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A role for the endocannabinoid enzymes monoacylglycerol and diacylglycerol lipases in cue-induced cocaine craving following prolonged abstinence

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Abstract

Following exposure to drugs of abuse, long-term neuroadaptations underlie persistent risk to relapse. Endocannabinoid signaling has been associated with drug-induced neuroadaptations, but the role of lipases that mediate endocannabinoid biosynthesis and metabolism in regulating relapse behaviors following prolonged periods of drug abstinence has not been examined. Here, we investigated how pharmacological manipulation of lipases involved in regulating the expression of the endocannabinoid 2-AG in the nucleus accumbens (NAc) influence cocaine relapse via discrete neuroadaptations. At prolonged abstinence (30 days) from cocaine self-administration, there is an increase in the NAc levels of DAGL, the enzyme responsible for the synthesis of the endocannabinoid 2-AG, along with decreased levels of MAGL, which hydrolyzes 2-AG. Since endocannabinoid-mediated behavioral plasticity involves phosphatase dysregulation, we examined the phosphatase calcineurin after 30 days of abstinence and found decreased expression in the NAc, which we demonstrate is regulated through the transcription factor EGR1. Intra-NAc pharmacological manipulation of DAGL and MAGL with inhibitors DO-34 and URB-602, respectively, bidirectionally regulated cue-induced cocaine seeking and altered the phosphostatus of translational initiation factor, eIF2α. Finally, we found that cocaine-seeking 30 days after abstinence leads to decreased phosphorylation of eIF2α and reduced expression of its downstream target NPAS4, a protein involved in experience-dependent neuronal plasticity. Together, our

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Author contributions

SM, PHG, CTW, and DMD conceptualized experiments. SM, PGH, JAM, SAT, MI, CTW, KE, SM, CL, and CA performed behavioral experiments. SM, PHG, and MI conducted biochemical experiments. SM and PHG performed statistical analyses. SM, PHG, CW and DMD wrote the manuscript with review from all authors.

Competing Interests

The authors declare that they have no competing interests.

findings demonstrate that lipases that regulate 2-AG expression influence transcriptional and translational changes in the NAc related to drug relapse vulnerability.

Introduction

Drug addiction is a neuropsychiatric disease in which individuals experience recurring episodes of relapse following prolonged periods of abstinence[1]. Escalated cocaine craving may contribute to persisting relapse vulnerability, which is triggered by drug-associated cues and mediated by neuroadaptations in brain regions that govern reward processing, including the nucleus accumbens (NAc)[2, 3]. Transcriptional and translational alterations in the NAc mediate long-term neuroplasticity and subsequent behavioral adaptations that occur following exposure to drugs of abuse[4, 5]. Although these neuroadaptations in the NAc have been thought to mediate perpetual relapse vulnerability, the precise mechanisms are not well understood[2].

Endocannabinoid signaling within the neural “reward circuitry” influences motivated behaviors[6], including following cocaine exposure[7–9]. Endocannabinoid signaling in the brain primarily comprises metabotropic receptors, endogenous ligands, and enzymes that synthesize and degrade the ligands[10, 11]. Endocannabinoids include anandamide and 2-arachidonoyl glycerol (2-AG)[11, 12], the most predominant ligand for cannabinoid type 1 (CB1) receptors[13, 14]. 2-AG is produced through the metabolism of 1,2-diacylglycerol by sn1-specific diacylglycerol lipase (DAGL)[15–17] and is hydrolyzed by monoacylglycerol lipase (MAGL)[18]. Endocannabinoid signaling mediates long-term depression (LTD) of glutamatergic synaptic transmission in the NAc[19]. This endocannabinoid-mediated LTD is altered and is part of the synaptic remodeling that occurs during prolonged abstinence from cocaine self-administration[2, 20, 21]. While previous studies demonstrate that drug seeking intensifies, or incubates, during abstinence[22], the functional or behavioral significance of the changes induced by LTD or the role that the endocannabinoids may play in such plasticity remains undetermined. Moreover, it is unclear how endocannabinoid signaling in the NAc may underlie transcriptional and/or translational changes during abstinence.

The immediate early gene for early growth response 1 (EGR1) encodes a master transcriptional regulator that is essential for cocaine-induced cellular and behavioral plasticity[23, 24]. The regulation of EGR1 itself is mediated in part through endocannabinoid signaling as well as calcineurin, a neuron-enriched phosphatase[25–27], suggesting that EGR1 may be an essential hub in the neurobiological adaptations induced by endocannabinoids. Furthermore, endocannabinoid-mediated acquisition and consolidation of memory are thought to involve altered regulation of downstream phosphatases[28].

The phosphostatus of eukaryotic initiation factor 2 α-subunit (eIF2α), an integral component of protein synthetic machinery, is critical for the initiation of protein translation[29]. Typically, phosphorylation of eIF2α halts translational machinery whereas dephosphorylation of eIF2α leads to increased protein synthesis[30]. Endocannabinoid-dependent synaptic remodeling has been linked to transcriptional and translational changes[31], and endocannabinoid-mediated presynaptic plasticity involves the phosphatase calcineurin[32]. Moreover, calcineurin promotes auto-phosphorylation of PKR-like ER kinase

(PERK), which in turn increases the phosphorylation of eIF2α and attenuates protein translation[33]. The phosphostatus of eIF2α has been shown to regulate experience-dependent translational and behavioral plasticity[34–40]. Cue-induced cocaine seeking following prolonged abstinence reduces the levels of phosphorylated eIF2α (p-eIF2α) in the NAc, and pharmacologically inhibiting eIF2α dephosphorylation attenuates cue-induced cocaine seeking[41]. Although eIF2α dephosphorylation increases general translation, there is reduced translation of a subset of target mRNAs containing open reading frames such as those for Neuronal PAS domain protein 4 (NPAS4) and activating transcription factor 4 (ATF4) that are thought to facilitate behavioral adaptations[34, 35, 38, 39, 41, 42].

