UNIVERSITY OF CALIFORNIA SAN DIEGO

Cortical Dynamics Underlying Cocaine Seeking Behavior

A Thesis submitted in partial satisfaction
of the requirements for the degree Master of Science

in

Biology

by

Patrick Honma

Committee in Charge:

Professor Byungkook Lim, Chair
Professor Takaki Komiyama
Professor Nicholas Spitzer

2019

The Thesis of Patrick Honma is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California San Diego

2019

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to thank Dr. Byungkook Lim for giving me the opportunity to work in his lab as an undergraduate volunteer and as a MS student, where he has served as a wonderful mentor over the past three years.

I would like to acknowledge Dr. Nicholas Spitzer and Dr. Takaki Komiyama for serving on my thesis committee.

I would like to thank the other members of Byungkook Lim's lab, as they have been instrumental in my success as a student. I'd especially like to thank Eric Wang for serving as my mentor and for assisting me with the heavy computations for wide-field calcium imaging.

ABSTRACT OF THE THESIS

Cortical Dynamics Underlying Cocaine Seeking Behavior

by

Patrick Honma

Master of Science in Biology

University of California San Diego, 2019

Professor Byungkook Lim, Chair

Cocaine addiction is one of the leading causes of drug-related deaths and overdose in the United States, and there are currently no cheap and effective treatments for this disease. To combat this problem, it is necessary to study the underlying neuronal circuits and populations that underlie cocaine seeking behavior. Previous studies have demonstrated that the prefrontal cortex (PFC) becomes hypoexcitable following chronic compulsive drug seeking (Chen et. al. 2013), so we decided to see how the inputs and outputs of the prefrontal cortex are modulated

following chronic cocaine seeking. To answer both questions, we designed a novel head-fixed cocaine self-administration behavioral setup and utilized this model to answer two main questions: 1) how are the inputs to the prefrontal cortex modulated following cocaine addiction? 2) How are the neocortical populations downstream of the prefrontal cortex modulated following cocaine seeking behavior?

Introduction

The abuse of cocaine is on the rise for young adults, and there are currently no FDA-approved medications to treat this addiction. Substance use disorder is defined by six or more symptoms from the 11 DSM 5 symptoms, such as taking large amounts of the substance, cravings, continual use despite negative consequences, development of withdrawal symptoms, or development of tolerance (Hartney 2018). According to the National Survey on Drug Use and Health (NSDUH), there was an estimated 1.5 million users of cocaine above the age of 12 as of 2014. In the same study, over 900,000 Americans met the criteria for abuse of cocaine within the past 12 months, and over 500,000 emergency room visits were due to cocaine-related incidents (NIDA 2014). Understanding the underlying mechanisms mediating cocaine addiction is necessary to develop an effective treatment and hopefully combat the drug problem in the United States.

Cocaine works by chemically hijacking the reward system, by binding to dopamine transporters (DAT) that reuptake dopamine into dopaminergic neurons. Based on evidence in the literature, cocaine appears to alter dopaminergic activity in the mesocorticolimbic circuit, where it stimulates dopaminergic signaling (Hummel and Unterwald 2002). Recent evidence has turned towards other neuronal projections that may be mediating cocaine seeking behaviors, such as cortical and thalamic regions. One group showed that lesions of the mediodorsal nucleus of the thalamus attenuated cocaine seeking behavior (Weissenborn et al, 1998). Further evidence has implicated hypoactivity of the prelimbic cortex as a key feature of compulsive cocaine seeking (Chen et al, 2013). Upon optogenetic stimulation of the prelimbic cortex, this group was able to successfully rescue cocaine seeking behavior. Due to PFC's connections to other regions of the cortex, we also wanted to study how cortical populations are modulated following cocaine

addiction. To do this, we applied a cortex-wide calcium imaging approach (Chen et al, 2017, Gilad et al, 2018, Allen et al, 2017) to image neocortical populations simultaneously. The development of a novel head-fixed cocaine self-administration behavioral paradigm has allowed us to uncover how alternate pathways may be mediating cocaine addiction.

Methods

Animals

All procedures, housing, and handling of mice was done under the approval of the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Diego. Mice were maintained on a 12-hr/12-hr reverse light/dark light cycle with mouse chow and water ad libitum. Adult, C57BL/6J wild-type and Thy1-GCaMP6f mice were purchased from Jackson Laboratory. For all behavioral experiments, animals were single housed following implantation of the jugular vein catheter and head bar. For all experiments, mice were 8-12 weeks old male and females. No significant differences in sex were observed in behavioral performance.

Viral Generation

AAV vector plasmids were prepared using standard molecular cloning methods.

Stereotaxic Injections

Male and female mice were housed in a 12hr/12hr reverse dark/light cycle prior to surgery. Mice (8-12 weeks old) were anesthetized with a mixture of ketamine (100 mg/kg) and dexmedetomidine (0.5 mg/kg) and head-fixed in the stereotaxic apparatus (David Kopf Instruments). A heating pad with regulated temperature was used to maintain a stable body temperature, and a heating pad was used for post-operation recovery. Viral injections were targeted utilizing the Paxinos and Franklin mouse brain atlas (Paxinos and Franklin, 2008) coordinates. Coordinates were adjusted 0.5 mm anterior relative to the Paxinos and Franklin Atlas. Carprofen (5.0 mg/kg) was injected intraperitoneal (i.p.) for analgesia every 12 hours, and anti-sedan (150 mg/kg) was administered subcutaneously to wake up the animals. Mice were

monitored in a regulated heat pad (Harvard Apparatus) while recovering from anesthesia. Following 5 days of post-operation monitoring and recovery, experiments were conducted.

For retrograde tracing experiments, male or female wild-type mice (8-12 weeks old) were infused unilaterally in either the prelimbic cortex (PL) or mediodorsal thalamus (MD) with RV-mRuby2 and RV-eGFP virus. The coordinates for PL were (AP, 2.1 mm; ML, +/- 0.25 mm; DV, -1.75 mm) and the coordinates for MD were (AP, -1.36 mm; ML, +/- 0.5 mm; DV, -3.55 mm).

For pharmacological behavioral experiments, adult (8-12 weeks old) male or female WT mice were injected with 250 nL of either AAV-Hsyn-HM4Di-mCherry or AAV-eGFP bilaterally into MD. The same coordinates as stated above were used. Following viral injection, a 4 cm long head bar was implanted over the cerebellum and dental cement (C&B metabond; Parkell) was used to secure the head bar in place. Finally, absorbable sutures (Oasis) were used to close the incision.

For electrophysiology experiments, 6 week old Ai14 (+/+) male mice were bilaterally injected with 250 nL of RG-EIAV-CAG-Cre into the PL using the same coordinates as above. Mice were given five days of post-op monitoring before administering I.P. injections of cocaine.

Jugular Vein Catheter Implantation

Either male or female mice (8-12 weeks) were anesthetized using isoflurane gas and maintained under anesthesia using a breathing tube. The jugular vein catheter method was adapted from Kmiotek et. al. 2012 (Kmiotek et. al., 2012). The catheter was inserted into the jugular vein and the tubing pushed subcutaneously behind the mouse's neck and attached to the infusion button. The incisions were closed with absorbable sutures (Oasis) and placed in a heating chamber to recover fully. Carprofen (5 mg/kg) was administered i.p. every 12 hours and post-op monitoring was conducted for 5 days. The jugular vein catheter (Access Technologies)

and infusion buttons (Instech) were flushed daily with 0.1% heparinized saline solution. Experiments were conducted following 5 days of post-op monitoring.

