprecipitation were performed to maximize recovery). The combined bound fraction 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. Samples were run on 4-12% Bis-Tris gels (Bio-Rad) and transferred to PVDF membranes for immunoblotting. Membranes were then washed in dH₂O and blocked with 1% normal goat serum with 5% nonfat dry milk in 0.05% Tween-20 in TBS, pH 7.4, for 1 h at room temperature. Membranes were incubated overnight at 4°C with antibodies to 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), p-Ser (1:120, sc-81514, Santa Cruz Biotechnology, Dallas, TX), Homer1b/c (1:200, sc-55463, Santa Cruz Biotechnology, Dallas, TX), or Homer2 (1:500, H00009455-B01P, Abnova, Taipei, Taiwan). The mGlu5 dimer band (~260 kDa) was analyzed because it represents the functional pool of these receptors (Jingami et al., 2003).

GAPDH was used as a loading control when immunoblotting total tissue homogenates. Secondary antibodies to rabbit or mouse IgG light chain were used (211-032-171 or 115-035-174, Jackson ImmunoResearch Laboratories, West Grove, PA) in co-IP studies 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 with TotalLab (Life Sciences Analysis Essentials). Data were excluded only if imperfections in the gel or blot interfered with analysis. Across all blots, data points for two samples were removed from analysis. These consisted of one cocaine and one saline data point from the synaptoneurosome preparation.

**Synaptoneurosome preparation**

Rats were decapitated and bilateral NAc tissue (primarily core) was rapidly dissected from a 2 mm coronal slice prepared with a brain matrix (ASI Instruments, Warren, MI). Immediately
following dissection, synaptoneurosomes were prepared according to published protocols (Most et al., 2015; Workman et al., 2015; Werner et al., 2018). NAc punches were homogenized in 500 μl of homogenization buffer [HB; 20 mM HEPES, 0.5 mM EGTA, 1X Proteasome Inhibitor Cocktail Set 1 (Millipore)]. Homogenates were passed through a 100-μm-pore filter and then through a 5-μm-pore filter (Millipore; both filters were prewashed with HB). After homogenates were passed through each filter, filters were washed with 50 μl of HB, and the washes were added to homogenates to maximize yield. Homogenates were then centrifuged at 14,000 μg for 20 min at 4°C. The pellet, which contains the synaptoneurosomes, was frozen on dry ice, stored at -80°C, and ultimately lysed in lysis buffer [0.605 g Tris-HCl, 0.25 g sodium deoxycholate, 0.876 g NaCl, 1 μg/ml PMSF, 5 ml of 20% SDS, and 1X Protease Inhibitor Cocktail Set 1 (Millipore) in 100 ml dH2O] for immunoblotting. NAc synaptoneurosomes prepared from individual rats (10 μg protein/lane) were mixed 1:1 with 2x sample treatment buffer (161–0737, Bio-Rad) and analyzed by SDS-PAGE and immunoblotting. Beta-tubulin was used as a loading control.

DGL Activity

Rats were decapitated and bilateral NAc was rapidly dissected as described above for synaptoneurosome preparation. Lipase activity was subsequently assayed using fluorescence resonance energy transfer (FRET) as adapted from a previous report (Johnston et al., 2012). Briefly, 600 ul of homogenization buffer [50 mM HEPES pH 7.0, 250 mM sucrose, 1 mM Roche PhosStop (Millipore Sigma, Burlington, MA), 0.5 mM tris (2-carboxyethyl)phosphine (TCEP), 0.01 mM leupeptin, 0.001 mM pepsin] was added to punched NAc tissue and macerated by a 27-gauge needle. The solution was homogenized with an electric pestle and then centrifuged for 30 minutes. Supernatant was removed from the pellet and discarded. The pellet was resuspended in 300 ul of membrane resuspension buffer (same as homogenization buffer but without sucrose), then vortexed and briefly sonicated on ice. Using a Bradford assay, protein
concentration of the punched NAc tissue was determined. A 96-well plate was then prepared on ice with protein samples in triplicate at 40 ug total protein per well. As a negative control, tissue from cerebellum was run in the absence and presence of the DAGL inhibitor DO34 (2 uM). The FRET reporter compound (compound #17 in Johnston et al., 2012) (MRJ20), a FRET-based substrate for DGL, was made in-house (Vanderbilt Chemical Synthesis Core, Nashville, TN). MRJ20 was added to the assay buffer (50 mM HEPES pH 7.0, 0.5 mM TCEP, 1 mM PhosStop) to reach a final concentration of 2 uM. The assay buffer was then vortexed and sonicated on ice for 2 minutes. The plate was then taken to a pre-warmed (BioTek Synergy H4) fluorescent plate reader, and the recently sonicated assay buffer was pipetted into the wells at 150 ul. The reader maintained a constant 37°C temperature and took fluorescent measurements every minute for 30 minutes. Samples were then analyzed for Relative Fluorescent Units (RFU) over the 30 minutes, and a line of best fit for RFU/minute determined relative slopes (ΔRFU/minute) for each sample (GraphPad Prism).