In this study, we examined the roles of DAGL and MAGL in the NAc in mediating the expression of cocaine seeking during prolonged abstinence following cocaine self-administration. Additionally, we sought to determine how DAGL and MAGL may regulate transcriptional and translational mechanisms following prolonged abstinence. These findings provide novel insight into the long-lasting neural adaptations that lead to relapse vulnerability.

Materials and Methods

Subjects

Male Sprague-Dawley rats (250–275 g; Envigo, Indianapolis, IN) were habituated to a colony room with constant temperature and humidity levels for 5 to 7 days. Rats were singly housed and had *ad libitum* access to food and water. Behavioral testing was performed during the dark phase of the 12-h light-dark cycle. This study was conducted in accordance with the guidelines set up by the Institutional Animal Care and Use Committee of the State University of New York at Buffalo.

Drugs

Cocaine hydrochloride, gifted by the National Institute on Drug Abuse (Bethesda, MD), was dissolved in sterile 0.9% saline (1.75 mg/mL). Pump durations/injection volumes were adjusted daily according to the body weights to deliver 0.5 mg/kg/infusion (inf). The DAGL inhibitor DO-34 (1 μg/0.3μL) (Aobious, Gloucester, MA) was dissolved in dimethyl sulfoxide (DMSO) as a stock solution and used at a final concentration of 10% DMSO and 90% saline. URB-602, a noncompetitive inhibitor that preferentially inhibits MAGL and potentiates 2-AG levels[43] was used to inhibit MAGL. URB602 (300 pmol/0.3 μL) (Tocris, Bristol, UK) was dissolved in a solution containing 10% DMSO in saline. The pharmacological agents and doses were selected from previously published studies[43, 44].

Jugular catheterization and patency testing

Rats were implanted with chronic indwelling jugular catheters as described previously[45, 46] and given 4 days to recover from surgery. Catheters were flushed daily with 0.2 mL of a solution of enrofloxacin (4 mg mL^{-1}) in heparinized saline (50 IU mL^{-1} in 0.9% sterile saline) to preserve catheter patency. One day prior to behavioral testing, each animal received an intravenous infusion of ketamine hydrochloride (0.5 mg/kg in 0.05 mL), and

an appropriate behavioral response (loss of muscle tone and righting reflexes) was used to verify catheter patency. Only rats with patent catheters were included in data analyses.

Acquisition of extended access self-administration

After the rats recovered from catheter surgery, they were assigned to self-administer either saline or cocaine during the dark phase in test chambers (MED Associates, St. Albans, VT) that were equipped with two snout-poke holes. During the acquisition, rats were trained for 1 h/day for 5 days. A response in the active hole resulted in an intravenous injection of saline or cocaine (0.5 mg/kg/inf) using a fixed ratio of 1 (FR1) schedule of reinforcement that was increased daily to an FR5 schedule. Infusions were accompanied by a 5-s illumination of the stimulus light above the active snout-poke hole, with the house light being extinguished for the duration of the infusion and the following 30-s timeout period. Responses to the inactive hole resulted in no programmed consequences. The criterion for the acquisition of cocaine self-administration was an average of 10 cocaine infusions per day during the last 3 days of the 5-day acquisition phase[24, 47].

Extended-access self-administration training

Following the acquisition phase, rats were allowed to self-administer either saline or cocaine (0.5 mg/kg/inf) in the training phase (6 h/day) using an FR5 schedule of reinforcement for 10 days. This procedure is commonly used in models of persistent and elevated drug seeking (i.e., incubation)[48, 49]. After each session of 6 h/day, the catheters were flushed and the rats returned to their home cages in the colony room. Only rats that responded for an average of 50 infusions or more in the last 5 sessions were used for further experiments[24, 47].

Cannulation and microinjection procedure

On abstinence day 22 (AD22), rats were implanted with bilateral guide cannulas as described previously[45, 46]. Briefly, animals were anesthetized with ketamine and xylazine (60 and 5 mg/kg, respectively, intraperitoneally) and positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Cannulas (C235G-2.4; Plastics One, Roanoke, VA) were implanted in the NAc using the following coordinates: AP, +1.8 mm from bregma; ML, +1.2 mm; DV, −6.5 mm[46, 50]. Cannulas were fixed to the skull with an acrylic resin and stainless-steel screws. Animals were handled daily and sham injected during the recovery period (AD23 to AD27) to habituate them to the microinjection procedure. Microinjections were carried out on AD28, AD29, and AD30. Microinjections were infused at a rate of 0.5 μL/min using a microsyringe (Hamilton, Reno, NV) connected to an infusion pump. At the end of the procedure, the syringe remained connected to the cannula for 3 min to allow the diffusion of the drug. One hour after the final microinjection (AD30), rats were placed in the operant chambers and tested in the cue-induced cocaine-seeking test as described below. A chronic microinjection schedule for DAGL and MAGL inhibitors was performed to ensure changes in gene expression levels that typically cannot be achieved through an acute injection.

Abstinence and cue-induced seeking

After training, catheters were flushed, and rats were returned to their home cages to undergo abstinence for 1 (AD1) or 30 (AD30) days. For studies in which rats received intracranial injections, rats were returned to the operant chamber for a 60-min test to measure cue-induced seeking 1 h after the microinjection. Cue tests were performed following forced abstinence (AD1 or AD30) under extinction conditions (i.e., no drug availability when the cue was present) as previously described[24]. The total number of active responses was used as the operational measure of cocaine seeking. We report active responses for seeking tests in figures, and inactive responses are reported in Supplementary Table 1.

