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The incubation of cocaine craving is dissociated from changes in glial cell markers within prefrontal cortex and nucleus accumbens of rats

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1. Introduction

Cocaine use disorder (CUD) is characterized by impulsivity, compulsive drug- seeking and -taking with high rates of relapse, even after an extended period of abstinence, and the lack of effective treatment options impede lasting recovery [1,2]. Persistent cocaine-induced neuroadaptations that increase the probability of relapse have been identified in a number of animal models of substance use disorder (SUD) [e.g., 3-5]. However, a relatively small preclinical literature exists pertaining to the role of glial cell types in CUD-related brain pathology [c.f., 6-8]. Such research remains critical as chronic cocaine use is consistently correlated with reduced white matter volume within the prefrontal cortex (PFC) of humans [9-13]) that is theorized to results in disrupted connectivity/efficiency of signaling both

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The authors have no conflicts of interest to declare.

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Supplementary materials

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within the PFC proper and corticolimbic projections and contribute to the abnormal drive for cocaine in CUD.

Historically considered “support cells” for neurons, glial cells have essential roles in the regulation of neurotransmission, conduction of nerve impulses, neurotransmitter metabolism (including release), supply of energy metabolites, development and formation of synaptic connections, and repair [14-16]. Astrocytes, oligodendrocytes (OLs), and microglia also express receptors for many neurotransmitters and peptides (e.g., DA, glutamate, & GABA), rendering these cell types sensitive to neurochemical changes resulting from environmental insults, including cocaine administration [17]. Indeed, withdrawal from repeated cocaine administration increases the expression of the astrocytic marker GFAP (glial fibrillary acidic protein), along with significant modifications in astrocyte number, size and shape complexity, within the PFC, its major efferent the nucleus accumbens (NAC) and dentate gyrus [e.g., 18,19].

While astrocytes do not show classical electrical signaling, they have a form of calcium signaling initiated both spontaneously and through both ionotropic and metabotropic receptors on their cell surface [20], the latter of which activate IP3-dependent pathways [21]. The calcium waves generated can propagate between cells via the exchange of IP3 through gap junctions composed of connexin 30 and 43 proteins (Cx30 and Cx43, respectively); hemichannels composed of these proteins are known to release ATP into the extracellular space leading to further calcium wave spread among neighboring astrocytes [22,23]. Based on the above, we examined for the short- and longer-term effects of a history of extended cocaine-taking upon the expression of astrocyte-specific proteins involved in glutamate recycling and intracellular communication, specifically, glutamine synthetase (GS), Cx30 and Cx43.

Microarray analyses of post-mortem tissue from the nucleus accumbens (NAC) of humans with CUD revealed reduced expression of myelin-related genes including: myelin basic protein (MBP), proteolipid protein (PLP), myelin oligodendrocyte basic protein (MOBP), and myelin and leukocyte T-cell differentiation protein 2 [24]. Importantly, the abundance of MBP transcripts alone was sufficient to classify subjects as cocaine users with 80% accuracy [24]. Such findings, coupled with evidence for white matter loss in the PFC of humans with CUD [9-12], prompted us also to examine for changes in oligodendrocyte-specific proteins relating to myelin integrity in our cocaine self-administration model.

Finally, microglial cell activation has been linked to depression, a common disorder co-morbid with SUDs [e.g., 25-27]. Indeed, alcohol, methamphetamine, depression and stress all enhance innate immune gene induction within microglia [14]. Cocaine exposure *in vitro* or *in vivo* increases inflammation related-transcripts and markers of oxidative stress [28-32]. Stress has also been widely used as a mechanism to initiate relapse to drug-seeking in cocaine-experienced rodents, and both stress and drugs of abuse lead to microglial activation that promotes microglial proliferation [33,34]. To examine possible changes in microglial cell numbers following cocaine self-administration, we determined the levels of microglial specific protein, CD11b, an integrin important in migration, adhesion, and phagocytosis and a marker often used for identifying these cells types [35].

It was hypothesized that long-access to cocaine self-administration would lead to changes in glial protein expression within the NAC and PFC. Additionally, it was hypothesized that if changes in glial function contribute to drug craving, then the drug-elicited alterations in protein expression should correlate with the magnitude of craving during withdrawal from cocaine. To address these hypotheses, we examined tissue from rats expressing an “incubation of cocaine craving” [36,37] for the levels of glial markers at both early and protracted withdrawal.

2. Materials and methods

2.1. Subjects

Adult (275-325 g) male Sprague-Dawley rats (Charles River Laboratories, Hollister, CA) were housed in pairs in a colony room controlled for temperature (25 °C) and humidity (71%), under a 12-hr reverse cycle room (lights on at 20:00 hrs). Rats were given *ad libitum* access to food and water, except during lever response training for food reinforcement. Rats were allowed to acclimate to the colony room for three days following arrival then were handled daily till self-administration procedures were complete. All animal procedures were in accordance with the guidelines of the National Institutes of Health *Guide for Care and Use of Laboratory Animals* (Publication 80-23, revised 1996) and were reviewed and approved by the University of California Santa Barbara, Institutional Animal Care and Use Committee.

2.2. Experiment 1 tissue

In all, two immunoblotting experiments were conducted, the first of which (Experiment 1) was conducted on tissue collected from a prior behavioral study of incubated cocaine-seeking, the details of which are provided in Ben-Shahar et al. [38] and are similar to those detailed below for Experiment 2. Experiment 1 was designed to test the hypothesis that an incubation of cocaine craving is associated with changes in glial cell markers. Thus, for Experiment 1, tissue was compared between rats that underwent daily 6-h cocaine (COC-6h) or saline (SAL-6h) intravenous self-administration sessions for a period of 10 days. At 3 or 30 days withdrawal (WD3 or WD30, respectively), the rats underwent a 2-h test for cue-elicited cocaine-seeking to index an incubation of cocaine craving. Immediately following these tests, rats were decapitated, brains removed and the tissue from the ventromedial PFC (vmPFC) and dorsomedial PFC (dmPFC), as well as the core and shell subregions of the NAC were dissected out over ice (see Fig. 1) and stored frozen at –80 °C until time of immunoblotting, which occurred within 6-9 months from dissection (see Ref. 38 for details).

