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Role of Perirhinal to Prefrontal Cortex Reciprocal Connection in Methamphetamine-Induced Memory Deficits

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by

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Department of Neuroscience

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Dedication:

To David and Jonathan, who never think I'm less than great.

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Key of Abbreviations

Abbreviation	Term
SUD	Substance Use Disorder
MUD	Methamphetamine Use Disorder
PFC	Prefrontal Cortex
BLA	Basolateral Amygdala
vSUB	Ventral Subiculum
PVT	Paraventricular nucleus of the Thalamus
NOR	Novel Object Recognition Task
DSM-V	Diagnostic and Statistical Manual of Mental Disorders
GFP	Green Fluorescent Protein
mGluR5	Metabotropic glutamate receptor 5
SA	Selfadministration
CNO	Clozapine-N-Oxide
PL	Prelimbic Region of Prefrontal Cortex
IL	Infralimbic Regions of the Prefrontal Cortex
PRH	Perirhinal Cortex

Abstract:

Chronic use of methamphetamine (meth) disrupts cortical processing across multiple cognitive domains including impulsivity, decision making and memory. Our laboratory has consistently shown that extended access to contingent meth self-administration reliably produces memory deficits in novel object recognition (NOR) tasks designed to test the “what” component of episodic memory in a rodent model. This type of memory is dependent on an intact function of the perirhinal cortex (PRH). However, the ongoing role of the prefrontal cortex (PFC) in this task and the directionality of communication between the PRH and the PFC is not entirely understood. A set of four experiments were designed to characterize the bi-directional connection between the PFC and the PRH during the exploration of novel vs. familiar objects and whether the manipulation of the circuit will restore recognition memory following chronic meth self-administration (SA). Male and female rats were infused with retrograde GFP-tagged adeno-associated virus (AAV) in the PRH (Experiment 1) and the PFC (Experiment 2). Three weeks later rats were tested for NOR with half the animals exploring two familiar objects and half exploring a novel object. Brain tissue was processed for co-labeled cells containing both GFP and c-Fos, an indicator of neuronal activation. Rats spent more time exploring novel vs. familiar objects. During novel object exploration, animals that explored novel objects had more co-labeled cells that project from the PRH to the PFC, but not in cells that project from PFC to the PRH. A dual viral approach was utilized in the second set of

experiments to activate the PRH to PFC pathway following meth SA (Experiment 3) or inhibit the PRH to PFC pathway in meth naïve animals (Experiment 4). AAV viral vectors containing CRE-dependent Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) were infused into the PRH while retrograde AAV encoding CRE recombinase was infused into the PFC. A recognition memory deficit was established in meth SA rats and restored through activation of excitatory DREADDS (Experiment 3). In meth naïve animals a NOR memory deficit was also induced by activation of inhibitory DREADDS within the same circuit (Experiment 4). In conclusion, this data set suggests communication from the PRH to the PFC directs novel object recognition.

Chapter 1: Introduction

The use of psychoactive substances is as old as human civilizations. Such substances have been integral parts of religious ceremonies, medicinal treatments, and used by members of the population at large since prehistoric times (1). As society has changed over millennia so has the relationship humans have with these substances. The current concept of addiction as it is known today was first described as early as the 17th century. According to current pathological definitions, outlined in the DSM-V, substance use disorder is classified as the “chronically relapsing, compulsive pattern of drug-taking” and can vary in severity from mild to severe (2). Addiction can be thought of as a cyclic pattern of thinking and behavior and can be further broken down into stages (3). The three stages of the addiction cycle include an initial binge/intoxication stage, followed by withdrawal/negative affect, where unpleasant symptoms drive the user to the third stage of preoccupation/anticipation or drug craving (3). Continual cycling can lead to a substance use disorder (SUD) and can result in significant changes in the brain.

Section 1: Methamphetamine Use Disorder (MUD)

Methamphetamine, a psychostimulant, was synthesized from ephedrine by a Japanese pharmacologist in the late 19th century. Since that time, the population’s relationship with meth has been complicated. As our understanding of action in the body and abuse potential has changed, so has the legality and

availability of the drug (4). Meth is predominantly metabolized in the liver and excreted in urine. Depending on route of administration and individual variation, meth can be found in the body 8-24 hours after use (5). Chronic use has not been found to alter metabolism, thus increasing dosages among users is likely due to changes in the brain. Since 2015, the number of users and overdoses has increased worldwide. Driven by its' relatively low cost and high purity levels, the market for meth use has grown significantly in Asian countries with steady use throughout North America (6,7). Clinical characteristics of MUD include altered sleep patterns, paranoia, hallucinations, distractibility, motor hyperactivity, psychosis, and cardiovascular problems (8). Indeed, long term use can lead to cardio and neurovascular problems that often lead to premature death from strokes (8). As a result, chronic meth use is associated with various individual and societal burdens including health care, economic strains, and homelessness. Meth dependence is further complicated by its' high relapse rates following sustained periods of abstinence (8).

Section 2: Effects of Chronic Meth Use on the Brain

Meth diffuses across the blood-brain barrier to enter the brain where it disrupts the monoamine system, including dopamine, serotonin, and norepinephrine neurotransmitter systems (9). Once inside neurons, this drug is responsible for altering the release and production of monoamine molecules via multiple mechanisms. Meth reverses monoamine transporters in the cell membrane that are responsible for the removal of these molecules from the synaptic cleft. Meth

also reverses monoamine-containing vesicular transporters to stimulate extensive premature monoamine release into the cytosol and synaptic cleft. Meth can further interrupt the balance of monoamine metabolism and catabolism by decreasing degradation proteins and increasing precursor molecules. Together these changes result in an abundance of monoamine molecules in the synaptic cleft and little within the cell and an impaired means of correcting the depletion (10). The binding of vesicles to the cell membrane can also lead to the premature or continual release of glutamate, the primary excitatory molecule in the brain, into the synaptic cleft. When glutamate binds its receptors on the post-synaptic cell, changes in Ca++, a secondary messenger, occur within the cell (11). This stimulates downstream processes in the cell that can lead to stress on the endoplasmic reticulum, the organelle responsible for the proper folding of proteins necessary for normal cellular function. Combined these effects can lead to excitotoxicity in the cell (12). Chronic methamphetamine use can induce other types of stress in the cells of the brain as well. Reactive oxygen species are common byproducts of dopamine auto-oxidation, which occurs as a result of an overabundance of dopamine in the synaptic cleft and can lead to oxidative stress (14). Meth can bind directly to microglial cells producing a rapid response and release of cytokines from in the brain as a response. An abundance of cytokines in the brain can lead to detrimental neuroinflammation (15,16). Such cellular changes can have global impacts on how the brain functions.

Section 3: Memory Deficits Associated with MUD in Humans

Whereas small doses of stimulants have positive effects on human concentration and alertness, large quantities resulting from chronic use can be detrimental to the brain (17). Circuitry responsible for drug addiction overlaps with some limbic and cortical regions involved in learning and memory. As a result of drug-related changes, deficits in learning and memory have been shown in meth users and understanding these deficits has become important for the overall treatment of meth users. Clinical studies have shown that meth users show signs of cognitive dysfunction in impulsivity, attention, as well as episodic and working memory following a period of drug abstinence (18). Compared to healthy controls, participants with methamphetamine use performed worse on tasks of attention, episodic and spatial memory (19-21). Specifically, patients that had recently relapsed following a period of drug abstinence performed more poorly than even consistent users or abstinent users suggesting a crucial timepoint for therapies (20, 21). Furthermore, meth users have shown deficiencies in metamemory or the ability to know and understand memory capacity (23-25). Memory retention inability impacts the ability of abstinent meth users to maintain daily functioning behavior outside of drug use (24). As cognitive deficits have become more understood, cognitive training has become a viable therapeutic strategy in humans with methamphetamine addiction (23-26). The deficits in executive function and memory are likely contributing factors to the high relapse potential

seen in chronic users of meth and could therefore be potential targets for new behavioral or pharmacological therapies.

Section 4: Brain Regions involved in Episodic Memory

Understanding the brain regions involved in different neuronal processes is essential to how we interpret when things have changed from baseline. The neuronal substrate for memory has, for many years, been attributed to hippocampal function (27). Anatomically the hippocampus is connected to several other brain regions. These regions include several cortical areas such as the prefrontal cortex, a portion of the frontal cortex that is responsible for higher order thinking and executive function broadening our understanding of memory (28). Other cortical regions include areas known collectively as the parahippocampal regions and include the entorhinal cortex and perirhinal cortex (29,30).

Memory can be split into two categories, declarative and non-declarative. Non-declarative memory includes information that is not consciously recalled, such as emotional responses and habits, whereas declarative memory is recalled consciously and includes facts and events (29). Episodic memory is a form of declarative memory that encodes environmental and situational information of a certain event. This type of memory is the “what, when, and where” of an event (29).

Many studies have been conducted to determine exactly which brain regions contribute to such memories in rodent models (31-36). Different behavioral paradigms can be used to mimic events in a controlled setting. Rodent tasks developed specifically to test single components of episodic memory are object recognition (what), object in place (where), temporal order (when) (37). During each task there is a sampling phase where the animal is allowed to explore the environment with given objects in specific arrangements, two objects in object recognition and temporal order and four objects in objects in place. For temporal order only there is a second sampling phase with two new objects. Following a period of memory retention, the animals are exposed to a test period where a specific component of the task as been changed. For object recognition (Schematic 1A), one of the objects as been replaced with a new object. The order in which the animals saw the objects has been changed during test period of temporal order (Schematic 1B), and finally, the location of two objects have been switched during the testing phase of object in place (Schematic 1C). The time the animal spends exploring the different object is considered the recognition of that change in environment. Task descriptions can be found in Schematic 1. Another task used to assess episodic memory is spatial location. This task can involve allowing the animal to swim in a pool of water until they find the platform on which they can escape and measures latency to find that platform in subsequent trials. This task can also be performed using changes in object location as well.