Food reinforcement

Food self-administration was conducted as previously described with minor modifications[46]. Briefly, rats were food restricted to 10% of their body weight. Behavioral training was conducted daily for 1 h over a 10-day period in 2-lever operant chambers placed within sound-attenuating ventilated boxes (Coulbourn Instruments, LLC, Allentown, PA) during the dark phase of the 12-h light/dark cycle. Rats were trained to obtain a reward of a maximum of 50 sucrose pellets (45-mg pellet; Bio-Serv, Flemington, NJ) following lever press responses under an FR1 schedule. The reinforcement schedule was progressively increased to FR5 over the 10-day period. Twenty-two days after training (AD22), rats underwent bilateral cannulation targeted at the NAc. After 5 days of recovery (AD23 to AD27), rats received bilateral intra-NAc injections of DO-34, URB-602, and vehicle on AD28, AD29, and AD30. One hour after microinjections on AD30, rats were tested for cue-induced food seeking under extinction conditions, where an active lever press delivered the cue light previously paired with food pellet reward without dispensing any food reward.

Locomotor activity

One hour after receiving microinjections of DO-34, URB-602, or vehicle on AD29, rats were placed inside transparent plastic cages (40 × 40 × 30 cm), and the locomotor activity was recorded by an infrared motion-sensor system (AccuScan Instruments, Inc., Columbus, OH). Versa Max animal activity software monitored the distance rats traveled during a 1-h test.

Immunoblotting

On AD1 and AD30, rats from different experimental groups were euthanized by rapid decapitation. Brains were removed and sliced into 1-mm-thick sections using a brain matrix (Braintree Scientific, Inc., Braintree, MA), and 2-mm-diameter tissue punches from the NAc shell regions were collected and rapidly frozen on dry ice. Western blotting was carried out as previously described[45, 46]. Briefly, punches were homogenized in 25 mmol/L Tris (pH 8.0) and 0.25 mol/L sucrose buffer. Total protein was extracted, and 30-μg samples were loaded onto 10% Tris-sodium dodecyl sulfate (SDS) polyacrylamide gels for electrophoretic separation. Bands were then transferred to nitrocellulose membranes and blocked with Rockland buffer (Rockland Immunochemicals, Inc., Limerick, PA). Membranes were incubated overnight at 4°C with primary antibodies diluted in blocking buffer (Rockland Immunochemicals, Inc.), including anti-DAGL (1:200, ab81984; Abcam, Cambridge,

MA), anti-MAGL (1:200, ab24701; Abcam), anti-eIF2α (1:333, #9722; Cell Signaling Technologies, Inc., Danvers, MA), anti-phospho eIF2α (Ser51, 1:150, #9721; Cell Signaling Technologies, Inc.), anti-EGR1 (1:100, sc515830; Cell Signaling Technologies, Inc.), anti-NPAS4 (1:1,000; gifted by the Zhen Yan lab at the University at Buffalo and procured from Michael Greenberg at Harvard), anti-ATF4 (1:10, #118150; Cell Signaling Technologies Inc.), anti-calcineurin (1:2,000, ab3673; Abcam), and anti-β-actin (1:10,000, #3700; Cell Signaling Technologies, Inc.). Membranes were incubated with IRDye secondary antibodies (1:5,000; LI-COR, Inc., Lincoln, NE) for 1 h at room temperature. The blots were imaged using an Odyssey infrared imaging system (LI-COR, Inc.) and quantified by densitometry using ImageJ (National Institutes of Health, Bethesda, MD). β-Actin was used as a loading control.

RNA extraction and qPCR

For quantitative PCR (qPCR), NAc tissue punches were collected from animals at AD30 as previously described[24]. Total RNA was extracted with TRIzol reagent (Ambion, Austin, TX) and purified using the E.Z.N.A. MicroElute Total RNA kit (Omega Bio-Tek, Norcross, GA). RNA concentrations were determined with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and reverse-transcribed using a SuperScript III first-strand synthesis system (Invitrogen of Thermo Fisher Scientific). mRNA expression was measured using IQ SYBR green SuperMix (Bio-Rad Laboratories, Hercules, CA), and quantification was conducted on an iQ5 system (Bio-Rad Laboratories). All reactions were run in triplicates, and quantifications were analyzed by a relative threshold cycle quantification method using *Gapdh* as a housekeeping gene. For *EGR1* and *calcineurin*, the primer sequences used were 5′-TGGACAACTACCCCAAACTGG-3′ (forward) and 5′-AAAAGGACTTGGTCAGGTG-3′ (reverse) and 5′-TAACTTTCGAGCCAGCCCAG-3′ (forward) and 5′-CGGAAATGGAACGGCTTTCA-3’ respectively. For *Gapdh*, the primer sequences were 5′-AACGACCCCTTCATTGAC-3′ (forward) and 5′-TCCACGACATACTCAGCA-3′ (reverse).