2.3. Experiment 2 lever response training

The rats in Experiment 2 were first trained to lever press for food re-inforcement using identical methods to those described by our group (45 mg pellets; Noyes, Lancaster, NH; see Ref 38) on a fixed ratio 1 (FR1) schedule in sound-attenuating operant conditioning chambers (30 × 20 × 24 cm high; Med Associates Inc., St. Albans, VT) during daily 1-hr training sessions. Rats were food-deprived 24-hr prior to the initiation of training and maintained on a restricted diet for the duration of food training. The operant chambers contained two retractable levers with a stimulus light located above each lever, a food pellet

dispenser located outside the operant chamber, a food trough between the levers, a house light on the opposite wall to the levers, a speaker connected to a tone generator (ANL-926, Med Associates), and a fan to provide ventilation and mask extraneous noise. During the session, pressing the active lever resulted in the delivery of a food pellet and pressing the inactive lever had no programmed consequence. Rats that failed to reach the criterion of a minimum of 100 responses on the active lever during two consecutive 1-hr sessions, received additional food training sessions until criterion was reached. Following successful acquisition of lever-pressing behavior (5-7 days of 1-hr daily sessions), food was freely available for the remainder of the study.

2.4. Experiment 2 surgery

Following lever response training, rats underwent surgical implantation of chronic intravenous (IV) catheters as previously described by our group [e.g., 38-40]. Under ketamine/xylazine anesthesia (56.25 and 7.5 mg/kg, respectively, administered intramuscularly (IM); Abbott Laboratories, North Chicago, IL), rats were implanted with a chronic silastic catheter (13 cm long; 0.3 mm inner diameter, 0.64 mm diameter; Dow Coming Corporation, Midland, MI) into the right jugular vein. Each catheter ran subcutaneously around the shoulder to back where it was secured to a threaded 22-gauge metal guide cannula (Plastics One, Roanoke, VA), which surfaced from the midline of the animal's back between the shoulder blades. A plastic plug covered the open end of the cannula to protect from contamination, and a metal bolt was secured around the threading of the cannula to prevent rats from chewing the catheter port. The cannula was held in place with a small square of Bard Mesh (C. R. Bard Inc., Cranston, RI) to which it was cemented, and the mesh was laid flat subcutaneously on the animal's back. Banamine (2 mg/kg, subcutaneous, Butler Schein Animal Health, Dublin, OH) was administered to treat post-surgical pain. All rats were allowed a minimum of 5 days for recovery and IV catheter patency was maintained by flushing daily with 0.1 ml of sterile heparin (60 IU/ml; Sagent Pharmaceuticals, Schaumburg, IL) and timentin/saline (100 mg/ml; Glaxo-SmithKline, Research Triangle Park, NC) or 0.1 Gentamicin/Cefazolin (1 mg/ml, Butler Schein Animal Health, and 5 mg/ml, Westward Pharmaceuticals, Eatontown, NJ, respectively).

2.5. Experiment 2 self-administration training

Following surgical recovery, rats (n=10-15 per group at the start of each experiment) were trained to self-administer IV cocaine (0.25 mg/0.1 ml/infusion; National Institute on Drug Abuse, Bethesda, MD) during daily 6-h sessions (COC-6h), or saline (0.1 ml/infusion) during 1-h sessions (SAL-1h), on a FR1 schedule of reinforcement. As in our prior work [e.g., 38-40], the shorter saline-access group was included in Experiment 2 to facilitate study throughput and was justified based on the results of a pilot immunoblotting experiment directly comparing the expression of certain glial cell markers between the SAL-6h tissue from Experiment 1 and the SAL-1h tissue from Experiment 2 (Supplemental Table 1) – a finding consistent with our prior studies of glutamate-related protein expression [e.g., 38-40].

At the beginning of each session, the animal's catheter was connected to a motorized pump (located outside of the sound attenuated chamber) via a liquid swivel as previously

described [e.g., 38]. Active lever responses elicited a 5-sec activation of the infusion pump and a 20-sec presentation of a visual (white light) and auditory (tone generator, 70 dB, 2 kHz) stimulus combination, during which additional responses had no consequences. Responses on the inactive lever were recorded but had no programmed consequences. Rats were trained to self-administer cocaine or saline for 10 sessions. To prevent overdose, the number of cocaine infusions permitted during the first two training sessions were limited at 100 (session 1) and 150 (session 2) and rats failing to meet self-administration criterion (minimum of 50 infusions/6-hr session for the last 3 days of training) were excluded from the study. All self-administration training and testing occurred during the dark phase of the light cycle. Following the 10 days of self-administration training, rats remained in the colony room for a minimum of 3 days of withdrawal.

2.6. Experiment 2 tests for cue-elicited cocaine-seeking

Prior studies from our laboratory indicate that many of the protein changes observed within the vmPFC of cocaine-incubated rats reflect factors associated with responding for the cocaine-associated cues as they are not observed in rats with similar cocaine self-administration histories left undisturbed in the home cage [e.g., 38-40]. As the results of Experiment 1 failed to indicate any time-dependent changes in the expression of glial cell markers (see Results below), we tested the rats in Experiment 2 only at the 3 WD time-point, with half of the rats subjected to a 2-hr test for cue-elicited cocaine seeking under extinction conditions and the other half of the rats left undisturbed in the home cage. For cue-testing, rats were tethered as during the self-administration training phase of the study and lever-press responses were recorded. Responses on the active lever elicited the tone + light compound stimulus previously paired with cocaine/saline infusions, but no infusions were administered during this test. Responses on the inactive lever had no programmed consequences, but were recorded. Upon the completion of the 2-h cue test session, Experiment 2 rats were anesthetized with 2-3% isoflurane, decapitated, and the vmPFC was dissected out over ice (see Fig. 1). Following three days withdrawal, the rats left undisturbed in their home cage (“untested” controls) were also anesthetized with isoflurane and the vmPFC removed. This region was selected for study in Experiment 2 as it exhibited the most robust saline-cocaine differences in protein expression as determined in Experiment 1 (see Results below).

