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Heroin self-administration and extinction increases prelimbic cortical astroglia-synapse proximity and alters dendritic spine morphometrics that are reversed by N-acetylcysteine

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Abstract

Both clinical and preclinical studies indicate that adaptations in corticostriatal neurotransmission underlie heroin relapse vulnerability. In animal models, heroin self-administration and extinction produce linked molecular and cellular adaptations in both astrocytes and neurons in the nucleus accumbens (NA) core that are required for cued relapse. For example, decreased expression of the glutamate transporter GLT-1 and reduced association of perisynaptic astrocytic processes with NAcore synapses allow glutamate overflow from prelimbic (PrL) cortical terminals to engage synaptic and structural plasticity in NAcore medium spiny neurons. Importantly, normalizing heroin-induced GLT-1 downregulation prevents glutamate overflow, medium spiny neuron plasticity, and relapse. Surprisingly, little is known about heroin-induced alterations in cortical astroglia and their interaction with neurons. Here we show that heroin SA followed by extinction leads to increased astrocyte complexity and association with synaptic markers in the PrL cortex in male Sprague-Dawley rats. Enhanced astroglial complexity and synaptic interaction were reversed during extinction by repeated treatment with N-acetylcysteine (NAC), an antioxidant drug previously shown to inhibit heroin seeking. We also show that dendritic spines of PrL cortical neurons projecting to the NAcore are enlarged, yet the density of spines is decreased, after extinction from heroin SA. Repeated NAC treatment prevented the decrease in spine density but not dendritic spine expansion. These results reveal circuit-level adaptations in cortical dendritic spine morphology that are related to heroin-induced alterations in astrocyte complexity and association at synapses and demonstrate that NAC reverses cortical heroin-induced adaptations in multiple cell types.

Introduction

Relapse remains a major clinical obstacle in treating opioid use disorders and is typically brought on by craving evoked by exposure to opioid-associated contexts or cues [1]. In humans, elevated activity in the corticostriatal circuit is a neural correlate of cue-evoked craving for heroin [2] and heroin-mediated dysfunction in corticostriatal circuity is faithfully recapitulated in animal models of heroin self-administration (SA) and relapse [3]. A general consensus from rodent models is that dysfunctional prefrontal cortical regulation of ventral striatal plasticity occurs as a result of chronic opioid exposure, allowing opioid-associated cues and contexts to drive drug seeking even after prolonged periods of abstinence [4].

The nucleus accumbens core (NAcore) is a ventral striatal region required for motivationally-relevant external stimuli to evoke motor programs linked to seeking reinforcers or rewards [5]. It has been repeatedly demonstrated that heroin SA in rodents alters glutamatergic plasticity in the NAcore in a manner that is mechanistically linked to relapse [6, 7]. For example, following extinction of heroin [8] or cocaine [9] SA, an inability to induce and maintain LTP and LTD in the NAcore is observed following stimulation of the prelimbic (PrL) cortex *in vivo*. These data demonstrate drug-mediated dysfunctional plasticity at PrL-NAcore synapses. Akin to cocaine [10], heroin SA and extinction produces reduce glutamate clearance in the NAcore due to downregulation of the high-affinity astroglial glutamate transporter, GLT-1 [11] and concomitant reductions in the proximity of GLT-1-containing perisynaptic astrocyte processes (PAPs) to NAcore synapses [12]. Interestingly, decreased astrocytic association with NAcore synapses is also observed following extinction of methamphetamine or cocaine SA [13, 14]. Together these data suggest that structural and cellular astrocyte dysfunction in the NAcore is a central aspect of the pathophysiology underlying relapse vulnerability [15]. As such, reduced GLT-1 expression coupled with decreased proximity of astrocyte PAPs to synapses likely synergistically facilitate glutamate spillover to extrasynaptic sites following synaptic release. Consistent with dysfunctional plasticity at PrL-NAcore synapses, cue- and heroin-primed seeking promotes sustained elevation of glutamate levels in the NAcore specifically due to release from PrL cortical afferents [16]. This elevated glutamate release and spillover facilitates LTP-like increases in synaptic strength, including an upregulation of NR2B-containing NMDA receptors and enlargement of dendritic spine heads, on NAcore medium spiny neurons (MSNs). Accordingly, it has been shown that normalizing drug-induced alterations in PrL-NAcore synaptic transmission, mediated in large part by astrocytic dysfunction, is a potent means for decreasing relapse vulnerability [17].

One drug that is particularly effective at targeting drug-induced adaptations in astrocytes is the antioxidant drug, N-acetylcysteine (NAC). NAC is a cystine pro-drug that has shown some promise clinically [18] but has been more extensively investigated preclinically as a research tool to probe glutamate homeostasis [15]. NAC has been shown to significantly limit heroin [19, 20], cocaine [21, 22], nicotine [23], or methamphetamine [14] seeking in rats with a drug self-administration history. Mechanistically, NAC is thought to prevent relapse by reversing the drug-induced downregulation of GLT-1 in the NAcore. This hypothesis was first confirmed in studies in which cocaine downregulated GLT-1 expression in the NAcore. In these studies, preventing NAC-mediated restoration of GLT-1 expression in the NAcore following cocaine SA and extinction blocked NAC's ability to prevent relapse [10, 24]. While NAC does indeed facilitate extinction and prevent cued heroin seeking [19], the ability of NAC to normalize heroin-induced decreases in GLT-1 in the NAcore remains to be directly evaluated. However, additional drugs that upregulate GLT-1 expression (i.e. ceftriaxone) have been shown to normalize heroin-induced decreases in glutamate clearance in the NAcore, inhibiting relapse [11]. Importantly, ceftriaxone has also been shown to normalize cocaine-induced decreases in PAP association with synapses in the NAcore [13], suggesting a significant structure-function relationship underlying drug-induced decreases in PAP-synapse associations, alterations in glutamate clearance, and relapse.

While there is a substantial body of work regarding the consequences of heroin SA and extinction on neuronal and astroglial plasticity in the NAcore, little is known about the impact of heroin on neurons or astrocytes in the PrL cortex. This gap in our knowledge is particularly significant given that cue-induced glutamate release in the NAcore during heroin seeking requires activation of the PrL cortex [16]. While less is known about the relationship between cortical astrocytes and opiates, there is some literature describing opiate-induced adaptations in cortical astroglia. For example, chronic morphine treatment increases expression of glial fibrillary

acidic protein (GFAP), a canonical marker for astrocytes [25], in the frontal cortex [26]. These data suggest that morphine induces astrogliosis, yet addition of morphine to astrocytes *in vitro* decreases proliferation and increases complexity of astrocyte processes, an effect prevented by naloxone pretreatment [27]. These data suggest that heroin may impact astrocyte function in the PrL cortex; however, such adaptations have yet to be shown in vivo or linked to the degree to which astrocytes associate with synapses.

Here we demonstrate that, in contrast to heroin-induced alterations in PAP interactions at NAcore synapses, heroin SA and extinction *increase* the association of PAPs with synaptic markers in the PrL cortex. This increased PAP-synaptic proximity was accompanied by increased complexity of the GFAP cytoskeleton of PrL cortical astrocytes. Importantly, both adaptations were normalized by repeated NAC treatment during extinction. Finally, heroin SA and extinction increased the size of dendritic spine heads but decreased dendritic spine density in apical tuft dendrites of PrL-NAcore neurons. Interestingly, only the heroin-induced decrease in density was normalized by chronic NAC treatment. These results reveal that, in addition to normalizing astrocyte dysfunction in the NAcore, NAC normalizes heroin-induced astroglia and neuronal adaptations in the PrL cortex.

