ABSTINENCE FROM COCAINE SELF-ADMINISTRATION IN RATS ALTERS PRELIMBIC CORTICAL ASTROCYTE-NEURON INTERACTIONS

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Abstract

Substance use disorder (SUD) represents an insidious neuropsychiatric disorder characterized by high relapse rates, even after prolonged abstinence. Rodent models of self-administration (SA) have established that dysfunctional glutamatergic transmission in the prelimbic cortex (PLC) projection to the nucleus accumbens core (NAcore) mediates cue-induced craving, driving relapse to cocaine seeking after abstinence. Excitatory synapses are housed in sub-cellular structures referred to as dendritic spines, and synaptic plasticity is shaped by the interaction of the non-neuronal cells, astroglia, with pre and post-synaptic compartments. Accordingly, recent studies have shown that cocaine SA and abstinence increases dendritic spine diameter on dendrites within the distal apical tuft of PLC-NAcore neurons, and cocaine SA followed by extinction has been demonstrated to decrease astrocyte morphological features and their association with a synaptic marker in the NAcore, but not the PLC. In this study, we examined the effects of cocaine SA and abstinence on spine morphology and the colocalization between PLC-NAcore dendritic spines, or a non-specific synaptic marker, and astrocytes. Male rats received intravenous catheters followed by an intra-NAcore microinjection of a retrogradely transported adeno-associated virus (AAV), expressing Cre recombinase in neurons projecting to the NAcore. Rats then received an intra-PLC microinjection of a viral cocktail used to fluorescently label PLC-NAcore neurons and astroglia membranes in the PLC. Male rats underwent cocaine SA or received yoked saline infusions followed by one week of forced homecage abstinence and were transcardially perfused. Immunohistochemistry was conducted on coronal sections containing the PLC followed by high-resolution confocal imaging and 3D reconstruction assays for astrocyte proximity

to Synapsin I puncta (non-specific synapses) and PLC-NAcore dendrites and spines. Here we show that while cocaine SA followed by one week of abstinence decreased the degree to which astrocytes associate with Synapsin I, cocaine SA increased PLC-NAcore dendritic spine head volume as well as the degree to which astrocytes associate with these spines. Thus, astrocyte association with PLC-NAcore neurons is enhanced after abstinence from cocaine SA, whereas the association with non-specific synapses is decreased. Future studies will examine whether astrocyte association with dendritic spines on neurons of pathways that serve dichotomous functions relative to PLC-NAcore, such as PLC projections to the paraventricular nucleus of the thalamus, are differentially altered following cocaine SA and abstinence.

Introduction

Prevalence and societal impact of illicit drug use

The use of mind-altering drugs, particularly psychostimulants, is not a modern phenomenon and has played a key role in the religious and economical aspects of many cultures worldwide. Whether for recreation, religious experiences, or self-medication, the use of drugs has been occurring for centuries and persists today. Illicit drug use has impacted several cultures worldwide in the modern era. In the United States, an estimated 30.5 million people were considered current drug users in 2017, with 7.5 million people diagnosed with a substance use disorder (SUD). 7.5 million people equates to approximately 2.8% of the national population aged 12 or older, demonstrating the significant impact illicit drug use has on the United States (SAMHSA, 2017).

The fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM) outlines the criteria for diagnosing SUD, all of which exemplify the significant impairment and distress brought about by drug addiction. Failure to satisfy work, school, or home obligations along with significant time spent seeking out drugs otherwise dedicated to fulfilling other responsibilities demonstrates the impact addiction can have on a person and community (American Psychiatric Association, 2013). A common misconception is that the abuse of illicit drugs affects only the individual; however, society as a whole suffers in many ways as a result of these practices. In 2007, the use of illicit drugs was found to cost the United States $193 billion when taking crime, healthcare, and loss of productivity into consideration (NDIC, 2011). Ten years later, the number of illicit drug users has increased which is likely predictive of an unprecedented, negative, economic impact (SAMSHA, 2017).

In a recent annual report, the Substance Abuse and Mental Health Services Administration (SAMHSA) reported that 2.2 million people over the age of 12 were current cocaine users in 2017, with nearly one million of those people qualifying to be diagnosed with cocaine use disorder (SAMHSA, 2017). Accordingly, cocaine addiction remains one of the most difficult of the SUDs to treat. This is due to the relatively high rates of relapse in cocaine-addicted patients, even after prolonged periods of abstinence (Hanlon et al., 2013; Sinha et al., 2011). Relapse is a major issue related to the treatment of drug addiction in general, and several different stimuli that become associated with drug-induced euphoria can act as "triggers" which can themselves drive craving for drugs (Perry et al., 2014). Because of this, there has been significant research into potential treatments for cocaine addiction. Yet, there is no FDA-approved pharmacotherapeutic for cocaine addiction. However, several treatment strategies have recently shown potential promise for the treatment of cocaine addiction.

Clinical findings of cocaine use

The use of cocaine effects several structures throughout the brain involved with limbic processing, higher-order cognition, and learning (Breiter et al., 1997). The understanding of the specific brain regions implicated in cocaine addiction is ongoing and expands beyond the previously implicated reward pathway (discussed below) typically linked with drug-induced maladaptation in reinforcement learning and relapse. Both the limbic system and basal ganglia play a role in motivated behavior which explains why they have been implicated in the transition from recreational cocaine use to chronic cocaine use, and eventually cocaine use disorder (Ono et al., 2000; Kalivas and O'Brien, 2008). Clinical neuroimaging has not only revealed the structures affected by chronic

cocaine use, but also neural activation states during intoxication, abstinence, and during cocaine-associated cue exposure. Regional activation varies depending on the state of the user, whether it be during active using, craving, or abstinence. Activation in a state of intoxication often opposes activation in a state of withdrawal, providing an interesting interaction between state and activation.

Of the many regions implicated in cocaine use disorder, and relapse to cocaine use, the prefrontal cortex (PFC) has been repeatedly shown to be implicated in maladaptive alterations in the brain that lead to unmanageable drug use (Kalivas et al., 2005; Kalivas and Volkow, 2011). The PFC is involved in many advanced cognitive tasks, including decision making, working memory, and self-control (Goldstein and Volkow, 2011). Impaired PFC activity is implicated in the persistent drug seeking and relapse observed in chronic cocaine users (Kalivas, 2008). Clinical neuroimaging studies using positron emission tomography (PET) have revealed changes in neuronal activity in the PFC, in terms of glucose metabolism, during different states of cocaine use and withdrawal. During abstinence, activity in the PFC has been shown to be decreased in cocaine withdrawn patients (Volkow et al., 1991) and decreases are also induced by methylphenidate in cocaine addicted patients (Volkow et al., 2005). In drug-naïve patients, cues paired with biological reinforcers elicit increased PFC activity and this biological paired cue-induced increase in activity is attenuated in cocaine-addicted patients (Garavan et al., 2000). In addition, measuring cerebral blood flow (CBF), cocaine users have been shown to have an increase in activity in limbic regions, including the PFC, and a decrease in activity in basal ganglia regions while receiving visual drug cues, correlating with self-reported craving for cocaine (Childress et al., 1999). Moreover,

transcranial magnetic stimulation of the dorsolateral PFC in human cocaine-addicted patients decreases self-reported craving (Bolloni et al., 2018; Terraneo et al., 2016). Thus, one prevailing hypothesis is that a key underlying mechanism of cocaine addiction is the misattribution of positive valence for drug-paired stimuli relative to stimuli associated with natural rewards (Dackis and O'Brien, 2001).