2.7. Experiment 2 tissue homogenization

vmPFC tissue samples were homogenized in QIAzol lysis reagent (Qiagen, Louisville, KY), using the Qiagen TissueRuptor, and separated into aqueous and organic phases by the addition of chloroform and centrifugation at 12,000 *g*. The organic phase of the tissue preparation with QIAzol lysis reagent was further processed for the extraction of total protein. Any remaining aqueous phase was removed and isopropanol added to precipitate protein followed by centrifugation at 12,000 *g*. The supernatant was removed and the protein pellet was incubated in 0.3 M guanidine-hydrochloride in 95% ethanol, followed by centrifugation at 7500 *g*, the supernatant removed and the process repeated twice. The protein pellet was air dried, re-suspended in 10 M urea and 50 mM DTT in water, broken up with a needle and incubated at room temperature for 1-hr. Samples were then incubated at 95 °C for 3 minutes, sonicated in short bursts, and centrifuged at 10,000 *g*. 5 ul of loading

dye consisting of 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris HCl, was added to 20 μ l of each sample, vortexed and loaded into a Bis-Tris gradient gel (4-12%; Invitrogen). Following electrophoresis, proteins were transferred to PVDF membranes and further processed via immunoblotting procedures as described below.

2.8. Immunoblotting

For both experiments, protein samples (20 μ g/lane) were subjected to SDS-polyacrylamide gel electrophoresis and non-reduced when run on Bis-Tris gradient gels (4-12%) and Tris-Glycine gradient gels (4-20%) (Invitrogen, Carlsbad, CA) or reduced when run on Tris-Acetate gradient gels (3-8%) (Invitrogen). Bis-Tris gradient gels were used for separation of Cx30, Cx43, GS, MBP, MOG, and PLP, Tris-Glycine gradient gels for claudin-11, Cx32, and MAG and, Tris-Acetate gradient gels were used for separation of CD11b, CNPase, Cx47, NF155, NG2+, and UGT8. Proteins were transferred to wet polyvinylidene difluoride (PVDF, BioRad) membranes, and were pre-blocked with phosphate-buffered saline containing 0.1% (v/v) Tween-20 and either 5% (w/v) bovine serum albumin (for CD11b, Cx30, Cx43, Cx47, MBP, MOG, and NG2+), 1.5% normal horse serum, or 5% (w/v) non-fat dried milk powder (for all other proteins) for no less than 2h at room temperature or overnight at 4 °C, followed by a 2-h incubation with primary antibodies. The following primary antibodies were used: rabbit- anti-CD11b (Novus Biologicals, Littleton, CO; Catalogue number: NB110-89474B; RRID:AB_1659994; 1:500 dilution); mouse-anti-CNPase (Millipore, Temecula, CA; Catalogue number: MAB326R; RRID:AB_94780; 1:500 dilution); rabbit-anti-Cx30 (Invitrogen, Carlsbad, CA; Catalogue number: 71-2200; RRID:AB_2533979; 1:500 dilution); mouse-anti-Cx32 (Chemicon International, Millipore; Catalogue number: MAB3069; RRID:AB_2110769; 1:500 dilution); mouse-anti-Cx43 (Millipore; Catalogue number: MAB3067; RRID:AB_11210474; 1:1000 dilution); rabbit-anti-Cx47 (Santa Cruz Biotechnology, Santa Cruz, CA; Catalogue number: sc-30335; no RRID available; 1:500 dilution); rabbit-anti-GS (Thermo Fisher Scientific, Rockford, IL; Catalogue number: PA1-46165; RRID:AB_2263362; 1:10,000 dilution); mouse-anti-myelin associated glycoprotein (Millipore; Catalogue number: MAB1567; RRID:AB_2137847; 1:1000 dilution); rabbit-anti-MBP (GenWay Biotech, San Diego, CA; Catalogue number: 18-783-77273; RRID:AB_1026855; 1:1000 dilution); goat-anti-MOG (LifeSpan Biosciences, Seattle, WA; Catalogue number: LS-B2419; RRID:AB_2145554; 1:1000 dilution); mouse-anti-Neurofascin (R&D Systems, Minneapolis, MN; Catalogue number: MAB3235; RRID:AB_10973333; 1:500 dilution); rabbit-anti-NG2 Chondroitin Sulfate Proteoglycan (Millipore; Catalogue number: AB5320; RRID:AB_11213678; 1:1000 dilution); rabbit-anti-oligodendrocyte specific protein (claudin-11; Abcam, Cambridge, MA; Catalogue number: ab53041; RRID:AB_2276205; 1:1000 dilution); mouse-anti-PLP (LifeSpan BioSciences; Catalogue number: LS-B2867; RRID:AB_1936047; 1:1000 dilution); rabbit-anti-UGT8 (Proteintech Group Inc., Chicago, IL; Catalogue number: 17982-1-AP; RRID:AB_2214254; 1:500 dilution). Membranes were washed, incubated with a horseradish peroxidase-conjugated anti-rabbit secondary antibody (Millipore; 1:40,000-1:100,000 dilution), anti-mouse secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA; 1:40,000 dilution), or anti-goat secondary (Jackson Immuno Research Laboratories, West Grove, PA; 1:20,000 dilution) for 90 min at room temperature, washed again, and immunoreactive bands were detected by enhanced chemiluminescence

using either ECL Plus (Amersham Biosciences, GE Healthcare Life Sciences, Pittsburgh, PA) or Pierce SuperSignal West Femto (Thermo Fisher Scientific, Rockford, IL). A rabbit anti-calnexin polyclonal primary antibody (Enzo Life Sciences Inc., Farmington, NY; Catalogue number: ADI-SPA-860-F; RRID:AB_11178981; 1:1000 dilution) was also used to index protein loading, transfer, and as a reference protein for comparison. The levels of immunoreactivity for all proteins were quantified using Image J (NIH, Bethesda, MD).