**Statistical Analyses**

Data are expressed as mean ± SEM. Groups were compared with unpaired (between group) or paired (within group) t-tests. Pearson correlation coefficients were used to assess the linear correlation between the magnitude of incubation of craving and DGL activity. Differences between experimental groups were considered statistically significant when p < 0.05.

**Results**

*Incubation of cocaine craving is not associated with changes in DGL expression or its association with mGlu5 receptors and Homer scaffolding proteins*

Three cohorts of rats underwent extended-access saline or cocaine self-administration (10 sessions of 6 hours/day; the three cohorts are depicted in Fig. 1A top, Fig. 1A bottom, and Fig. 9.
In all cases, biochemical analyses of NAc were conducted on or after WD40, when incubation of cocaine craving has plateaued (Lu et al., 2004) and stable CP-AMPAR elevation (Wolf and Tseng, 2012) and loss of mGlu5-dependent synaptic depression (McCutcheon et al., 2011; Scheyer et al., 2018) have been observed. The core subregion was analyzed, due to its critical role in the incubation of cocaine craving (Conrad et al., 2008; Guillem et al., 2014; Loweth et al., 2014). In NAc homogenates prepared from the first cohort of rats (Fig. 1A top), we found that DGL levels did not differ between cocaine and saline groups (Fig. 1B top). NAc tissue from the second cohort (Fig. 1A bottom) was used to prepare synaptoneurosomes, a subcellular fraction enriched for the postsynaptic density (Hollingsworth et al., 1985; Quinlan et al., 1999; Most et al., 2015; Workman et al., 2015), on WD40. DGL levels in NAc synaptoneurosomes likewise did not differ between cocaine and saline groups (Fig. 1B bottom).

NAc tissue from the third cohort was used to assess DGL’s associations with Homer2 and mGlu5 by immunoprecipitating DGL and measuring bound Homer and mGlu5 levels (Fig. 2). mGlu5 and its association with Homer scaffolding proteins are necessary for 2-AG signaling at excitatory synapses (Roloff et al., 2010) and DGL associations with Homer scaffolding proteins are required for membrane-bound DGL activity (Jung et al., 2007). Although DGL does not directly bind to mGlu5, we detected bound mGlu5 in DGL immune complexes (Fig. 2B and Extended Data Fig. 2-1A) and also detected bound DGL in mGlu5 immune complexes (Extended Data Fig. 2-1A), presumably via intermediate Homer interactions. Homer1b/c was not detected in our DGL immune complexes in NAc core (Extended Data Fig. 2-1B,C). We therefore focused on Homer2, which has been implicated in cocaine’s actions in the NAc (Szumlinski et al., 2008). DGL associations with Homer2 and mGlu5 were unaffected by cocaine self-administration and prolonged withdrawal (Fig. 2B). These results suggest that impairments in physical association between these components of the 2-AG signalosome are
not responsible for impaired mGlu5-dependent synaptic depression after incubation of cocaine craving.

**Incubation of cocaine craving is associated with increased interactions between CaMKII and DGL**

The loss of mGlu5-dependent synaptic depression after incubation of cocaine craving may be due to interactions that modulate DGL activity. For instance, CaMKIIα and CaMKIIβ bind DGL, and 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). Furthermore, increased CaMKII activity is implicated in stress-induced impairment of endocannabinoid-mediated synaptic depression in the lateral habenula (Park et al., 2017). To determine whether interactions between DGL and CaMKII are affected during late withdrawal from cocaine self-administration, different aliquots of the same tissue samples used for the experiment depicted in **Fig. 2** were used to immunoprecipitate DGL and measure bound CaMKII by immunoblotting.

DGL associations with total CaMKIIα (t_{14}=2.59, \( p=0.021 \)), total CaMKIIβ (t_{14}=2.30, \( p=0.037 \)), phosphorylated (Thr286) CaMKIIα (t_{14}=2.14, \( p=0.050 \)) and phosphorylated CaMKIIβ (t_{14}=2.59, \( p=0.014 \)) were all increased in the cocaine group relative to saline controls (**Fig. 3**). CaMKII phosphorylates DGL on two serine residues (Shonesy et al., 2013). Therefore, to determine if CaMKII associations with DGL also led to detectable increases in DGL phosphorylation, other aliquots of these DGL immunoprecipitated samples were subjected to SDS-PAGE followed by immunoblotting with an antibody that recognizes phosphorylated serine residues. A band was detected at the molecular weight of DGL (~100 kDa) but its intensity did not differ between saline and cocaine groups (Extended Data **Fig. 3-1**). However, these data are not conclusive.