Misattribution of cocaine-related stimuli contributes to relapse because recognition of previously neutral stimuli as rewarding activates dysregulated pathways implicated in processing rewarding stimuli; increasing drug seeking. The PFC as well as the ventral striatum is densely innervated by dopaminergic neurons originating in the ventral tegmental area (VTA) of the midbrain (Buchta et al., 2017). The PFC, in turn, sends a glutamatergic projection to several downstream structures, but the most implicated in craving and relapse to cocaine use is the projection to the ventral striatum, including the nucleus accumbens (NAc) (Koob and Volkow, 2016; Cooper et al., 2017). This circuit becomes dysfunctional following chronic cocaine use and such dysregulation is thought to underlie the transformation to compulsive drug use (Buchta and Riegel, 2015). Other brain regions also undergo maladaptive plasticity that drives relapse, including the amygdala. Accordingly, dysfunctional glutamatergic transmission between the PFC and the basolateral amygdala (BLA) results in increased drug seeking once misattribution of the valence of drug-related cues is established (Volkow, 2002). Additionally, glutamatergic projections from the PFC to the dorsal striatum, specifically the caudate, regulate motor activity, and drug seeking, when exposed to cocaine-related cues (Koob and Volkow, 2016).

Interestingly, in individuals considered long-term abstainers, there is greater activation in the PFC compared to controls during inhibition tasks, which require enhanced PFC activity (Connolly et al., 2012). Thus, a dampening of PFC activity during withdrawal (referred to as hypofrontality) from cocaine may predispose cocaine-addicted patients to have heightened activation during presentation of cocaine-paired stimuli. Clinical findings provide valuable insight into the brain regions involved with cocaine exposure and altered gross anatomical activity within those regions. However, in order to develop effective pharmacotherapies for treating cocaine-use disorder, a greater understanding of the cellular mechanisms implicated in addiction requires preclinical animal models.

The dopaminergic system and cocaine

The primary mechanism of action of cocaine in the brain is at the dopamine active transporter (DAT), which is located presynaptically on dopamine-releasing terminals. Cocaine, along with other stimulants, has been found to increase dopamine levels by binding and blocking dopamine transporters (Ritz et al., 1987). When the synapse is saturated with dopamine, postsynaptic receptors are continually activated leading to alterations in the amount of dopamine receptors present without cocaine on-board (Ferris et al., 2011; Siciliano et al., 2016). This leads to a sequence of post-synaptic signaling events in medium spiny neurons (MSNs) within the NAc that, through altered gene transcription, produce enhanced encoding of cocaine-associated stimuli (Taniguchi et al., 2017). Specifically, activation of dopamine D1 receptors by cocaine has been shown to be required for cocaine-induced alterations in MSN plasticity which ultimately mediate relapse (Calipari et al., 2016). Sustained levels of dopamine exceeding optimal levels due

to cocaine administration produces the euphoric feelings often reported by individuals (Volkow et al., 1999).

The dopamine system is critically involved in the encoding of reward-predictive stimuli, as well as the attribution of positive valence to otherwise neutral stimuli (Bromberg-Martin et al., 2010). The pathway encoding these stimuli primarily involves the VTA, nucleus accumbens core (NAcore), and PFC (Cooper et al., 2017). Accordingly, when animals are exposed to a stimulus perceived as rewarding, dopaminergic neurons in VTA are activated and release dopamine in areas involved with reward and reinforcement learning, like the NAcore and PFC (Lammel et al., 2012). Dopamine signaling in these two brain regions is implicated in reward and reinforcement learning (Anderson and Pierce, 2005). Interestingly, reward-predictive cues that are normally perceived as neutral, when paired several times with a reward, can elicit dopamine neuron firing by themselves (Shultz et al., 1997). Dysfunction in dopaminergic regulation of reward-related behavior has been implicated in the maladaptations within the brain following chronic cocaine, as discussed below.

Preclinical models of drug addiction

There are three rodent models of drug addiction typically employed to study cocaine addiction. Cocaine conditioned place preference (CPP) involves the pairing of cocaine with a particular environment and a vehicle with a different environment. After pairing, the associations can be extinguished by placing the animal in the drug-paired side, but without cocaine. CPP can then be assessed by injecting the animal with cocaine and evaluating the degree to which the animal prefers the cocaine-paired environment (Otis and Mueller, 2017; Taniguchi et al., 2017). Alternatively, behavioral sensitization is

a method of studying addiction that examines locomotor activity in response to repeated drug administration. In this model, animals become progressively more hyperactive in response to injections of cocaine over several days. Animals can then undergo a forced abstinence period whereby cocaine injections are not given. Locomotor sensitization can then be assessed by giving an animal an injection of cocaine and recording their locomotor activity which is elevated relative to the previous days of cocaine injections (Kalivas, 1995; Pierce et al., 1996).

Both cocaine conditioned place preference and behavioral sensitization involve experimenter-administered cocaine, thus limiting their face validity for modeling addiction. Rodent models of self-administration (SA), as the name implies, requires the animal to make the choice to take the cocaine, thereby simulating the conscious choice that humans make to take drugs. Animals learn to associate pressing a lever with a cue and a drug infusion, and this association can then be manipulated to study withdrawal and craving. Rodent models of SA have the most construct and face validity when generalizing results to humans because humans consciously make the choice to administer cocaine (Spanagel, 2017).

SA is largely regarded to be among the most valid models for studying craving and drug-seeking induced by cues, which provides insight into the high relapse rates observed in cocaine users (Bossert et al., 2013). Interestingly, cocaine seeking increases as a result of extended withdrawal periods, so it is important to understand the effects of abstinence in order to determine an effective treatment in humans (Grimm et al., 2001; Kelamangalath and Wagner, 2009). To model withdrawal and relapse, rodents can either be exposed to forced abstinence or extinction training followed by reinstatement of drug-

seeking. Forced abstinence involves removing rodents from the environment where they experienced drug exposure for a period of time then measuring drug seeking behavior induced by re-exposure of the animal to the SA context with or without the presence of conditioned cues. Presses on the formerly active lever is typically used as an index of drug seeking. Alternatively, extinction and reinstatement without abstinence is also useful for understanding cue-induced craving (Scofield et al., 2016b). Extinction training after cocaine SA involves the disassociation of the paired cues and drug infusion that previously resulted from a behavior. Once the behavior is extinguished, the animal undergoes a session whereby active lever presses results in conditioned cue presentation, but no cocaine. Animals display reinstated drug seeking if cumulative active lever presses are greater than extinction lever pressing (Spanagel, 2017).

Using these models, several preclinical studies have revealed compelling results linking dysfunction in PFC activity and structure to relapse. The prelimbic cortex (PLC), in general, undergoes maladaptive plasticity following cocaine SA and abstinence (Siemsen et al., 2018) or extinction (Parrilla-Carrero et al., 2018). Preclinical studies have found that cells in layer V of the PLC become “hyperexcitable” following cocaine CPP, which primes the brain for retrieval of cocaine-associated memories (Otis et al., 2018). Using cocaine SA, it has been shown that restoring the hyperexcitability normalizes the electrophysiological activity of these cells and reduces drug-seeking behaviors (Parrilla-Carrero et al., 2018). Moreover, it is now understood that the downstream projections arising from the PFC in the striatum (dorsal and ventral caudate and putamen) are implicated in the enhanced craving and unmanageable desire to seek and use cocaine (Hanlon et al., 2016). In particular, the ventral striatum, described herein as the NAc, has

been most implicated in craving for cocaine (Wilcox et al., 2011). The NAc is involved in the regulation and execution of motivated behaviors by way of dopaminergic inputs and glutamatergic outputs (Wolf, 2016). Thus, the PFC projection to the NAc is likely involved in enhanced encoding of cocaine-associated stimuli relative to stimuli associated with natural rewards (Wilcox et al., 2011).