Cranial Window Implantation

Adult male and female (8-12 week old) Thy1-GcAMP6s mice were anaesthetized using an i.p. injection of a ketamine (100 mg/kg) and dexmedetomidine (0.5 mg/kg) cocktail. The skin above the skull was removed to expose the entire surface of the skull. The head bar was implanted above the cerebellum using dental cement (C&B metabond; Parkell). A thin layer of cryanoacrylate glue (Krazy Glue) was applied to the surface of the skull and a molded glass cover slip was placed on top. Dental cement (C&B metabond; Parkell) was applied around the edge of the cover glass to seal the cover glass in place. Mice were administered antisedan (150 mg/kg) subcutaneously. Carprofen (5.0 mg/kg) was administered i.p. once every 12 hours for analgesia.

Behavior

Self-Administration Behavioral Paradigm

To assess cocaine seeking behavior, we designed a novel head-fixed behavioral paradigm for cocaine self-administration. The behavioral apparatus consisted of 3D printed head-fixed apparatus and levers attached to joysticks. The levers, speaker, and variable speed syringe pump (Med Associates; PHM-100VS-2) were attached to an Arduino Mega 2560. The trial structure (90 trials) consisted of an initial cue period (20 s) in which mice were trained to press the left lever (active press), which would activate the self-administration pump and initiate the inter-trial interval period (30 s). Pressing during the inter-trial interval did not result in activation of the self-administration pump or cue. Behavioral testing lasted for 33 days, which consisted of a habituation phase, acquisition phase, withdrawal, and cue-induced reinstatement. For habituation

(3 days), mice were water restricted to 1 mL of water per day and received a drop of water if they pressed either the left or right lever. During the acquisition phase (12 days), mice were given water ad libitum and received a single infusion of cocaine (0.6 mg/kg) when pressing the left lever. Mice were not run during the withdrawal phase (14 days), but the catheters were flushed with 0.9% heparinized saline. During the reinstatement phase (4 days), mice were ran on the same behavior paradigm but had no access to cocaine until the last day. Control groups (saline) were ran under the same behavioral paradigm but given 0.9% saline.

***Ex Vivo* Electrophysiology**

To assess the electrophysiological changes occurring in the mediodorsal thalamus, Ai14 (+/+) mice were injected bilaterally with EIAV-Cre as described above. After 5 days of post-op monitoring, mice were administered single i.p. injections of cocaine (5.0 mg/kg) for 5 straight days. 10 days after the last injection, mice were anesthetized with isoflurane gas and perfused with choline buffer solution. The choline solution consisted of 25 mM NaHCO3, 1.25 mM NaH2PO4, 2.5 mM KCl, 7 mM MgCl2, 25 mM glucose, 0.5 mM CaCl2, 110 mM choline chloride, 11.6 mM ascorbic acid, and 3.1 mM pyruvic acid. 250 μm coronal brain slices were collected using a Leica VT1200 vibratome and recovered for 30 min in a recovery chamber at 31ºC consisting of 118 mM NaCl, 2.6 mM NaHCO3, 11 mM glucose, 15 mM HEPES, 2.5 mM KCl, 1.25 mM NaH2PO4, 2 mM sodium pyruvate, 0.4 mM sodium ascorbate, 2 mM CaCl2, and 1 mM CaCl2. Slices were maintained in the recovery chamber at room temperature for 1 hour before transferring to bath for recordings. Slices were transferred to a recording chamber, which was perfused with oxygenated ACSF consisting of 125 mM NaCl, 25 mM NaHCO3, 2.5 mM KCl, 1.25 mM NaH2PO4, 11 mM glucose, 1.3 mM glucose, 1.3 mM MgCl2, and 2.5 mM CaCl2 at 28ºC. Neurons labeled with RG-EIAV-CAG-Cre were visualized using a 40X water-

immersion objective (Olympus). Patch pipettes were pulled from borosilicate glass (G150 TF-4; Warner Instruments) and filled with internal solution containing 135 mM K-gluconate, 5 mM KCl, 10 mM HEPES, 0.1 mM EGTA, 2 mM MgCl2, 2 mM Mg2+-ATP, and 0.2 mM Na+-GTP, pH 7.35 (290 mOsm, pH 7.4). Recordings were made using a MultiClamp 700B amplifier and PClamp software (Molecular Devices).

For measuring firing frequency, steady-state current was injected in +20 pA increments from -100 pA to 400 pA. 10 µm NBQX and 100 µM picrotoxin were present for all recordings. Action potential threshold was measured as the change in voltage from rest. Peak amplitude was measured as the change in voltage from threshold to peak amplitude. The after hyperpolarization (AHP) was the change in voltage and time from the threshold to minimum after peak amplitude. Half-width was measured as the width at half the max peak amplitude.

***In Vivo* Pharmacology**

To determine the role of the MD in cocaine seeking behavior, male and female (8-12 weeks old) mice were bilaterally injected with AAV-hsyn-HM4Di-mCherry or AAV-eGFP (controls) into the MD. Groups were both ran on the head-fixed cocaine self-administration behavioral paradigm. For three consecutive days before reinstatement, mice were given i.p. injections of 0.9% saline to habituate them to the injections. On day one of reinstatement, the groups were injected with either saline or CNO 30 minutes prior to the start of the trials. Sites of injection were confirmed by fixing tissue following completion of behavior.

Histology

Mice were anaesthetized using isoflurane gas and perfusing with 20 mL 0.9% NaCl and 20 mL of 4% paraformaldehyde (PFA) in 1X phosphate-buffered saline (PBS). Brains were fixed overnight at 4º C and washed with 1X PBS the following day. The brains were sliced into 60µm

coronal sections using a Leica VT1000 vibratome and mounted on slides (Superfrost plus) with DAPI Fluoromount-G.

Imaging

Slides were imaged using the Olympus VS120 fluorescent microscopy slide scanner.

Wide-Field Calcium Imaging

To study calcium activity during cocaine self-administration, Thy1-GcAMP6s mice were implanted with a cranial window, jugular vein catheter, and head bar as described above. Mice were trained using the same behavioral paradigm as above, but imaging was conducted on days 1, 3, 4, 8, 12, 16, and 28. To avoid photobleaching, mice were only imaged for 20 minutes of their trial (starting at 5 minutes into the trial). Fluorescent activity was aligned to the onset of pressing, which was obtained through outputs from the Arduino. Excess noise during imaging trials prevented the use of images throughout all imaging days, so we decided to analyze the first day of cocaine/saline ("acute") and last day of reinstatement seeking ("chronic").

Using the raw image stacks from the different animals, principal component analysis followed by independent component analysis was used to define eight functionally and anatomically distinct regions of interest. The number of pixels per module was calculated to ensure that modules were consistent throughout the experiment. Using a decoding machine learning algorithm generated in the laboratory, modules were assessed for movement related activity and decoding related activity. A decoding accuracy of 0.5 indicated random chance. A representative correlation matrix between various regions of interest was generated using fluorescent activity in the various populations. Maximum dF/F and time to half-max were calculated by averaging the fluorescence for each region of interest during movement trials at the acute and chronic stages of the experiment. Finally, the maximum amplitude and frequency of

spontaneous fluctuations were calculated by examining trials in which animals did not move. The peaks were counted and quantified above a certain threshold.