Whereas the lysis buffer for initial homogenization of tissue contained a reagent for inhibiting
phosphatases (20 mM NaF), our washing buffer for processing the bound fraction during the
immunoprecipitation protocol did not, because at the time the tissue was processed, we did not
intend to use it to detect protein phosphorylation. Therefore, DGL might have been
dephosphorylated during the lengthy immunoprecipitation protocol (see Materials and
Methods).

DGL activity is reduced in the NAc core after incubation of cocaine craving

Based on earlier studies (Shonesy et al., 2013; Park et al., 2017), the observation of increased
CaMKII association with DGL suggests that DGL activity will be reduced after prolonged
withdrawal from extended-access cocaine self-administration. To test this, a new cohort of rats
was generated (9 saline and 9 cocaine) (Fig. 4A). Cue-induced seeking tests were conducted
on WD1 and WD40 to confirm incubation of craving ($t_{16}=3.748, p=0.002$; Fig. 4B). On WD45,
NAc tissue (primarily core) was harvested and snap frozen. DGL activity was subsequently
assayed using FRET (Johnston et al., 2012). Compared to saline controls, the cocaine group
was found to have reduced DGL activity ($t_{14}=2.54, p=0.024$; Fig. 4C). Furthermore, in this initial
cohort there was a trend for an inverse relationship between DGL activity in late withdrawal
(WD40) and the magnitude of incubation of craving for rats in the cocaine group, expressed as
the ratio of active hole nose-pokes during 30 min seeking tests conducted on WD40 versus
WD1 for each rat ($p=0.08$; Fig. 4D).

We attempted to replicate this using an additional cohort of late withdrawal cocaine rats (Fig.
5A) for which incubation of craving was demonstrated (Fig. 5B). In this second cohort, we
observed no significant relationship between DGL activity and the magnitude of incubation (Fig.
5D). We also assessed this relationship after combining the two cohorts. The second cohort
was run over a year after the first, and required re-establishment of the assay. Perhaps for this
reason, absolute RFU/min values for the two cohorts differed substantially (compare Fig. 4C
with Fig. 5C and Extended Data Fig. 5-1). Because the second cohort did not contain saline controls (they were not run because our focus was on understanding the correlation between DGL activity and incubation in the cocaine group), we could not combine the two cohorts by normalizing to saline control values. As an alternative approach, mean values (ΔRFU/minute) were calculated for each cohort, and individual rat values were expressed as percent of the cohort mean. We found no significant difference between DGL activity and incubation when the cohorts were combined in this manner (Fig. 5E). Overall, these results show that DGL activity is reduced in the NAc core after prolonged withdrawal from extended-access cocaine self-administration, and that the level of DGL activity in late withdrawal cocaine rats does not correlate with the magnitude of incubation.

Discussion

Enduring vulnerability to relapse is encoded by synaptic plasticity in the NAc (Luscher, 2016; Scofield et al., 2016; Wolf, 2016). An important form of synaptic depression in the NAc is elicited by postsynaptic mGlu5 stimulation, resulting in generation of eCBs which travel in a retrograde fashion to stimulate presynaptic CB1 receptors and reduce glutamate release (see Introduction). After incubation of cocaine craving, this mGlu5/CB1R-dependent synaptic depression is lost in the NAc core (McCutcheon et al., 2011; Scheyer et al., 2014; Scheyer et al., 2018). This is accompanied by reduced surface mGlu5 and reduced mGlu5-Homer interactions (Loweth et al., 2014). Coupled with intact synaptic depression in the NAc of cocaine incubated animals in response to direct CB1R activation (McCutcheon et al., 2011), these results suggest a postsynaptic locus for the loss of mGlu5/CB1R-dependent synaptic depression.