Although there is a clearly defined role for dopamine in mediating the acute, reinforcing qualities of cocaine, altered glutamate homeostasis in the NAc is linked to cue-induced craving and relapse following prolonged cocaine SA and extinction (Kalivas, 2009; Scofield at al., 2016a). In the NAcore, elevated extracellular glutamate levels from PFC projections prompt a rapid increase in dendritic spine head diameter and synaptic strength which is essential for cue-induced reinstatement of cocaine seeking (McFarland et al., 2003; Gipson et al., 2013). Due to the importance of glutamate homeostasis, releasing and clearing glutamate from the synapse is a vital mechanism implicated in addiction. Astrocytes have membrane transporters responsible for releasing glutamate in order to maintain optimal levels. The primary transporter involved is the cystine-glutamate antiporter, xCT, which after repeated cocaine use is less effective and results in reduced extracellular glutamate. The xCT transporter also provides glutamatergic tone on mGluR2 and mGluR3, glutamate receptors on presynaptic terminals (Baker et al., 2003). Such activation limits glutamate release from cortical terminals, and prevents cue-induced glutamate release and relapse (Baker et al., 2002).

Morphological changes of dendritic spines impact drug seeking and relapse

Dendritic spines are postsynaptic sub-cellular structures housing excitatory synapses. Dendritic spines are exceptionally dynamic; with the ability to modify their

shape, size, and density in response to synaptic activity levels on the scale of minutes (Amaral and Pozzo-Miller, 2009). Other physiological processes like development and disease also influence the structure of dendritic spines (Hayashi and Majewska, 2005). Synapse formation between a presynaptic compartment and postsynaptic dendritic spine is rapid and can occur within hours of new spine development (Zito et al., 2009). Embedded in the membrane of dendritic spines, there is a diverse population of proteins responsible for receiving incoming signals, initiating signaling cascades, and providing mechanical support during structural modifications and growth (Gipson and Olive, 2017). One such method of signaling at the dendritic spine is the influx of Ca^{2+} through NMDA and AMPA receptors. Calcium influx activates many immediate and modulatory neuronal processes through postsynaptic signaling proteins like CaMKII, Ras, Rho, and Cdc42 which regulate plasticity and growth (Higley and Sabatini, 2008; Murakoshi and Yasuda, 2012).

A single administration of cocaine can have significant effects on the morphology of neuronal cells and their projections, some of which persist for an extended period of time (Caffino et al., 2018). Modifications include the number of dendritic spines present, as well as the shape and size of the spines. These qualities influence the communication that occurs at the synapse between presynaptic and postsynaptic cells, as well as with associated glial cells. Specifically, dendritic spine density was found to decrease after a single administration of cocaine in the medial PFC of adolescent rats (Caffino et al., 2018) and PLC after cocaine SA and one week of abstinence (Siemsen et al., 2018). In the PFC projection to the NAcore, variation in spine density has been observed depending on the length of abstinence. Immediately after the final cocaine SA session, dendritic spine

heads on PLC neurons projecting to the NAc displayed a decrease in size but following a week of abstinence dendritic spine heads were found to increase in size (Siemsen et al., 2018). After nearly a month and a half of withdrawal, an increase in thin spines was observed followed by a decrease after 2 months of withdrawal (Christian et al., 2017). Dendritic spines demonstrate continued plasticity in response to all states of cocaine use. Further evidence is demonstrated in that after a period of abstinence from chronic cocaine use, withdrawal from acute cocaine administration results in biphasic modifications in the NAc with a nearly immediate increase in spine head diameter followed shortly after by a significant decrease in spine head diameter (Kalivas, 2009; Shen et al., 2009). This suggests that chronic cocaine use may lead to synaptic metaplasticity, where previous synaptic modifications alter future modifications observed when cocaine is reintroduced after a period of abstinence (Shen et al., 2009).

Astrocyte-dendritic spine interaction

Maintaining glutamate homeostasis at the synapse is vital because increased synaptic glutamate levels promotes disrupted communication between the PFC and NAcore, two principal components of addiction (Kalivas, 2009). The interaction of astrocytes at a synapse is the primary mechanism for maintaining optimal glutamate levels. Therefore, the degree to which astrocytes insulate a synapse and interact with dendritic spines is critical. These interactions are altered after cocaine use. There is evidence of reduced surface area and volume of astrocytes and decreased colocalization of astrocytes with synapses in the NAcore after cocaine SA and a period of extinction (Testen et al., 2018; Scofield et al., 2016b). Because of the role of glutamate in the NAc, the effects of cocaine exposure on the association of astrocytes with dendritic spines and

overall glutamatergic transmission within the reward pathway is essential to understand for the development of potential treatments.

Purpose and hypothesis

Based on the current understanding of mechanisms related to relapse after cocaine abstinence, there is precedent for further investigation into the interaction between astrocytes and dendritic spines within the PFC-NAc pathway that is altered by chronic cocaine exposure. Previous findings revealed alterations in dendritic spine size and density after cocaine SA and the opposite effect after a week of abstinence, leading to modified association of spines with astrocytes in the NAcore (Siemsen et al., 2018). Accordingly, we hypothesized that PLC dendritic spines in the PFC-NAc tract would exhibit an increase in size and density in addition to increased association with astrocytes.

Methods

Animals

Sixteen adult male Sprague Dawley rats, weighing between 275-325g at the time of surgery were used in this study (Charles Rivers Laboratories; Wilmington, MA, USA). Rats were given at least three days to acclimate to the humidity-controlled vivarium with standard rat chow (Harlan; Indianapolis, IN, USA) and water available *ad libitum*. Rats were housed individually on a 12hr reverse light/dark cycle (lights on at 6AM). All protocols complied with the Institutional Animal Care and Use Committee of the Medical University of South Carolina and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (8^{th} ed. 2011).

Surgery

Prior to inserting a silastic catheter (Fisher Scientific; Hampton, NH) in the right jugular vein, rats were given an i.p. injection of ketamine (66 mg/kg) and xylazine (1.33 mg/kg) for anesthesia and ketorolac (2.0 mg/kg) for analgesia. For viral microinjection, rats were positioned in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA). Rats received single microinjections within the NAcore and PLC using a Nanoject II (Drummond scientific, Broomall, PA, USA). The first was a microinjection (0.75μl/hemisphere) of a Canine adeno virus type 2 expressing a Cre-eGFP (CAV2-Cre-eGFP) fusion protein under the control of a CMV promoter ($3.6x10^{12}$ vg/ml) from Montpellier vector core (Montvellier, FR) into the NAcore (coordinates: +1.6mm AP from bregma, ±2.8mm ML from bregma, -7.1mm DV from skull, 10° angle). The other was a microinjection of an 1:1 mixture of AAV5-hSyn-DIO-mCherry (~$1.56x10^{12}$ vg/ml) and AAV5-GFAP-Lck-GFP (~$1.0x10^{12}$ vg/ml) from Addgene (Cambridge, MA, USA) into the PLC (coordinates: +2.8mm AP from bregma, ±0.6mm ML from bregma, -3.8DV from skull). Microinjections were performed over a five-minute period and to promote diffusion, injectors were left in place for 10 minutes and then retracted. After surgery, rats were given at least five days to recover with *ad libitum* food and water.

Self-administration and abstinence

In standard Med Associates operant boxes (Fairfax, VT, USA), rats underwent 12-14 days of cocaine SA or received yoked-saline infusions (2hrs/day with at least 10 infusions/day) on a FR1 reinforcement schedule. There are two retractable levers in the operant boxes, an active lever and inactive lever. When the active lever is pressed, light and tone cues are presented to the rats followed by a single infusion (200μg/50μl) of

cocaine hydrochloride (NIDA, Research Triangle Park, NC, USA). After the cocaine infusion, there was a 20s timeout period in which active lever presses elicited no cue or infusion. Inactive lever presses were also recorded but had no programmed consequence. Control rats received a 0.9% sterile saline infusion along with a light and tone cue when their yoked partner received a cocaine infusion.

After the final cocaine SA session, rats underwent one week of forced homecage abstinence with food and water provided *ad libitum*. They were then anesthetized with equithesin (~0.5ml, i.v.) and transcardially perfused with 150mL of ice-cold 0.1M phosphate buffer at 60 mL/min followed by 200mL of ice-cold 4% paraformaldehyde (pH 7.4) in 0.1M phosphate buffer. Brains were then removed and placed in the same fixative for 24hrs and then immersed in 20% sucrose solution until sectioning.