Results

Designing a Head-fixed Cocaine Self-Administration Model

To study how both the inputs and outputs of the prefrontal cortex are modulated following cocaine self-administration in mice, it is necessary to design a novel head-fixed behavioral paradigm that can answer both questions (Fig. 1A). The behavior experiment consisted of 4 phases: 1) Water training (Habituation), 2) Acquisition phase, 3) Withdrawal Phase, and 4) Reinstatement-Seeking Test. Each day of training consisted of 90 trials in which pressing the active lever resulted in a single 0.6 mg/kg injection of cocaine (Fig. 1B).

To test whether the behavioral paradigm recapitulated the normal cocaine seeking behaviors typical of cocaine addiction, five mice were run on cocaine and five mice were run on saline. Mice in both groups demonstrated high active lever pressing by the final day of the acquisition phase (Fig. 1C). During the reinstatement seeking test, cocaine treated mice demonstrated higher active lever pressing during the first two days. Saline and cocaine treated mice showed similar levels of active lever pressing during the final two days of reinstatement testing (Figure 1D). When looking at a discrimination index, calculated as (Active Press – Inactive Press)/(Total Press), cocaine treated mice showed a preference for the active lever throughout the first half of the acquisition phase. Midway through the acquisition phase, these mice tended to switch preference towards the inactive lever, which could be indicative of impulsivity resulting from chronic cocaine use (Fig 1.E). Together, these results demonstrate that the behavioral paradigm successfully recapitulated normal cocaine seeking behaviors.

Chronic Cocaine Causes Hyperexcitability of MD → PFC Neurons

To understand the role of MD→PFC neurons in mediating cocaine seeking behavior, it was necessary to study how the intrinsic excitability is affected after chronic passive administration of cocaine. Previous studies of compulsive drug seeking have demonstrated that prefrontal cortex neurons become hypoexcitable following compulsive drug seeking, so we hypothesized that mediodorsal thalamus neurons may be mediating these effects (Chen et al, 2013). The intrinsic excitability of PFC-projection MD neurons was assessed following withdrawal from cocaine. Ai14 (+/+) male mice were injected bilaterally in the PL with RG-EIAV-CAG-Cre to label PL-projection MD neurons (Fig. 2.B). Mice were given single I.P. injections of saline (control) or cocaine (5 mg/ml) for five consecutive days. After 10 days of no injections, mice were sacrificed, and brain tissue was collected for whole cell patch clamp electrophysiology (Fig. 2.A). Recordings were conducted in the presence of NBQX and PTX to block AMPA receptors and GABA synaptic inputs. When looking at the action potential properties of cocaine- and saline-treated mice, MD→PFC neurons of cocaine-treated mice showed a significant reduction in AHP Latency and AP Half-width, determinants of the frequency and firing pattern of neurons (Fig. 2.E,H). In addition, these same neurons showed higher excitability when current was injected, as measured by the firing frequency (Fig. 2.J). Together, these results demonstrate that chronic cocaine use increases intrinsic excitability of MD→PFC neurons.

Pharmacological Inhibition of Mediodorsal Thalamus Neurons Attenuates Cocaine Seeking

To test whether inhibiting activity of mediodorsal thalamus neurons could rescue chronic cocaine seeking, an adeno-associated virus (AAV) encoding inhibitory DREADDS (HM4Di) was bilaterally injected into the MD. After the withdrawal phase of the cocaine self-administration behavioral task, an i.p. injection of CNO (0.5 mg/ml) was delivered to activate the

inhibitory DREADDS (Fig. 3.A). Inhibition of these MD neurons attenuated cocaine seeking during the reinstatement seeking phase, which serves as evidence for the role of MD neurons in mediating cocaine seeking (Fig 3.C). Viral-mediated tracing done has demonstrated that large populations of mediodorsal thalamus neurons project to both the prelimbic cortex and the orbitofrontal cortex (Choi 2014). However, projection-specific manipulations of the PL-projecting MD neurons is needed to determine if this projection is in fact mediating cocaine seeking.

Cortex-wide Calcium Imaging

We monitored the activity of the mouse neocortex throughout the various phases of the addiction cycle using wide-field calcium imaging in combination with our head-fixed self-administration task. Mice in both the saline- and cocaine-treated groups were able to acquire the behavioral task, but cocaine treated mice showed quicker acquisition of the task. During imaging trials (Days 1, 3, 4, 8, 12, 16, and 28), the fluorescence of GCaMP6s in the dorsal surface of the neocortex was measured using an intact skull preparation with a wide-field microscope (Fig. 4.A). Wide-field calcium imaging allows for simultaneous recording of all neocortical populations while the animals are performing the behavior task. GCaMP6s is expressed in excitatory neurons and fluoresces green when calcium is detected. To determine anatomical regions of the neocortex, we used Principal Component Analysis (PCA) followed by Independent Component Analysis (ICA), which separated populations into eight functionally and anatomically distinct regions of interest. These included visual, sensory, motor, and premotor regions on both the left and right hemisphere (Fig. 4.B). The module size, measured as pixels per region, stayed constant from acute to chronic sessions for both groups and in all regions of interest, which indicates that the regions were well-defined (Fig. 4.C).

Because wide field imaging lacks single-cell resolution, it is difficult to understand what information is stored within the different cortical regions. Thus, we decided to utilize a machine learning decoding algorithm to understand what information is stored in each region. The wide-field fluorescent activity was able to decode movement activity with an accuracy above 50% but was unable to decode choice information with the same accuracy (Fig. 4D-E). In addition, when looking at an example correlation matrix of an example mouse (mouse 3), the strongest correlation was between the left and right corresponding hemispheres (Fig. 4F). To understand how much activity is occurring in each region, we quantified the maximum dF/F for trials in which the mice moved and compared acute and chronic sessions (Fig. 4G). Cocaine-treated mice showed higher levels of fluorescent activity compared to the saline-treated mice, but it is difficult to make any definitive conclusions based on the sample size. Finally, we quantified the time to half-max by looking at the time it took for fluorescent activity to reach half of the maximum amplitude (Fig. 4H).

To understand what is causing changings in fluorescence, we decided to look at endogenous fluorescent activity by looking at spontaneous fluctuations during trials in which mice did not move or make a choice. Peaks were quantified by looking at spontaneous fluctuations that exceeded a threshold dF/F (Fig. 5A). The amplitude and frequency of these fluctuations were averaged for each region across groups at both acute and chronic sessions (Fig. 5B-C).