Each immunoblotting gel contained samples from each of the different conditions so comparisons could be made across drug treatment and withdrawal time-point (Experiment 1) or cue-testing experience (Experiment 2). The immunoreactivity of each protein band was normalized to its respective calnexin immunoreactivity to control for loading. As in our prior work [e.g., 29-31], the immunoblotting data for each protein of interest are expressed as a percent of the average immunoreactivity of the SAL-6h 3WD control (for Experiment 1) or the SAL1h-No Cue control (for Experiment 2).

2.9. Statistical analysis

Two-way ANOVAs were conducted on the data to examine for Training Condition (COC vs SAL, or SAL1h vs SAL6h) X Withdrawal (3 or 30 days following the last self-administration session) interactions, or for Training Condition X Test (Tested vs Untested) interactions. Fisher's LSD tests were used to deconstruct significant interaction effects. Significance was set at $\alpha = 0.05$. Throughout, the data are presented graphically as box plots to illustrate the data mean and median, as well as the interquartile range (IQR), with the error bars representing 1.5 X IQR. Datapoints that lie outside the 1.5 X IQR are also indicated in each Fig. panel. All Data were analyzed with Prism 6 for Mac OS X, version 6 (GraphPad, Software Inc.).

3. Results

3.1. The incubation of cocaine craving is dissociated from reductions in the expression of certain glial-related proteins (Experiment 1)

3.1.1. Summary of Behavior—The behavioral data for the tissue employed in this study has been previously published [29] and indicated that rats with a 10-day history of 6-h access to IV cocaine exhibited a time-dependent intensification of responding on the active, cocaine-associated, but not the inactive, lever when assessed under extinction conditions (i.e., an incubation of cocaine craving). The average number of cocaine infusions across the last 3 days of self-administration training were similar for the rats tested on withdrawal day 3 and day 30 (for day 3, 125.3 ± 12.4 , for day 30, 121.3 ± 8.9 ; see Ref. 29 for full results, including graphical depictions of behavioral data).

3.1.2. Immunoblotting for glial-specific proteins—We first compared glial-specific protein expression between the COC-6h and SAL-6h rats tested for cue-elicited craving on WD3 versus WD30. A comparison of vmPFC protein levels indicated significant main effects of Training Condition for MBP (Fig. 2A) [$F(1,32)=5.96$, $p=0.021$], myelin oligodendrocyte glycoprotein (Fig. 2B) (MOG) [$F(1,35)=7.67$, $p=0.009$], and NG2+ (Fig. 2C) [$F(1,36)=4.52$, $p=0.040$]. The results of the ANOVA for these proteins did not indicate

significant main effects of Withdrawal or significant Training Condition X Withdrawal interactions (all p 's>0.05). Analyses of the other proteins examined within the vmPFC failed to reveal any group differences in protein expression (two-way ANOVAs, all p 's>0.05; Supplemental Table 2A). Moreover, no statistically significant group differences in protein expression were found within the dmPFC for any of the proteins examined (two-way ANOVAs: all p 's>0.05; Supplemental Table 2B).

Within the NAC core subregion, a comparison of protein levels revealed a significant main effect of Training Condition for Cx43 expression only (Fig. 3A) [$F(1,48)=7.836$, $p=0.008$; 2-way ANOVAs, all other p 's>0.05]. No statistically significant changes in protein expression were found within the NAC shell subregion for either Training Condition or Withdrawal for any of the proteins examined (two-way ANOVAs: p 's>0.05; see Supplemental Table 3).

3.2. Early withdrawal from cocaine reduces MBP expression within the vmPFC irrespective of cue-testing conditions (Experiment 2)

As the results of Experiment 1 revealed little interaction between IV cocaine history and withdrawal (Fig. 2), we tested rats at the WD3 time-point only in Experiment 2.

3.2.1. Behavior during the self-administration and cue-testing phases of Experiment 2—The behavioral data for Experiment 2 are summarized in Table 1. During self-administration training, cocaine rats earned significantly more infusions than saline controls, and the number of infusions earned were equivalent between the rats slated for cue-testing and those slated to remain in their home cage during withdrawal [Training Condition effect: $F(1,44)=1142$, $p<0.0001$; no Testing effect or Training Condition X Testing interaction, p 's>0.05]. Similarly, both cocaine groups pressed the active lever significantly more than saline controls as revealed by a significant main effect of Training Condition [$F(1,44)=404.8$, $p<0.0001$], but no Testing effect or interaction between these variables (p 's>0.05). Cocaine self-administering rats also pressed the inactive lever significantly less than saline controls revealed by a significant main effect of Training Condition [$F(1,42)=6.35$, $p=0.016$]. For the rats tested for cue-reinforced behavior, cocaine-experienced rats responded on both active and inactive levers significantly more during the 2-h cue-seeking test than did saline controls [Treatment x Lever, $F(1, 43)=18.47$, $p<0.0001$]. *Post-hoc* tests revealed that active lever presses emitted by cocaine-experienced rats was greater than their inactive lever presses (p 's<0.05), but the inactive lever presses did not differ between cocaine and saline groups (see Table 1).

3.2.2. Immunoblotting results of experiment 2—The protein extraction procedure for Experiment 2 yielded only a sufficient amount of vmPFC tissue to conduct immunoblotting on a limited number of proteins. Based on the results of Experiment 1, we opted to conduct immunoblotting for MBP protein expression. Akin to the results of Experiment 1, a two-way ANOVA revealed a significant main effect of Training Condition for MBP, which reflected *lower* expression of the 18.5 kDa MBP in cocaine rats versus saline controls [$F(1,32)=6.128$, $p=0.019$; Testing effect and Training Condition X Testing interaction, p 's>0.05] (Fig. 4).