Chromatin immunoprecipitation

Quantitative chromatin immunoprecipitation for EGR1 was performed as previously described[24] by processing bilateral NAc punches obtained at AD30 following cocaine self-administration. Four NAc punches from 2 rats were pooled for each sample (n = 5–6 samples/group) and fixed with 1% formaldehyde followed by quenching with 2 M glycine. Chromatin was solubilized with cell and nuclear lysis buffers and sheared using a Bioruptor 300 (Diagenode, Liège, Belgium) at 4°C with high sonication intensity 3 times at 30s on/30s off for 10 min. Fragment sizes of 250 to 1,000 base pairs were verified on a 2% agarose gel. Magnetic sheep anti-rabbit beads (Invitrogen) were incubated with 7.5μg of EGR1 antibody (#4153; Cell Signaling Technology) at 4°C overnight on a rotator. Magnetic bead/antibody complexes were then washed, and 70 μL of the slurry was incubated with the sheared chromatin sample for 16 h at 4°C; 10% of each sample of sheared chromatin was used as an input control. Samples were washed with LiCl and Tris-EDTA buffers and reverse cross-linked at 65°C overnight, with proteins and RNA removed by proteinase K (Invitrogen) and ribonuclease (Roche, Basel, Switzerland), respectively. Finally, DNA was

purified using a DNA purification kit (Qiagen, Hilden, Germany). Nonspecific binding was determined using an immunoglobulin G control.

qPCR was performed (iQ5 system; Bio-Rad Laboratories, Hercules, CA) to identify the binding of EGR1 to the proximal promoter region of the *calcineurin* gene using the primer sequences 5′-GCGGGACTGACTGGCACCC-3′ (forward) and 5′-GGGTGCCAGTCAGTCCCGC-3′ (reverse). All chromatin immunoprecipitation-qPCR primers were tested for efficiency before being used under experimental conditions. Amplification reactions were run in triplicates with iQ SYBR green (Bio-Rad Laboratories), and each sample was normalized to the immunoglobulin G control. Fold changes were calculated relative to the saline control.

Statistical analysis

Analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA). Self-administration training was analyzed using within-subject two-way repeated measures analyses of variance (ANOVAs), followed by Bonferroni *post hoc* comparisons. Cue-induced relapse locomotor activity and immunoblotting were subjected to Student *t* tests. Significance was set at a *P* value of <0.05, and data are presented as the means ± SEMs.

Results

Dysregulation of DAGL and MAGL in the NAc following prolonged, but not acute, abstinence from cocaine self-administration.

After acute abstinence (AD1) (Figure 1A, B) following extended-access self-administration of cocaine or saline (two-way repeated-measures ANOVA, drug × session interaction effect $F_{(9,144)} = 2.874$, $P = 0.00043$) protein levels of DAGL ($t_{(15)} = 0.9$, $P = 0.39$) and MAGL ($t_{(14)} = 0.52$, $P = 0.60$) (Figure 1C, D) were unaltered between cocaine and saline groups. On the contrary, after prolonged abstinence (AD30) from cocaine or saline self-administration (Figure 1A, B) (two-way repeated-measures ANOVA, drug × session interaction effect $F_{(9,135)} = 3.991$, $P = .00002$), DAGL protein levels (Figure 1E) were significantly increased ($t_{(15)} = 2.56$, $P = 0.022$) and MAGL protein levels (Figure 1F) were significantly decreased ($t_{(15)} = 2.81$, $P = 0.012$) in the NAc of cocaine-treated rats compared to their respective saline controls.

Reduced EGR1 transcriptional regulation in the NAc following prolonged abstinence.

Endocannabinoid signaling has been associated with changes in the expression of the phosphatase calcineurin and the transcription factor EGR1[25]. Therefore, we analyzed EGR1 and calcineurin levels in the NAc of rats at AD30 following extended-access cocaine self-administration. We found that EGR1 mRNA ($t_{(7)} = 2.88$, $P = 0.02$) (Figure 2A) and protein ($t_{(9)} = 2.28$, $P = 0.04$) (Figure 2B) levels were attenuated at AD30 in cocaine-treated rats compared to levels in their respective saline controls. Concomitant to the reduced expression of EGR1, we found a decrease in the binding of EGR1 to the gene encoding *calcineurin* ($t_{(8)} = 1.99$, $P = 0.04$) (Figure 2C), demonstrating that the transcriptional regulation of calcineurin by EGR1 may be altered. Based on the decreased binding of EGR1 to the *calcineurin* promoter, we probed the NAc expression of calcineurin at AD30. We found that mRNA ($t_{(10)} = 3.092$, $P = 0.005$) (Figure 2D) and protein ($t_{(12)} = 2.73$, $P = 0.01$) (Figure 2E)

expression levels of calcineurin were also attenuated in the NAc at AD30 in cocaine-treated rats compared to that in saline controls. In contrast, mRNA levels of *ERG1* ($t_{(10)} = 0.6938$, $P = 0.50$) (Supplementary Figure 1A) and *calcineurin* ($t_{(11)} = 0.09$, $P = 0.46$) (Supplementary Figure 1B) in the NAc were unaltered at AD1.

Decreased p-eIF2α and downstream target NPAS4 following prolonged abstinence.

To determine the potential role of reduced EGR1 and calcineurin expression, we measured phosphorylation levels of a known downstream target, eIF2α, in a new group of animals that underwent a cue-induced cocaine-seeking test on AD30 after they had learned to self-administer the drug (Figure 3A) (two-way repeated-measures ANOVA, drug × session interaction effect $F_{(9,110)} = 3.428$, $P = .0009$). Following the cue-induced cocaine-seeking test, AD30 cocaine rats showed significantly more total active responses than saline controls ($t_{(11)} = 4.94$, $p = 0.002$) (Figure 3B), and NAc tissues collected from these animals 1 h later had reduced phosphorylated p-eIF2α ($t_{(11)} = 1.938$, $P = 0.0393$) (Figure 3C) but no changes in total eIF2α protein expression levels ($t_{(11)} = 0.9362$, $P = 0.3693$) (Figure 3D) compared to that in saline controls. We then investigated NPAS4 and ATF4 protein levels, which are targets modulated by eIF2α[53, 41] and mediate activity-dependent translational changes[37, 51]. We found reduced NPAS4 protein expression ($t_{(10)} = 2.23$, $P = 0.04$) in cocaine-treated rats compared to that in saline controls (Figure 3E). However, no changes in ATF4 levels were observed following cocaine seeking ($t_{(11)} = 1.19$, $P = 0.12$) (Figure 3F), which aligns with previous findings[41].