Materials and methods

Animal subjects and surgery:

Male Sprague-Dawley rats (*N*=63) were purchased from Charles Rivers Laboratory (Wilmington, MA) and were single-housed upon arrival in a temperature and humidity-controlled vivarium with standard rat chow (Harlan; Indianapolis, IN) and water available *ad libitum*. Rats were maintained on a 12-hr reverse light/dark cycle (lights off at 6 AM). All animal use protocols were approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina and were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8th ed. 2011). At the time of surgery, all rats weighed 275-300g. On the day of surgery, rats were anesthetized with an intraperitoneal (i.p.) ketamine (66 mg/kg) and xylazine (1.33 mg/kg) injection, and received ketorolac (2.0 mg/kg, i.p.) for analgesia. A silastic catheter was implanted in the right jugular vein, attached to a cannula, which exited the animal's back. Rats in Experiment 1 received a bilateral intra-PrL cortical (coordinates- AP: +2.8, ML: +/-0.6, DV: -3.8 mm from bregma) microinjection of AAV5-GfaABC1D-Lck-GFP (0.75 ul/hemisphere, Titer: $1x10^{13}$ vg/ml) to label the fine membrane processes of astrocytes [13, 28]. Rats in Experiment 2 received a bilateral microinjection of AAV1-CAG-Flex-Ruby2sm-FLAG (titer: $5x10^{12}$ vg/ml) as well as intra-NAcore (coordinates-AP: +1.7, ML: +/- 1.6, DV: -7 mm relative to bregma) injection of rgAAV-hSyn-Cre-WPRE (0.75 ul/hem, titer: $7x10^{12}$) to specifically target PrL cortical-NAcore neurons for dendritic spine analyses [29]. Injections (0.75 ul/hemisphere) were performed over a period of 5 minutes (0.15 ul/minute) using a Nanoject II (Drummond Scientific, Broomall, PA) and injectors were left in place for 5 minutes to facilitate viral diffusion from the injection site. All constructs were purchased from Addgene (Cambridge, MA) except AAV5-GfaABC1D-Lck-GFP which was custom-packaged at the University of North Carolina Gene Therapy Center viral vector core (Chapel Hill, NC). Rats were allowed at least 5 days of post-operative recovery during which catheters were flushed daily with taurolidine citrate solution (TCS, Access Technologies, Skokie, IL) and food and water were available *ad libitum*. For each experiment, ≥2 independent cohorts were used.

Drugs:

Heroin hydrochloride was provided by the National Institute on Drug Abuse. N-acetylcysteine was purchased from Sigma (#A7250) and diluted in sterile saline to 100 mg/ml.

Heroin self-administration and extinction procedures:

All experiments were performed between the hours of 8AM and 6PM. All SA experiments were performed in standard MedPC operant chambers equipped with two retractable levers, a tone generator, and a cue light (Med Associates Fairfax, VT). The beginning of each session was signaled by illumination of the houselight and lever availability. Rats were trained for 14 days (3 hrs/day) to lever press for heroin on a fixed ratio 1 (FR1) schedule of reinforcement. On days 1-2, active lever presses (ALP) resulted in a light and tone cue delivery followed by a 100 μg/50 μl iv bolus of heroin hydrochloride dissolved in sterile saline. On days 3-4, rats received a 50 μg/50 μl bolus followed by a 20 sec. timeout period, and on days 5-14 rats received a 25 μg/50 μl bolus (maintenance

dose). Yoked-saline animals were paired to a heroin SA animal; receiving non-contingent saline infusions in a temporally-paired manner. SA criteria were set at ≥1 infusion per session on days 1-4 whereas animals were required to receive ≥10 infusions per session on days 5-14. Following SA, all rats entered extinction whereby ALP resulted in no cue or drug delivery. All rats received 12-13 days of extinction training (3 hours/day). Beginning on day 4, a subset of animals received N-acetylcysteine (100 mg/kg, i.p.) or vehicle (0.9% NaCl) 30 minutes before each subsequent session, and all animals injected with N-acetylcysteine received exactly 13 days of extinction training. All yoked-saline animals received vehicle injections during extinction. This dose of NAC has previously been shown to reduce extinction responding on the active lever and provide an enduring reduction in cue- and primed- heroin seeking using the same heroin SA protocol [19].

Transcardial perfusions and immunohistochemistry:

Twenty-four hours after the final extinction session, rats were transcardially perfused with 150 ml of 0.1M phosphate buffer (PB), followed by 200 ml of 4% paraformaldehyde (PFA) in 0.1M PB. Brains were extracted and post-fixed in PFA for 24 hours. Sections (80 µm) containing the PrL cortex and NAcore were collected in 0.1M PBS containing 0.02% sodium azide with a vibrating microtome (Leica), and stored at 4ºC until processing.

Immunohistochemistry (IHC) was performed as previously described [13, 14, 30]. Briefly, 3 sections per animal were blocked with 0.1M PBS containing 2% (v/v) TritonX-100 (PBST) containing 2% (v/v) normal goat serum (NGS). Sections were then incubated overnight with gentle agitation at 4ºC with the following primary antisera: Experiment 1 – chicken anti-GFP (Abcam, 1:1000, ab13970 RRID:AB_300798) + mouse anti-GluA2 (Millipore, 2.13 ug/ml, MAB397 RRID:AB_11212990) or rabbit anti-Synapsin I (Abcam, 1:500, ab64581 RRID:AB_1281135), and in a separate run, rabbit anti-GFAP (Abcam, 1:1000, ab7260 RRID:AB_305808); Experiment 2 – mouse anti-FLAG (Sigma, 1:2000, F1804 RRID:AB_262044). Sections were then washed 3X5 minutes with PBST, and were incubated in species-specific secondary antisera (Invitrogen, all 1:1000) conjugated to Alexa Fluor 488 (RRID:AB_2534096, GFP), 594 (RRID:AB_2534091, FLAG), or 647 (RRID:AB_2535804, GFAP, Synapsin I, and GluA2) diluted in PBST with 2% NGS for 5 hours at room temperature with gentle agitation. Sections were washed 3X5 minutes in PBST, then mounted on Superfrost plus slides with Prolong Gold antifade (ThermoFisher Scientific). Slides were stored at 4ºC until imaging (< 2 months).

Confocal microscopy:

For all high-resolution confocal microscopy experiments, a Leica SP8 confocal microscope equipped with HyD detectors for enhanced sensitivity was used. Laser power, gain, and pinhole size (≤1 Airy Unit) were empirically determined to obtain optimal saturation values and subsequent deconvolution results. Laser power and gain values were then held relatively constant for the remainder of the experiment. For all experiments, Alexa488-mediated Lck-GFP detection was carried out using an Argon 488nm laser, Alexa-594-mediated FLAG detection was carried out using an OPSL 552nm laser, and Alexa647-mediated GluA2, GFAP, and Synapsin I detection was carried out using a Diode 638nm laser. In experiment 1a, fields of GFAP+ cells were imaged in layers II/III and V of the PrL cortex at a 4096x4096 frame size, 0.1 µm Z-step size, 1X digital zoom, with a line average of 4 using a 63X oil-immersion objective (1.4 N.A.). In experiment 1b, Lck-GFP-expressing astrocytes that were visually isolated from neighboring astrocytes in layers II/III and V as well as GluA2/Synapsin I puncta were sampled using a 63X oil-immersion objective (1.4 N.A.) at a 2048X2048 frame size, 0.3 µm Z-step size, 1.5X digital zoom, with a line average of 4. Care was taken to ensure that the majority of each astrocyte in the Z-plane was imaged. In experiment 2, apical tuft dendrites of layer V PrL-NAcore neurons were imaged as previously described [30]. Briefly, dendritic spine segments were imaged only if they met the following criteria: 1: they could be traced back to the cell body of origin, 2: they were unobscured by neighboring dendritic segments, and 3: they were past the bifurcation of the proximal dendrite (terminating in layers I and II). Dendritic spine segments (~50 µm) were collected at 1024x512 frame size, 0.1 µm Z-step size, 4.1X digital zoom, with a line average of 2.

Image analysis:

All image analyses were performed by an investigator blind to experimental groups. Following acquisition, confocal Z-stacks were deconvolved using Huygens software (Scientific Volume Imaging, Hilversum, NL) and then exported to Bitplane Imaris (v. 9.1) for digital rendering and analysis. Complexity of GFAP arbors were analyzed as previously described[14]. Briefly, Z-stacks were cropped to 12 μm in the Z plane to identify fields of cells with homogenous signal intensity, avoiding large areas of vasculature. We used a previously published semi-automated approach to skeletonize GFAP arbors belonging to individual, non-overlapping astrocytes. In doing so, we set the minimum diameter of a GFAP filaments to 1 μm. Iterative processing of GFAP skeletons was performed in order to accurately skeletonize each astrocyte within the field. We exported and analyzed astrocyte structure using the following outputs from the Imaris filament function: the number of astrocytes in each image, the total sum of the GFAP filament length for each cell per image, the average GFAP diameter of each cell per image, the average number of branch points for each cell per image, and the average number of 3D Sholl's intersections (1 μm radius) per cell, per image. The average number of Sholl's intersections was then binned by 5 μm radii from 0-50. Data is expressed as an average for each cell per image, or an overall image average as specified in the results.