Immunohistochemistry

80μm coronal sections containing the PLC and NAcore (AP + 2.52-4.2mm from bregma) were sliced using a Leica cryostat then stored in 0.1M PBS with 0.01% sodium azide at 4°C until immunohistochemistry was performed. Three to four PLC and NAcore sections were blocked in 0.1M phosphate-buffered saline with 2% Triton X-100 (PBST) and 2% normal goat serum (NGS) at room temperature for 2hrs. Next, the tissue was incubated in chicken anti-mCherry primary antisera (1:3000, LSbiosciences) to label PLC neurons projecting to the NAcore overnight at 4 degrees Celsius overnight. In separate sections, astrocyte-GFP was labeled with a chicken anti-GFP primary antibody (AbCam, 1:1000) and rabbit anti-Synapsin-I (AbCam, 1:500). Sections were washed in PBST (3X5 minutes), then incubated in secondary antisera conjugated to Alexa-594 (mCherry), Alexa-488 (GFP), or Alexa-647 (Synapsin-1) in a species-appropriate manner for two

hours at room temperature. Sections were then washed in PBST (3X5 minutes) and mounted on superfrost plus slides using ProLong Gold antifade.

Confocal microscopy

Dendritic spine imaging

Confocal Z-series data sets were imaged using a Leica SP8 laser-scanning confocal microscope (Wetzler, Germany). mCherry immunoreactivity was excited using an Optically Pumped Semiconductor Laser (OPSL) 552 nm laser line and GluA1/2 were excited with a diode 638 nm laser line. To image dendrites, laser power, gain, pinhole, and optical section thickness were set empirically and held constant. Images were taken using a 63x oil immersion objective and had a frame size of 1024 x 512, 3.5x digital zoom, 700 Hz acquisition rate, and 0.1 μm step size. Following image acquisition, files were deconvolved using Huygens essential deconvolution software (Scientific Volume Imaging, Hilversum, The Netherlands).

Whole cell astrocyte imaging

In adjacent sections, astrocytes were imaged along with Synapsin-I puncta. Astrocytes were imaged preferentially in layers II/III and V of the PLC as previously performed (Testen et al., 2018). Images were acquired using a Leica SP8 laser-scanning confocal microscope equipped with Hybrid Detectors (HyD, Leica Microsystems). A 63X oil immersion objective (N.A. 1.4, Leica Microsystems) was used for individual astrocyte image acquisition. Z-stacks were acquired at a 2048X2048 pixel frame size, 1.5X digital zoom, and a 0.3 μm Z-step size. These parameters were chosen based off of the recommendations of Huygens Deconvolution software. GFP was excited using an Argon 488 nm laser line and Synapsin-I was excited using a diode 638 nm laser line. Z-stacks

typically consisted of ~200 individual optical sections. Following acquisition, Z-stacks were deconvolved using Huygens software.

Imaris 3D reconstruction, morphological analysis, and colocalization

Colocalization

The colocalization module of Imaris was used to determine the degree to which astrocyte processes contact Synapsin-I on a whole-cell level, as well as dendrites and dendritic spines of PLC-NAcore neurons. Colocalization occurs when two fluorophores are within a given distance of each other, and this distance is dependent on the confocal system utilized (~130 nm in our hands). Thus, colocalization can be used as an index of astrocyte-dendritic spines interaction as well as gross estimates of astrocyte synaptic contact. Colocalization also depends on the signal intensity of each fluorophore. Thus, all signal intensity thresholds were set automatically using the Costes method (Dunn et al., 2011). This allows for a non-biased estimate of colocalization, eliminating the need to subjectively set signal intensity thresholds.

Whole-cell astrocyte colocalization with Synapsin-I

To analyze the association of whole cell astrocytes with Synapsin I, a surface was built on the raw GFP channel to isolate it from the surrounding background signal and to measure astrocyte surface area and volume. Using the colocalization module, colocalization between the masked GFP channel and the raw Synapsin I channel was calculated using the Costes method as described above. If the observed correlation between the astrocyte and Synapsin I channels was less than 0.1, the colocalization channel was built. If the observed correlation was greater than 0.1, then Z-stacks were cropped in the Z-dimension and the colocalization was recalculated. A 3D space-filling

model was then built on the colocalization channel. To compare cocaine animals with saline controls and normalize the observed colocalization to the varying astrocyte sizes, the colocalization volume was normalized to the size of each astrocyte, producing a percent of region of interest (ROI) colocalization metric.

Dendritic spine morphological analyses

To complete morphological analyses, deconvolved Z-stacks were imported into Bit-Plane Imaris (Zurich, Switzerland, version 9.0). Z-stacks were initially cropped in 3D to isolate individual dendrites. The filament module was used to semi-manually draw a filament, which included the dendrite, and all identified dendritic spines. Spheres representing the dendritic spine heads were generated by Imaris through a filament analysis from which spine head volume and diameter were measured. Dendritic spine density was calculated as the number of spines normalized to the length of the dendrite (μm).

Astrocyte process colocalization with PLC-NAcore neurons

Initially, a channel was built using the colocalization module between the raw astrocyte channel and the raw filament channel with an observed correlation less than 0.1. This generates a colocalization channel on the whole filament, and a surface was then built on that channel. The focus of this study was on the interaction of astrocytes with dendritic spines heads, therefore colocalization was cropped to only the spine heads. To accomplish this, the spine head spheres generated earlier were expanded 200nm to create a larger sphere surface surrounding the spine heads. A new spine head colocalization channel was built based on the expanded spine head surface, masked to the raw colocalization channel and a surface was built on the cropped colocalization

channel. The volume of the cropped colocalization was normalized to the volume of the expanded spine head sphere to determine the percent colocalization.

Statistical analysis

Behavioral data were analyzed with a two-way repeated measures ANOVA with cocaine versus saline treatment as a between-subjects factor and SA session as a within-subjects factor. When a significant interaction or a significant main effect of treatment was detected, a Bonferroni-corrected pairwise comparison was completed. A two-tailed t-test with or without Welch's correction was used to compare two groups based on a single variable. Cumulative frequency distribution data of spine head volume and coverage were analyzed using a Kolmogorov-Smirnov non-parametric test to reveal any shifts in the population. Data are expressed as the mean ± SEM and significance was established as $p < 0.05$.

Results

Experimental design and cocaine SA

Figure 1 shows a PLC-NAcore neuron in a field of astrocytes and a proximal apical dendrite with the associated astrocyte representative of what was imaged for this experiment. A breakdown of the steps involved in determining astrocyte-neuron interaction is also included demonstrating the expansion of dendritic spine heads to restrict colocalization to the spine heads. After IV catheter implantation and viral surgery, rats underwent 12-14 days of cocaine SA or received yoked-saline infusions followed by one week of homecage abstinence (Fig. 2a). Figure 2b illustrates the number of both active and inactive lever presses as well as cocaine infusion data. A two-way repeated-measures ANOVA indicated a significant main effect of treatment ($F(1,13)=30.52$,

p<0.0001), but not a significant time by treatment interaction (F(11,143)=1.194, p=0.29). Bonferroni-corrected pairwise comparison test indicated a significant difference between cocaine and saline on days 5 (p<0.0001), 8-10 (p<0.05), as well as day 12 (p<0.05).