Pharmacological manipulation of DAGL and MAGL bidirectionally regulate cue-induced cocaine seeking and eIF2α phosphorylation following prolonged abstinence.

2-AG influences reward-related behaviors[7, 40], and we found that the enzymes that regulate 2-AG, MAGL, and DAGL are altered during prolonged abstinence from extended-access cocaine self-administration. Therefore, we used pharmacological tools to examine the role of these enzymes in cocaine craving following prolonged abstinence (Figure 4A). In one experiment, a group of rats was pseudorandomly divided into vehicle and DAGL groups following extended-access cocaine self-administration based on drug infusions (two-way repeated-measures ANOVA, treatment effect, $F_{(1,19)} = 0.02$, $P = 0.9646$ [Figure 4B]). Rats then received intra-NAc microinjections of the DAGL inhibitor DO-34 or vehicle on AD28, AD29, and AD30. One hour after the microinjections on AD30, rats were returned to operant chambers for a cue-induced cocaine-seeking test. Inhibition of DAGL reduced total active responses compared to the vehicle group ($t_{(16)} = 2.22$, $P = 0.0411$) (Figure 4C). Interestingly, DAGL inhibition also increased *ERG1* mRNA expression ($t_{(9)} = 1.86$, $P = 0.04$) (Figure 4D) and p-eIF2α protein levels ($t_{(9)} = 2.71$, $P = 0.02$) (Figure 4E) compared to that in vehicle controls.

In another experiment, a separate group of animals was pseudorandomly divided following cocaine self-administration (two-way repeated-measures ANOVA, treatment effect, $F_{(1,16)} = 0.1624$, $P = 0.65$ [Figure 4F]). In contrast to that with DAGL, inhibition of MAGL via URB-602 microinjections on AD28, AD29, and AD30 significantly increased responding in a cue-induced cocaine-seeking test at AD30 compared to the vehicle-treated rats ($t_{(16)} = 2.57$, $P = 0.0204$) (Figure 4G). MAGL inhibition also downregulated *EGR1* mRNA (t_8

= 3.082, $P = 0.01$) (Figure 4H) and p-eIF2α protein levels ($t_{14} = 2.19$, $P = 0.040$) (Figure 4I). Overall, locomotor activity was not affected by intra-NAc microinjections of DO-34 ($t_{(16)} = 0.37$, $P = 0.71$) (Supplementary Figure 2B) or URB-602 ($t_{(16)} = 0.389$, $P = 0.70$) (Supplementary Figure 2D).

To investigate whether NAc endocannabinoid signaling contributes to non-drug rewards, we trained a separate group of rats to self-administer food and then pseudorandomly divided the rats into vehicle, DO-34, and URB-602 groups (Supplementary Figure 3A) based on their lever press responses ($F_{2,18} = 0.88$, $P = 0.43$) (Supplementary Figure 3B). As in previous experiments, we administered microinjections on AD28, AD29, and AD30. One hour after the microinjection on AD30, rats were returned to operant chambers for a seeking test under forced abstinence conditions. Total active lever presses did not differ between vehicle, DO-34, and URB-602 ($F_{2,16} = 0.03$, $P = 0.96$) groups (Supplementary Figure 3C). Additionally, overall locomotion did not differ between the treatment groups ($F_{2,16} = 0.57$, $P = 0.57$) (Supplementary Figure 3D). These data suggest that the effect of endocannabinoid signaling in the NAc after prolonged abstinence is specific to drug-seeking behaviors.

Discussion

Here, we present evidence that pharmacological manipulation of DAGL and MAGL in the NAc regulates cue-induced cocaine seeking during prolonged abstinence, which may occur via transcriptional and translational changes. Our data demonstrate a temporally dependent change in DAGL and MAGL protein expression after 30 days, but not 1 day, of forced abstinence from extended-access cocaine self-administration. Furthermore, levels of p-eIF2α and its translational target NPAS4 were attenuated following cue-induced cocaine seeking on AD30, suggesting p-eIF2α-mediated dysregulated protein translation may underlie relapse vulnerability. At AD30, cocaine-treated rats also had lower calcineurin and EGR1 expression and EGR1-mediated transcriptional control of calcineurin, a modulator of the phosphostatus of eIF2α, suggesting altered transcriptional programming of EGR1. Finally, we demonstrate that regulating 2-AG synthesis and degradation through pharmacological manipulation of DAGL and MAGL, respectively, governs expression of cue-induced cocaine seeking bidirectionally. Overall, our findings demonstrate a pivotal role of the 2-AG lipases in influencing dysregulated transcriptional and translational mechanisms governing neuroadaptations underlying relapse vulnerability following exposure to and long-term abstinence from cocaine (Figure 5).

2-AG-mediated endocannabinoid signaling in the NAc is known to influence behavior related to drugs of abuse and natural rewards[7]. As the direct measurement and manipulation of 2-AG *in vivo* can be challenging and often not feasible[52], targeting the enzymes that regulate 2-AG levels is a suitable alternative. Our findings are consistent with previous studies showing dysregulation of MAGL and DAGL levels in multiple brain regions after cocaine exposure[53–55]. However, we found that the dysregulation of DAGL and MAGL in the NAc is specific to prolonged and not acute abstinence from cocaine self-administration and was associated with altered transcriptional and translational processes, which are known to be key mediators of the neurobiology of addiction-like behaviors. Intra-NAc inhibition of DAGL at AD30 attenuated cue-induced cocaine seeking. Conversely, intra-NAc

microinjections of URB-602 potentiated cue-induced cocaine seeking at AD30 and may have even exacerbated abstinence-dependent cocaine craving. Pharmacological inhibition of DAGL reversed (increased) the reduced levels of EGR1 and p-eIF2α observed in the NAc of cocaine-treated rats at AD30 (Figure 1). This relative directional change needs to be viewed in the context of both global neuroadaptations and abstinence in the rats that administered cocaine in comparison to their saline controls (Figure 1), whereas the effects of DAGL were observed by comparing rats that self-administered cocaine and were treated with or without the inhibitor at AD30 (Figure 4).