The whole-cell astrocyte synaptic contact assay was performed as described previously [13, 14], with minor modifications. Briefly, confocal Z-stacks were cropped in 3D to the border of each cell to isolate each astrocyte. A 3D space-filling model was generated from the GFP signal from each virally labeled astrocyte. We then masked out all GFP signal outside of each cell to eliminate background. The colocalization module of Imaris was used to perform co-registry analyses as previously described. A signal intensity threshold was automatically set according to the Costes method [31] for each channel (Channel 1: GFP, Channel 2: Synapsin I or GluA2). Images that could not be assigned automatic thresholds, due to heterogenous signal intensity distribution through the Z-plane of the dataset, were not analyzed. Voxels that contained signal above threshold for each channel were considered colocalized. The greater number of co-registered voxels indicates a greater degree of astrocyte association at synapses on a whole-cell level. In a subset of images, we also digitally modeled each Synapsin I or GluA2 puncta to determine the effect of treatment on the number of puncta within each image, normalized to the dataset volume. Data exported included the percent of the dataset colocalized normalized to the saline control group (% colocalized), and the number of Synapsin I/GluA2 puncta normalized to the dataset volume.

Dendritic spine morphology and density were analyzed as previously described [30]. Briefly, the filament extension of Imaris was used to manually trace each dendritic shaft and semi-automatically label corresponding dendritic spines. Care was taken to ensure that each dendritic spine head was modeled accurately. Variables exported included the average dendritic spine head diameter (d_H) of each segment and the dendritic spine density (spines/μm). We also binned the spine head diameters (0.2 μm bins) to generate a frequency distribution.

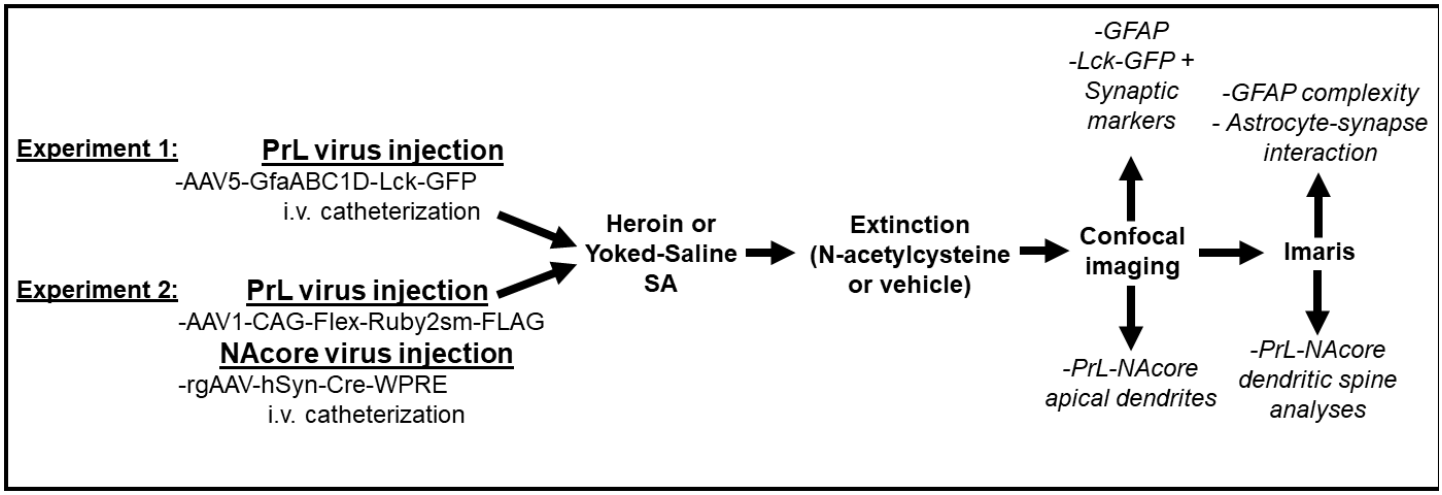


Figure 1. Diagram illustrating the experimental timeline for each experiment.

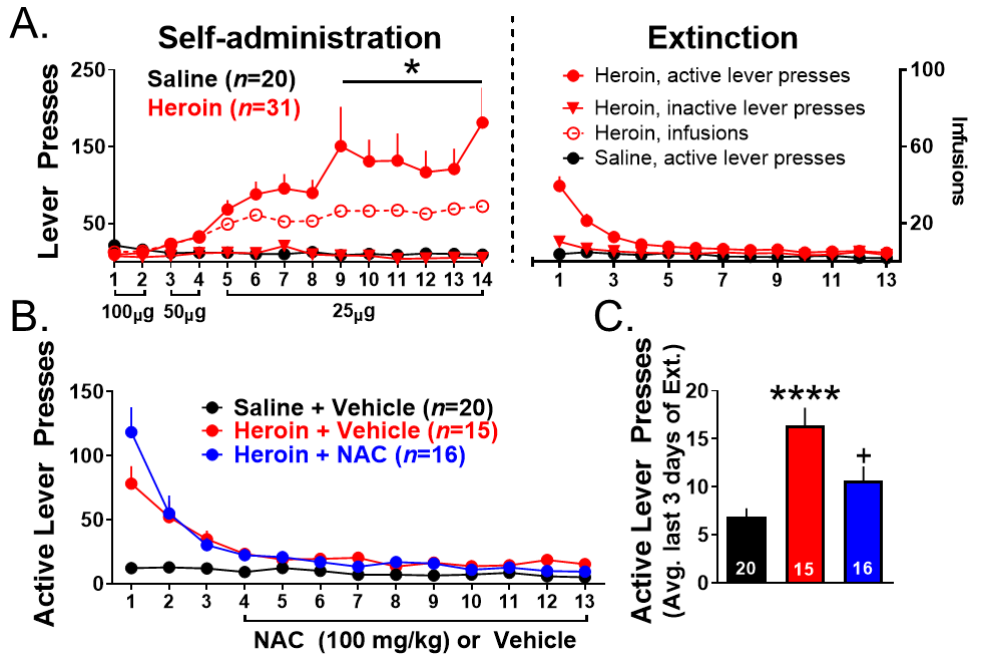


Figure 2. NAC reduces extinction responding. A. Self-administration and extinction behavior for Heroin and Yoked-Saline rats. Lever presses are shown on the left y axis and infusions are shown on the right y axis. *p<0.05 compared to saline active lever presses. B. Active lever presses during extinction for Yoked-Saline, Heroin animals injected with vehicle, and heroin animals injected with NAC. C. Average active lever presses over the last 3 days of extinction for the three experimental groups. ****p<0.0001 compared to Yoked-Saline, +p<0.05 compared to Heroin + Vehicle. The number of animals within each group are shown within the bars.

Data is expressed as animal averages; collapsing all dendritic spine morphometric data across segments sampled from each animal.

Experimental design:

Figure 1 shows the experimental timelines. Experiment 1 was designed to examine the effects of heroin self-administration and extinction, with or without NAC administration, on GFAP complexity in the PrL cortex (1a) as well as astrocyte-synapse proximity (1b). Experiment 2 was designed to examine whether dendritic spine morphometric properties were altered in PrL-NAcore neurons after extinction of heroin SA with or without NAC administration.

Statistical analyses:

All statistical analyses were performed with Graphpad Prism software (v. 8.0, La Jolla, CA). When analyzing three groups across a single dependent variable, a one-way ANOVA was performed followed by Tukey's multiple comparison test if a significant effect of treatment was revealed. Normality of data within each group was analyzed using Shapiro-Wilk test. If one or more groups for each variable analyzed did not pass the Shapiro-Wilk test, a non-parametric Kruskal-Wallis test was used. In this instance, post hoc tests controlled for the false discovery rate (FDR) using the two-stage linear step-up procedure of Benjamini, Krieger,and Yekutieli if a significant effect was revealed. If data were sampled from a normal distribution, but between group standard deviations were not equal, a Brown-Forsythe-corrected one-way ANOVA was performed followed by Dunnett's T3 test when a significant effect of treatment was revealed. When three groups were compared across a repeated measure, a repeated measure one-way ANOVA was applied followed by Tukey's multiple comparison test comparing the mean of the repeated measure between groups. Dendritic spine frequency distribution data as well as self-administration data were analyzed with a two-way repeated measure ANOVA followed by Tukey's multiple comparison test when a significant interaction was revealed. Unless otherwise stated, each data point represents an animal average derived from multiple images or SA/extinction sessions. Statistical outliers were detected according to the ROUT method (Q=1%) and these data were removed from analyses as specified in the results. All data is expressed as the mean +/- SEM and significance was set at p<0.05.