3. Discussion

The present study was conducted to determine the impact of a history of extended cocaine-access and withdrawal upon changes in glial-specific markers within PFC and NAC subregions. Overall, cocaine self-administration produced relatively few changes in the total protein expression of many of the markers investigated herein. Interestingly, despite an incubation of cue-elicited drug-seeking behavior during protracted withdrawal, the observed changes in glia-specific protein expression were independent of the duration of withdrawal from cocaine. Thus, the relatively few glia-related protein changes observed early during withdrawal persisted for at least 30 days, indicating that a history of extended access to cocaine produces certain enduring “glioadaptations” that are likely to impact brain function of relevance to the neurobiology of CUD.

In Experiment 1 (Fig. 3A), a history of cocaine-taking produced a persistent decrease in the astrocyte-specific gap junction protein Cx43 within the NAC core. This finding is consistent with other reports in the literature indicating an effect of withdrawal from cocaine upon the functional status of astrocytes [18,41]. As mentioned at the outset of this report, GFAP expression is elevated within the PFC, NAC core and shell at 3 weeks withdrawal from a repeated cocaine injection regimen. As GFAP provides intermediate filament scaffolding, this finding was hypothesized to influence astrocytic process stability [18]. Moreover, experimenter-administered cocaine is reported to increase GFAP expression within the dentate gyrus, along with significant increases to cell numbers, size, and shape complexity [19]. Additionally, cocaine-induced increases in glutamate signaling within the NAC core might also interact with astrocytic ionotropic and metabotropic glutamate receptors to alter astrocyte function [42]. When stimulated, these receptors cause increases in cytoplasmic calcium concentrations within astrocytes; propagation of calcium waves is a form of intra- and inter-cellular signaling and can affect gene transcription through calcium-dependent second messenger pathways [20]. Through the formation of gap junctions and hemichannels, Cx43 mediates calcium wave propagation [22,23]. Therefore, an early and persistent reduction in Cx43 following extended cocaine-access would be predicted to reduce calcium signaling both within and between astrocytes of the NAC core. Theoretically, impaired calcium signaling could result in immediate effects upon calcium-dependent enzyme activation, including those involved in regulating gene transcription, protein translation, synthesis and turnover [e.g., 22,23], which could be envisioned to have both short- and longer-term effects upon protein expression/function within astrocytes. A change in astrocytic calcium signaling could also result in alterations in astrocytic release of glutamate and ATP by calcium-dependent exocytosis, leading to changes in neuronal excitability [20,43]. The functional relevance of altered astrocytic calcium signaling for behavior still requires direct investigation.

In Experiment 1 (Fig. 2), we also detected an early and persistent reduction in the oligodendrocytic proteins MBP, MOG and NG2+ within the vmPFC of cocaine self-administering rats. Such findings from an animal model of cocaine-taking are consistent with clinical reports of decreased white matter integrity in humans with CUD [9-13]. George et al. [44] also reported decreases in NG2+ immunoreactive oligodendrocytes within the PFC that correlated with deficits in working memory following long-access

to cocaine. These authors suggested decreased labeling of NG2+ as indicative of decreased oligodendrocyte numbers; however, this particular protein is more commonly used to identify oligodendrocyte progenitor cells [45]. Therefore, a reduction in NG2+ immunoreactivity could reflect a combined decrease in mature and progenitor oligodendrocytes, or progenitors only. Alternatively, NG2+ positive cells have also been found to differentiate into gray matter astrocytes, neurons, or remain as a small population of mature NG2+ glial cells distinct from oligodendrocytes [46]. Therefore, changes in NG2+ levels are not specific to a particular cell type nor a reflection of an effect of cocaine solely on oligodendrocytes.

MOG is a minor component of compact myelin that is expressed during the later stages of oligodendrocyte development and has been proposed as a marker for mature cells [46,47]. The fact that both MOG and NG2+ expression is reduced within the vmPFC following cocaine self-administration in Experiment 1 (Fig. 2) would suggest a reduction in both mature and progenitor oligodendrocytes, complementing the findings of George et al. [44]. This raises an interesting hypothesis that reduction in white matter observed in humans with CUD, might reflect stunted oligodendrocyte maturation and subsequent improper myelination of axons [9]. Unfortunately, as the tissue processing techniques conducted in Experiment 2 were optimized for MBP protein detection and there was insufficient tissue to replicate the results from Experiment 1 for proteins other than MBP. Thus, the co-regulation of MOG and NG2+ by a history of long-access cocaine self-administration requires replication in future studies in order to provide a more solid basis for the study of these proteins in SUD-related changes in brain and behavior.

MBP is a major protein component of the myelin sheath that is vital for the formation of myelin and the major dense line [45,48]. It is notable that the cocaine-induced reduction in vmPFC MBP expression observed in rats tested for cue-reinforced responding on WD3 in Experiment 1 (Fig. 2) was replicated in a separate cohort of rats in Experiment 2 (Fig. 4), conducted several years later. Moreover, the results of Experiment 2 also indicated that a cocaine-induced reduction in vmPFC MBP can be observed also in rats left undisturbed in the home cage during withdrawal (Fig. 4). Thus, history of extended cocaine access is sufficient to elicit a deficit in MBP within vmPFC and re-exposure to the drug-associated stimuli are not required to induce this deficit in protein expression. The cocaine-induced reduction in MBP expression observed within the vmPFC might reflect increased protein degradation, or alternatively, decreased gene transcription. As the biogenesis and maintenance of the myelin sheath remains poorly understood [49-51], it will be important to determine in future work the mechanisms through which withdrawal from long-access cocaine self-administration lowers MBP expression. As alterations in MBP phosphorylation and expression impact myelin compaction and structure to influence the conduction velocity and efficiency of action potentials critical for normal neuronal communication [48,52], cocaine-induced changes in MBP levels within the vmPFC may be key to perturbations in brain function in CUD.