Using these methods, studies have been conducted using localized lesions to damage specific brain areas to determine if these regions contribute to the behavior being investigated. Historically, these studies focused on three key areas in the brain, the hippocampus, the perirhinal cortex and the prefrontal cortex (29-33). Damage to different areas leads to different performances by the rodents in the varying tasks. These experiments demonstrated that all three areas investigated were involved in recalling the “where” in the object-in-place task and “when” of objects in temporal order task, but that only the perirhinal cortex was necessary for the “what”, that is the recall of objects themselves (34-35). Though these studies helped to indicate the involved brain regions, they do not offer clues about how the different regions are communicating with one another. Later studies, using optogenetic methods where special light-activated channels control neuronal activation, were able to determine that glutamatergic projections from the prefrontal cortex impact a rodent’s performance on an object task but not object recognition (36). This study suggests that PFC-->PRH circuit is not responsible for object recognition. Such findings prompt the need to expand our knowledge about the circuitry underlying these types of memory.

Section 5: Methamphetamine, Episodic Memory and the Perirhinal Cortex

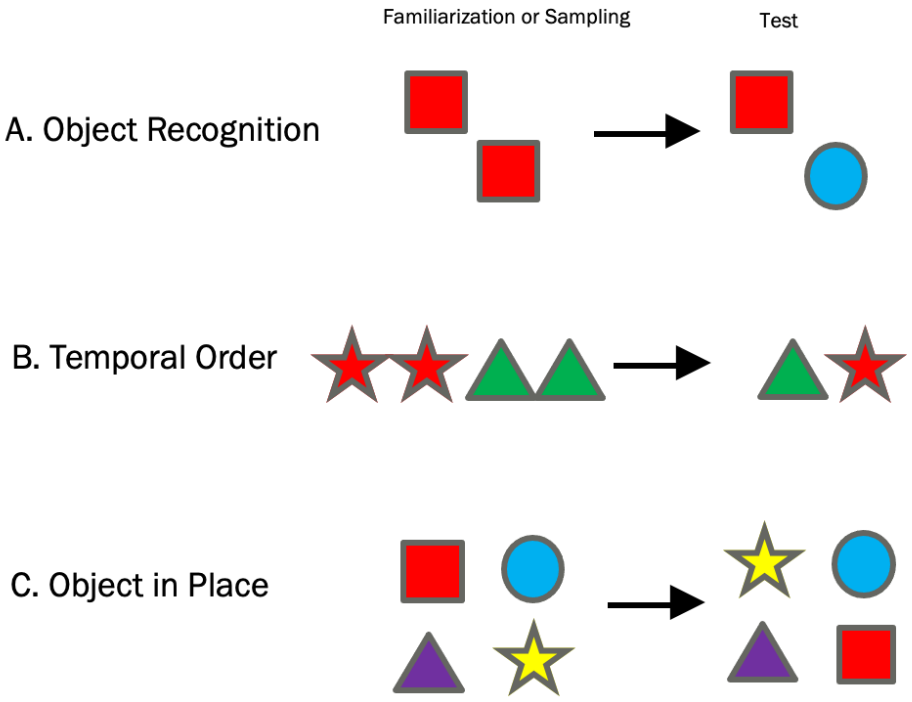
Rodents in models of methamphetamine addiction have shown deficits in attention, impulsivity and episodic memory similar to what is seen in humans (38). Following neurotoxic regimens of methamphetamine administration, rats have been found to have decreases in recognition memory performance on

object recognition as well as object in place tasks but not in spatial location tasks (39-43). Long access to meth-induced changes in object recognition memory while short access meth self-administration did not (39). These investigations showed behavioral and biochemical changes as a result of non-contingent and binge doses of meth given to rats. Specifically, changes in monoamine transporters were found in the PRH and HPC of animals that performed poorly on object recognition memory and spatial memory tasks, respectively (40).

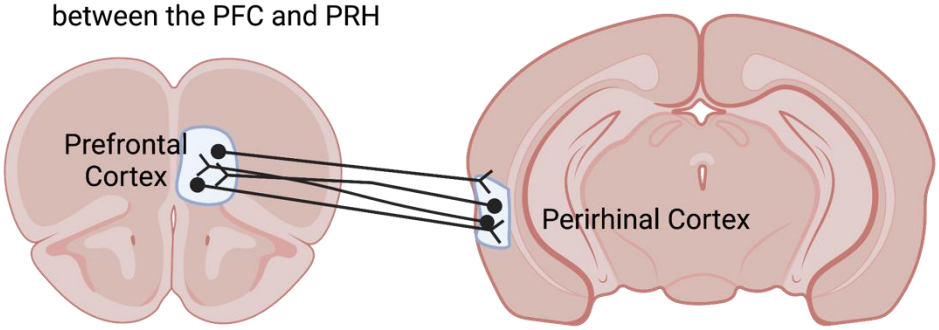
Such studies have directed work from the Reichel lab. The PRH has been shown to be dysregulated following chronic methamphetamine in both a rodent long access model as well as a binge non-contingent model of drug taking (41-44). Long-access meth has been shown to decrease the natural proclivity of a rodent to explore objects that are novel (44,45). Previous work from the Reichel Lab has shown that chronic methamphetamine can lead to downregulation of metabotropic glutamate receptor 5 expression(mGluR5) (45) and disruption of long-term depression specifically in this region (46). Such meth-induced deficits were restored by direct infusion of a mGluR5 positive allosteric modulator into the PRH (45). Anatomically the PRH is connected to many other regions that have been dysregulated in MUD including another region in the memory and addiction circuitry, the PFC. The PFC has been shown to be a key player in addiction biology (47) and the reciprocal connections in the rat PRH can be seen in Schematic 2 (30). As this interplay of addiction and memory occurs in both the PFC and the PRH it is important to determine if this circuitry plays a critical role in

object recognition behavior. **I hypothesize that it is an intact projection from the PRH to the PFC that is essential for object recognition and that this pathway is damaged by chronic methamphetamine use.** To determine the involvement of the PFC and PRH during the task, the reciprocal connections will be characterized following the task and the circuitry from PRH to PFC will be manipulated during the NOR task using dual viral approach to activate or inhibit the pathway.

Schematic 1: Pictorial Descriptions of Episodic Memory Tasks



Schematic 2: Anatomical projections between the PFC and PRH



Chapter 2: Methods

Subjects: Age-matched male (250-275g) and female (225-250g) Sprague Dawley rats (Envigo, Indianapolis, IN, USA) were used in the following experiments.

Viral Infusions: Animals were sedated at an appropriate anesthetic plane for intracranial infusions using a Kopf digital stereotax. For experiments 1 and 2, AAVrg-hSyn-HI-enhanced green fluorescent protein (eGFP)-Cre-WPRE-SV40 [71012vg/ml; Addgene#105540]) was infused into the PRH and PFC, respectively. A dual viral approach was utilized in experiments 3 and 4, where the viral construct above was infused into the PFC while Cre-dependent DREADDs (Gq-DREADD: AAV2-hSyn-DIO-hM3D(Gq)-mCherry Addgene #44361 or Gi-DREADD: AAV2-hSyn-DIO-hM4D(Gi)-mCherry Addgene #44362) were infused into the PRH. Viral infusions were completed using a Nanoinject II at a volume of 50.6 nl per injection every 30 s at a rate of 23 nl/second for a total of 0.5 ul per hemisphere followed by an additional 5 min to allow the12njectted virus to diffuse prior to removal of the pipette. Stereotaxic coordinates for all intracranial surgeries are as follows, PFC: AP+2.8 mm; ML, ± 0.64 mm; DV –4.5 mm and PRH: AP –4.8mm; ML +5.0mm; DV –7.5mm at a -10° angle. Animals in experiment 1,2, and 4 all underwent a period of viral incubation for three weeks in the home cage. Animals in experiment 3 underwent methamphetamine self-administration following surgeries.

Novel Object Recognition: The NOR behavioral task was conducted using a large circular arena (100cm in diameter) with walls constructed of poster paper secured to the side of the arena via Velcro. Curtain walls were constructed surrounding the arena to block outside environmental distractions with four soft lights used within the curtained area. A camera with Ethovision software was suspended above the arena for analysis of tasks. The behavioral task included two days of habituation where each animal was allowed to explore only the arena for 10 minutes. On the third day, animals underwent a 5-minute period of sampling where they were exposed to two objects, either two curved PVC pipes, or two travel shampoo bottles. Animals were returned to the home cage following sampling for a retention period of 90 minutes, after which animals were returned to the arena with one familiar object and one novel object counterbalanced for placement. As a control in experiments 1 and 2, a subset of animals was only exposed to familiar objects and were never shown a novel object. Videos of sampling and test behavior were recorded with Ethovision software and manually scored for object interaction. Interactions with objects were designated by nose or front paw interactions. Animals climbing over the object were not scored as interacting with object. Recognition index (RI) of novel object was determined by the equation RI= Time spent with novel object/Total time with both objects. Animals in experiment 3 or 4 were injected with 10 mg of clozapine-N-oxide (CNO) or saline solution immediately after the sampling period.

Catheter Surgery and Methamphetamine Self-Administration: Animals in only experiment 3 underwent intravenous jugular catheterization for drug self-administration following intracranial surgeries. These surgeries included sedation at the same anesthetic plane as stereotaxic surgeries. Magnetic single port catheters (SAI Infusion Technologies) were fed subcutaneously from the back of the animal to the right jugular vein where they were secured into the vein by sutures. Following recovery, these animals were placed in drug self-administration chambers from MedPC and allowed to lever press for 6 hours per day for 18 days on a fixed ratio of 1, where 1 lever press equated to 1 infusion of meth at a dose of 0.4 mg/ml. Following SA, animals underwent a period of forced home cage abstinence for 7 days.