Decrease in colocalization of astrocytes with Synapsin I in PLC

To determine the degree to which cocaine SA alters astrocyte association with Synapsin I in the PLC, we analyzed whole cell astrocytes in cocaine and saline SA rats. One animal was excluded due to lack of virus expression. 31 astrocytes from 7 saline SA rats and 29 astrocytes from 7 cocaine SA rats were analyzed. Animal average data from cocaine and saline SA had different variances in terms of astrocyte association with Synapsin I (F(6,6)=6.871, p=0.034). A Welch's-corrected two-tailed t-test revealed a significant decrease in colocalization of astrocytes with Synapsin I in cocaine SA rats compared to saline rats (t(7.71)=2.475, p=0.04) (Fig. 3a). Data resulting from normalizing astrocyte surface area to volume, an index of astrocyte complexity, showed no difference in variance between groups (F(6,6)=1.256, p=0.789). There was no significant change in surface area:volume ratio in cocaine SA rats compared to saline rats (t(12)=0.316, p=0.758) (Fig. 3b). Figure 3c depicts representative astrocytes and colocalization with the Synapsin I marker in cocaine and saline animals.

Increase in volume of PLC dendritic spine heads

To determine the degree to which cocaine SA impacts dendritic spine volume of PLC neurons projecting to the NAcore, we imaged and analyzed dendritic spine segments from cocaine and saline SA rats. Two animals were excluded due to a lack of virus expression. 24 spine segments from 5 saline SA animals (1,705 individual spines) and 28 spine segments from 5 cocaine SA animals (1,921 individual spines) were analyzed.

Data from cocaine and saline SA animals showed different variances regarding spine head volume ($F(27,23)=3.341$, $p=0.005$). A Welch's-corrected two-tailed t-test revealed that cocaine SA rats showed significantly larger spine head volume compared to saline rats ($t(42.99)=2.412$, $p=0.02$) (Fig. 4a). Data from cocaine and saline SA animals showed similar variances regarding spine head diameter ($F(27,23)=1.484$, $p=0.34$). A two-tailed t-test revealed that cocaine SA rats showed a nonsignificant increase in spine head diameter compared to saline rats ($t(50)=1.717$, $p=0.092$) (Fig. 4b). We also analyzed population shifts within these two groups by analyzing the cumulative frequency of spine head volume using each spine as an individual data point. We found that cocaine SA followed by one week of abstinence produced a significant rightward shift in spine head volume according to Kolmogorov-Smirnov test (K-D=0.116, $p<0.0001$) (Fig. 4c). We also analyzed dendritic spine density by normalizing the number of spines on each dendrite to the length of the dendrite. Dendritic spine density data showed similar variances ($F(27,23)=1.008$, $p=0.994$). A two-tailed t-test revealed no significant difference in dendritic spine density between cocaine SA and saline SA rats ($t(50)=0.77$, $p=0.445$) (Fig. 4d). Figure 4e shows representative dendrites in cocaine and saline animals.

Increase in colocalization of astrocytes with PLC dendritic spines

To determine the degree to which cocaine SA altered the association of astrocytes with dendritic spines of PLC neurons, the colocalization was calculated. Data from cocaine and saline animals displayed no difference in variance in terms of spine head coverage ($F(27,23)=1.899$, $p=0.122$). A two-tailed t-test demonstrated a significant increase in spine head coverage in cocaine SA rats compared to saline rats ($t(50)=2.09$, $p=0.042$) (Fig. 5a). We also evaluated population shifts in cocaine and saline animals by

analyzing the cumulative frequency of spine head coverage with the percent colocalization on each spine represented as an individual data point. This resulted in the analysis of 1673 individual spines from saline animals and 1762 individual spines form cocaine animals. We observed a significant rightward shift in spine head coverage in rats exposed to cocaine SA and one week of abstinence according to a Kolmogorov-Smirnov test (K-D=0.07. *p*=0.0004) (Fig. 5b). When animal averages of spine head coverage were analyzed, there was no significant difference in variance between groups (F(4,4)=2.544, p=0.388). A two-tailed t-test revealed that cocaine SA rats exhibited a nonsignificant increase in spine head coverage compared to saline rats (t(8)=2.188, p=0.06) (Fig. 5c). Data from saline and cocaine animals regarding the percent of dendritic spines with detectable spine head coverage showed similar variances (F(23,27)=1.002, p=0.9874). A two-tailed t-test demonstrated no significant difference in the number of dendritic spines with detectable spine head coverage (t(50)=0.8159, p=0.4184) (Fig. 5d). Animal average data showed similar variances between cocaine and saline animals (F(4,4)=1.210, p=0.8578) and no significant difference in the percent of spines with detectable astrocyte coverage (t(8)=0.080, p=0.3118) (Fig. 5e). Figure 5f shows the colocalization between dendritic spine heads and astrocytes in cocaine and saline animals.

Discussion

Summary of findings

Here we show that cocaine SA followed by one week of abstinence decreased the degree to which PLC astrocytes associate with general synapses, yet there was no discernable change in astrocyte morphological complexity as measured by astrocyte surface area normalized to volume. Interestingly, we found that PLC astrocytes increase

their contact at dendritic spines of PLC-NAcore neurons. This was associated with an increase in dendritic spine head volume, but not dendritic spine density. This study, to the best of our knowledge, is the first to examine cocaine-induced adaptations in astrocyte association with dendritic spines in a pathway-specific manner.

Morphological changes in dendritic spines of PLC-NAcore neurons following cocaine exposure

Dendritic spines are complex structures that house individual excitatory synapses and are typically thought of as the sites of synaptic plasticity (Harris, 1999). Measuring evoked currents following the exposure of isolated dendritic spines to glutamate revealed that the size of dendritic spines is linked to synaptic potentiation with larger spines producing a greater current (Matsuzaki et al., 2001). Because of this characteristic, it is critical to understand cocaine-induced changes of spine morphology and glutamatergic transmission in the PLC-NAcore pathway in order to gain a better understanding of the cellular mechanism involved in cocaine addiction and relapse.

Cocaine SA alters the morphology of dendritic spines in the PFC and NAcore which may result in both immediate and persistent modifications in the PLC-NAcore pathway (Robinson and Kolb, 1999). Our observed increase in spine head volume in PLC neurons is consistent with previous findings (Siemsen et al., 2018). However, we did not observe any change in dendritic spine density, which contracts with previous reports.

Previous findings indicate that cocaine SA and abstinence decreases layer II/III PLC neuron distal apical spine density (Radley et al., 2015) as well as PLC-NAcore distal apical spine density (Siemsen et al., 2018). This apparent discrepancy may be accounted for by the region of the apical dendrite analyzed. For example, stress-induced hypotrophy

of dendritic spines seems to be specific to the distal apical tuft of PLC pyramidal neurons (Liu and Aghajanian, 2008). Moreover, inputs to the distal apical tuft and proximal apical dendrite differ in their origin. The distal apical tuft of PLC pyramidal neurons receive a mixture of monoaminergic, orexinergic, and glutamatergic inputs. Specifically, orexinergic inputs, presumably arising from the lateral hypothalamus (Sakurai et al., 1998) to the distal apical tuft have been shown to target presynaptic, glutamatergic, axonal boutons arising from the midline thalamus and this input directly regulates calcium signaling in dendritic spines of layer V PLC neurons (Lambe et al., 2003). However, ventral hippocampal inputs to the proximal apical dendrite have been previously identified, whereas BLA inputs largely target the basal dendrites of layer V PLC neurons (Flores-Barrera et al, 2014). Taken together, these data suggest that PLC-NAcore neurons likely undergo differential structural plasticity depending on the precise domain of the apical dendrite analyzed, and may explain contrasting results regarding cocaine-induced regulation of PLC neuronal morphological plasticity.

Increased size of spine heads after cocaine exposure has also been observed in the NAcore (Dobi et al., 2011). Previous findings show that cocaine SA and extinction leads to a transient enhancement of NAcore MSN AMPA/NMDA ratio, an index of synaptic potentiation, during cue-induced reinstatement (Gipson et al., 2013). Both the transient elevation in dendritic spine head diameter and AMPA/NMDA ratio tightly correlate with cocaine seeking behavior (Gipson et al., 2013; Smith et al., 2017). Further evidence for the involvement of this pathway was revealed when optogenetically silencing the PLC neurons projecting to the NAcore after cocaine SA and extinction was found to block cue-induced reinstatement and prevent cue-induced transient synaptic potentiation

in the NAcore (Stefanik et al., 2016). Because the size of spine heads is correlated with synaptic potentiation (Matsuzaki et al., 2004), these findings collectively suggest that the PLC-NAcore pathway may undergo cocaine-induced synaptic plasticity leading to pathway-dependent synaptic potentiation following cocaine SA and abstinence.