Here, we assessed the postsynaptic mGlu5 signaling complex that mediates the generation of 2-AG [the 2-AG signalosome; (Piomelli, 2014)] in the NAc core subregion. No changes were found in DGL protein levels or its physical association with mGlu5 and Homer
proteins (Figs. 1 and 2), indicating that loss of mGlu5/CB1R-dependent synaptic depression does not reflect reductions in physical associations between these components of the 2AG-signalasome. However, we observed increased associations between DGL and CaMKII (Fig. 3), an interaction previously demonstrated to result in DGL phosphorylation and reduced DGL activity (Shonesy et al., 2013). For these biochemical studies (Figs. 1-3), we did not demonstrate incubation of craving in the rats utilized, but we made measurements at withdrawal times (WD>40) when incubation is reliably demonstrated after the same regimen (e.g., (Conrad et al., 2008; Loweth et al., 2014); Figs. 4 and 5 herein). Next, we measured enzymatic activity of DGL, the enzyme that catalyzes 2-AG production (Piomelli, 2014). As might be expected from our observation of increased DGL-CaMKII association and previous results mentioned above (Shonesy et al., 2013), we observed a decrease in DGL activity in NAc core tissue obtained from rats demonstrated to have undergone incubation of cocaine craving compared to saline controls (Fig. 4). Although we lack evidence for a causal relationship between the increased DGL-CaMKII association and the observed reduction in DGL activity, it is tempting to speculate that the increased DGL-CaMKII association may contribute to the loss of mGlu5/CB1R-dependent synaptic depression after prolonged cocaine withdrawal. Finally, we found that DGL activity in late withdrawal (WD40 or WD42) was not significantly correlated with the magnitude of incubation (expressed as the ratio of active hole nose-pokes during seeking tests conducted on WD40 or WD42 versus WD1) for individual cocaine rats (Figs. 4 and 5).

We note that there are several limitations to our study. First, we did not include cocaine WD1 rats or yoked cocaine controls in our biochemical studies. These groups would be necessary to establish that observed changes are specific to incubation and not merely the result of cocaine self-administration or cocaine exposure, respectively. A second limitation of our study is that the use of homogenates or synaptoneurosomes precludes detection of potentially cell type-specific (D1 vs D2 MSN) or input-specific adaptations in DGL activity. This could be one reason why we failed to identify a clear role for DGL adaptations in the incubation
of craving. For example, one prominent incubation-related adaptation, synaptic insertion of CP-AMPARs, can occur on both D1 and D2 MSNs (Terrier et al., 2016; Wolf, 2016) but demonstrates input specificity (Lee et al., 2013; Ma et al., 2014; Pascoli et al., 2014; Terrier et al., 2016). A third limitation is that saline controls were not included in the experiment shown in Fig. 5, as discussed extensively in Results. Finally, male rats were used for this study, in accordance with the NIH grant supporting this project. Given recent work showing sex differences in the incubation of cocaine craving (Kerstetter et al., 2008; Nicolas et al., 2019), future studies should explore similar questions in female rats.

Role for CaMKII in loss of mGlu5/CB1R-dependent synaptic depression after incubation of cocaine craving

CaMKII is a Ca\(^{2+}\) activated enzyme that is best known for its critical role in early and late phases of long-term potentiation (LTP) but also participates in many other mechanisms regulating synaptic function (Lisman et al., 2002; Lisman et al., 2012; Hell, 2014; Bayer and Schulman, 2019). There are four isoforms derived from four genes (α, β, γ and δ), with the CaMKII\(\alpha\) and CaMKII\(\beta\) isoforms mainly expressed in the brain. The regulatory site of each isoform contains a phosphorylation site (Thr286 for CaMKII\(\alpha\) and Thr287 for CaMKII\(\beta\)) that can generate autonomous kinase activity (see reviews cited above).

We explored a role for CaMKII in the loss of mGlu5/CB1R-dependent synaptic depression after incubation of craving based on studies in dorsal striatum showing that CaMKII\(\alpha\) and CaMKII\(\beta\) bind DGL, 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). Furthermore, increased CaMKII activity is implicated in stress-induced impairment of endocannabinoid-mediated synaptic depression in the lateral habenula (Park et al., 2017). Consistent with these prior findings, we
found increased associations between DGL and both total and phosphorylated CaMKIIα and CaMKIIβ in NAc core tissue from cocaine rats (examined at an incubation-relevant late withdrawal time) versus saline controls. Furthermore, we observed a significant reduction in DGL activity in rats that had undergone incubation of craving compared to saline controls. These results suggest that CaMKII contributes to loss of mGlu5/CB1R-dependent synaptic depression after incubation. In theory, slice physiology experiments could directly test this relationship by determining if acute CaMKII inhibition restores mGlu5/CB1R-dependent synaptic depression in NAc core MSNs from rats that have undergone incubation of cocaine craving. A practical concern is that commercially available inhibitors are not effective against the autophosphorylated enzyme, although new inhibitors are under development (Nassal et al., 2020).