Numerous studies have established roles of transcriptional and epigenetic mechanisms in neuroadaptations associated with addiction phenotypes[56]. We validated findings from our previous study[24] showing that EGR1 expression is attenuated in the NAc in cocaine-treated rats on AD30, which may regulate cocaine seeking through a ubiquitin mechanism involving chromatin remodeler INO80[24]. Calcineurin is known to mediate endocannabinoid actions, and EGR1 is modulated by the calcineurin pathway. However, it is unknown if EGR1 could act in a negative feedback loop that regulates calcineurin activity. Here, we found decreased expression of calcineurin and EGR1 in the NAc and reduced binding of EGR1 to the calcineurin gene promoter in cocaine-treated rats on AD30. Interestingly, calcineurin expression decreases with repetitive noncontingent cocaine administration[57], and activation of calcineurin reduces cue-induced reinstatement in the amygdala[58], thereby implicating calcineurin as a possible target of chronic cocaine-elicited neuroadaptations. Our data show that following prolonged abstinence, there is reduced transcriptional control of calcineurin expression, which was likely responsible for reduced eIF2α regulation, perhaps through a PERK mechanism, as described previously[59].

eIF2α is involved in the regulation and expression of cocaine-seeking behaviors[41]. Accordingly, we observed a reduction of eIF2α phosphorylation in the NAc following re-exposure to a drug-paired cue in rats that had undergone prolonged abstinence. This reduction was accompanied by diminished levels of NPAS4, a protein regulated by eIF2α that undergoes rapid experience-dependent translation and affects synaptic mechanisms underlying memory formation[42, 60, 61]. However, the expression levels of another target of eIF2α, ATF4, which has been shown to mediate the reconsolidation of drug-induced memory in the amygdala[62], was not altered, suggesting that there are cell- and region-specific eIF2α targets mediating behavioral adaptations.

Previous studies have implicated both CB1 and CB2 (endocannabinoid type 2) receptors in altering behavioral and neurobiological mechanisms associated with psychostimulant exposure[63–64]. For example, studies have reported loss of endocannabinoid-mediated LTD following cocaine exposure, which may also regulate the seeking of drugs of abuse[65–70]. Interestingly, altered endocannabinoid-mediated LTD is thought to occur due to a loss of MAGL activity, 2-AG elevations, and partial CB1 receptor desensitization/downregulation in MAGL knockout mice[71]. It is, therefore, possible that the increase in cocaine-seeking behavior at AD30 induced by inhibition of 2-AG degradation observed here is occurring through the CB1 receptors; however, further studies are required to define the precise cellular mechanisms.

Desensitization and downregulation of CB1 receptor levels have been shown to alter immediate early genes, such as those encoding EGR1 and c-Fos, which vary across brain regions and exposures to diverse environmental stimuli[72, 73]. EGR1 is sensitive to such activity-dependent synaptic remodeling[74–79] and could explain, at least in part, the cocaine-induced temporal changes in the NAc due to DAGL and MAGL manipulations. The endocannabinoid pathway has, moreover, been linked to protein translation and memory formation[40, 80, 81] that relies on alterations in both pre-[81] and post-synaptic protein translational machinery. Because endocannabinoid mechanisms also play a critical role in non-neuronal cell types and interneurons in the NAc[82, 83], future causal studies that examine cell-type and compartmentalization specificity are needed to parse out the intricate role of endocannabinoid signaling following exposure to drugs of abuse. Furthermore, it is important to note that our findings are based on the use of male subjects. This is particularly germane, as there are reported sex differences in endocannabinoid signaling that are thought to play a vital role in behavioral responding[84, 85].

In summary, this study reveals that DAGL and MAGL act as modulators of cue-induced cocaine seeking during prolonged abstinence, which may involve EGR1 and p-eIF2α. This proposed mechanism incorporates DAGL and MAGL regulation of EGR1 transcriptional control of calcineurin activity, which presumably alters eIF2α-mediated translational control of NPAS4. This study is the first to report evidence of the role of manipulating endocannabinoid lipases to influence cocaine-induced behavioral plasticity following prolonged abstinence from extended-access cocaine self-administration. In the current context of the investigation, it cannot be claimed that the dysregulated endocannabinoid lipases and the resulting molecular changes are specific to cocaine incubation, per se. However, the molecular alterations we observed are more specific to prolonged abstinence from cocaine self-administration, which appears to foster maladaptive plasticity that regulates relapse vulnerability. Due to the role of DAGL and MAGL in mediating the biosynthesis of 2-AG, these findings provide evidence of the importance of 2-AG turnover by drugs of abuse as one of the many regulating factors modulating relapse vulnerability, which has implications for the development of new pharmacotherapies for treating drug addiction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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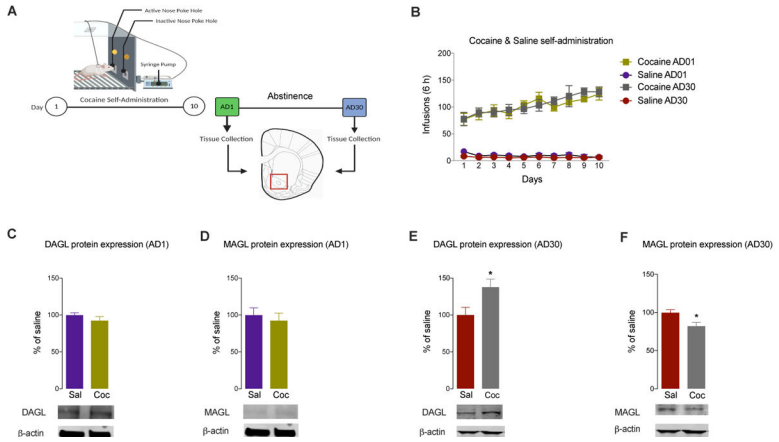

Figure 1. Dysregulation of endocannabinoid lipases in the NAc following self-administration of cocaine.