Results

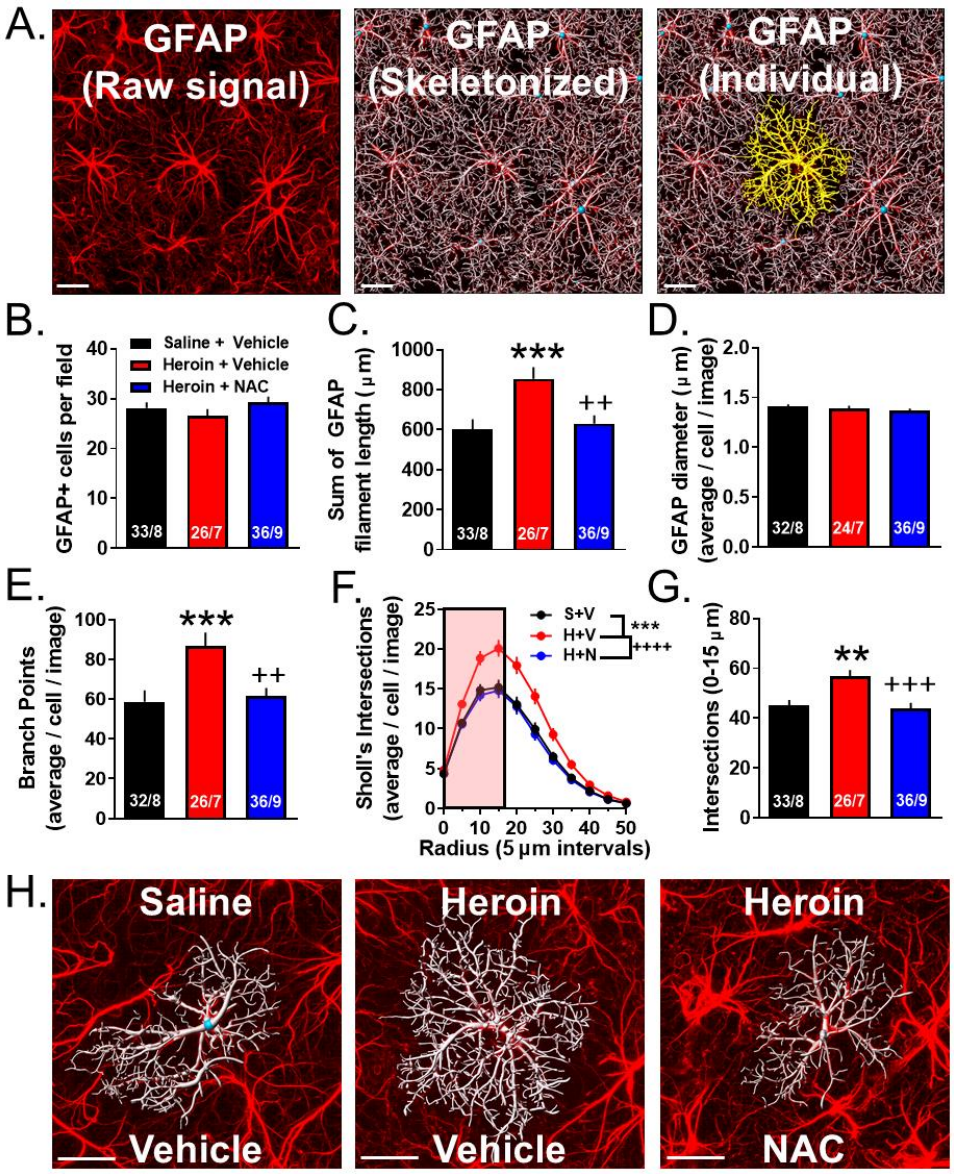
Heroin self-administration and extinction: Experiments 1 and 2

Of the 63 initial animals in Experiments 1 and 2, 12 animals did not finish SA due to catheter failure. **Figure 2A** shows self-administration and extinction data for heroin SA (N=31) and Yoked-Saline (N=20) rats used for all experiments. When comparing heroin and Yoked-Saline active lever presses across SA sessions, a two-way

repeated measures ANOVA revealed a significant group by time interaction ($F(13,367)=5.11$, $p<0.0001$). Bonferroni's multiple comparison test revealed that heroin SA animals pressed the active lever significantly more than Yoked-Saline controls in sessions 9-14 ($p<0.05$). There was no difference between groups (that were subsequently divided by treatment during extinction) in the average number of heroin infusions earned over the last 3 days of SA (Heroin + Vehicle: 29.38 +/-2.80, Heroin + NAC: 25.75+/-4.87). **Figure 2B** shows extinction training data for Yoked-Saline animals injected with Vehicle (n=20), and Heroin SA rats injected with Vehicle (n=15) or NAC (n=16) during the last 10 days of extinction. **Figure 2C** shows active lever presses averaged across the last 3 days of extinction for each group. A one-way ANOVA revealed a significant effect of treatment ($F(2,48)=12.15$, $p<0.0001$). Tukey's multiple comparison tests revealed that Heroin SA animals showed significantly more lever pressing during the last 3 days of extinction than Yoked-Saline rats ($p<0.0001$) and that NAC significantly decreased lever pressing to Yoked-Saline values (p=0.019).

Experiment 1A – Heroin SA and extinction increased astroglial GFAP arbor complexity, an effect that was prevented by repeated NAC treatment during extinction

Of the initial 10 Yoked-Saline rats used in this experiment, 2 were not included due to technical issues related to IHC processing, 2 Heroin + Vehicle rats were not included due to technical issues related to IHC, and one Heroin + NAC animal was excluded due to lack of analyzable images. **Figure 3A** shows a representative field of GFAP+ cells in the PL cortex (left); the same image is shown with an overlaid Imaris-based skeletonization (white) of each GFAP+ cell (middle), and an individual GFAP+ cell (yellow) isolated from the other cells (right). Using this high throughput approach, we analyzed GFAP complexity in 3-7 Z-series data sets from 8 Yoked-Saline rats, 7 Heroin + Vehicle rats, and 9 Heroin + NAC rats using several different metrics. We first analyzed the average number of GFAP+ cells per field. A one-way ANOVA revealed no effect of treatment ($F(2,92)=1.23$, p=0.30, **Figure 3B).** For complexity analyses, we first analyzed the total sum of GFAP filament length within each Z-series data set **(Figure 3C).** A Shapiro-Wilk test indicated that data in Yoked-Saline ($W=0.9$, p=0.0005) and Heroin + NAC ($W=0.94$, p=0.04) groups were non-normally distributed. Accordingly, a Kruskal-Wallis non-parametric ANOVA was used which indicated a significant difference between groups ($K\text{-}W=12.99$, p=0.002). The two-stage linear step-up procedure revealed that Heroin increased total GFAP filament length per image when compared to Yoked-Saline (q=0.0007), which was normalized by NAC treatment during extinction (q=0.004). We next analyzed the average GFAP filament diameter for each cell per z-series data set **(Figure 3D).** One Z-series data set from Yoked-Saline and 2 Z-series data sets from the Heroin + NAC group were identified as statistical outliers. A Brown-Forsythe test revealed that the standard deviations were significantly different between groups ($F(2,89)=3.26$, p=0.04). As such, these data were analyzed using a Brown-Forsythe-corrected ANOVA which revealed there was no significant effect of treatment ($F(2,74.38)=0.626$, p=0.54). We next analyzed the average number of branch points of each cell, within each z-series data set **(Figure 3E).** One Z-series data set from the Yoked-Saline group was identified as a statistical outlier. A Shapiro-Wilk test indicated that data from the Yoked-Saline group was non-normally distributed. Accordingly, a Kruskal-Wallis test was used which revealed a significant difference between groups ($K\text{-}W=14.61$, p=0.0007). The two-stage linear step-up procedure revealed that Heroin increased the average number of branch points within GFAP filament arbors compared to Yoked-Saline (q=0.0002) which was normalized by NAC treatment during extinction (q=0.003). We also performed a 3D Sholl's analysis for each cell within each image using 1 µm 3D concentric sphere radii



(which were then compiled into 5 µm bins) and analyzed the average number of intersections as a function of distance from the center of each cell (**Figure 3F**). A repeated measures one-way ANOVA revealed a significant difference between group means ($F(2,20)=19.23$, $p<0.0001$). Tukey's multiple comparison test revealed that Heroin increased the average number of Sholl's intersections compared to Yoked-Saline ($p=0.0002$), which was normalized by NAC treatment during extinction ($p<0.0001$). In a subsequent analysis, we focused on the first 15 µm from the center of each cell (**Figure 3G**). In these data, a one-way ANOVA revealed a significant difference between groups ($F(2,92)=8.41$, $p=0.0004$). Tukey's multiple comparison test revealed that Heroin increased the number of Sholl's intersections in this region when compared to Yoked-Saline ($p=0.003$), which was normalized by NAC treatment during extinction ($p=0.0007$). **Figure 3H** shows an individual cell with a representative skeletonized GFAP arbor from each treatment group.