Immunohistochemistry: Ninety minutes following NOR test period, all animals were transcardially perfused, and brains were removed. Tissue was sliced on a cryostat at 40-50um and were subjected to immunohistochemistry protocols for amplification of GFP and c-FOS or GFP, c-FOS, and mCherry.

Microscopy and Analysis: Tissue was analyzed using both scanning laser confocal and epifluorescent microscopy. Cells of interest were determined by co-localization of GFP and c-FOS in Experiments 1 and 2 and mCherry and c-FOS in Experiments 3 and 4.

Chapter 3: Tracing Activated Pathways during Object Recognition Memory

Section 1: Introduction

Chronic methamphetamine use in humans has been shown to cause memory deficits, specifically in episodic memory. Episodic memory encodes the spatial and contextual details of a given event. The brain regions responsible for this type of memory have been found to be the triangulation of circuitry between the medial prefrontal cortex, the perirhinal cortex and the hippocampus (31-36). Such studies show the PRH to be the primary substrate in object recognition memory. Though these studies have provided the broad regional input in episodic memory, our knowledge base thus far lacks details pertaining to neuronal activation following a memory task and information about the directionality of such activation. In order to determine sources of neuronal input into the PRH and PFC, I utilized an established method of virally induced neuronal tracing followed by activation during a novel object recognition behavioral task. Neurons with terminals located in brain regions of interest were visualized as the virus used transduces at the axon terminal and is transported back to cell body where it is expressed. This strategy was used to identify projections into the PRH or PFC and allow for the understanding of which specific cells were activated during the task.

Section 2: Results

For experiment 1, a retrograde AAV viral construct with enhanced GFP-tagged CRE recombinase was infused into the PRH by stereotaxic surgery. Animals were subjected to the NOR paradigm after three weeks of viral incubation. During this task, animals were sorted into two groups as a control for neuronal activation from sensory and motor cortices. One group was not exposed to a novel object but rather two of the same objects, like in the sampling period. The second group underwent a typical NOR setup. Animals from each group explored both objects equally during sampling, also called familiarization, (Figure 1A, $t(32) = 0.2580$, $p = 0.7980$, unpaired t-test). In a two-way interaction analyzing the time each group spent with each object, animals that previously saw the same two objects spent the same amount of time with both objects whereas animals in the second group spent more time exploring a novel object than the same familiar object, Figure 2B. The overall two-way ANOVA was significant $F(1,15) = 7.077$, $p = 0.0178$. The main effect of object was significant $F(1,15) = 5.121$, $p = 0.0389$. The main effect of group was not significant $F(1,15) = 0.0348$, $p = 0.8545$. Post hoc Šídák multiple comparisons showed that the mean difference in time spent between objects was only significantly different in the novel group ($p = 0.0032$). In Figure 1C, the recognition index of each group is defined as object exploration above chance where the dotted line indicates interactions due to chance alone. Animals that saw a novel object interacted with the object more than by chance alone (Wilcoxon signed rank test, $t(7) = 5.712$, $p = 0.0007$) while the animals that

saw the same object did not (t (6) =0.3314, p=0.7516). Following the assay outlined in the methods, animals were euthanized 90 minutes following the behavior to ensure peak c-Fos expression (48). Native unamplified GFP was imaged along with c-Fos following immunohistochemistry for expression patterns in the PFC. Due to the anatomical nature of the projections outlined in Chapter 1 as well as possible differing actions of subregions, the PFC was further separated into the prelimbic (PL) and the infralimbic (IL) for investigation. Cell counts among the groups were all analyzed with two-tailed unpaired t-tests. GFP in this experiment was used as a marker of viral transduction and was used to normalize for viral transduction across groups. As such, the number of cells that were Fos+GFP+ was normalized as a percentage of the total number of GFP+ cells in the region.

In the PL of the PFC there were no differences in the number of c-fos expressing cells between animals that saw the familiar object and animals that saw the novel object (two tailed unpaired t test; t (14) =0.3089, p=0.7620, Figure 2A). There was no difference in the number of cells labeled with GFP between the groups (two tailed unpaired t-test; t (14) =1.750, p=0.1019, Figure 2B). There was found to be no difference in the number of cells with overlapping labeling of GFP and c-fos between the groups as seen in Figure 2C (two-tailed unpaired t-test, t (14) =1.386, p=0.1875). This profile persists when normalized to total GFP expression in the region, there were no differences in the percent c-fos+GFP+ cells (two tailed unpaired t test; t (14) =0.1151, p=0.9100, Figure 2D). Figure 2E shows

representative images of GFP and c-Fos-expressing cells from each group, the yellow arrow indicates a co-labeled cell. A similar pattern of GFP and c-fos expression was seen in the IL. In Figure 3A, c-fos expression was not found to be different between animals that saw the familiar objects or the novel objects (two-tailed unpaired t-tes, t (12) =0.6713, p=0.5148). The number of GFP-containing cells did not differ between the two groups (two-tailed t-test, t (14) =1.757, p=0.1008, Figure 3B). Unlike the PL, there was found to be a greater number of co-labeled Fos+GFP+ cells in the IL of the animals that saw the novel object versus those that did not (two-tailed t-test, t (13) =3.895, p=0.0018, Figure 3C). Once normalized, this effect was lost. There was no difference between control and novel groups (two-tailed t-test, t (14) =0.1703, p=0.8672, Figure 3D). Representative images of GFP and c-Fos expressing cells from each group in the IL are shown in Figure 3E.

The same method was utilized to trace and determine the input into the PFC during object recognition memory (Experiment 2). GFP-tagged retrograde AAV virus was infused into the PFC via stereotaxic surgery. The virus was allowed to incubate for at least 3 weeks. During familiarization or sampling, animals did not explore one object more than the other (Figure 4A, two-tailed unpaired t-test, t(26) =1.419, p=0.1678). The overall two-way ANOVA was not significant F (1,13) =2.227, p=0.1595. The main effect of object was not significant F (1,13) =3.033, p=0.1052. The main effect of group was significant F (1,13) =8.017 p=0.0142. However, when compared to chance exploration, rats that saw novel objects had

significantly higher preference ratios indicating a preference for the novel object , Figure 4C ($t(4) = 4.838$, $p=0.0084$, one-sample t and Wilcoxon test). Animals in this experiment were euthanized following NOR for c-Fos analysis in PRH. There was no difference in the number of c-Fos containing cells between animals in novel and control groups (Figure 5A, two-tailed unpaired t-test, $t(11) = 1.527$, $p=0.1550$). A significant difference in the number of GFP labeled cells was found, where there were more labeled cells in the control group (Figure 5B, $t(12) = 3.197$, $p=0.0077$) than in the test group. A significant difference between groups of co-labeled Fos+GFP+ cells was not found (two-tailed unpaired t-test, $t(11)=0.6270$, $p=0.5434$, Figure 5C), but when number of co-labeled cells was normalized to the total number of cells expressing GFP, a significantly greater number of co-labeled cells was found in animals that saw the novel object than in animals than saw the same objects (unpaired t-test, $t(11)=2.768$, $p=0.0183$, Figure 5D). Figure 5E shows the representative images of GFP and c-Fos expressing cells from the PRH.

Section 3: Discussion

Previous work has suggested that episodic memory is the result of the coordination of the PFC, HPC, and PRH regions of the brain. The behavioral profiles for the animals were as expected showing that the task used exploits a rat's natural proclivity for exploring new objects in an environment. The control group used in these studies also behaved as expected by investigating the same two familiar objects equally. Using a retrograde viral tracer to map the inputs in

the PRH and activation following the NOR behavior showed that receiving inputs in the PRH do not change when an animal is presented with a novel object versus an object that has already been seen. The difference in the number of Fos+GFP+ cells between animals that saw a novel object versus those that did not was significant in the IL but not in the PL of the PFC. A lack of difference between the groups suggests that circuitry from PFC to PRH does not direct NOR behavior. Cell counts for the opposite direction, PRH-->PFC, did show a difference between the two groups. Statistically, no differences were found between the two groups in the number of Fos cells or Fos+GFP+ cells. The difference between the groups is apparent after the normalization to total number of GFP cells. The results indicate a greater percentage of Fos+GFP+ cells in the PRH of the animals that saw the novel object compared to the control. This normalization step became more important as a result of the significant difference in the number of GFP+ cells with the control group expressing higher numbers. As the groups were randomly assigned after surgeries and surgeries were performed in a uniform manner across the cohort this is likely an experimental artifact. However, the difference in the groups following normalization suggests that the PRH-->PFC circuitry conducts the flow of information in this task. That the PRH is the central substrate for object recognition data is congruent with data from the lesion studies previously published (31-36).

Activated and tracer neurons did not overlap in every occurrence. This suggests that only a subset of neurons in each brain region are involved in this task. This method of viral tracing is not without its caveats. Though AAVs are often reliable they do spread and are not always cell type specific. Cellular penetration is also not always guaranteed therefore some cells may have undergone relatively less transfection than others at time of testing. As mentioned earlier, in each region visualized for inputs into the PFC, the GFP expression was greater in the control group than in the novel group. This is perplexing since animals were randomly assigned into groups after surgery therefore differences in viral transfection of the retrograde tracer should not exist here. Some possible explanations are inaccurate stereotaxic coordinates or complications with infusion instruments resulting in different viral load having been injected into the control animals.