Alterations in astrocyte morphology after cocaine SA in the PLC and NAcore

Astrocytes are recognized as active regulators of synaptic strength and stability through the release of glutamate at synapses in the PLC-NAcore pathway (Scofield et al., 2016a). Previous studies have found that cocaine SA and extinction reduced astrocyte volume and surface area in the NAcore (Scofield et al., 2016b), but not the PLC (Testen et al., 2018). These modifications were reversed after ceftriaxone administration due to its ability to upregulate GLT-1 transporters on astrocytes to facilitate glutamate homeostasis (Scofield et al., 2016a). Our findings are consistent with Testen and colleagues regarding a lack of cocaine-induced change in astrocyte morphology. However, in contrast to Testen and colleagues (2018), we show herein that astrocytes retract from general synapses following cocaine SA and one week of abstinence. Two particular points of interest may explain this discrepancy. First, Testen et al. (2018) utilized PSD-95 as a synaptic marker in their studies. PSD-95 is a post-synaptic protein implicated in regulation of AMPA and NMDA receptor trafficking ay synapses, as well as protein scaffolding (Dosemeci et al., 2007; Bats et al., 2007). PSD-95 seems to be a marker largely for excitatory synapses. This is because overexpression of PSD-95 is sufficient to augment excitatory (i.e. glutamatergic) neurotransmission while downregulating inhibitory (i.e. GABAergic) neurotransmission (Prange et al., 2004). Interestingly, PSD-95 more strongly associates and labels stable, as opposed to

transient, dendritic spines (De Roo et al., 2008). Thus, Testen and colleagues were likely investigating astrocyte contact at mature excitatory synapses. In contrast, Synapsin-I is a generic marker for presynaptic terminals that has long been recognized as an essential and conserved component of synaptic release machinery (Hirokawa et al., 1989; Takei et al., 1995; Gitler et al., 2004). Thus, Synapsin-I serves as a marker for gross estimates of astrocyte-synaptic contact, whereas PSD-95 is likely more specific for mature excitatory synapses, and this difference is likely critical for interpreting differences in astrocyte-synapse contact.

Interaction of dendritic spine heads and astrocytes in the PLC-NAcore pathway

The interaction of dendritic spine heads and astrocytes is critical for maintaining glutamate homeostasis so any cocaine-induced changes in the association between neuronal and non-neuronal cells in the PLC-NAcore pathway could have significant consequences. We observed an increase in astrocyte coverage within the PLC-NAcore pathway but because we observed no difference in the number of spines with coverage between saline and cocaine animals, we concluded that there was an overall increase in spine head coverage after cocaine SA and abstinence. Cocaine SA and extinction resulted in decreased colocalization with Synapsin I in the NAcore (Scofield et al., 2016b) which is consistent with our findings in the PLC following cocaine SA and abstinence.

Abstinence versus extinction

Different circuits and brain regions are engaged following abstinence and extinction from cocaine SA because extinction involves eliminating the association between the drug and paired cues. This is an important distinction because most humans are not exposed to extinction training and only experience abstinence, a period of

withdrawal that is often still accompanied by exposure to drug-paired cues. The PLC-NAcore projection has been implicated in relapse after abstinence from cocaine SA (Ma et al., 2014). The circuitry involved in modulating these behaviors after reinstatement of cocaine-seeking after extinction also involves regions like the BLA and hippocampus (McLaughlin and See, 2003; Fuchs et al., 2005). The dorsolateral caudate-putamen (dlCPu) has been implicated in context-induced relapse. Relapse was previously though to involve the PLC, however one study found that this process was independent of the PLC because only inactivation of the dlCPu attenuated cocaine-seeking after abstinence and context-induced reinstatement after extinction (Fuchs et al., 2006). In this study we exposed rats to a week of abstinence following cocaine SA and we found increased spine head size and astrocyte association with PLC-NAcore neurons consistent with similar studies (Siemsen et al., 2018). This seems contradictory to the previously described findings because the morphological changes we observed in the PLC-NAcore pathway likely indicate a contribution of this region to drug-seeking and relapse. Future studies should evaluate the behavioral consequence of disrupting cocaine-induced enhancement of PLC-NAcore astrocyte association at dendritic spines.

Conclusion and future directions

Our findings that cocaine SA and abstinence result in reduced contact of astrocytes at general synapses, yet enhanced contact of dendritic spines PLC-NAcore neurons, reveals the importance of examining pathway-specific adaptations. Future experiments should examine alternative pathways arising from the PLC that may undergo differential plasticity to drive relapse. A projection from the PLC to the paraventricular nucleus of the thalamus (PVT) is an alternative pathway with dichotomous functions to

the PLC-NAcore pathway (Otis et al., 2017). Both pathways are involved in the reward and seeking, however the PLC-PVT projection was predicted to play a role in the acquisition and encoding of reward-predictive cues while the PLC-NAcore projection was predicted to play a role in the expression of these reward-seeking behaviors (Otis et al., 2017). Due to their roles in reward-seeking, further studies into both pathways would provide valuable insight into the neural mechanisms engaged in drug-seeking and relapse.

Figures

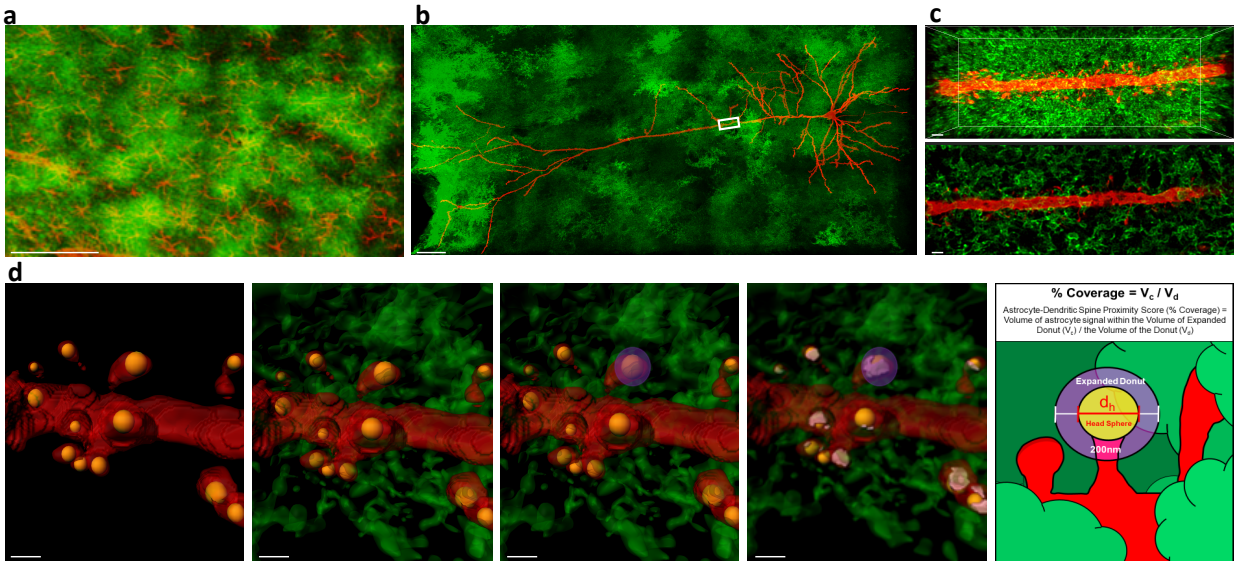

Figure 1. Representative neurons imaged and methodology for determining astrocyte-neuron interactions. a. Field of astrocytes in prelimbic cortex. Scale bar is equivalent to 150 μm. **b.** Field of astrocytes with isolated prelimbic cortical neuron. Scale bar is equivalent to 50 μm. **c.** 3D (top) and slice view (bottom) of apical dendrites used for analysis. Scale bars equivalent to 2 μm. **d.** Representation of detection of astrocyte-dendritic spine interaction using Imaris where spine head was expanded 200 nm and colocalization within the expanded sphere was calculated. Scale bar is equivalent to 0.7 μm.