Figure 3. NAC normalizes heroin-induced alterations in PrL cortical astrocyte complexity. A. Representative field of GFAP+ astrocytes skeletonized for analysis. Left-Raw GFAP signal from the confocal microscope. Middle–Skeletonized GFAP. Right-Isolated GFAP+ astrocyte from the field of cells. B. There was no difference between groups in the number of GFAP+ cells per field. C. Heroin increased the total length of the GFAP cytoskeletal filament which was normalized by repeated NAC treatment. D. There was no difference between groups in the average diameter of the GFAP filament. E. Heroin increased the average number of branch points which was normalized by repeated NAC treatment. F. Frequency distribution of the number of Sholl's intersections as a function of distance from the center of the cell. Heroin increased the number of Sholl's intersections averaged across all bins which was normalized by repeated NAC treatment. G. Average number of Sholl's intersections across the first 4 bins (0, 5, 10, and 15 µm). Heroin increased the number of Sholl's intersections which was normalized by chronic NAC treatment. H. Representative skeletonized GFAP+ cells for each treatment group. **$p<0.01$, ***$p<0.001$ compared to Yoked-Saline, ++$p<0.01$, +++$p<0.001$, ++++$p<0.0001$ compared to Heroin + Vehicle. Numbers within each bar denote the number of images taken / the number of animals used. Scale bars indicate 20 µm.

Experiment 1B – Heroin SA and extinction increased astrocyte association with multiple synaptic markers; an effect that was prevented by repeated NAC treatment during extinction

We performed two separate astrocyte-synaptic contact analyses using PrL cortical sections collected from the three experimental groups: Yoked-Saline (*n*=10), Heroin + Vehicle (*n*=9), and Heroin + NAC (*n*=10). In the first set, astroglial structure was visualized with viral vector-mediated membrane targeted GFP expression achieved with the AAV5-GfaABC1D-Lck-GFP (Lck-GFP) viral vector and further amplified with GFP IHC whereas excitatory synapses were labeled with GluA2 IHC as described previously [32]. The second set of astrocyte-synaptic contact analyses was performed identically, except that synapsin I IHC was used as a general synaptic marker [13]. We imaged an average of 3-5 cells/animal for both GluA2 and synapsin I analyses. For the GluA2 analyses, the following were excluded: 1 Yoked-Saline animal was missing localization of Lck-GFP virus expression, 1 Heroin + Vehicle animal had insufficient Lck-GFP virus expression, and 1 Heroin + NAC animal had no more available tissue and 2 had a lack of sufficient number of analyzable cells after imaging.

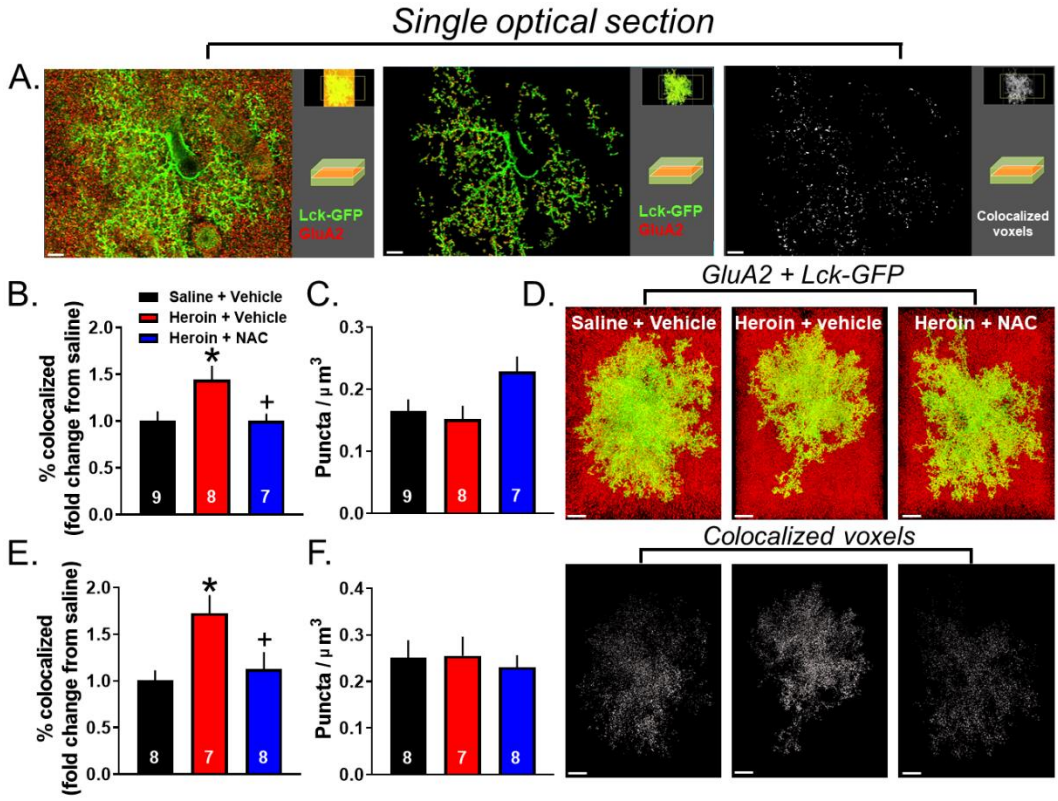


Figure 4. NAC normalized heroin-induced increases in PrL cortical astrocyte PAPs-synapse association. A. A single optical section from a representative Lck-GFP-labeled astrocyte and GluA2 puncta. Left-Raw signal from confocal microscope. Middle-GluA2 confined within the proximity of the astrocyte membrane. Right-Colocalized voxels within the proximity of the astrocyte membrane. B. Heroin SA and extinction increased the percent colocalization of GFP and GluA2 which was normalized by repeated NAC treatment. C. There was no difference between groups in the number of GluA2 puncta normalized to the dataset volume. D. Representative astrocytes from the three groups. Top-Raw signal from the confocal microscope. Bottom-Colocalized voxels within the region occupied by each astrocyte. E. Heroin SA and extinction increased the percent colocalization of GFP and Synapsin I which was normalized by repeated NAC treatment. F. There was no difference between groups in the number of Synapsin I puncta normalized to the dataset volume. Numbers within each bar denote the number of animals used. *p<0.05 compared to Yoked-Saline, +p<0.05 compared to Heroin + Vehicle. Scale bars indicate 5 µm (A) and 10 µm (D).