These experiments show that it is activation of the PRH and the communication from the PRH to the PFC that is more imperative in this specific task as denoted by the lack of significance in the number of co-labeled neurons in the PFC following the behavior. This is further supported by the significant number of co-labeled activated neurons in the PRH following the retrograde tracing from the PFC.

Figure 1: NOR Behavioral Data for Animals Infused with AAVrg into PRH

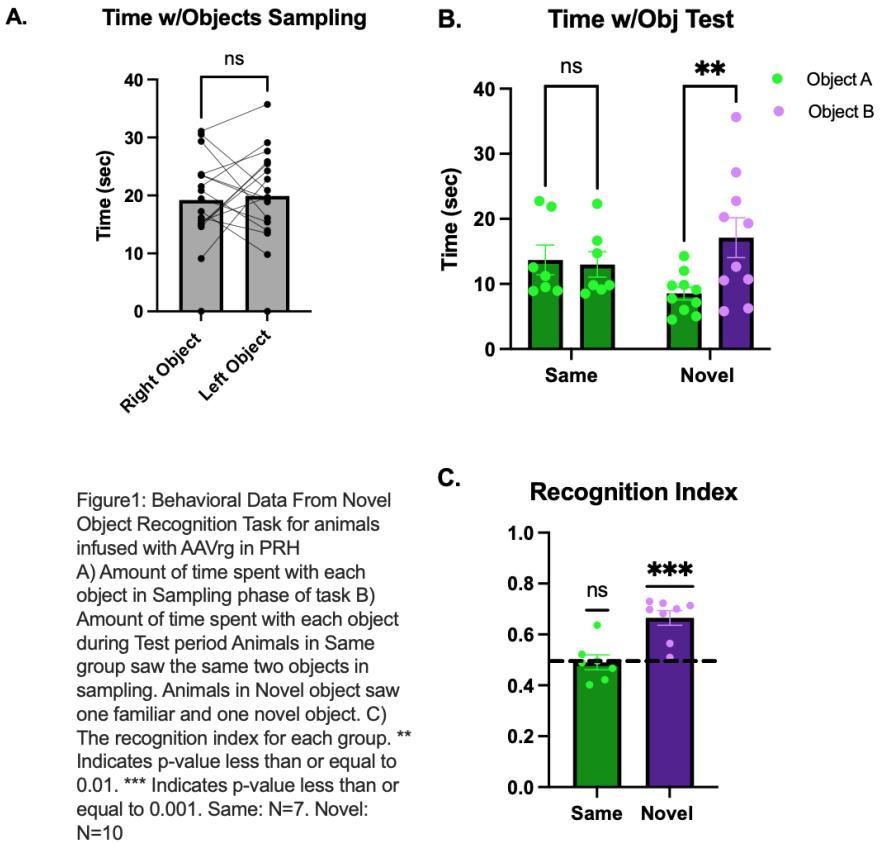


Figure1: Behavioral Data From Novel Object Recognition Task for animals infused with AAVrg in PRH
A) Amount of time spent with each object in Sampling phase of task B) Amount of time spent with each object during Test period Animals in Same group saw the same two objects in sampling. Animals in Novel object saw one familiar and one novel object. C) The recognition index for each group. ** Indicates p-value less than or equal to 0.01. *** Indicates p-value less than or equal to 0.001. Same: N=7. Novel: N=10

Figure 2: Cell Counts for PFC-PL Projections to PRH

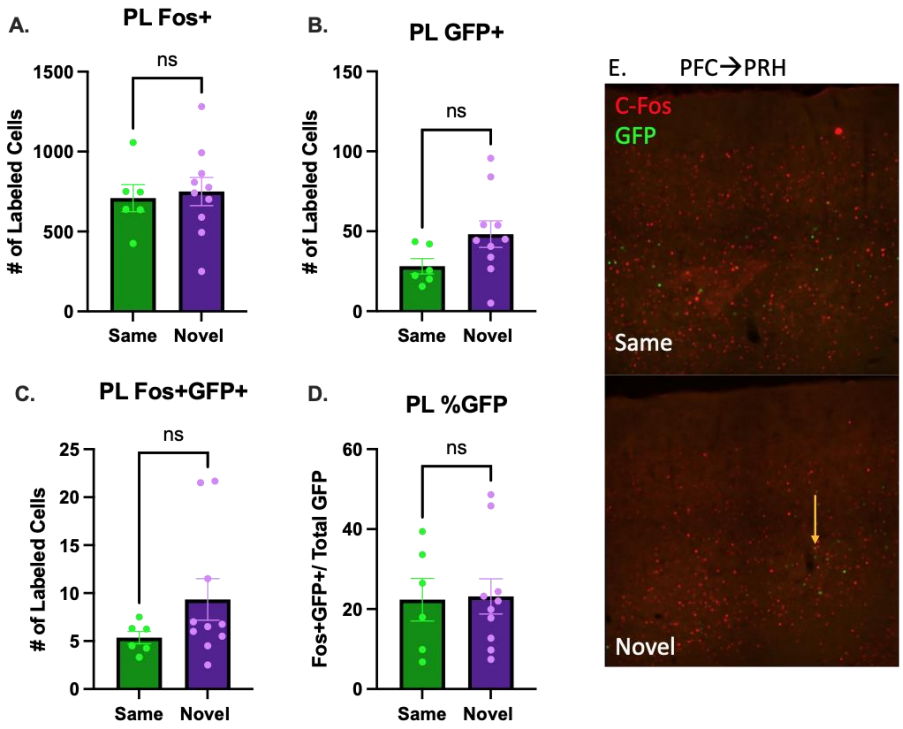


Figure 2: Number of Cell Labeled with GFP Tracer and c-Fos activation
A) Number of cells expressing c-Fos in PFC-PL B) Number of cell expressing GFP in PFC-PL C)Number of cells expressing c-Fos and GFP in PFC-PL D) Number of cells expressing c-Fos and GFP as a percentage of the total GFP in PFC-PL E) Representative images of fluroescent cells in PFC-PL of each group, yellow arrow indicates a co-labeled cell. Same N=6. Novel N=10.

Figure 3: Cell Counts for PFC-IL Projections to PRH

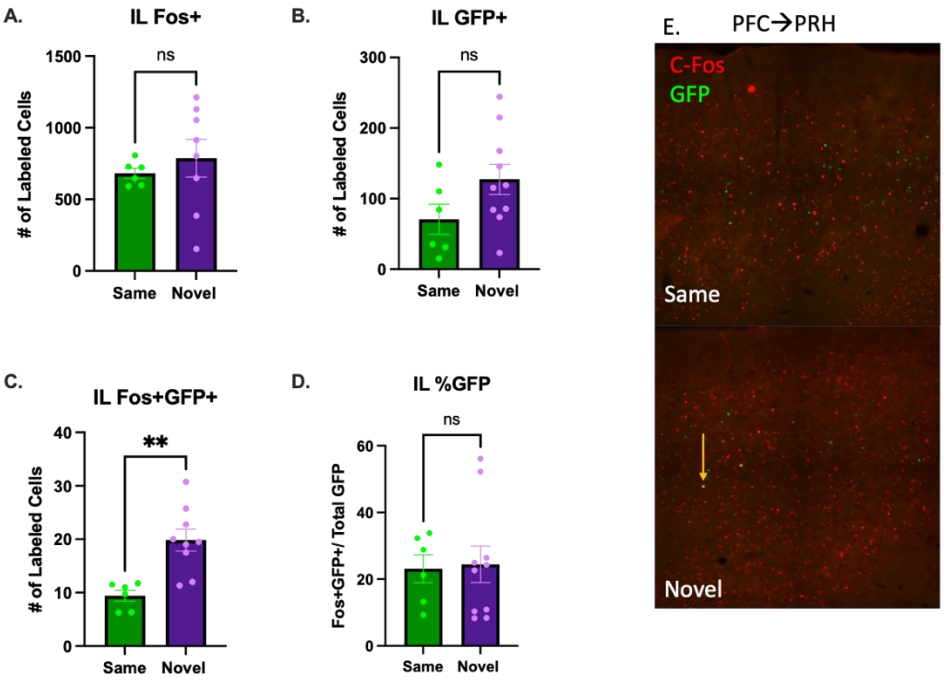


Figure 3: Number of Cell Labeled with GFP Tracer and c-Fos activation in PFC-IL
A) Number of cells expressing c-Fos in PFC-IL B) Number of cell expressing GFP in PFC-IL
C) Number of cells expressing c-Fos and GFP in PFC-IL D) Number of cells expressing c-Fos and GFP as a percentage of the total GFP in PFC-IL E) Representative images of fluroescent cells in PFC-IL of each group, yellow arrow indicates a co-labeled cell. ** Indicates p-value less than or equal to 0.01. Same: N=6. Novel: N=10.