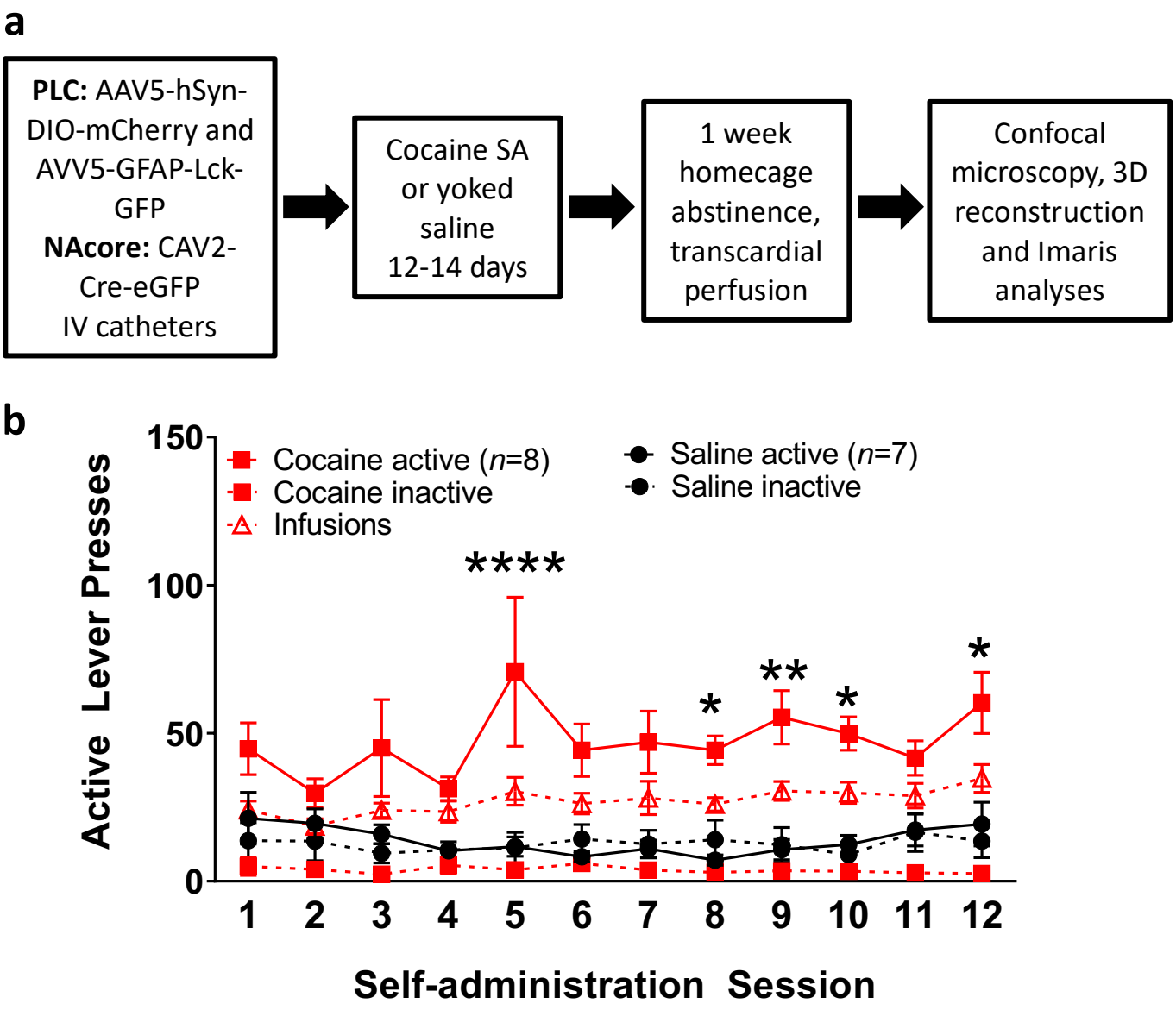

Figure 2. Experiment timeline and cocaine self-administration data. a. Timeline of experiment. **b.** Cocaine SA data for the last 12 days of self-administration demonstrating higher active lever presses in cocaine SA rats (red).

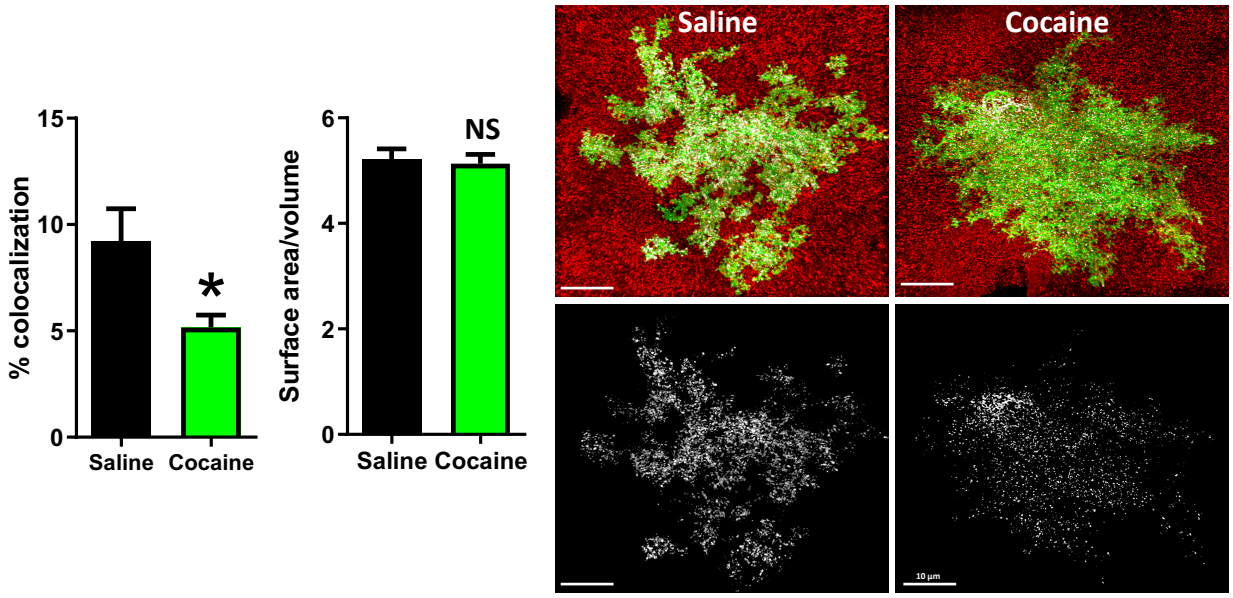

Figure 3. **Cocaine SA significantly decreased the degree to which astrocytes contact synapses. a.** Cocaine SA decreased the colocalization between astrocytes and Synapsin-I but not **b)** astrocyte morphology as measured by astrocyte surface area normalized to astrocyte volume. **c.** Representative astrocyte (green) and adjacent Synapsin-I (red) puncta for saline (left) and cocaine SA (right) rats. Colocalized voxels (below) are shown in white for each group. Scale bar is equivalent to 10 μm. *p<0.05, NS=p>0.1.