Figures

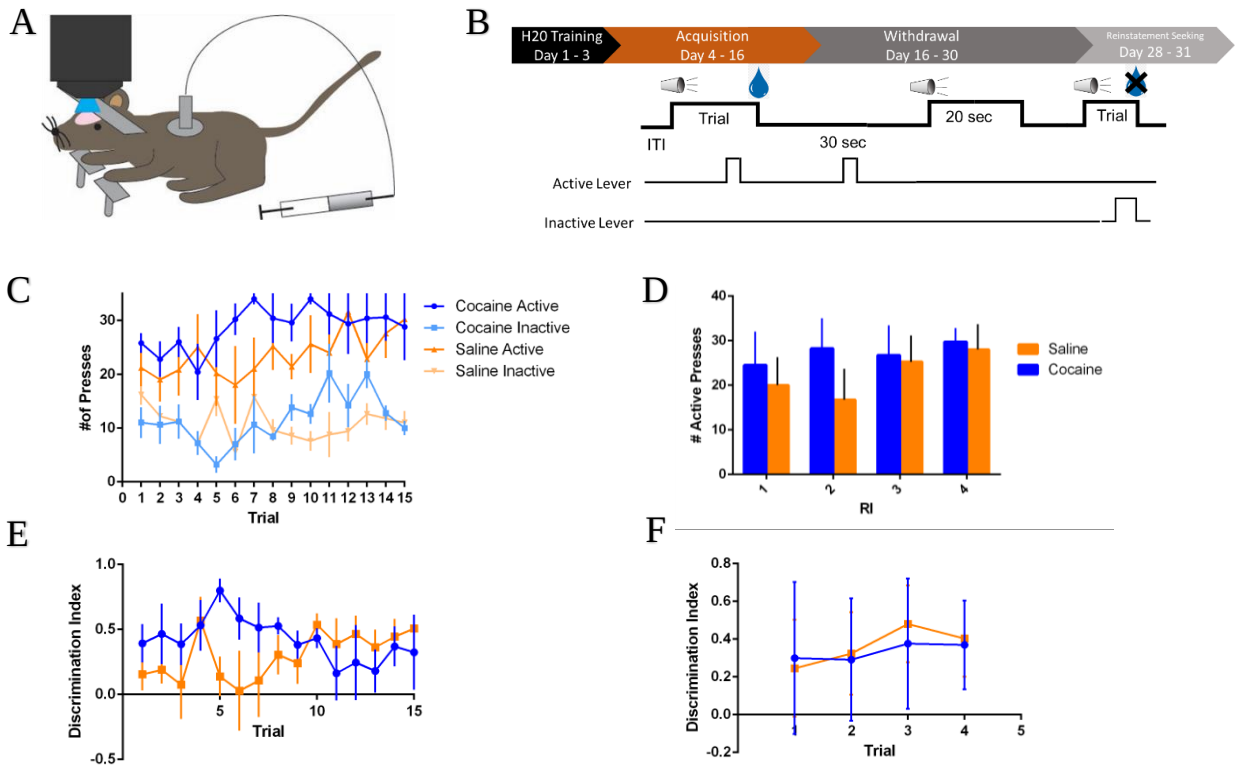


Figure 1. Designing a Head-Fixed Cocaine Self Administration Model. (A) Behavioral setup for the head-fixed cocaine self-administration task, which allows for simultaneous wide-field calcium imaging and cocaine self-administration. **(B)** Experimental schematic and trial structure for the self-administration task. Each session consisted of 90 trials, in which mice received a shot of cocaine (0.6 mg/kg) when pressing the active lever during the cue period. **(C)** Acquisition period data, in which the first 3 trials are H20 training (n=5 cocaine; n=5 saline). **(D)** Comparison of the cocaine- and saline-treated mice during the reinstatement seeking tests, measured in number of active presses. **(E-F)** Discrimination index during each session during the acquisition and reinstatement seeking test phases.

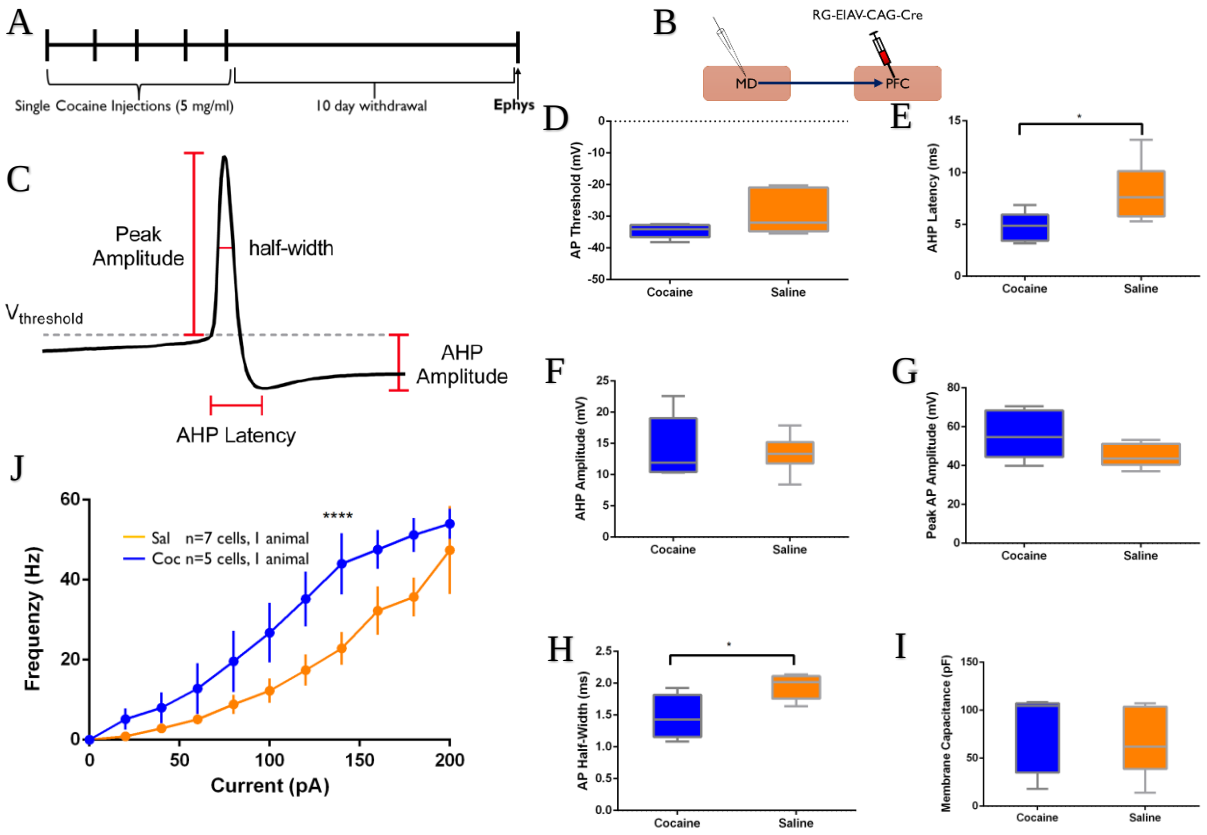


Figure 2. Chronic Cocaine Causes Hyperexcitability of MD→PFC Neurons. (A) Experimental timeline for chronic cocaine electrophysiology experiments. **(B)** Schematic for labeling MD→PFC neurons in acute slices. **(C)** Schematic showing how action potential (a.p.) properties were quantified. All measurements were conducted in the presence of NBQX and picrotoxin. **(D-I)** Action potential threshold (D), After-Hyperpolarization (AHP) latency (E, $p < 0.05$), AHP Amplitude (F), Peak action potential amplitude (G), Action potential half-width (H, $p < 0.05$), and membrane capacitance (I). **(J)** Spikes produced in MD→PFC neurons as a result of current injected. $p < 0.0001$; (n = 7,5 cells from 1,1 animal for saline- and cocaine-treated mice). Cocaine- and saline-treated mice were significantly different. Two-way ANOVA with Sidak test for multiple comparisons. All data presented as mean ± SEM. *$p < 0.05$, **$p<0.01$, ***$p<0.001$. All data was ran on an unpaired t test, except for (J).