(**A**) Experimental timeline for 10-day self-administration training and withdrawal (AD1 and AD30). (**B**) Number of infusions during self-administration training for animals receiving saline (Sal) or cocaine (Coc) that underwent one of the two forced abstinence periods (AD1 or AD30; $n = 8–10$/group). DAGL and MAGL protein expression levels in the NAc after an acute (AD1) (**C** and **D**, respectively) or prolonged (**E** and **F**, respectively) abstinence period (AD30) ($n = 8–10$/group). All data are presented as means ± SEMs with statistical significance (*) at $P < 0.05$.

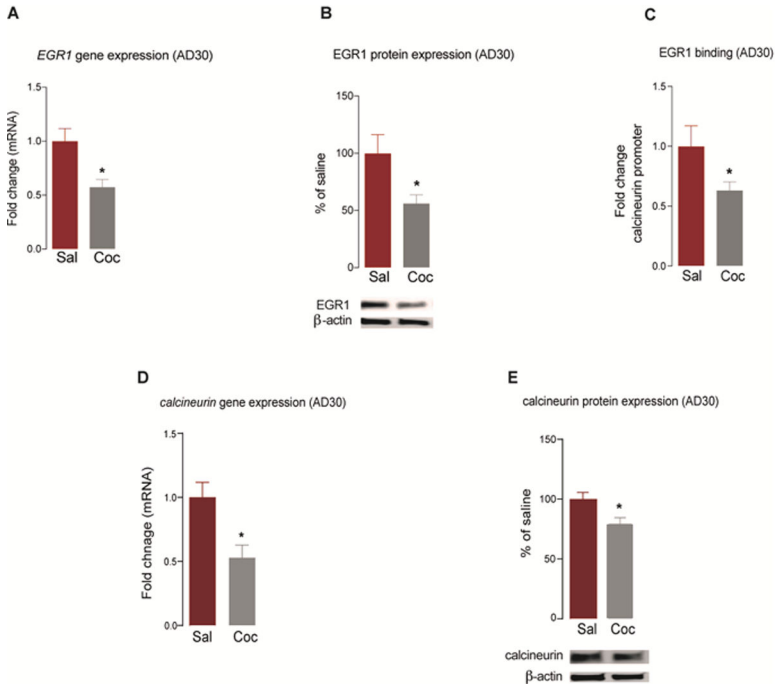

Figure 2. Reduced EGR1 transcriptional regulation in the NAc following prolonged abstinence.

Gene (**A**) and protein (**B**) expression of EGR1 at AD30 (n = 5–7/group). (**C**) Fold change in EGR1 binding to *calcineurin* gene promoter (n = 5/group). Gene (**D**) and protein (**E**) expression of calcineurin at AD30 (n = 5–7/group). All data are presented as means ± SEMs with statistical significance (*) at $P < 0.05$.

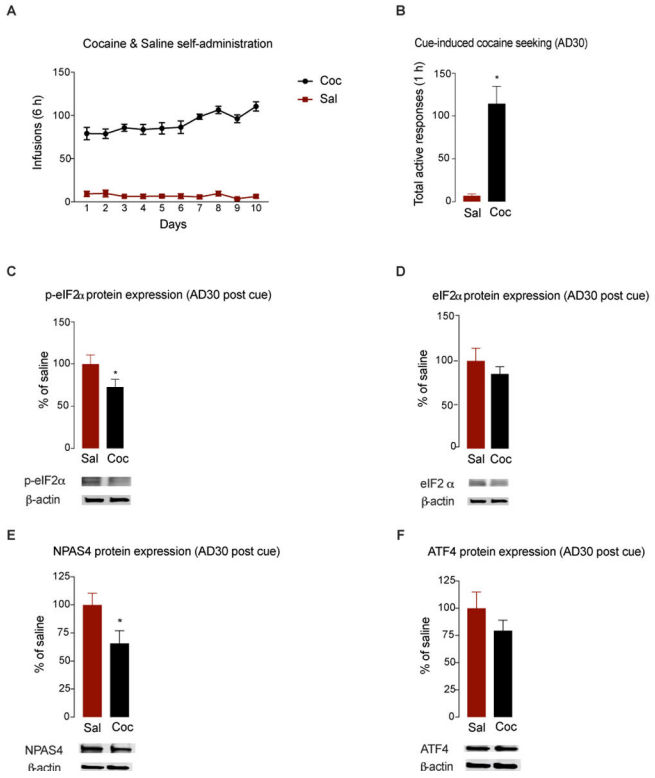

Figure 3. eIF2α phosphorylation is reduced after cue-induced cocaine seeking on AD30.

(**A**) Number of infusions during self-administration training for animals receiving saline (Sal) or cocaine (Coc). (**B**) Total active responses to a previously drug-paired cue on AD30 by animals trained to administer saline or cocaine. Phosphorylation (**C**) and total protein (**D**) levels of eIF2α in the NAc on AD30 following cue exposure. Protein levels of NPAS4 (**E**) and ATF4 (**F**) in the NAc on AD30 following cue exposure (n = 6–7/group). All data are presented as means ± SEMs with statistical significance (*) at $P < 0.05$.