It is curious that the cocaine-induced reduction in oligodendrocyte-related markers occurred selectively within the vmPFC, with no discernable effects detected in the more dorsal dmPFC (Supplemental Table 2) or either NAC subregion (Supplemental Table 3). Although

we were careful to avoid sampling from the rostral corpus callosum while dis-ecting out the dmPFC and vmPFC (see Fig. 1), it is possible that the vmPFC samples contained trace amounts of corpus callosum, perhaps facilitating detection of cocaine's effects upon oligodendrocyte/myelin-associated proteins. However, our samples of the NAC core contained the anterior commissure and no changes in oligodendrocyte markers were detected therein. The activity of both the vmPFC [38-40] and NAC core [c.f., 53] are required for the expression of incubated cocaine-seeking. Thus, it will be important to determine in future work how a history of cocaine self-administration impacts myelin-related protein expression specifically in white matter within these regions and to examine for the time-dependency of any such effects.

Both *in vivo* and *in vitro* cocaine exposure elevates inflammation-related transcripts and innate immune signaling. CD11b serves as a marker of microglial cells involved in innate immune signaling. While we failed to detect any change in CD11b expression following cocaine self-administration herein (Supplemental Table 2), other markers of microglial activation may be more sensitive indicators of an effect of cocaine on this glial cell type. For example, Ahmed et al. [28] reported increased IL-6, IL-4, and TNF mRNA within the PFC of rats with 18 days of short (1-h) or extended access (6-h) cocaine self-administration, and Piechota et al. [32] reported increased IL-6 α and TNF α within the caudate-putamen and NAC following a single injection of cocaine (25 mg/kg, IP) [see Ref 29 for detailed discussion]. Administration of other drugs of abuse also result in enhanced immune gene expression, including ethanol and methamphetamine [e.g., 54,55]. Thus, future studies should assess an array of immune-related proteins to more accurately examine for cocaine-induced changes in microglial function as it relates to the manifestation of drug-elicited changes in SUD-related behavior.

In summary, the results of the present study indicate that, in rats, long-access cocaine self-administration leads to early and persistent reductions in glial subtype-specific proteins, particularly, within vmPFC that may mediate the hypofunction of this area. Future studies need to directly examine the effect of cue-testing on expression levels of other glial-specific proteins, in order to better understand the role played by pharmacological and non-pharmacological factors in mediating the effects of cocaine self-administration upon the functional integrity of non-neuronal cells within brain regions exhibiting anomalous function in CUD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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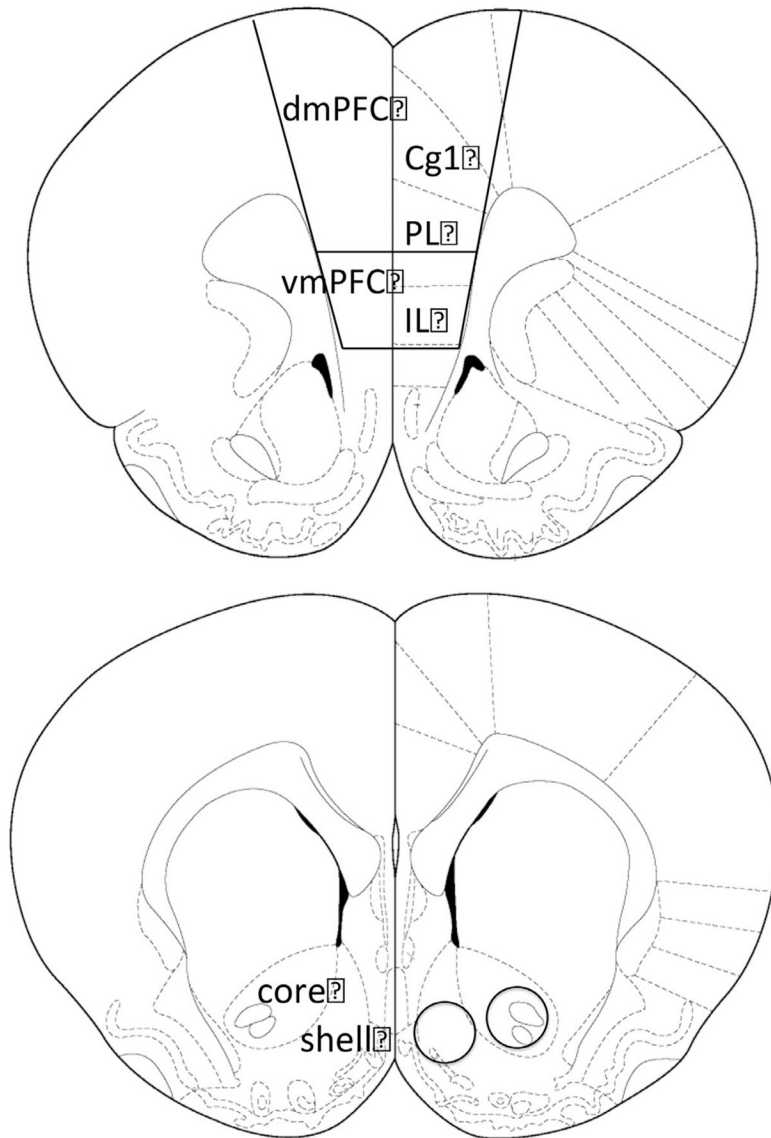


Fig. 1. Depiction of the gross dissections of PFC and NAC subregions for the immunoblotting studies conducted in this report.