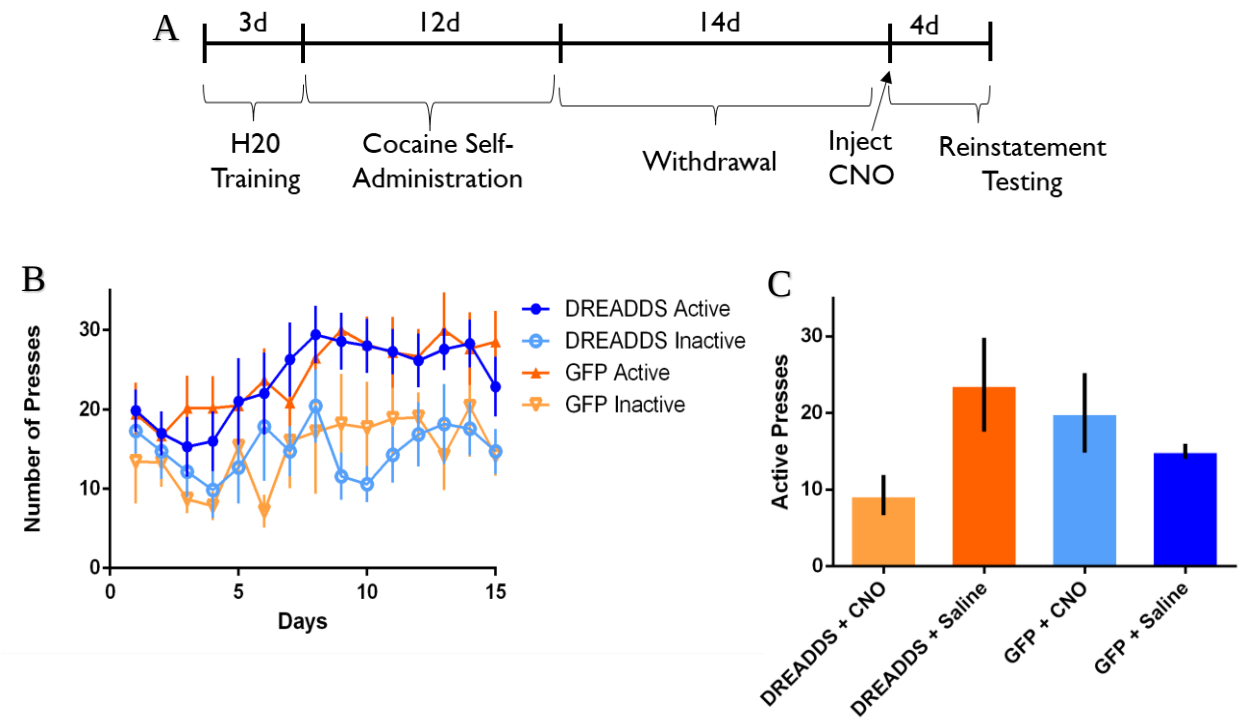
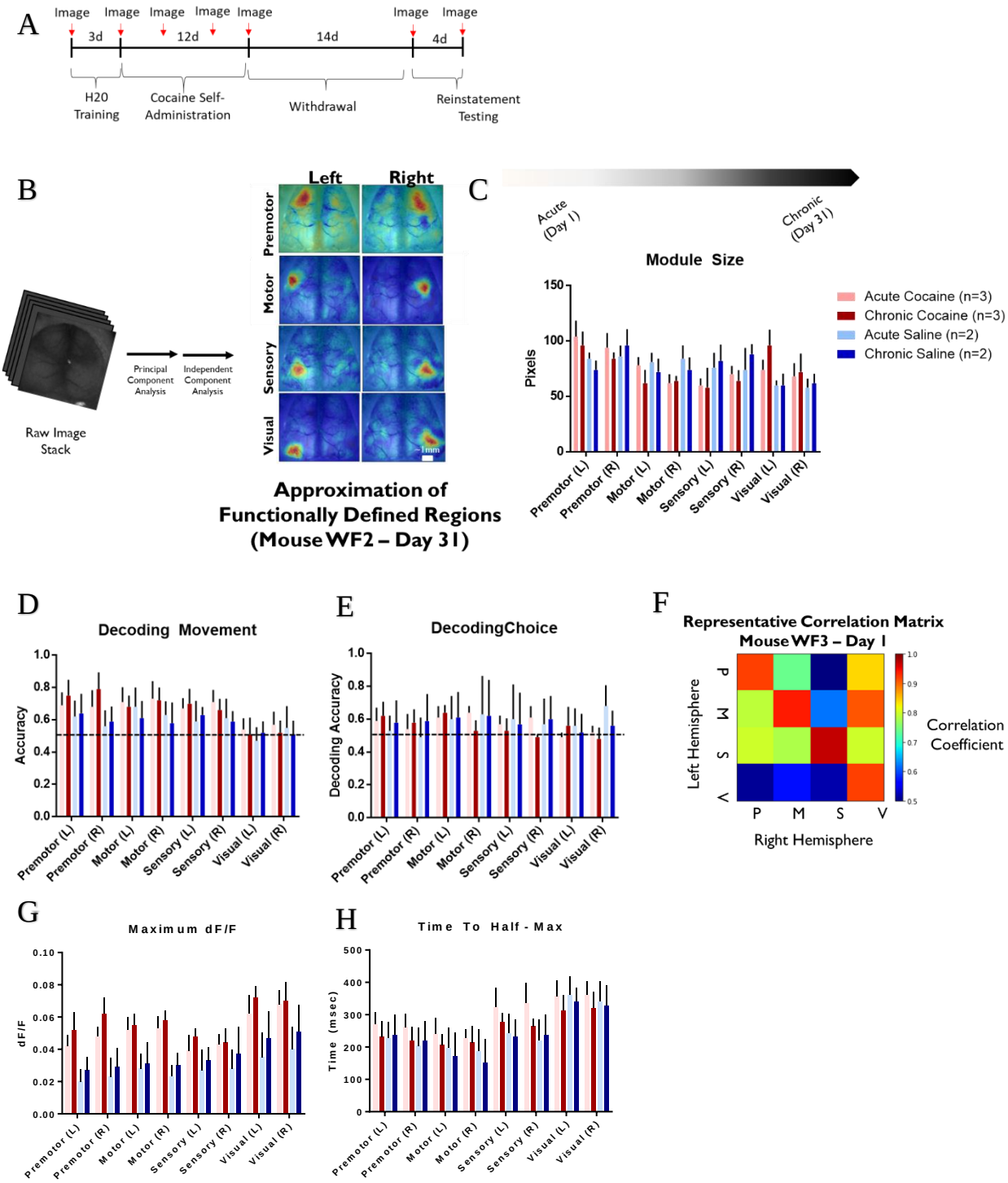


Figure 3. Pharmacological Inhibition of Mediodorsal Thalamus Neurons Attenuates Cocaine Seeking. (A) Experimental timeline for cocaine self-administration and pharmacological inhibition experiments. **(B)** Number of presses during each session of the H20 training (Days 1-3) and acquisition phase (Days 3-15). **(C)** Number of active presses during the first day of reinstatement seeking testing (Day 28). n= 7 DREADDS, n=6 GFP.