Figure 4A shows a representative PrL cortical Lck-GFP-labeled astroglia (green) and punctate signal corresponding to GluA2 (red). To aid in the visualization of the punctate GluA2 synaptic marker signal, a section within the center of an astrocyte from an individual optical section from the many images that comprise each Z-series data set is shown. The left panel shows the raw signal, the middle panel shows GluA2 signal contained within the 3D surface of the astrocyte membrane, and the right panel shows colocalized voxels. Within each panel, a smaller depiction of the full z-series data set is visible in the top right corner, with a white box depicting the field seen within the single optical section view. **Figure 4B** shows the percent of the co-registry of the GFP and synaptic marker signals across the three treatment groups, a metric of astrocyte-synaptic

interaction [13, 14, 33, 34]. Since our experimental measurements utilized both a general synaptic marker and an excitatory synaptic marker, all data were normalized to Yoked-Saline controls. This was done for ease of comparison between markers and cohorts of animals. However, it is important to note that although baseline values differed slightly from cohort to cohort, the effect of heroin was reproducible across both cohorts (**Figure S1A-D**). Shapiro-Wilk's test indicated that data from the Heroin + Vehicle group (W=0.78, *p*=0.018) were non-normally distributed. Accordingly, these data were analyzed using a Kruskal-Wallis non-parametric test which revealed a significant effect of treatment (K-W=6.97, *p*=0.03). The two-stage linear step-up procedure indicated that the Heroin + Vehicle group showed increased co-registry of GFP and GluA2 when compared to the Yoked-Saline group (*q*=0.024) which was normalized by NAC treatment during extinction (*q*=0.021). We next determined whether this effect was due to an increased number of GluA2 puncta within each dataset. While a one-way ANOVA revealed a marginally significant effect of treatment (F(2,21)=3.30, *p*=0.057, **Figure 4C),** this effect appears to be carried by the Heroin + NAC group which did not display increased astrocyte-synaptic interactions. Further, post hoc analysis did not reveal a significant difference between groups. **Figure 4D** shows representative 3D-reconstructed astroglia for each treatment group with GluA2 puncta (top) as well as the extent of synaptic contact as visualized by white GFP and GluA2 colocalized voxels (bottom).

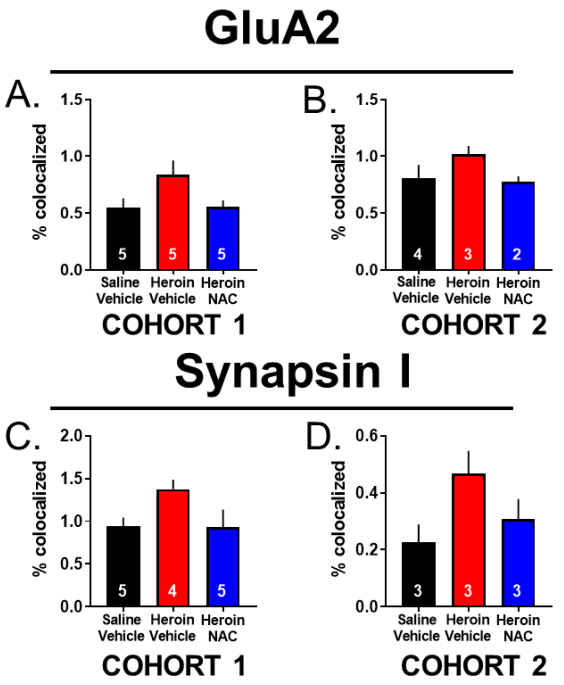


Figure S1. In Experiment 2b the two independent cohorts showed the same effect. A-B. Cohort 1 (A) and cohort 2 (B) showed the same effect of percent colocalization for GluA2. C-D. Cohort 1 (C) and cohort 2 (D) showed the same effect of percent colocalization for Synapsin I.

As described above, we also performed the same imaging and analysis for Lck-GFP and Synapsin I, using tissue from the same animals to determine whether the observed effect was specific to synapses containing the GluA2 subunit of AMPA receptors. Two Yoked-Saline animals, two Heroin + Vehicle animals, and two Heroin + NAC animals were removed from the analysis for the following reasons: Yoked-Saline + Vehicle: off-target Lck-GFP virus expression (*n*=1), lack of images satisfying Costes' threshold assignment (*n*=1); Heroin + Vehicle: lack of available tissue (*n*=1) or insufficient Lck-GFP virus expression (*n*=1); Heroin + NAC: lack of a sufficient number of analyzable cells after imaging (*n*=1), and technical issues related to IHC (*n*=1). A one-way ANOVA revealed a significant main effect of treatment (F(2,20)=5.29, *p*=0.014, **Figure 4E**). Tukey's multiple comparison test revealed that Heroin SA and extinction increased astrocyte-synaptic interaction as assayed by Lck-GFP-Synapsin 1 colocalization when compared to Yoked-Saline controls (*p*=0.016), which was normalized by NAC treatment during extinction (*p*=0.049). Akin to analyses performed for GluA2, we also analyzed the number of Synapsin I voxels in a subset of images from each animal **(Figure 4F)**. Shapiro-Wilk's test indicated that data from Yoked-Saline controls (W=0.8, *p*=0.03) and Heroin + NAC (W=0.81, *p*=0.035) were non-normally distributed. A Kruskal-Wallis test indicated no difference between groups (K-W=0.023, *p*=0.98).

Experiment 2 – Heroin SA and extinction impacts spine density and spine head diameter on PrL-NAcore neurons; an effect that was partially reversed by repeated NAC treatment during extinction

Others have shown that Heroin SA decreases the number of dendritic spines on PrL cortical neurons after one month of abstinence [35]. Here, we sought to determine whether heroin SA and extinction altered dendritic spine morphometrics specifically in PrL cortical neurons projecting to the NAcore. We imaged 5-8 dendritic spine segments from Yoked-Saline (*n*=10), Heroin + Vehicle (*n*=6) and Heroin + NAC (*n*=6) animals after extinction. **Figure 5A** shows a representative layer V PrL-NAcore neuron fully labeled with the highlighted area indicating the region sampled at the apical tuft. **Figure 5B** shows the dendritic spine segment sampled from the highlighted region in 5A. **Figure 5C** shows the filament-processed segment for spine detection and **Figure 5D** shows a color-coded map of dendritic spine d_H, with warmer colors indicating larger values. We first analyzed the average dendritic spine d_H across the three treatment groups. A one-way ANOVA revealed a significant effect of treatment ($F(2,19)=20.70$, $p<0.0001$, **Figure 5E**). Tukey's multiple comparison test revealed that Heroin SA and extinction increased the average dendritic spine d_H ($p<0.0001$), which was not altered by NAC treatment during extinction ($p=0.0007$). Next, we investigated whether this effect was specific to smaller or larger spines. To do so, we binned the spine d_H (0.2 µm bins) and generated a frequency distribution. A two-way repeated measures ANOVA revealed a significant treatment by bin interaction ($F(8,76)=8.144$, $p<0.0001$**, Figure 5F**). Tukey's multiple comparison test revealed that Heroin SA and extinction, regardless of NAC treatment, decreased the proportion of smaller spines (<0.2 – $p<0.0001$; 0.2-0.4 – $p<0.05$), but increased the proportion of spines with larger d_H (0.4-0.6 – $p<0.01$) compared to Yoked-Saline rats. Interestingly, the proportion of spines with d_H between 0.6-0.8 was