Our findings are not the first to implicate CaMKII in neuroadaptations in the NAc after cocaine self-administration. For example, CaMKIIα but not CaMKIIβ mRNA levels increased in NAc shell after cocaine self-administration and in cocaine yoked controls relative to saline self-administering animals, likely via histone H3 acetylation; the increase correlated with motivation for cocaine measured using a progressive ratio schedule, while knockdown of CaMKIIα in the NAc shell decreased motivation for cocaine (Wang et al., 2010). In another study, cocaine self-administration followed by extinction training did not alter CaMKII phosphorylation in NAc shell or core, however, cocaine-primed reinstatement was associated with an increase in NAc shell CaMKII phosphorylation, and intra-NAc shell CaMKII inhibition via KN-93 attenuated reinstatement (Anderson et al., 2008). During abstinence, increased CaMKIIα phosphorylation in the NAc was found 24 h after discontinuing short-access cocaine self-administration relative to yoked saline controls, but this was not observed in a yoked cocaine group, immediately after discontinuing self-administration, or after self-administration and 7 days of abstinence (Caffino et al., 2014). In an incubation of cocaine craving study in which rats self-administered saline or
cocaine under extended-access conditions, increased ratios of phosphorylated to total CaMKIIα and CaMKIIβ were found in NAc postsynaptic density fractions from cocaine rats on WD45 versus cocaine rats on WD1 and saline rats (Ferrario et al., 2011). CaMKII is also implicated in responding to other drugs of abuse. For example, CaMKII activity in NAc shell is required for the enhanced amphetamine intake and sensitized locomotor responding observed in rats previously sensitized to non-contingent amphetamine (Loweth et al., 2008; Loweth et al., 2010; Loweth et al., 2013).

How might CaMKII become activated during abstinence? While classical studies identified CaMKII activation following acute stimulation in slices (i.e., in conjunction with LTP), CaMKII may also contribute to homeostatic plasticity (Chen et al., 2014; Hell, 2014). Most notably, in cultured hippocampal neurons, prolonged AMPAR blockade (24 h) increases CaMKIIβ activity, which in turn scales up synaptic levels of homomeric GluA1 receptors; when blockade is discontinued, a presynaptic component of homeostatic adaptation (increased release probability) is revealed in response to Ca$^{2+}$ entry via CP-AMPARs and resulting retrograde signaling (Thiagarajan et al., 2002; Thiagarajan et al., 2005; Groth et al., 2011). It has been suggested that AMPAR upregulation during cocaine abstinence reflects a homeostatic scaling up of synaptic strength, triggered by relative inactivity during abstinence versus the period of cocaine exposure; while this may normalize synaptic transmission during abstinence, it has the unfortunate consequence of enhancing excitatory responses to drug-related cues when they are presented after abstinence (Boudreau and Wolf, 2005; Conrad et al., 2008; Sun and Wolf, 2009). Likewise, the observation that sustained food restriction upregulates reward responding through upregulation of NAc CP-AMPARs has been hypothesized to represent a homeostatic response (Carr, 2020). The finding of increased CaMKII phosphorylation in NAc postsynaptic density fractions prepared after incubation of cocaine craving (Ferrario et al., 2011) combined with increased phosphorylation of the DGL-bound CaMKII pool in the present study.
suggests that CaMKII may play a role after cocaine withdrawal that to some extent parallels that
observed after AMPAR blockade in hippocampal neurons (above). The reduction in DGL activity
that is observed in parallel with CaMKII activation could be “collateral damage” or it could
contribute to the homeostatic response by removing a braking effect on excitatory synaptic
transmission. It is also possible that CaMKII activation after incubation of craving is functionally
relevant to DGL regulation, but that different homeostatic cascades are involved in CP-AMPAR
upregulation (Wang et al., 2018; Loweth et al., 2019).

Relationship between mGlu5/CB1-dependent synaptic depression and cocaine seeking
As DGL activity was reduced in rats that underwent incubation of cocaine craving compared to
saline controls (above), we tested for a correlation between DGL activity and the magnitude of
incubation of craving (expressed relative to baseline craving on withdrawal day 1) for individual
cocaine rats in two separate cohorts. Because expression of incubation requires excitatory
synaptic transmission onto NAc core MSN (Wolf, 2016), and DGL activity leading to 2-AG
formation leads to reduced glutamate transmission (i.e., synaptic depression), we hypothesized
that cocaine rats with the lowest DGL activity might show the strongest incubation of craving.
However, we failed to observe a significant correlation between DGL activity and cocaine
seeking. This does not rule out a contribution of reduced DGL activity to incubation. It is
possible that reduction of DGL activity below a threshold level plays a permissive role in the
enhancement of cue-induced cocaine seeking.