Figure 4. Behavior and Wide Field Calcium Imaging. (A) Experimental schematic of the wide-field calcium imaging during the cocaine self-administration behavioral experiment. **(B)** Schematic of how regions of interest were determined based on raw image stacks followed by Principal Component Analysis (PCA) followed by Independent Component Analysis (ICA). **(C)** Module sizes of the 8 modules for acute and chronic saline- or cocaine-treated mice. **(D)** Average accuracy of decoding movement, where an accuracy of 0.5 is chance. **(E)** Average accuracy in decoding choice, where an accuracy of 0.5 is chance. **(F)** Correlation matrix of an example mouse (mouse 3), of the left and right hemisphere regions. **(G)** Average max amplitude during movement trials, measured as dF/F, for each region of interest for cocaine- and saline-treated mice. **(H)** Average time to half of the maximum dF/F of movement trials at chronic and acute stages of cocaine or saline. n=3 cocaine, n=2 saline.


A
Image
Image
Image
Image
Image
Image
Image
3d
12d
14d
4d
H20 Training
Cocaine Self-Administration
Withdrawal
Reinstatement Testing
B
Left
Right
Premotor
Motor
Sensory
Visual
Raw Image Stack
Principal Component Analysis
Independent Component Analysis
Approximation of Functionally Defined Regions (Mouse WF2 – Day 31)
C
Acute (Day 1)
Chronic (Day 31)
Module Size
Pixels
Acute Cocaine (n=3)
Chronic Cocaine (n=3)
Acute Saline (n=2)
Chronic Saline (n=2)
Premotor (L)
Premotor (R)
Motor (L)
Motor (R)
Sensory (L)
Sensory (R)
Visual (L)
Visual (R)
D
Decoding Movement
Accuracy
E
DecodingChoice
Decoding Accuracy
F
Representative Correlation Matrix
Mouse WF3 – Day 1
Left Hemisphere
Right Hemisphere
Correlation Coefficient
G
Maximum dF/F
dF/F
H
Time To Half-Max
Time (msec)

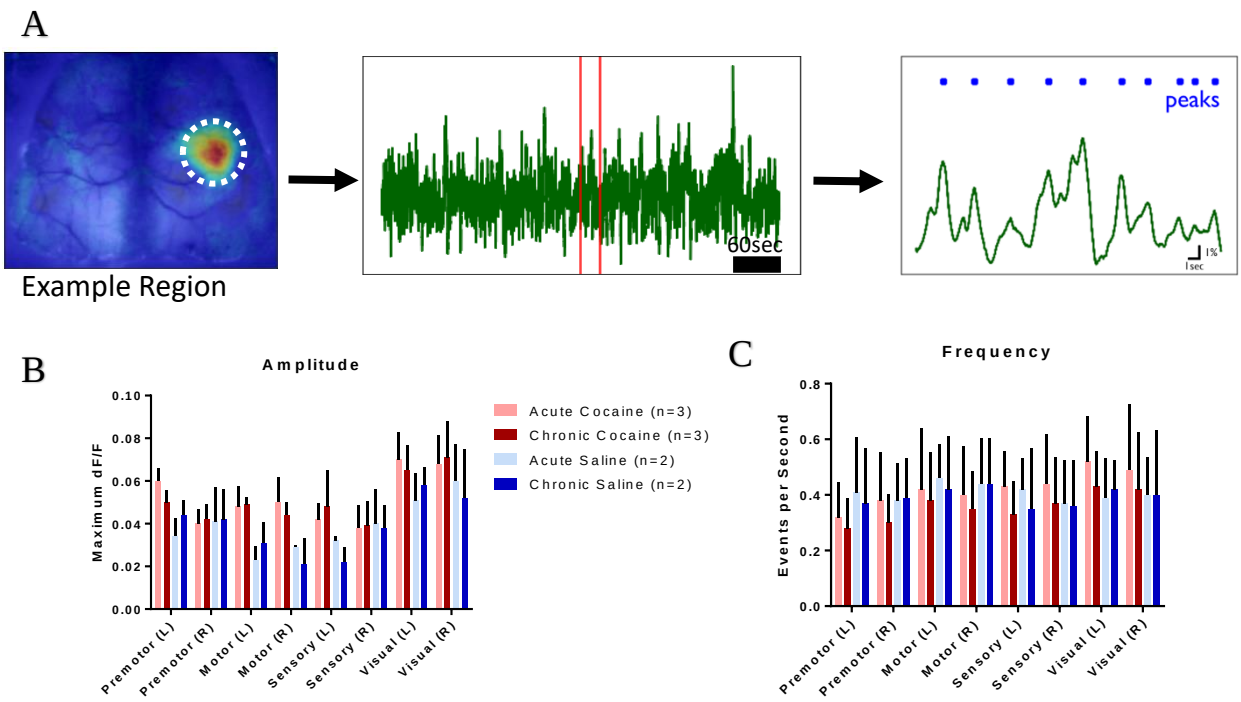


Figure 5. Spontaneous Fluctuations of Cortical Populations. (A) Diagram showing how spontaneous fluctuations were monitored in individual regions of interest. Peaks were quantified above a threshold. **(B)** Average maximum dF/F of spontaneous fluctuations (trials in which mice did not move), at different time points for cocaine and saline mice. **(C)** Average frequency of spontaneous events in the 8 regions of interest at different time points. n=3 cocaine, n=2 saline.

Discussion

Establishing a Cocaine Self-Administration Model

A substance-use disorder is defined by excessive use of a substance that often results in cravings, tolerance, and use even in the presence of negative consequences (Hartney 2018). When studying specific drugs of abuse, like cocaine, it is necessary to recapitulate the key features of the addiction cycle. These include administration of the drug, feelings of reward, a withdrawal period, and cravings. In addition, the route of administration plays a large role when designing cocaine addiction behavioral paradigms. Lecca's group demonstrated that active administration of cocaine through a jugular vein catheter significantly increased the dopamine levels compared to passive administration of cocaine (Lecca et al., 2006). We decided to utilize the same active cocaine self-administration paradigm when designing our cocaine seeking behavioral setup.

Intravenous cocaine self-administration studies are widely used in the study of cocaine addiction, as the model most accurately recapitulates cocaine seeking behavior seen in addiction. We successfully adapted this model in designing our head-fixed cocaine self-administration behavioral paradigm, as evidenced by the mice's performance in the reinstatement seeking test (Fig. 1D). However, one caveat of our experiment was that the saline-treated mice performed at similar levels as the cocaine-treated mice. This could be due the fact that mice found the saline somewhat rewarding or that the mice were simply learning to perform the task over time. The cue-induced trial structure was originally designed to allow for alignment of imaging to cue periods for wide-field calcium imaging, but future behavioral paradigms will need to look at a self-initiated task to better study drug seeking behavior. However, dissecting out the neuronal

populations and circuits that underlie this complex behavior was essential in studying how cocaine produces long-term seeking behaviors.