Figure 4: NOR Behavioral Data for Animals with rgAAV Infused into PFC

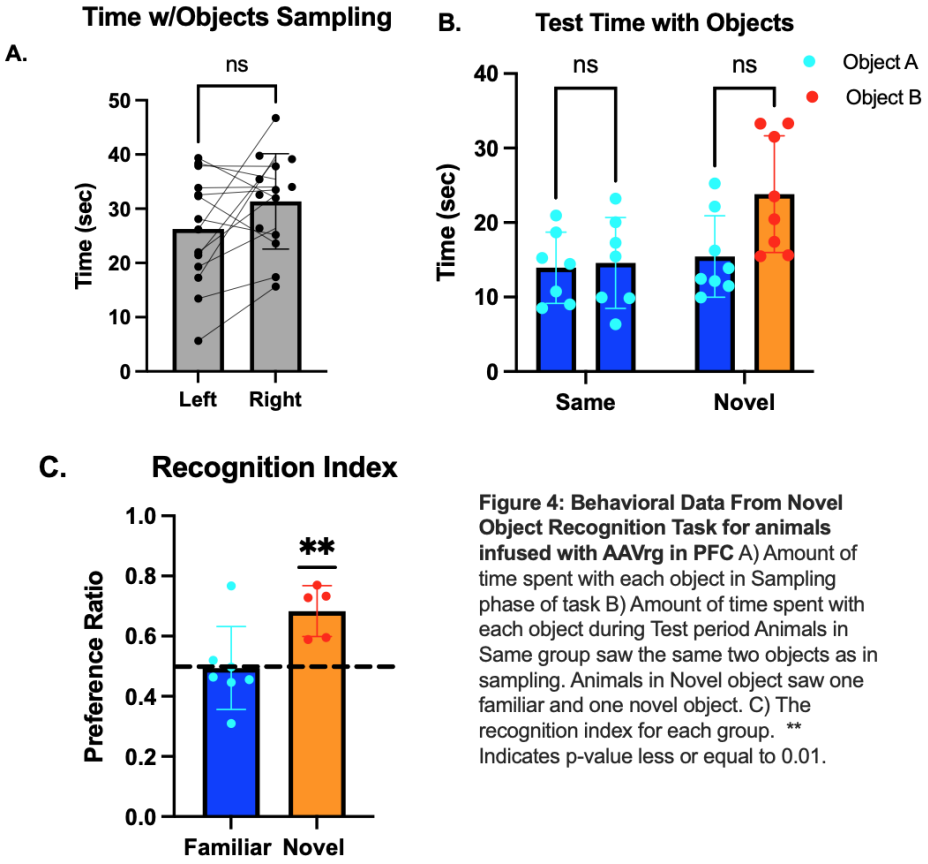


Figure 4: Behavioral Data From Novel Object Recognition Task for animals infused with AAVrg in PFC A) Amount of time spent with each object in Sampling phase of task B) Amount of time spent with each object during Test period Animals in Same group saw the same two objects as in sampling. Animals in Novel object saw one familiar and one novel object. C) The recognition index for each group. ** Indicates p-value less or equal to 0.01.

Figure 5: Cell Counts in PRH Projection to PFC

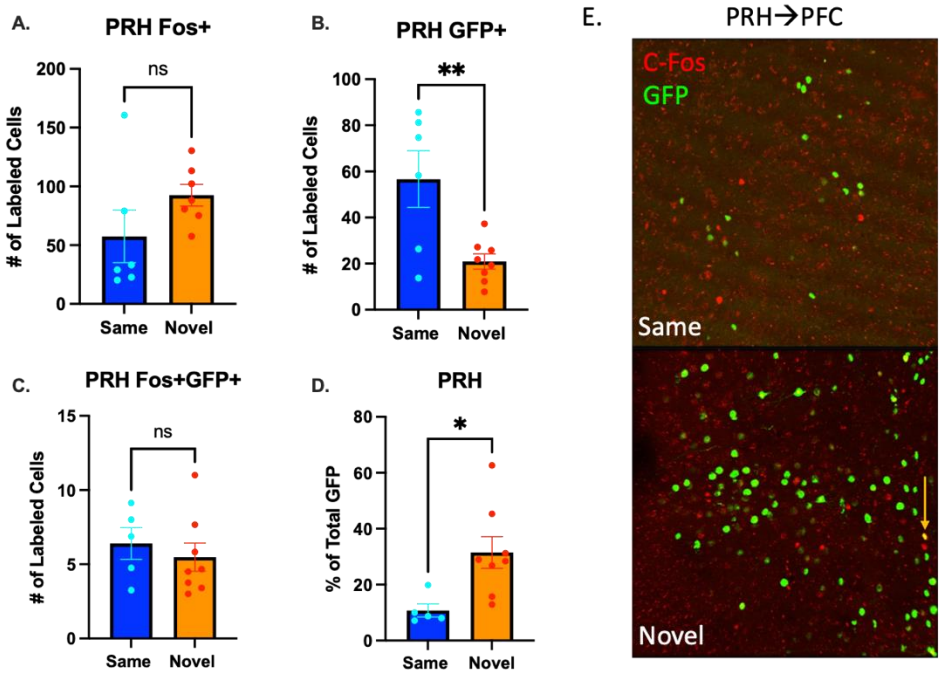


Figure 5: Number of Cell Labeled with GFP Tracer and c-Fos activation
A) Number of cells expressing c-Fos in PRH. B) Number of cell expressing GFP in PRH. C)Number of cells expressing c-Fos and GFP in PRH. D) Number of cells expressing c-Fos and GFP as a percentage of the total GFP in PRH. E) Representative images of fluroescent cells in PFC-PL of each group, yellow arrow indicates a co-labeled cell. * Indicates p-value of less or equal to 0.05. ** Indicates p-value less or equal to 0.01.

Chapter 4: Chemogenetic Manipulation of PRH-PFC Pathway in NOR

Section 1: Introduction

Changes in the PRH following chronic meth exposure contribute to the deficit seen in recognition memory behavior in rodents. A technique used activate or inactivate brain areas or circuits are designer receptors activated exclusively by designer drugs (DREADDS). These receptors are mutated muscarinic aceytlcholine, G protien coupled receptors that only have binding affinity with specific ligands. The receptors are introduced in vivo by viral vectors. The specific action on the infected cell is determined by the subunit coupled with the G protein and can stimulate or inhibit a cell. The specific drug that has been used to activate this type of virally transduced receptor, clozapine-*N*-oxide (CNO). Chemogenetic activation of the PRH has been shown to restore object recognition in the lab in a non-circuit specific manner (50). These previous studies examined sole manipulation of PRH in the NOR task but not the circuitry involved. DREADDS that are only expressed in the presence of CRE recombinase can inform us about pathway specificity when manipulated in the NOR task. The following experiments use an excitatory DREADD, where the receptor is coupled with a Gq protein for activating the cell, and an inhibitory DREADD, which is coupled with a Gi protein, for depressing activity in the cell. The experiments below were used to determine whether object recognition could be restored in animals that have a history of meth use and disrupted in meth-naïve animals.

Section 2: Results

Animals in experiment 3 underwent intracranial surgery to infuse a CRE-dependent Gq DREADD into the PRH and a retrograde CRE virus into the PFC. Afterward, these animals were implanted with indwelling jugular vein catheters for methamphetamine self-administration. Figure 7 shows the acquisition curve over 18 days of SA. The overall two-way ANOVA was not significant $F(17,233) = 1.138$, $p=0.3187$. The main effect of day on intake was significant $F(17,233) = 4.922$, $p<0.0001$. The main effect of sex was not significant $F(1,14=0.1289$, $p=0.7250$. Following 7 days of forced abstinence in the home cage, animals were tested in the NOR paradigm. Animals equally explored both objects in the sampling period ($t(30) = 1.425$, p=).1643). The DREADD activating drug, CNO, was given to animals immediately following sampling with two of the same objects. During the testing phase, animals that received saline vehicle injection did not significantly explore the novel object more than the familiar. Animals that received the CNO injection explored the novel object, object B, significantly more than the familiar object, object A. The overall two-way ANOVA was not significant $F(1,14) = 1.832$, $p=0.1974$. The main effect of object was significant $F(1,14) = 11.77$, $p=0.0041$. The main effect of group was not significant $F(1,14) = 0.4486$, $p=0.5139$. The group that received CNO injection had a recognition index significantly above chance alone ($t(7) = 3.430$, $p=0.0110$, one sample t and Wilcoxon test), while vehicle animals did not (Figure 7C, one sample t and Wilcoxon test, $t(5) = 0.9328$, $p= 0.3937$). There was no difference in the

recognition index between the groups in an unpaired t test. As in Experiment 1 and 2, animals were euthanized 90 minutes following behavioral testing for c-Fos analysis. A difference in the number of mCherry, an indicator of DREADD expression following CRE recombination, labeled cells were not found between the groups injected with vehicle versus those injected with CNO ($t(8) = 0.6169$, $p = 0.5545$, Figure 8A). There was also no difference in the number of co-labeled cells for mCherry and c-fos between CNO and vehicle groups (two-tailed, unpaired t-test, $t(8) = 2.196$, $p = 0.0594$). Similar to Experiments 1 and 2, the number of mCherry expressing cells was used to normalize for viral transfection so that when the number of co-labeled cells were expressed as a percentage of the total number of mCherry cells, animals that received the CNO injection had a greater number of co-labeled cells than those that received vehicle (two-tailed unpaired t-test, $t(7) = 3.481$, $p = 0.0103$, Figure 8C). Figure 8D shows the relative placements of viral infusions among the animals, differences in experimental conditions are not shown. Figure 9 shows a representative overlay of mCherry and c-fos expressing cells from an animal injected with CNO.

In experiment 4, an inhibitory DREADD was used instead of an excitatory DREADD. Animals were intracranially infused with Gi DREADD in the PRH and a retrograde CRE virus in the PFC to move back along the projections to the PRH. These animals were housed in the home cage vivarium until they reached a similar age to animals in Experiment 3. Animals in this experiment did not show a difference in time spent with each object during sampling (Figure 10A, two-

tailed unpaired t-test, t (28) =0.5513, p=0.5858). As with Experiment 3, these animals were injected with CNO immediately following the sampling phase. After 90 minutes retention animals were tested. The overall two-way ANOVA was not significant F (1,12) =1.878, p=0.1956. The main effect of object was not significant F (1,12) =3.262, p=0.0960. The main effect of group was not significant F (1,12) =0.3629, p=0.5581. The group of animals that received an injection of CNO activating the inhibitory DREADDS had a significantly lower recognition index than due to chance alone (one sample t and Wilcoxon test, t (7) =2.667, p=0.0321. While the group of animals that received a vehicle injection did not have a recognition index significantly higher than chance alone (on sample t and Wilcoxon test, t (5) =2.204, p= 0.0787) there was a significant difference in the group recognition indices between the vehicle and CNO groups (two-tailed unpaired t-test, t (12) =3.315, p=0.0031). There was no difference in the number of mCherry or co-labeled Fos+mCherry+ expressing cells between the two groups (Figure 11A and 11B two-tailed unpaired t-test, t (10) =1.444, p=0.1792, t (10) =1.470, p=0.1724, respectively). When normalized to the total percentage of mCherry expressing cells in the groups, there was a significant decrease in the number of co-labeled cells in the group that received the CNO injection in comparison to the group that received vehicle injection (Figure 11C, two-tailed unpaired t-test, t (10) =2.002, p=0.0366). Figure 11D shows the placement and general spread of the viral infusions into the PRH.