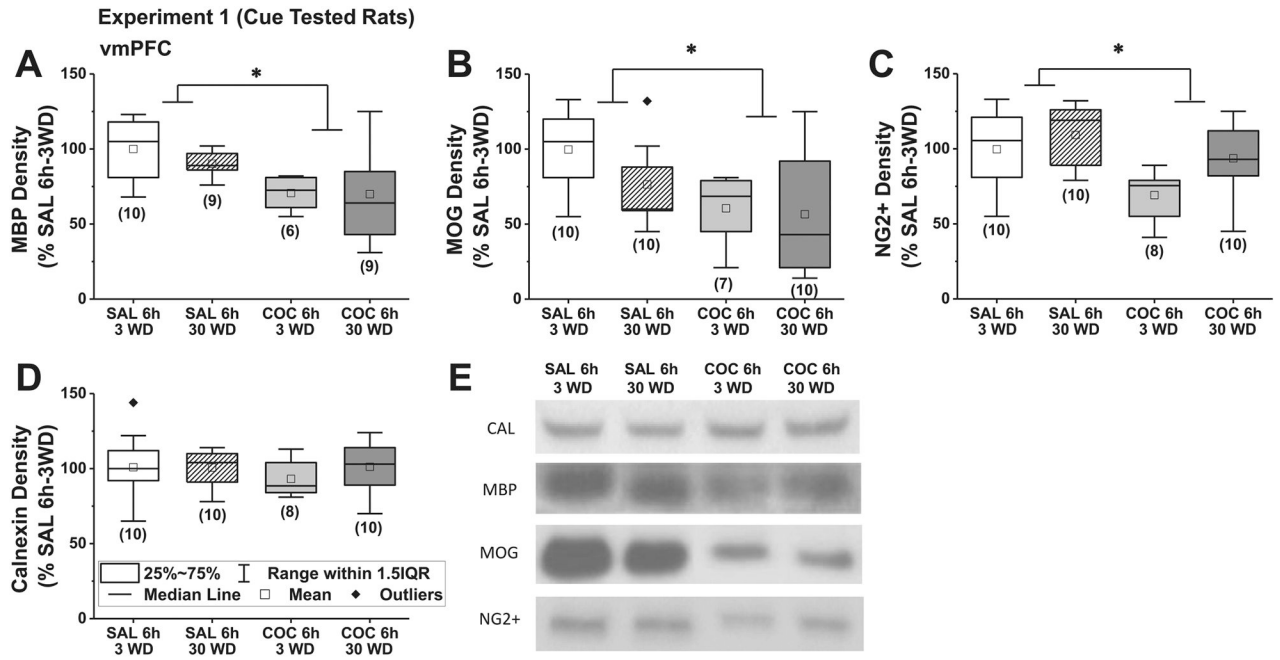


Fig. 2.

A history of cocaine self-administration produces a time-independent reduction in MBP, MOG and NG2+ expression within the vmPFC (Expt 1). Rats self-administered cocaine (COC) during extended access (6h) procedures and underwent a 2-h cocaine-seeking test prior to sacrifice. Relative to saline (SAL) self-administering controls tested on withdrawal day 3 (3WD), cocaine-experienced rats exhibited decreased expression of MBP (A), MOG (B), and NG2+ (C) within the vmPFC on 3WD - an effect that persisted into protracted withdrawal (30WD). For panels A-C, the optical density for each protein within a treatment condition is represented as a percent of the SAL 6h-3 WD condition. (D) Calnexin (CAL) was used as a reference protein to control for differences in gel loading, and was not significantly affected by Training Condition or Withdrawal conditions. (E) Representative immunoblots from SAL 6h-3 WD, SAL 6h-30 WD, COC 6h-3 WD and COC 6h-30 WD treatment conditions. * $p < 0.05$, Training Condition main effect of COC versus SAL.

Experiment 1 (Cue Tested Rats)

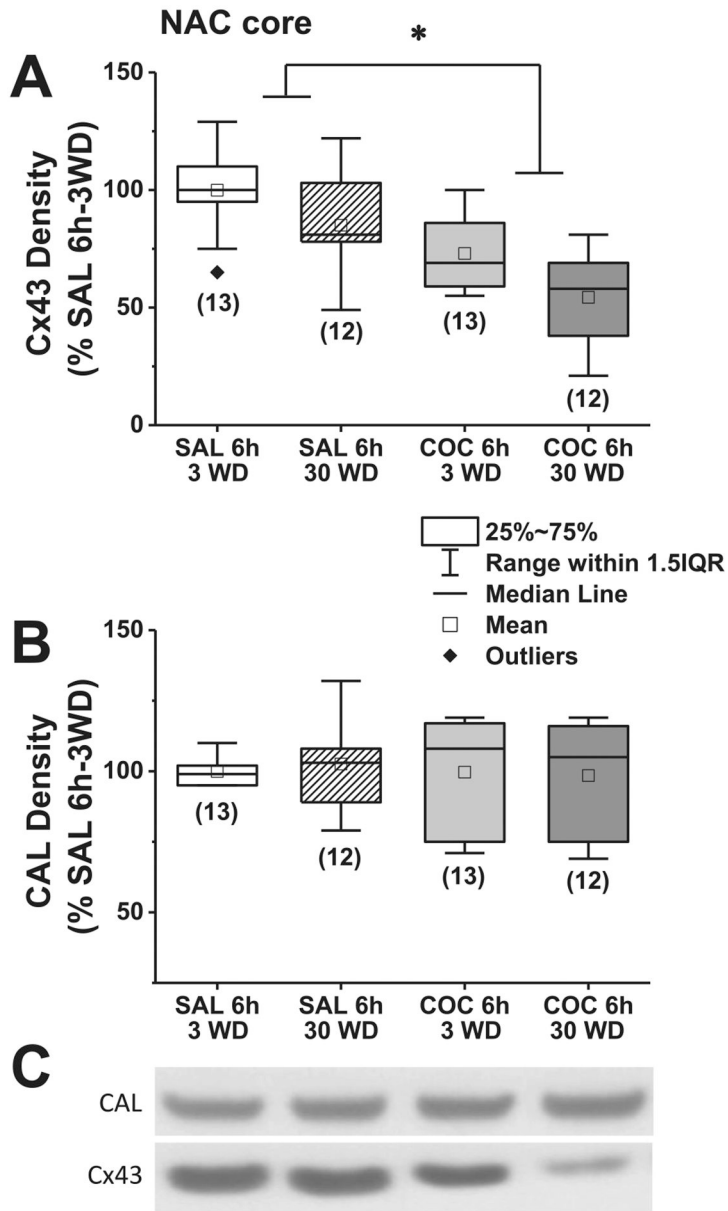


Fig. 3. A history of cocaine self-administration produces a time-independent reduction in Cx43 within the NAC core (Expt 1).