Dissecting Circuits Underlying Cocaine Seeking Behavior

Cocaine acts on the terminals of presynaptic dopaminergic neurons, where it blocks the reuptake of dopamine and causes increased dopamine signaling at the targets of these dopaminergic neurons. Thus, it is important to look to these targets when understanding how cocaine produces its long-term addictive properties. There are two main sites of dopamine in the brain, the ventral tegmental area (VTA) and the substantia nigra pars compact (SNr), which project to regions such as the nucleus accumbens (NAc), prefrontal cortex (PFC), and the ventral pallidum (VP). It is commonly believed that the NAc encodes the rewarding properties of cocaine, but it is still unclear which regions encode long-term cocaine seeking behavior (Kalivas et. al., 1993). The prefrontal cortex is a viable target for cocaine seeking behavior, as the VTA sends dopaminergic projections to this region. Studies have demonstrated that chronic cocaine self-administration causes hypoactivity in this region, and manipulations of this region were successful in attenuating seeking behavior (Chen et. al., 2013). In addition, other groups have used fMRI to demonstrate that the PFC and other cortical regions become altered following cocaine administration in humans (Breiter et. al., 1997). A paper from Martin-Iverson's group demonstrated that 6-hydroxydopamine lesions of VTA→PFC dopaminergic fibers failed to attenuate cocaine self-administration (Martin-Iverson et. al., 1986). Thus, we hypothesized that the VTA modulates the PFC through an alternate pathway, such as through the ventral pallidum or mediodorsal thalamus.

Current studies in the Lim Laboratory are looking at the role of VP Drd3+ receptors in mediating cocaine seeking behaviors. There is convincing behavioral, anatomical, and

electrophysiological data suggesting that D3 signaling in the VP are mediating cocaine seeking. In addition, anatomical tracing has revealed a large population of neurons projection from the VP to MD and a population of neurons projection from the MD to PFC. Based on this evidence, we decided to look at the mediodorsal thalamus's role in mediating cocaine seeking behavior, as it is the "missing link" in the VTA→PFC indirect pathway. Using patch-clamp electrophysiology, we were able to study how chronic cocaine modulates the electrophysiological properties of the MD→PFC neurons. Consistent with our hypothesis, these neurons became hyperexcitable following chronic cocaine and not saline. Although the action potential properties and firing frequency were altered, we do not know whether changes in excitability of the MD neurons are due to intrinsic properties or synaptic inputs. Further electrophysiological tests are needed to fully understand how MD→PFC neurons are modulated following chronic cocaine, such as looking at the E/I ratio and postsynaptic currents.

Rescuing Cocaine Seeking Behaviors

Currently, there are no readily available treatments for cocaine addiction, but new technology has brought the field closer to a treatment. The rise of deep brain stimulation (DBS) has been applied to a variety of neuropsychiatric disorders, including cocaine dependence. A recent study demonstrated that deep transcranial magnetic stimulation (dTMS) of the dorsolateral prefrontal cortex (DLPC) was able to reduce craving in cocaine use disorder patients, but definitive conclusions could not be made based on the sample size. Several other groups have also demonstrated that transcranial magnetic stimulation, deep brain stimulation, and other brain stimulation techniques of the prefrontal cortex have showed promising results in reducing cocaine craving (Rachid 2018). Further tests are still needed, as the safety of the approach is in

question and further circuit-based approaches are being researched. We decided to utilize a circuit-based approach to chemogenetically rescue cocaine-seeking behavior.

Using designer drugs specifically activated by designer drugs (DREADDs), we were able to chemogenetically inhibit mediodorsal thalamus neurons at a specific timepoint. The DREADDs system works by encoding a designer receptor in a specific anatomical region, such as the MD, where it will couple with a G-protein (Gi). Upon administration of Clozapine-N-oxide (CNO), the drug will bind to the designer receptor and activate the Gi-couple pathway to inhibit the neurons where it is expressed. By inhibiting the MD neurons before reinstatement seeking tests, we were able to prevent cocaine seeking behavior as evidenced by their lack of pressing during the seeking tests. These results were surprising, given the fact that electrophysiology data demonstrated that MD→PFC neurons become hyperexcitable following chronic cocaine use. Previous studies demonstrated hypoexcitability of PFC neurons, so MD neurons may be synapsing onto inhibitory neurons in the PFC. In fact, anatomical studies have demonstrated that the MD exerts strong influence on PFC pyramidal cells by way of parvalbumin (PV) inhibitory interneurons (Rotaru et al., 2005). Thus, MD neurons may be driving cocaine-induced PFC hypoexcitability through these PV cells in the PFC. Projection-specific and cell-type specific DREADDs manipulations are still needed to understand the role of MD→PFC neurons in mediating cocaine seeking behavior. This circuit-specific chemogenetic inhibition could serve as a safe and effective way of combating cocaine addiction.

Cortex-Wide Calcium Imaging During Cocaine Seeking

The prefrontal cortex has been shown to project to various regions throughout the cortex (Barbas and Zikopoulos 2007). We wanted to understand how these outputs are modulated following chronic cocaine use, as it has been shown that the PFC becomes modulated following

this treatment. To do this, we employed wide-field calcium imaging to simultaneously record the fluorescent activity of all neocortical populations. This strategy has been widely used (Allen et al., 2017, Chen et al., 2017, Gilad et al., 2018, Makino et al., 2017) to understand how cortical dynamics are modulated following motor learning, short term memory, and other behavior tasks. Thy1-GCaMP6s mice express GCaMP6s in mostly excitatory neurons. GCaMP6s is a genetically encoded calcium indicator (GECI), consists of calmodulin (CaM), a peptide sequence, and an EGFP molecule. In the presence of calcium, GCaMP isomerizes and fluoresces green. We adapted this imaging strategy to understand how various cortical populations are modulated following different stages of cocaine addiction.

Mice were imaged for 20 minutes per session at various timepoints throughout the cocaine self-administration behavioral paradigm. Problems arose in the middle sessions, as noise prevented proper acquisition of imaging sessions. The microscope setup was adjusted to allow for a higher signal: noise ratio. Thus, we were forced to only look at the first and last day of imaging, characterized as acute and chronic days. Principal component analysis followed by independent component analysis allowed us to define functionally and anatomically distinct regions of interest based on individual pixel activity. We defined 4 regions on both the left and right hemisphere, which included the sensory, motor, visual, and premotor areas. However, these cortical populations also consist of subregions that were not segregated in our study as a result of the poor noise with our initial microscope setup. Future studies will need to utilize the new microscope setup to define further subregions, as various subregions have distinct functionality.

One con of wide-field calcium imaging is the lack of single cell resolution, so it is difficult to understand what information is encoded in the fluorescent activity. To get around this problem, we ran the fluorescent activity through a machine learning decoding algorithm that

could distinguish what kind of activity was encoded in each region of interest. Interestingly, we found that fluorescent activity in the regions decoded movement activity but was unable to distinguish choice-related activity. Additionally, when running a correlation matrix between the different regions of an example mouse (mouse 3), we found that there were greatest correlations between corresponding regions of the left/right side. This is in line with our decoding choice data, as the left and right hemispheres seem to be encoding the same information. We hypothesized that there were two possibilities for this finding: 1) Mice engaged both sides of their body when deciding on a single left or right choice and thus, the activity in the hemispheres could not distinguish left from right choice. 2) The two hemispheres demonstrate a mirror effect in their activity, so both hemispheres are activated when making either a left or right choice. One way to get around the first possibility would be to modify the head-fixed setup to give the animals more postural support so that they can better isolate which paw to engage when making a choice.