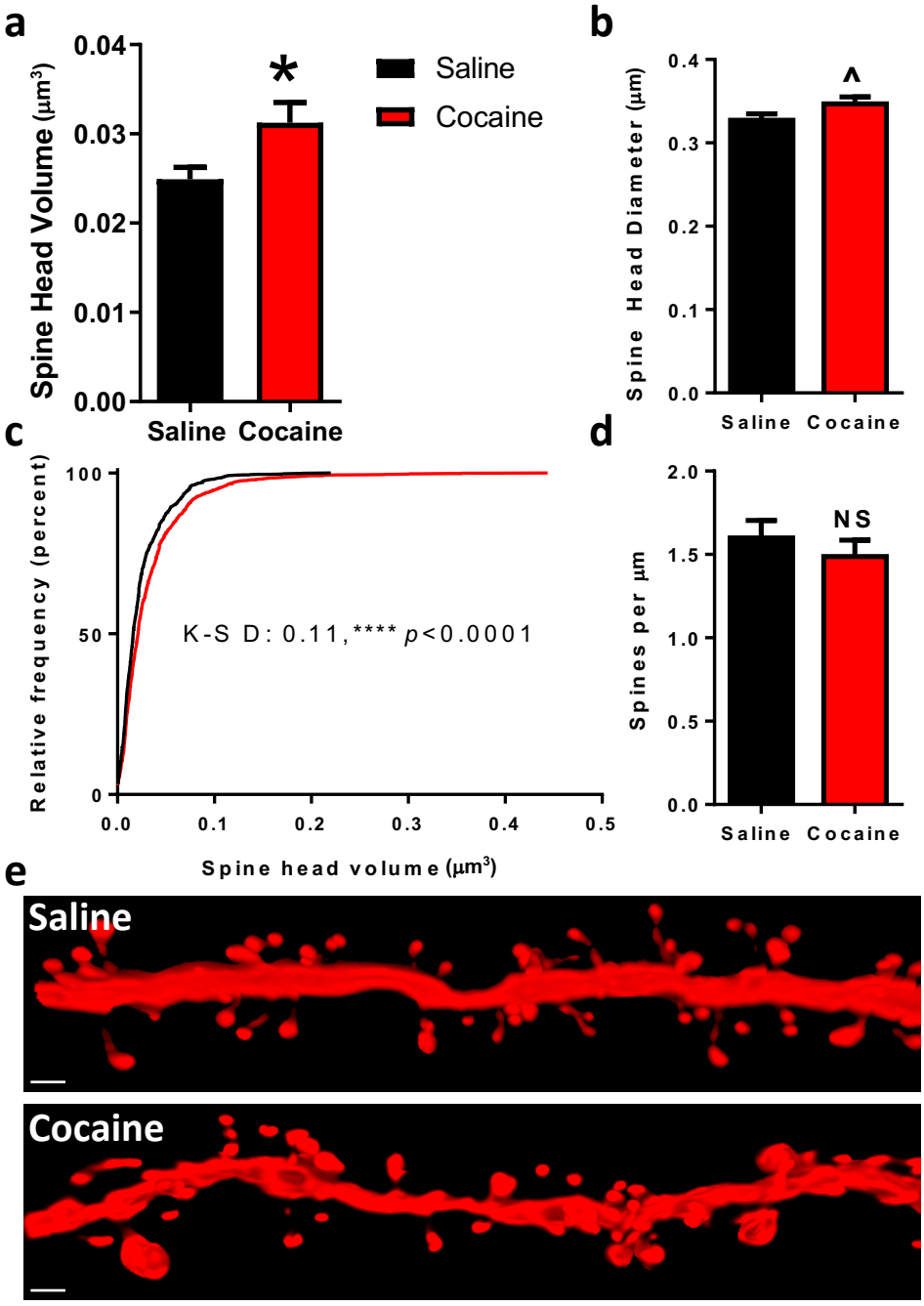

Figure 4. Cocaine SA followed by abstinence increased PLC-NAcore spine head volume. **a.** Cocaine SA increased spine head volume, but not **b)** spine head diameter. **c.** Cumulative frequency distribution reveals a rightward shift in spine head volume in cocaine SA rats. **d.** There was no difference in dendritic spine density. **e.** Representative PLC-NAcore dendrites from cocaine and saline animals. Scale bar is equivalent to 1 µm. *p<0.05, ^p between 0.05 and 0.1, NS=p>0.1.

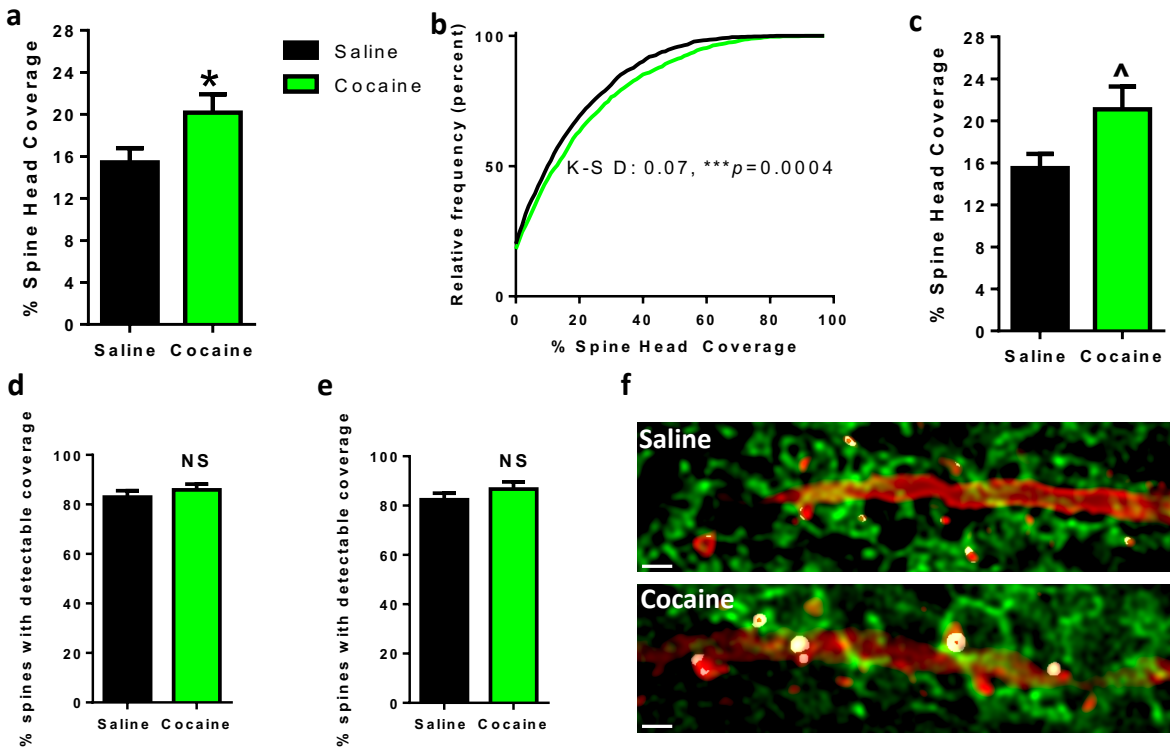

Figure 5. Cocaine SA followed by abstinence increased astrocyte association with PLC-NAcore dendritic spines. a-c. Cocaine SA followed by one week of abstinence increased the average spine head coverage of PLC-NAcore dendritic spines when **a)** each segment is treated as an individual data point, but only shows a trend towards significant difference when **c)** analyzing animal averages. **b)** Cumulative frequency distribution analyses reveals a significant rightward shift in the degree to which astrocytes associate with PLC-NAcore dendritic spines. **d-e.** No difference in percentage of spines with detectable astrocyte coverage when **d)** each segment was treated as an individual data point and when **e)** analyzing animal averages. **f)** Representative PLC-NAcore dendrites and spines showing elevated astrocyte colocalization (white). Scale bar is equivalent to 1 μm. *p<0.05, ^p between 0.05 and 0.1, NS=p>0.1.

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