Rats self-administered cocaine (COC) during extended access (6h) procedures and underwent a 2-h cocaine-seeking test prior to sacrifice. Relative to saline (SAL) self-administering controls tested on withdrawal day 3 (3WD), cocaine-experienced rats exhibited decreased expression of Cx43 within the NAC core on 3WD - an effect that persisted into protracted withdrawal (30 WD). The optical density for Cx43 (A) and the loading control protein calnexin (B) within a treatment condition are represented as a percent of the SAL 6h-3 days withdrawal condition. (C) Representative immunoblots from SAL 6h-3 WD, SAL 6h-30 WD, COC 6h-3 WD and COC 6h-30 WD treatment conditions. Calnexin (CAL) is used as a reference protein to control for differences in gel loading,

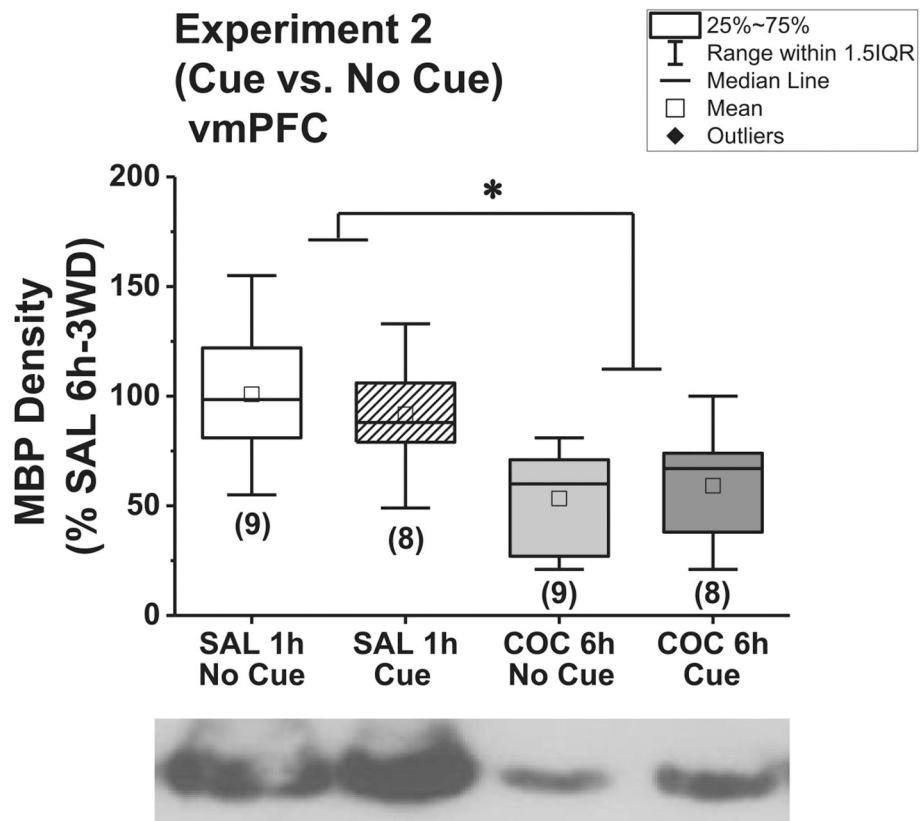
and is not significantly affected by Training Condition or Withdrawal conditions. * $p < 0.05$, Training Condition main effect of COC versus SAL.

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**Fig. 4.**

The opportunity to respond for cocaine-associated cues under extinction conditions does not influence the cocaine-induced reduction in MBP expression within vmPFC. (Expt 2). Rats self-administering cocaine (COC) during extended access (6h) procedures exhibited decreased expression of MBP within the vmPFC compared to saline (SAL 1h) self-administering controls. This was observed irrespective of behavioral testing (cue-tested) prior to sacrifice. Depicted data represent the optical densities for each protein within a treatment condition and are expressed as a percent of the SAL 1h-untreated condition. A representative immunoblot from SAL 1h-untreated, SAL 1h-cue-tested, COC 6h-untreated and COC 6h-cue-tested treatment conditions lies below the graphical data. * $p < 0.05$, Training Condition main effect of COC versus SAL.

Behavioral outcomes obtained from Expt 2, in which groups of rats were trained to self-administer saline (1 h/day) or cocaine (6 h/day) for 10 days. Half of the rats from each self-administration group were then tested for cue-reinforced responding at 3 days withdrawal (Tested), while the other half of the rats were left undisturbed in the home cage (Untested). Data represent the mean \pm SEM of the number of rats indicated in parentheses.

Table 1

Self-administration Phase	Saline-Untested (n=13)		Saline-Tested (n=11)		Cocaine-Untested (n=12)		Cocaine-Tested (n=12)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Infusions	5.03 \pm 1.17		4.15 \pm 0.68		94.97 \pm 3.77		97.64 \pm 3.63	
Active Lever Presses	7.97 \pm 1.90		6.58 \pm 1.28		117.67 \pm 8.04		125.00 \pm 7.61	
Inactive Lever Presses	4.08 \pm 1.23		2.61 \pm 0.35		1.21 \pm 0.48		1.30 \pm 0.70	
Cue Test Phase								
Active Lever Presses			11.36 \pm 2.53				96.58 \pm 16.80	
Inactive Lever Presses			7.417 \pm 1.69				16.42 \pm 3.21	