Section 3: Discussion

The use of pathway-specific excitatory and inhibitory DREADDs in the PRH during NOR produced the desired restoration and inhibition of recognition memory respectively. Activating the pathway through Gq DREADDS re-established object recognition memory in animals with a history of meth. Inhibiting the pathway disrupted recognition memory to lower novel object exploration than due to chance alone in rats that should otherwise be able to distinguish novel from familiar. This again suggests that the PRH-->PFC circuitry directs this behavior to indicate that there is something new and different about a previously experienced environment. It is possible that following input from the PRH, the PFC, the main hub for executive function, encode information about the salience of that novelty but if the information about the existence of a novel object is not received from the PRH the PFC cannot process whether the object should be explored. Animals with the inhibitory DREADDS were meth naïve animals so any impacts that meth could have on PFC are not present in these animals. It is solely the inhibition of neurons that signal the PFC from the PRH that resulted in the absence of recognition memory. Like experiment 3 where dual vial approach with DREADDS was also used, unilateral expression of the Gi DREADDS was found in some animals following tissue processing. Animals that were found to have this unilateral expression still had recognition indices indicative of a lack of novel object recognition. It is possible that the receptors

were expressed at a threshold lower than what is necessary for detection with the methods used, but still able to inhibit the pathway during the behavior.

As with the first set of experiments, the existence of some cells that express mCherry or c-fos but not co-labeled suggests that only a subset of projection neurons are involved in this particular behavior. As the PFC and PRH are also involved in other aspects of episodic memory, determining if there are sets of neurons for each component of episodic memory would be very important.

Additional subjects should be added to these studies to verify if the behavioral profiles persist. Other controls that should be added to these data sets include CNO and viral controls. Viral controls would ensure that the expression of foreign proteins such as mCherry do not impact the function of the neurons or are innately damaging enough to alter behavior. These animals could also be injected with CNO at the same time point as the experimental animals in these studies to ensure that the drug is inert in the brain when not in the presence of DREADDS. Each of these controls can be used to determine if the viral vector or drug has any off-target effects that could result in altered behavior.

Figure 6: Methamphetamine Acquisition By Sex for Gq DREADD infused Animals

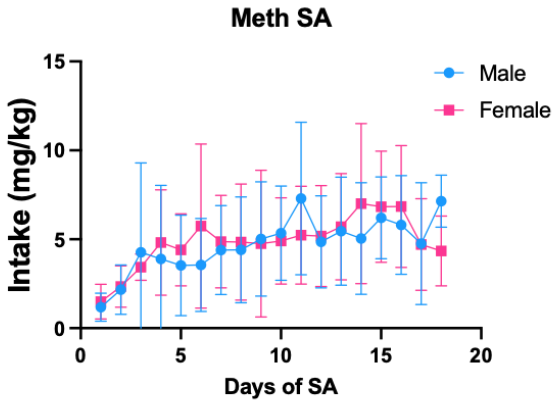


Figure 6: Aqusition Curve for Animals infused with Gq DREADD in PRH—>PFC Pathway Separated by Sex. Intake of Methamphetamine measured in mg/kg of animals during 18 days of SA, separated into CNO and vehicle groups *post hoc.*

Figure 7: NOR Behavioral Data for Gq DREADDS in PRH->PFC Pathway in Meth Animals

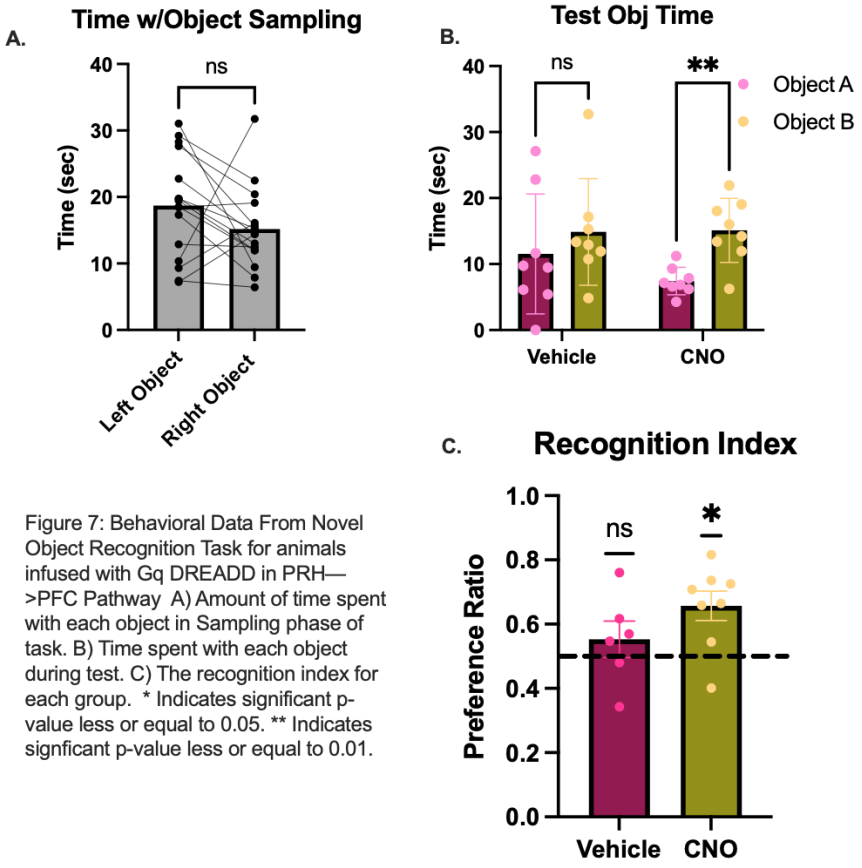


Figure 7: Behavioral Data From Novel Object Recognition Task for animals infused with Gq DREADD in PRH—>PFC Pathway A) Amount of time spent with each object in Sampling phase of task. B) Time spent with each object during test. C) The recognition index for each group. * Indicates significant p-value less or equal to 0.05. ** Indicates signficant p-value less or equal to 0.01.

Figure 8: Cell Counts and Infusion Placements for Animals with Excitatory DREADDS in PRH

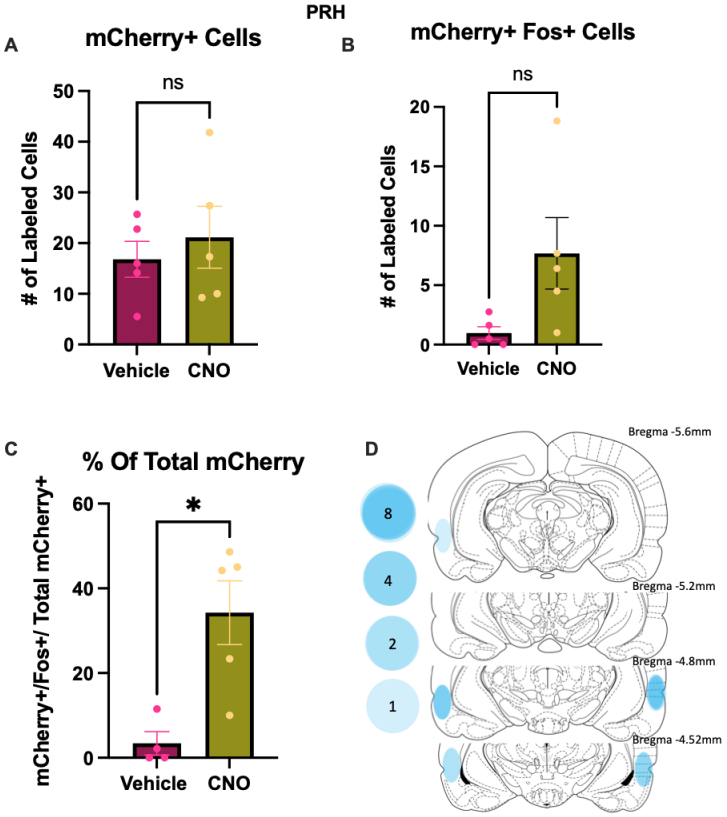


Figure 8: Number of Cells Label with mCherry DREADD and c-Fos
A) Number of cell in each group that are labeled with mCherry indicating DREADD expression. B) Number of cells co-labled with mCherry DREADD and c-Fos. C) Number of cells expressing mCherry DREADD and c-fos as a percentage of total mCherry-DREADD. D) Heatmap of infusion placements indicated by mCherry DREADD expression. * Indicates significant p-value less or equal to 0.05.