However, a number of lines of evidence argue against this possibility. First,
mGlu5/CB1R-dependent synaptic depression has been linked to the promotion of reward
seeking in mice with constitutive knockdown of mGlu5 in D1R-expressing MSN. These mice do
not express mGlu5-dependent synaptic depression or demonstrate cue-induced reinstatement
to cocaine or saccharin; however, after 2-AG elevation with monoacylglycerol lipase (MAGL)
inhibition, reinstatement to saccharin seeking is restored (Novak et al., 2010; Bilbao et al.,
Furthermore, it was found that DGL and MAGL were dysregulated after prolonged abstinence (30 days) from cocaine self-administration, and that intra-NAc administration of a DGL inhibitor at this withdrawal time reduced cue-induced cocaine craving, while inhibition of MAGL (to increase 2-AG levels) had the opposite effect (Mitra et al., 2021). We note that DGL levels were increased at WD30 in this prior study (Mitra et al., 2021), in contrast to the lack of change reported here. This is most likely due to the fact that Mitra and colleagues measured DGL in NAc shell whereas we analyzed NAc core; there were also significant differences in the cocaine self-administration regimen. While the work cited above focused on synaptic depression expressed presynaptically via CB1R stimulation, other studies link mGlu5-dependent reinstatement of cocaine seeking to postsynaptically expressed LTD mediated via AMPAR internalization (Schmidt et al., 2013; Benneyworth et al., 2019).

Additional evidence against a causal relationship between loss of mGlu5/CB1R-dependent synaptic depression and elevation of cue-induced drug craving comes from studies on the incubation of methamphetamine craving. DHPG-induced, mGlu5-dependent synaptic depression in the NAc core was lost during the first week of abstinence from extended-access methamphetamine self-administration, which corresponds to the rising phase of incubation of methamphetamine craving, but at later withdrawal times this synaptic depression was restored whereas craving remained at high ‘incubated’ levels (Murray et al., 2021). These results argue against a role for mGlu5/CB1R-dependent synaptic depression in maintaining incubated craving for methamphetamine. It is also interesting to note that associations between CaMKII and DGL were unaltered during the first week of methamphetamine withdrawal when mGlu5/CB1R-dependent synaptic depression was lost (Murray et al., 2021), in contrast to the present results with cocaine. This adds to mechanistic distinctions between incubation of cocaine and methamphetamine craving, although commonalities also exist (Murray et al., 2019).

Overall, these results argue that synaptic depression can promote (Novak et al., 2010; Bilbao et al., 2020; Mitra et al., 2021) or be dissociated from (Murray et al., 2021) drug or natural...
reward seeking. Thus, the functional significance of the observed reduction in DGL activity observed after prolonged cocaine withdrawal remains unclear. Likewise, no consensus has emerged from work that has tested the effect of manipulation of eCB signaling on seeking for cocaine and other drugs of abuse. While reducing endocannabinoid signaling through negative allosteric modulation has shown therapeutic promise, so too has enhancement of endocannabinoid signaling through inhibition of endocannabinoid degradation (Galaj and Xi, 2019). The complex nature of effects observed after pharmacological targeting of CB1Rs is likely to reflect their widespread expression, e.g., they are expressed not only on glutamate terminals but also on GABA terminals throughout the brain (Augustin and Lovinger, 2018). Furthermore, within the NAc, in addition to mediating synaptic depression onto MSN, endocannabinoids also mediate synaptic depression onto parvalbumin-containing interneurons that regulate MSN activity (Manz et al., 2020) and contribute to the modulation of dopamine release (Mateo et al., 2017).

Conclusion
The loss of mGlu5/CB1R-dependent synaptic depression in the NAc core after incubation of cocaine craving is likely due to multiple postsynaptic mechanisms, including reduced surface expression of mGlu5 and reduced mGlu5-Homer interactions (Loweth et al., 2014) and, as shown here, a reduction in DGL activity which may be due to increased CaMKII-DGL association. Although our findings and related work in the literature suggest that loss of mGlu5/CB1R-dependent synaptic transmission is unlikely to be a driver of the incubation of cocaine craving, it is expected that loss of a major form of synaptic plasticity would have significant consequences for NAc function. Future studies should continue to explore this problem.
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Figure Legends

Figure 1. DGL protein levels in the NAc core are unchanged after prolonged withdrawal from extended-access cocaine (Coc) self-administration. (A) Two cohorts of rats underwent extended-access self-administration of cocaine or saline (6 h/day for 10 days), destined for preparation of NAc core homogenates after withdrawal day (WD) 40 (WD>40) (top) or NAc synaptoneurosomes on WD40 (bottom). Shown are the number of infusions (left graphs) or number of active hole (AH) and inactive hole (IH) nose-pokes (right graphs) on the 10 days of self-administration training. N values are given in the figure. (B) DGL protein levels do not differ between saline and cocaine groups based on immunoblot analysis of NAc homogenates (top) or synaptoneurosomes (bottom). Data are expressed as % control (n = 7-9 rats/group; mean ± SEM). Representative lanes from immunoblots (cropped) are shown for a saline rat (left) and a cocaine rat (right). Arrows indicate bands analyzed and lines indicate location of molecular weight marker. Saline and cocaine groups were compared with unpaired t-tests (N.S.)