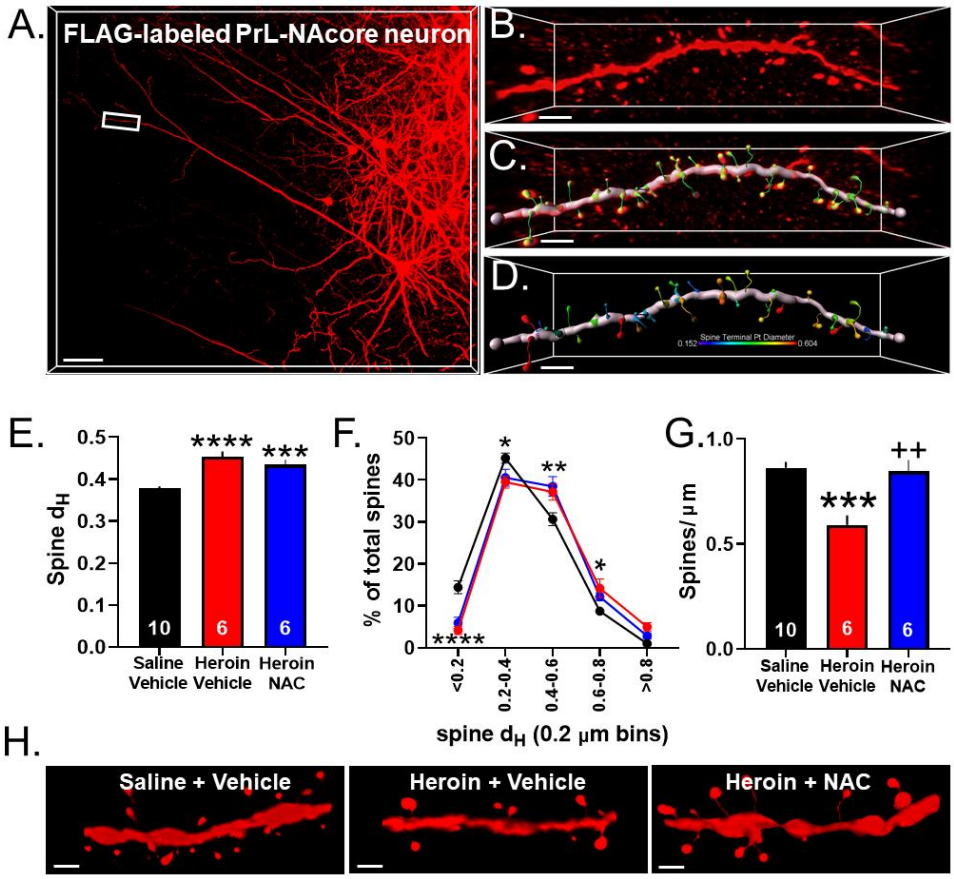


Figure 5. NAC normalized Heroin-induced decreases in PrL-NAcore apical spine density, but not head diameter. A. Left-Representative FLAG-labeled PrL-NAcore neuron. B-D. B) Dendritic spine segment taken from the apical tuft (boxed region in A), C) Filament processed spine segment, D) Heat map of dendritic spine head diameters along the segment – warmer colors indicate a greater head diameter. Note that most spines fall within the middle range of head diameters. E. Heroin SA and extinction increased the average spine head diameter of PrL-NAcore neurons which was not affected by repeated NAC treatment. F. Frequency distribution of the percent of total spines as a function of spine head diameter bin (0.2 µm). Heroin SA and extinction regardless of NAC treatment decreased the proportion of spines with smaller head diameters and increased the proportion of spines with larger head diameters. G. Heroin SA and extinction reduced the number of spines per µm of dendrite which was normalized by repeated NAC treatment during extinction. H. Representative spines from a 15 µm stretch of dendrite are shown. d_H=head diameter. Numbers within each bar denote the number of animals used. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ compared to Yoked-Saline. ++$p<0.01$ compared to Heroin + Vehicle. Scale bars indicate 70 µm (A), 3 µm (B-D), and 2 µm (H).

increased in the Heroin SA + Vehicle group (p=0.008) but there was no effect of NAC on this specific bin compared to Yoked-Saline (p=0.17). We next analyzed the average dendritic spine density in all three treatment groups from the same dendrite segments. A one-way ANOVA revealed a significant effect of treatment ($F(2,19)=13.46$, p=0.0002**, Figure 5G**). Tukey's multiple comparison test indicated that heroin SA and extinction decreased the average spine density compared to saline controls (p=0.0003), which was prevented by NAC treatment during extinction (p=0.002). These effects were likely not due to inherent differences in signal intensity within the spine heads as there was no difference between groups in the average FLAG intensity within spine heads when normalized to saline controls (Yoked-Saline: 100 +/- 2.53, Heroin + Vehicle: 103.2 +/-5.07, Heroin + NAC: 95.86 +/-2.00). Representative spine segments (15 µm cropped segments) are shown in **Figure 5H**.

Discussion

Our data demonstrate that Heroin SA followed by extinction enhances the fundamental cytoskeletal cellular complexity of astrocytes in the PrL cortex, a finding that was accompanied by increased association of membranous astrocytic processes, virally labeled with membrane-targeted GFP expression, with two distinct synaptic markers. Both of these effects, as well as heroin seeking during extinction, were normalized by repeated NAC treatment during the last 10 days of extinction training, confirming that NAC reduces responding during extinction as others have reported [19]. Apart from impacts on cortical astrocytes, heroin SA and extinction also increased the average spine d_H on apical tuft dendrites of PrL cortical neurons that project to the NAcore, an effect that was accompanied by decreased spine density. Interestingly, NAC treatment during extinction normalized heroin-induced decreases in dendritic spine density but did not impact dendritic spine d_H. These results suggest that the ability of NAC to prevent heroin relapse in preclinical models may not only be due to its action in the NAcore but may also include actions that normalize structural plasticity in astrocytes, as well as neurons within the PrL cortex that project to the NAcore.

Repeated NAC treatment facilitated extinction training

Our data provide the first preclinical evidence for NAC acting in a cortical region to facilitate extinction. Although NAC's ability to promote abstinence has yet to be tested in the clinic for heroin-addicted patients, preclinical data indicate that daily NAC treatment during extinction reduces extinction responding as well as cue–and heroin–induced drug seeking for up to 40 days after the last injection [19]. Although rats did not undergo reinstatement in the current study, our data demonstrate that active lever presses over the last 3 days of extinction training are reduced by repeated NAC treatment. Ultimately, NAC has shown promise preclinically in decreasing relapse for several drugs of abuse [14, 20, 22-24]. However, it is critical to note that NAC's ability to suppress relapse clinically, at least in the case of cocaine, is minimal to date [36]. The general consensus is that NAC is effective in increasing abstinence duration in patients who are already abstinent, but does not reduce cocaine craving per se [36-38]. Thus, NAC may not be a clinically useful therapeutic, but given its well-described mechanism of action it continues to be a powerful research tool for understanding the role of astrocyte dysfunction in addiction. Regarding treatment groups, in the current study we chose to omit Yoked-Saline animals injected with NAC for several reasons. Most importantly, NAC has been shown to have no effect on NAcore glutamate concentrations or cystine uptake through X_c^- in control animals [39]. Moreover, NAC treatment normalized increased glutamate concentrations in the anterior cingulate of cocaine-addicted patients but had no effect in healthy controls [40].

Repeated NAC treatment during extinction normalized astrocyte complexity and increased association of PAPs with synapses in PrL cortex induced by heroin SA and extinction

Here we provide evidence that extinction from heroin SA increased GFAP cytoskeletal complexity without altering the number of GFAP+ cells in the PrL cortex. This is likely not due to the induction of reactive astrocytes given there was no change in the average GFAP filament diameter, a canonical index of reactive astrocytes [41]. It is possible that this effect may be due to heroin's direct impact on astrocyte opioid receptors. Astrocytes express mRNA for all three opioid receptor subtypes and cortical astrocytes in particular express an abundance of mu opioid receptor mRNA [42], the primary receptor engaged by morphine [43]. Further, incubation of cortical astrocyte cultures with morphine enhances intracellular Ca^{2+} signaling [44], resulting in increased area occupied by astrocytes [45]. In addition, astrocytic mu opioid receptor expression has been directly linked to the rewarding properties of drugs of abuse whereby activation of mu opioid receptors on hippocampal astrocytes induces

conditioned place preference [46]. Thus, it is likely that one component of the observed increase in GFAP complexity in the PrL cortex of heroin-exposed rats is due to chronic tone on astrocytic mu opioid receptors. In support, a study performed in the early 1990's showed that cultured astrocytes treated with morphine increase their branching complexity; yet morphine decreased astrocyte proliferation [27]. Alternatively, chronic injections of morphine in rats have been shown to increase GFAP protein expression in the frontal cortex [26], although this study did not have a prolonged withdrawal period. While we did not causally link increased GFAP complexity to relapse behavior, repeated NAC treatment prevented the action of heroin at cortical astrocyte GFAP arbors and is known to reverse drug-induced deficits in astrocyte function linked to relapse propensity [39, 47]. Whether normalized cortical GFAP complexity is required for NAC's ability to suppress relapse remains to be determined experimentally.

Extinction from cocaine [13, 34], heroin [12], or methamphetamine [14] SA leads to decreased association of PAPs with synaptic markers in the NAcore. This finding can be interpreted as a withdrawal of PAPs, which are highly enriched in GLT-1 [48], from synapses [49, 50] as this phenomenon is often paired with decreased GLT-1 expression and reduced glutamate clearance [51]. We show here that extinction from heroin SA leads to increased association of PAPs with synaptic markers in the PrL cortex, an effect that was specific to heroin and was not observed following extinction of cocaine SA [34]. While the GFAP cytoskeleton represents a canonical maker for astrocytes and a means to interrogate astrocyte structure [52, 53] and activation states [54], it does not accurately recapitulate the membranous extremities of astrocytes [55]. Accordingly, alterations in the cytoskeletal properties of astrocytes at the level of GFAP arbors do not necessarily mandate that adaptations in astrocyte-synaptic interaction will be observed. As an example, methamphetamine SA and extinction decreased PAP-synapse association, but not GFAP cytoskeletal complexity, in the NAcore [14]. However, here we did observe that heroin-induced increases in GFAP complexity were accompanied by ~50% increase in the association of PAPs with synaptic markers in the PrL cortex following extinction from heroin SA. Interestingly, both adaptations in GFAP structure and astrocyte-synaptic interaction were normalized by repeated NAC treatment. One potential alternative explanation for our observations is that there was increased synaptogenesis in the PrL cortex after extinction of heroin SA, with no alteration in the motility of astrocytes. However, this is unlikely given that we did not observe any difference between groups in the number of GluA2 or Synapsin I puncta.