Figure 9: Representative Images of Co-labeled Cell in Animals with Excitatory DREADDS in PRH

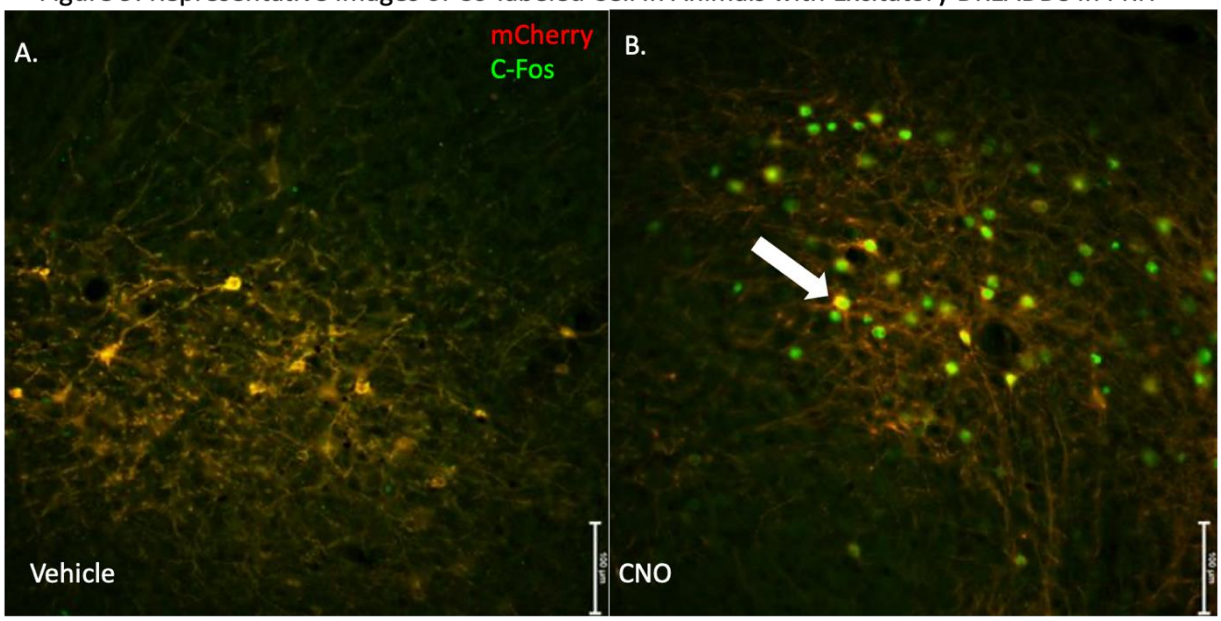


Figure 9: Representative Images of Gq DREADD and c-Fos expression in PRH

A) Image of mCherry and c-Fos in PRH of animal that received vehicle following NOR sampling phase
B) Image of mCherry and c-Fos in PRH of animal that received CNO following NOR sampling phase, white arrow indicates co-localization

Figure 10: NOR Behavioral Data for Meth Naïve Animals Infused with Inhibitory DREADDS in PRH-->PFC

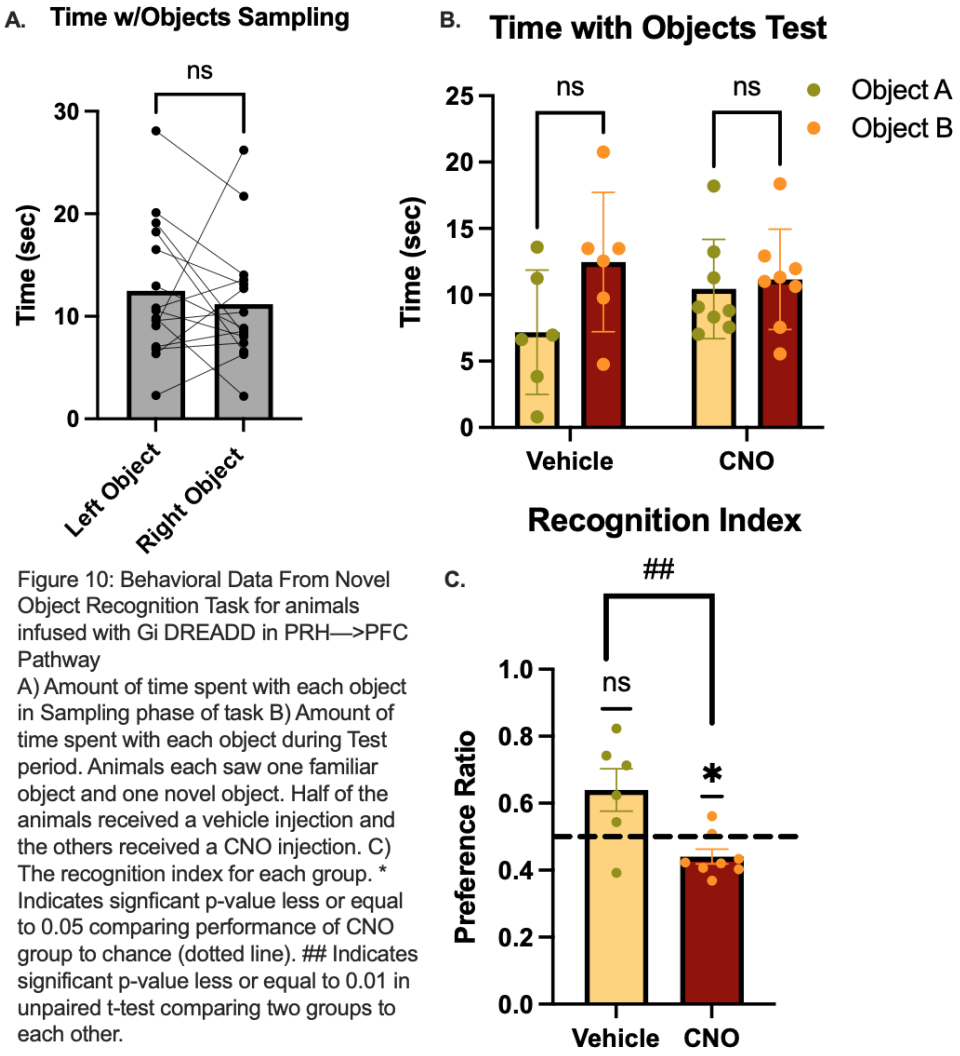


Figure 10: Behavioral Data From Novel Object Recognition Task for animals infused with Gi DREADD in PRH—>PFC Pathway
A) Amount of time spent with each object in Sampling phase of task B) Amount of time spent with each object during Test period. Animals each saw one familiar object and one novel object. Half of the animals received a vehicle injection and the others received a CNO injection. C) The recognition index for each group. * Indicates signficant p-value less or equal to 0.05 comparing performance of CNO group to chance (dotted line). ## Indicates significant p-value less or equal to 0.01 in unpaired t-test comparing two groups to each other.

Figure 11: Cell Counts and Infusion Placements for Animals with Inhibitory DREADDS in PRH

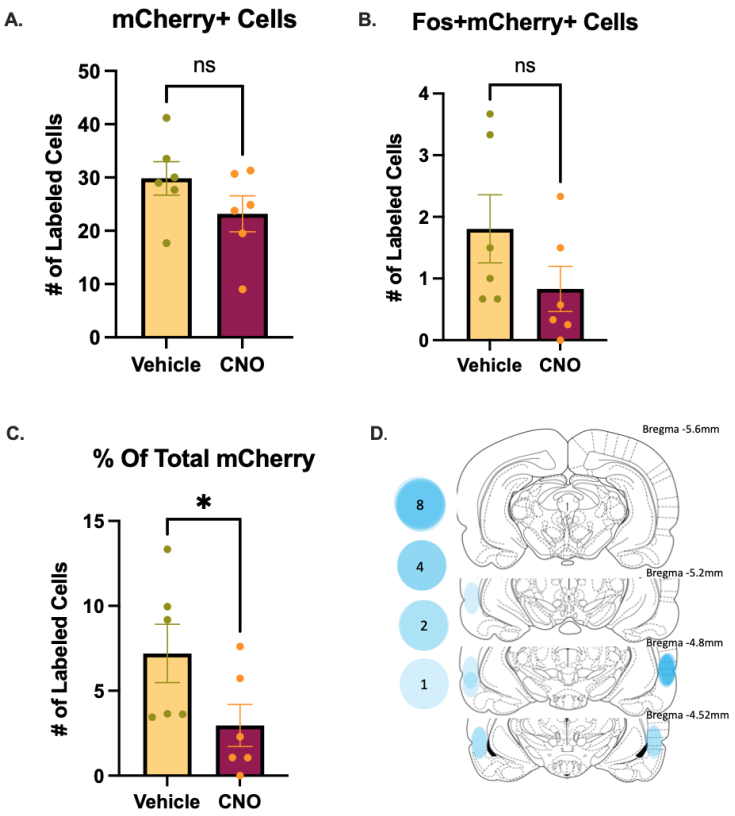


Figure 11: Number of Cells Label with mCherry Gi DREADD and c-Fos
A) Number of cell in each group that are labeled with mCherry indicating DREADD expression. B) Number of cells co-labled with mCherry DREADD and c-Fos. C) Number of cells expressing mCherry DREADD and c-fos as a percentage of total mCherry-DREADD. D) Heatmap of infusion placements indicated by mCherry DREADD expression. * Indicates significant p-value less or equal to 0.05.

b

Figure 12: Representative Images of Co-labeled Cell in Animals with Inhibitory DREADDS in PRH