Figure 2. Associations between components of the 2-AG signalosome in the NAc core are unchanged after prolonged withdrawal from extended-access cocaine (Coc) self-administration. (A) Rats underwent extended-access self-administration of cocaine or saline (6 h/day for 10 days), destined for preparation of NAc core homogenates on withdrawal day (WD) 55. Shown are the number of infusions (left graph) or number of active hole (AH) and inactive hole (IH) nose-pokes (right graph) on the 10 days of self-administration training. N values are given in the figure. (B) Co-immunoprecipitation experiments assessing the physical associations between DGL and mGlu5 or Homer2 in NAc tissue obtained on WD55 (see also Extended Data Fig. 2-1). DGL was immunoprecipitated from NAc core homogenates, and the bound fraction was immunoblotted for mGlu5 or Homer2. Data are expressed as % of saline (Sal) control (n = 9-10 rats/group; mean ± SEM). Representative lanes from immunoblots (cropped) are shown for a
saline rat (left) and a cocaine rat (right). Arrows indicate bands analyzed and lines indicate location of molecular weight marker. IB, immunoblot; IP, immunoprecipitation. Saline and cocaine groups are compared with unpaired t-tests (N.S.)

**Figure 3.** The association of CaMKII with DGL is increased in the NAc core after prolonged withdrawal from extended-access cocaine (Coc) self-administration. Co-immunoprecipitation experiments assessed the physical associations between DGL and phosphorylated or total CaMKIIα and CaMKIIβ in NAc core homogenates prepared on withdrawal day (WD) 55 (same rats for which behavioral data is shown in Fig. 2). See also Extended Data Fig. 3-1. Data are expressed as % of saline (Sal) control group (n = 6-10 rats/group; mean ± SEM; unpaired t-tests, *p < 0.05, #p = 0.0501 vs. saline). Representative lanes from immunoblots (cropped) are shown for a saline rat (left) and a cocaine rat (right). Arrows indicate bands analyzed and lines indicate location of molecular weight marker. IB, immunoblot; IP, immunoprecipitation.

**Figure 4.** Reduction of DGL activity in the NAc after prolonged withdrawal from extended-access cocaine (Coc) self-administration. (A) Training. Rats self-administered saline or cocaine for 6 hours/day for 10 days (n = 9 rats/group). Shown are the number of infusions (left graph) or number of active hole (AH) and inactive hole (IH) nose-pokes (right graph). (B) Seeking tests on withdrawal day (WD) 1 and WD40. During the 30 min test, AH nose-pokes led to contingent presentation of the 20-second light cue previously paired with cocaine infusion, but no cocaine. Shown are AH and IH nose-pokes during the seeking test (mean ± SEM; paired t-test, **p < 0.01 vs WD1). (C) DGL activity. Five days after the WD40 seeking test, bilateral NAc punches comprised primarily of NAc core were snap frozen. Fluorescence resonance energy transfer assay of DGL activity was then conducted and results expressed as ΔRFU/minute. The cocaine group shows reduced DGL activity (n = 7-9 rats/group; mean ± SEM; unpaired t-test, *p < 0.05 vs. saline). (D) Linear regression evaluating the relationship between DGL activity in NAc core
of cocaine animals vs. magnitude of incubation of craving expressed as WD40/WD1 AH nose-pokes (n = 7 rats; R = -0.692, p = 0.085). RFU, Relative Fluorescent Units.