Next, we wanted to understand how activity during movement trials was modulated at different timepoints of the addiction process and in different regions of interest. We found that there was a trend towards cocaine-treated mice showing increased fluorescent activity across the all regions, but it is difficult to make conclusions based on the sample size. If in fact there is an increase in fluorescent activity in these cortical populations, then it is likely that chronic cocaine increases activity of these populations during trials in which mice go to make a choice. Future studies will need to delve deeper into these individual populations to fully understand their role in encoding chronic cocaine seeking behaviors. Finally, we looked at time to half-maximum of the dF/F but did not see any significant differences between regions or groups.

Increases in fluorescent activity in a certain region could be due to several different reasons, such as an increase in the number of neurons recruited to a certain region or an increase in the amplitude of specific neurons in a certain region. To get around this discrepancy, we decided to look at endogenous activity. In trials in which the mice did not move, or go to make a choice, we looked at spontaneous fluctuations in their fluorescent activity. In general, the cocaine-treated mice showed an increase in the maximum amplitude of their spontaneous peaks, but it is also difficult to make conclusions based on the sample size. The frequency of events stayed relative constant among groups and regions of interest. This information may tell us that chronic cocaine alters the intrinsic firing properties of different cortical regions. Future studies will need to investigate specific regions at a single-cell resolution, such as with two-photon microscopy, to understand how individual neurons are modulated throughout the addiction process.

In summary, we have developed a novel head-fixed cocaine self-administration behavioral paradigm that allowed us to study how the inputs and outputs of the prefrontal cortex are modulated following chronic cocaine abuse. When looking at the inputs to the prefrontal cortex, we have uncovered an indirect pathway from the VTA to PFC that may be mediating cocaine seeking behavior. In particular, PFC-projecting MD neurons become hyperexcitable following chronic cocaine treatment, and chemogenetic inhibition of these neurons was able to attenuate cocaine seeking. When looking at the outputs of the prefrontal cortex, several neocortical populations show dynamic changes following chronic cocaine treatment. In line with recent literature, the inputs and outputs of the prefrontal cortex could serve as a potential site of cocaine addiction therapeutics.

References

Ariel Gilad, Yasir Gallero-Salas, Dominik Groos, Fritjof Helmchen. Behavioral Strategy Determines Frontal or Posterior Location of Short-Term Memory in Neocortex. *Neuron*, Volume 99, Issue 4 (2018). Pages 814-828.e7.

Barbas H, Zikopoulos B. The prefrontal cortex and flexible behavior. *Neuroscientist*. 2007;13(5):532-45.

Breiter HC, Gasic GP, Makris N. Imaging the neural systems for motivated behavior and their dysfunction in neuropsychiatric illness. In: Deisboeck TS, Kresh JY, editors. *Complex Systems Science in Biomedicine*. Springer Verlag; 2006. pp. 763–810.

Chen BT, Yau HJ, Hatch C, Kusumoto-Yoshida I, Cho SL, Hopf FW, Bonci A. Rescuing cocaine-induced prefrontal cortex hypoactivity prevents compulsive cocaine seeking. *Nature*. 496, 359-62 (2013).

Fady Rachid. Neurostimulation techniques in the treatment of cocaine dependence: A review of the literature. *Addictive Behaviors.* Volume 76, 2018. 145-155. ISSN 0306-4603. https://doi.org/10.1016/j.addbeh.2017.08.004.

H. Makino, C. Ren, H. Liu, A.N. Kim, N. Kondapaneni, X. Liu, D. Kuzum, T. KomiyamaTransf ormation of cortex-wide emergent properties during motor learning. *Neuron*, 94 (2017), pp. 880-890.e8.

Hans C Breiter, Randy L Gollub, Robert M Weisskoff, David N Kennedy, Nikos Makris, Joshua D Berke, Julie M Goodman, Howard L Kantor, David R Gastfriend, Jonn P Riorden, R.Thomas Mathew, Bruce R Rosen, Steven E Hyman,Acute Effects of Cocaine on. Human Brain Activity and Emotion. *Neuron*. Volume 19, Issue 3. (1997) 591-611. https://doi.org/10.1016/S0896-6273(00)80374-8.

Hartney, E. A Guide to DSM 5 Criteria for Substance Use Disorders. *Verywell Mind.* 2019 January 29. https://www.verywellmind.com/dsm-5-criteria-for-substance-use-disorders-21926.

Hummel, M. and Unterwald, E. M. (2002), D_1 dopamine receptor: A putative neurochemical and behavioral link to cocaine action. *J. Cell. Physiol*., 191: 17-27. doi:10.1002/jcp.10078.

Kalivas, P.W. & Duffy, P. (1993) Time course of extracellular dopamine and behavioral sensitization to cocaine. I. Dopamine axon terminals. *J. Neurosci.*, 13, 266–275.

Kmiotek EK, Baimel C, Gill KJ. Methods for intravenous self administration in a mouse model. *J Vis Exp*. 2012;(70):e3739. Published 2012 Dec 8. doi:10.3791/3739

Lecca, D., Cacciapaglia, F., Valentini, V. Differential neurochemical and behavioral adaptation to cocaine after response contingent and noncontingent exposure in the rat. *Psychopharmacology* (2007) 191: 653. https://doi.org/10.1007/s00213-006-0496-y

Mark, G.P., Hajnal, A., Kinney, A.E. & Keys, A.S. (1999) Self-administration of cocaine increases the release of acetylcholine to a greater extent than response-independent cocaine in the nucleus accumbens of rats. *Psychopharmacology (Berl.)*, 143, 47–53.

Martin-Iverson, M.T., Szostak, C. & Fibiger, H.C. *Psychopharmacology* (1986) 88: 310. https://doi.org/10.1007/BF00180830

NIDA. (2016, May 6). Cocaine. Retrieved from https://www.drugabuse.gov/publications/research-reports/cocaine on 2019, January 7

D.C. Rotaru, G. Barrionuevo, S.R. Sesack. Mediodorsal thalamic afferents to layer III of the rat prefrontal cortex: Synaptic relationships to subclasses of interneurons. *J Comp Neurol*, 490 (2005), pp. 220-238

T.-W. Chen, N. Li, K. Daie, K. Svoboda. A map of anticipatory activity in mouse motor cortex. *Neuron*, 94 (2017), pp. 866-879.e4

W.E. Allen, I.V. Kauvar, M.Z. Chen, E.B. Richman, S.J. Yang, K. Chan, V. Gradinaru, B.E. Deverman, L. Luo, K. Deisseroth. Global representations of goal-directed behavior in distinct cell types of mouse neocortex. *Neuron*, 94 (2017), pp. 891-907.e6.

W.E. Allen, I.V. Kauvar, M.Z. Chen, E.B. Richman, S.J. Yang, K. Chan, V.Gradinaru, B.E. Dev erman, L. Luo, K. Deisseroth. Global representations of goal-directed behavior in distinct cell types of mouse neocortex. *Neuron*, 94 (2017), pp. 891-907.e6.

Weissenborn, R., Whitelaw, R., Robbins, T. Excitotoxic lesions of the mediodorsal thalamic nucleus attenuate intravenous cocaine self-administration. *Psychopharmacology* (1998) 140: 225. https://doi.org/10.1007/s002130050761