It has been estimated that a single astrocyte makes contacts with ~100 dendrites and ~100,000 synapses in the rodent brain [56]. Our astrocyte-synaptic coregistration data could be interpreted either as each astrocyte contacting a greater number of synapses in the PrL cortex after extinction or that each astrocyte may be embracing individual synapses to a greater extent. Additionally, some combination of these scenarios may also be occurring. Each of these potential outcomes would produce an overall increase in coregistry of astrocyte membrane and synaptic marker signals. Astrocyte ensheathment of synapses shapes excitatory transmission by establishing the three-dimensional space in which neurotransmission occurs and, by extension, regulating the profiles of synaptic and non-synaptic receptor activation on a synapse-by-synapse basis. Further, the degree to which astrocytes associate, and physically interact, with synapses is shaped by neuronal activity in an extremely dynamic manner [57, 58]. As an example, whisker stimulation has been shown to increase GLT-1 expression in the barrel cortex, which is associated with increased ensheathment of PAPs at enlarged dendritic spine heads [59]. Additionally, cue-induced reinstatement of heroin seeking has been associated with dynamic changes in PAP-synapse association in the NAcore during the course of the reinstatement session that was directly linked to relapse behavior [12]. Together these data demonstrate that astrocytes physically respond to changes in neurotransmission with physiologically and behaviorally relevant consequences. Moreover, the fidelity of synaptic transmission is tightly controlled by PAP insulation of synapses; too little association is linked to glutamate overflow [12-14] whereas too much insulation can actually impede AMPA receptor-mediated excitatory transmission through enhanced glutamate uptake [60], a means of astroglial regulation of synaptic function initially postulated to occur during sleep by Cajal almost 150 years ago [61]. Further experimentation will be needed to determine where astrocytic synapse ensheathment in the PrL cortex from heroin-experienced animals lies on this functional continuum. Whether PAP association with synapses has reached a "ceiling" and is impeding AMPA receptor transmission or whether the increased association leads to a solidification of excitatory transmission in a supraphysiological manner is yet to be determined.

Extinction from heroin SA altered dendritic spine morphological profiles of prelimbic cortical neurons projecting to the nucleus accumbens core

Using similar viral vector-based pathway-specific labeling of dendritic spines, we previously found that cocaine SA followed by one week of abstinence decreased the density of apical tuft dendritic spines of PrL cortical neurons projecting to the NAcore; yet the remaining spines were enlarged and concomitantly displayed increased GluA1 and GluA2 immunoreactivity in the spine heads [30]. A previous study found that heroin SA and one month of abstinence decreased the density of dendritic spines of PrL cortical layer V pyramidal neurons, with no reported alterations in morphometric properties of the spines themselves [35]. To accompany the astrocyte-synapse interaction data discussed above, we investigated potential alterations in dendritic spine morphological profiles in PrL cortical neurons projecting to the NAcore. Akin to one week of abstinence from cocaine SA, we discovered that extinction from heroin SA decreased the density of dendritic spines along dendrites at the apical tuft of PrL-NAcore neurons, yet these neurons showed an increase in the average spine head size. These data are largely consistent with studies demonstrating repeated treatment of cultured cortical neurons with morphine leads to destabilization of pre-existing spines [62]. In our data set, heroin-mediated decreases in dendritic spine density were prevented by repeated NAC treatment; yet the heroin-evoked increases in spine head diameter were not. Generally, an increase in the number of spines, or an increase in the average spine head size, is interpreted as evidence for synaptic potentiation [63-66]. Thus, this finding is initially counter-intuitive given that NAC normalized the decrease in density, but NAC-treated animals retained enlarged dendritic spines following heroin SA and extinction, suggesting that NAC is driving synaptic potentiation in the PrL-NAcore circuit. Future studies should examine whether heroin SA and extinction alter the electrophysiological properties of PrL-NAcore neurons and whether repeated NAC treatment during extinction has an impact on such alterations. Regardless, given that NAC is known to reverse drug-induced deficits in astrocyte function [18], it is likely that the reduced spine density following extinction of heroin SA and the normalization of which by NAC is due to normalized astrocyte function, perhaps through altering the release patterns of astrocyte-derived neuromodulators.

It is well-documented that astrocytes release a variety of neurotransmitters, neuromodulators, and growth factors that can have a profound impact on neuronal plasticity, deemed gliotransmission (for reviews see [67, 68]). For example, thrombospondin-1 (TSP-1) released from astrocytes activates α2δ-1 on neurons to induce spinogenesis and excitatory synapse formation [69], dysfunction in which is linked to reduced dendritic spine density in down syndrome models [70]. It is possible that the heroin-induced decrease in spine density could be linked to dysfunctional gliotransmission following extinction, and that NAC's ability to drive spinogenesis could be linked to normalization of such dysfunction. For example, repeated morphine administration in mice downregulates TSP-1 gene expression in the cortex 24 hours after the final injection, as well as in cultured astrocytes following repeated morphine treatment [71]. One mechanism by which heroin may impact TSP-1 expression is through increased oxidative stress given that chronic heroin exposure induces a state of oxidative stress in human abusers [72] and also in cultured cortical neurons [73, 74]. Moreover, cultured primary astrocytes reduce TSP-1 gene expression under increased oxidative stress conditions, which is prevented by NAC [75]. Given that one well-documented mechanism by which NAC counteracts oxidative stress is increased glutathione synthesis [76], it is possible that heroin-induced adaptations in TSP-1 expression due to increased oxidative stress, and normalization of which by NAC, may be linked to the altered dendritic spine morphometric features observed herein. Overall, it is likely that NAC's ability to normalize heroin-induced adaptations in astrocyte-synapse proximity and GFAP cytoskeletal architecture has a direct influence on structural plasticity in PrL-NAcore neurons, perhaps through indirect or direct mechanisms at neurons. Thus, the degree to which PAPs associate with dendritic spines of PrL-NAcore neurons, whether this is altered following extinction of heroin SA, and whether NAC has an impact are areas of future investigation.

Methodological considerations

Canonically, GFAP is thought only to be present in the major branches and endfeet of astrocytes and not to be present in all non-reactive astrocytes in adult healthy CNS tissue [77]. However, our data are potentially at odds with this view as we found consistent fields of astrocytes in all three experimental groups and did not observe gaps that could potentially represent GFAP-negative astroglia. As described above, GFAP does not extend into

the finest membranous processes, which are the primary domains of astrocyte-neuron interactions; the motility of which are regulated by actin cycling [78]. The ability to use membrane-targeted expression of GFP driven by astrocyte-specific promoters in conjunction with immunohistochemical detection of synaptic proteins has gained traction as an assay to determine the proximity of PAPs to synapses (see [50] and [49] for reviews). In this study, we used two different synaptic markers; Synapsin I and GluA2 which label generic pre-synaptic terminals (Synapsin I) and excitatory post-synaptic AMPA receptors (GluA2), respectively. However, it is important to note that with an approximate minimum resolution of 150 nm, we are not attempting to distinguish between pre-and post-synaptic elements; separated by an approximate distance of 10 nm [79]. We are merely using IHC detection of these proteins as a means to label synapses. However, the fact that both antibodies yielded increased coregistration of synapses with astrocytic membranes in heroin animals can be viewed as a means to exclude epiphenomenological or potentially off-target effects from each individual antibody.

Conclusions

In conclusion, our data indicate that heroin SA and extinction elicit hypertrophic complexity and synapse association of astroglia in the PrL cortex that are associated with altered dendritic spine morphological features in PrL-NAcore neurons. Our data fill a much-needed gap in the field by focusing on the upstream portion of the canonical relapse circuit to accompany what has been observed in the NAcore. Moreover, these findings point to an additional node of this circuitry whereby NAC can modify heroin-induced adaptations in the PrL cortex in addition to its well-defined modification of NAcore synaptic pathology. Ultimately, astroglia and neuronal adaptations in the PrL cortex may be a target for informing future drug discovery for relapse prevention pharmacotherapies.

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