**Figure 5.** DGL activity in the NAc after prolonged withdrawal from extended-access cocaine (Coc) self-administration does not correlate with the magnitude of incubation of craving. (A) Training. Rats self-administered cocaine for 6 hours/day for 10 days (n = 9 rats). Shown are the number of infusions (left graph), and number of active hole (AH) and inactive hole (IH) nose-pokes (right graph). (B) Seeking tests on withdrawal day (WD) 1 and WD42. Shown are AH and IH nose-pokes during the 30 min seeking test (mean ± SEM; paired t-test, **p < 0.01 vs WD1). (C) Five days after the WD42 seeking test, bilateral NAc punches comprised primarily of NAc core were snap frozen and DGL activity was determined using fluorescent resonance energy transfer (also see Extended Data Fig. 5-1). (D) Linear regression evaluating DGL activity vs. magnitude of incubation of craving expressed as WD42/WD1 AH nose-pokes. No significant correlation between DGL activity and incubation was found (n = 9 rats; R = 0.393, N.S). (E) Combined linear regression evaluating DGL activity vs. magnitude of incubation of craving from both cocaine cohorts (Fig. 4A and Fig. 5A). From comparison of Fig. 4C and Fig. 5C, it is apparent that absolute RFU/min values for the two cohorts differed substantially (the cohorts were run over a year apart). To enable the data from the cohorts to be combined, mean values (∆RFU/minute) were calculated for each cohort and individual rat values (∆RFU/minute) were expressed as percentage of cohort mean (Fig. 5A cohort in black, n = 7; Fig. 4A cohort in gray, n = 9; R = 0.199, N.S.). RFU, Relative Fluorescence Units.

**Extended Data Figure Legends**

**Extended Figure 2-1.** Assessment of associations between DGL, mGlu5, and Homer1b/c in the nucleus accumbens (NAc) of drug-naive animals. (A) Co-immunoprecipitation experiments
assessing the association between DGL and mGlu5. Left panel shows results of an experiment in which DGL was immunoprecipitated from NAc core homogenates, followed by immunoblotting for mGlu5 in the starting material (SM; homogenate), Bound (B) or immunoprecipitated fraction, and unbound (UB) fraction. mGlu5 is present in all fractions. Right panel shows results after immunoprecipitating mGlu5 from the same starting material and immunoblotting for DGL. Although DGL is more abundant in Starting Material and Unbound fractions, it is detectable in the Bound fraction. (B,C) Co-immunoprecipitation experiments assessing the association between DGL and Homer1b/c. Although Homer1b/c is present in the starting material (B), it is not detected in the Bound fraction after immunoprecipitation of DGL (C); the arrow in panel C shows the molecular weight at which the Homer1b/c band should have been observed. IB, immunoblot; IP, immunoprecipitation.

**Extended Figure 3-1.** Assessment of DGL phosphorylation in the nucleus accumbens (NAc) after prolonged withdrawal from extended-access cocaine (Coc) self-administration. Rats underwent extended-access self-administration of cocaine or saline (6 h/day for 10 days), destined for preparation of NAc core homogenates on withdrawal day (WD) 55 (see Fig. 2 for behavioral data). DGL was immunoprecipitated from NAc core homogenates (6 saline rats, 9 cocaine rats), and the Bound fraction was immunoblotted with an antibody that recognizes phosphorylated serine residues (p-Serine). No group difference was found (t13=2.54, p=0.897). However, as noted in the main text, these data are not conclusive. Because we did not originally plan to assess DGL’s phosphorylation state using this tissue, some of the buffers used in the immunoprecipitation protocol did not contain phosphatase inhibitors. Thus, DGL may have been dephosphorylated during the lengthy immunoprecipitation protocol. IB, immunoblot; IP, immunoprecipitation.

**Extended Figure 5-1.** DGL activity measurements in nucleus accumbens (NAc) core of cocaine...
rats and in two control groups. Rats self-administered cocaine (Fig. 5A) and received cue-induced seeking tests on withdrawal day (WD) 1 and WD42 (Fig. 5B). Five days after the WD42 seeking test, bilateral NAc punches comprised primarily of NAc core were snap frozen and DGL activity was determined using fluorescent resonance energy transfer. Triplicate values for each of 9 cocaine rats are shown, along with duplicates for two control groups: Cerebellum tissue (CB) and Cerebellum tissue plus the DGL inhibitor DO34 (CB + DO34). RFU, Relative Fluorescent Units.
Fig 1

A  Self-administration training
WD>40 Total DGL (N = 9 coc / 7 sal)

WD40 synaptoneurosomes (N = 8 coc / 8 sal)

B  DGL levels

% Saline

150
125
100
75
50
25
0

Total WD>40

Synapto WD40

100 kD
Fig 2

A  
Self-administration training
WD55 DGL IPs (N = 10 coc / 10 sal)

B  
DGL IP

---

Infusions (6 h)  
Training day

Nose-pokes (6 h)  
Training day

---

% Saline

mGlu5 WD55  
Homer2 WD55
Fig 3

DGL IP, CaMKII IB (WD55)

60 kD  |  50 kD
---|---
α     | β

DGL-CaMKIIα
DGL-pCaMKIIα
DGL-CaMKIIβ
DGL-pCaMKIIβ

% Saline

Sal  |  Coc

*  |  #  |  *  |  *