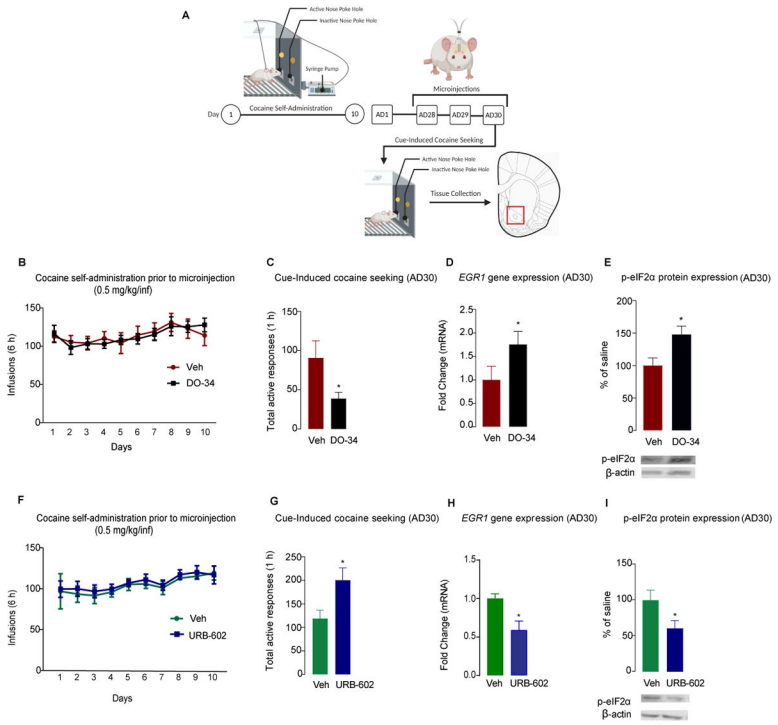

Figure 4. Pharmacological inhibition of DAGL and MAGL in the NAc bidirectionally alters cue-induced cocaine seeking and translational regulation.

(**A**) Experimental timeline for 10-day self-administration training, abstinence periods, intra-NAc microinjections, cue-induced seeking test, and tissue collection. (**B**) Number of infusions during self-administration training for animals that later received DO-34 or vehicle (Veh). (**C**) Total active responses to a previously drug-paired cue on AD30 by animals receiving DO-34 or vehicle. (**D**) *EGR1* gene expression in the NAc at AD30 following cue exposure in animals receiving DO-34 or vehicle microinjections. (**E**) eIF2α phosphorylation levels in the NAc at AD30 following cue exposure in animals receiving DO-34 or vehicle. (**F**) Numbers of infusions during self-administration training for animals that later received URB-602 or vehicle (Veh). (**G**) Total active responses to a previously drug-paired cue on AD30 by animals receiving URB-602 or vehicle. (**H**) *EGR1* gene expression in the NAc at AD30 following cue exposure in animals receiving URB-602 or vehicle microinjections. (**I**) eIF2α phosphorylation levels in the NAc at AD30 following cue exposure in animals receiving URB-602 or vehicle. All data are presented as means ± SEMs (n = 10–11/group) with statistical significance (*) at $P < 0.05$.

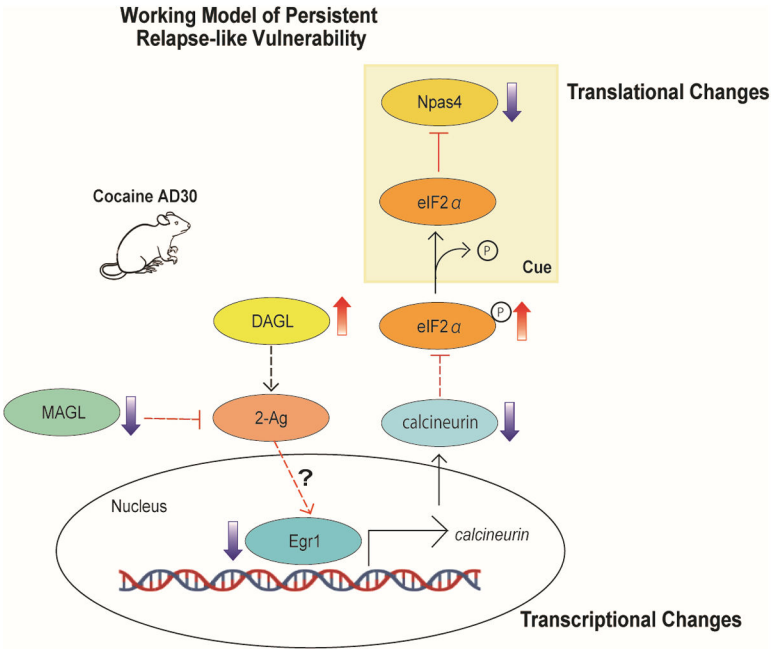

Figure 5. Proposed working model of endocannabinoid lipase-mediated neuroadaptations following prolonged abstinence from cocaine self-administration.

Prolonged abstinence from cocaine self-administration results in an imbalance in the endocannabinoid lipases characterized by an upregulation of DAGL and downregulation of MAGL in the NAc. Furthermore, EGR1 expression, EGR1 binding to the promoter of *calcineurin*, and calcineurin expression are reduced at AD30. This dysregulation of NAc endocannabinoid lipases coupled with dampened EGR1 transcriptional regulation on calcineurin facilitates reduced expression of translational initiator p-eIF2α and its downstream target NPAS4 upon exposure to drug-associated cues. eIF2α phosphorylation regulates activity-dependent protein synthesis of select targets underlying behavioral adaptations, and our findings elucidate a mechanism of endocannabinoid-mediated translational changes underlying cue-induced escalated cocaine craving. Red dashed lines indicate probable, but yet untested, cellular mechanisms.