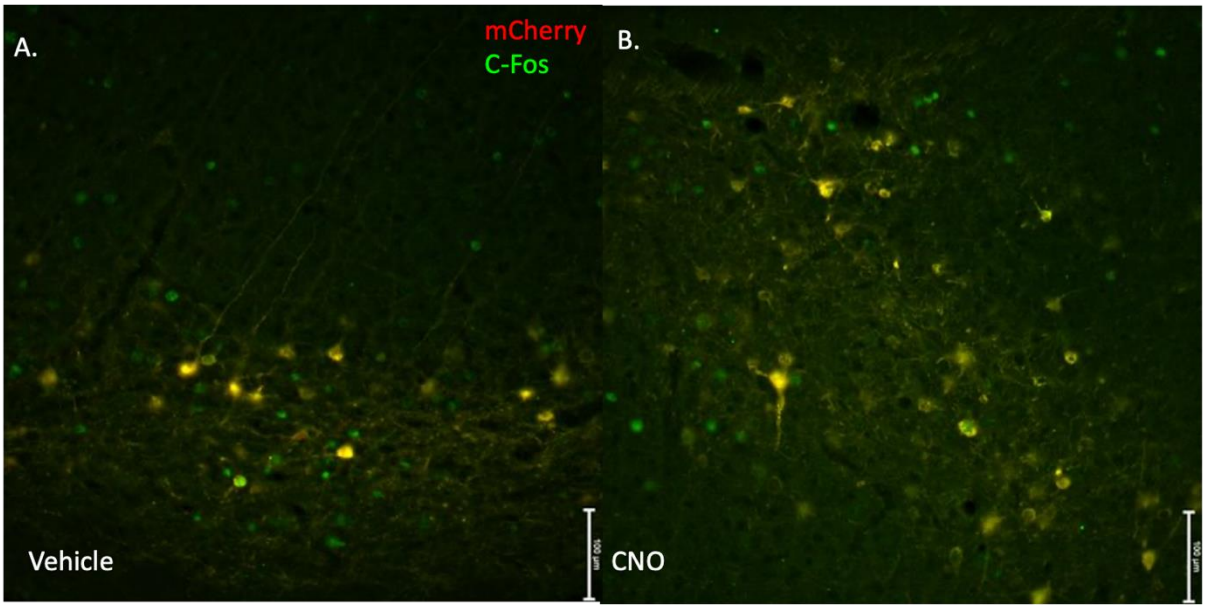


Figure 12: Representative Images of Gi DREADD and c-Fos expression in PRH

A) Image of mCherry and c-Fos in PRH of animal that received vehicle following NOR sampling phase
B) Image of mCherry and c-Fos in PRH of animal that received CNO following NOR sampling phase

Chapter 5: Final Discussion

Section 1: Conclusions and Significance

The experiments described above indicate that circuitry between the PRH and the PFC is indeed involved in object recognition memory. The first set of experiments showed that, though there are reciprocal connections between these brain regions, the PRH projection to the PFC is more involved than that of the projection from the PFC to the PRH as demonstrated by the greater difference in co-labeled activated cells. The second set of experiments further showcases the importance of this unidirectional communication in object recognition memory and how changes to the PRH can prevent the broadcast of signals from the PRH to other brain regions. Excitation of the PRH-->PFC circuitry restored novel object recognition following chronic meth SA, historically seen to induce deficits, while inhibition of this circuitry resulted object recognition deficits in naïve rats mimicking meth use.

Previous work conducted over the last few decades has shown that PFC, PRH, and HPC are essential substrates for recognition memory (29-33). Specifically, lesion studies have shown the necessity of an intact PRH in order for rodents to reliably determine when an object is novel in a given environment (31-36). The studies outlined here show that it is not just the PRH that is important but the communication pathway from the PRH to the PFC that is important. Though this fits within the previously held theories that the triangulation of PFC, PRH, HPC

circuitry is the basis for recognition memory, the data from these studies suggests that outward communication from the PRH to the PFC is responsible for specifically object recognition memory.

The data presented suggests a broader role for brain regions receiving input from the PRH than previously thought in objection recognition memory. Previously, it has been understood that a novel object in a given environment triggers the activation of the PRH alone and that processing of this information was thought to occur here, leading to the exploration seen in the behavioral outcomes. Given the current findings outlined, the PRH establishes recognition of novel objects in a given environment directing exploration of these objects.

Section 2: Sex as a Biological Variable and Limitations

In the described studies, both male and female rats were used, though the data was eventually collapsed between the sexes. This was due to the lack of significant differences in the behavioral measures between the sexes. For novel object recognition alone, these similarities are consistent with what has been previously seen. Female rats have been observed to have higher amounts of meth intake overall than males (50). No differences in intake were observed in my experiments but only a small portion of represented animals received meth. Given larger sample size it is possible that differences in drug intake could be observed, but that the measures of object recognition would still be the equal between the sexes following chronic exposure.

Limitations with the experiments are that the overall sample size per group is low, differences in viral transfection, and differences in tissue processing leading to loss. Each experiment had 8-10 animals used at the beginning of each experiment, but over the duration of the data collection, some samples were lost. Though each surgical procedure conducted was kept as uniform in nature as possible, minute changes in equipment and stereotaxic surgery could have resulted in animal loss or differences in transfection of the virus. Given that the results of these experiments were based in cell counts from viral expression this had the potential to skew the results, thus prompting normalization steps. Though the behavior was restored, the tissue collection following experiments showed that several animals had unilateral expression of DREADDS, indicated by mCherry fluorescence. This suggests that only one pathway was available for activation. This data is congruent with previous lesion studies that indicate that only one intact PRH was needed for object recognition.

Section 3: Future Directions

Through the circuitry approach, projections between the PRH and PFC were identified though not all were found to be involved in NOR behavior. Further characterization of the specific group of neurons involved and the impact chronic meth can have on them is needed. Future areas of investigation could include determining which region, PRH or PFC, is involved in the salience of the novel objects or the role of the projections from other regions, such as the PVT, in NOR behavior following chronic meth.

Object recognition memory is reliant on an intact pathway from PRH-->PFC. The PRH and its projections are damaged as a result of chronic meth use. Loss of episodic memory such as recognition memory is prevalent in abstinent and recently relapsed human users of meth (18). As such this is a potential target for enhancement to prevent future relapse events.

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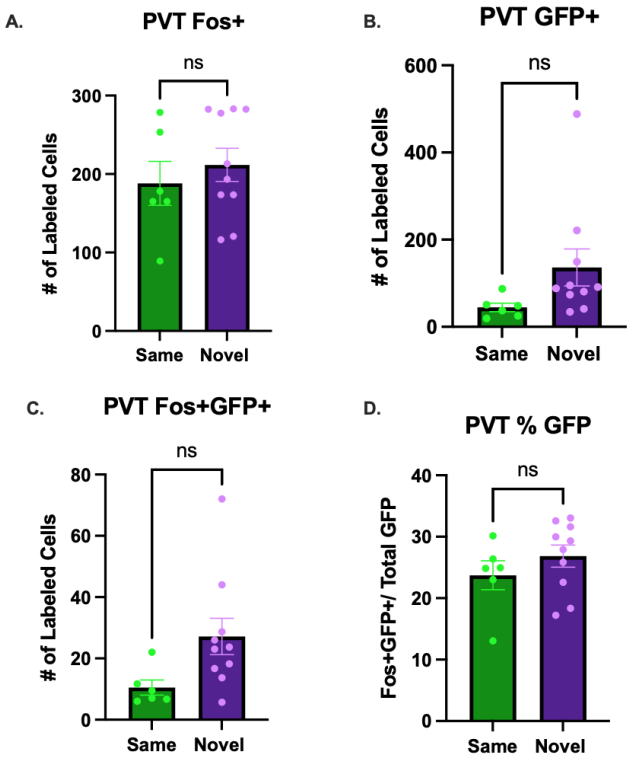
Appendix A: Brain Regions Projecting to PRH

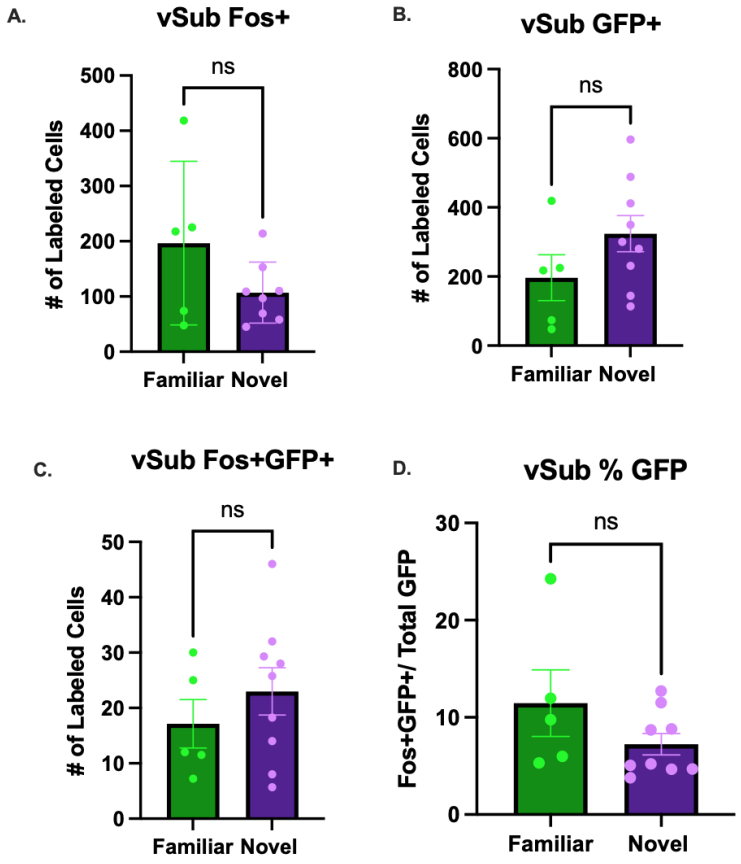
As activation mapping of NOR behavior is limited, a cursory look of other brain regions containing GFP and c-fos labeled cells was conducted from subsets of whole brain. Other regions found to project to the PRH include the paraventricular thalamus, ventral subiculum and CA1 of the hippocampus. These structures included portions of the HPC, an expected locale, but also the PVT. The PVT acts as a relay station for rewarding and threatening responses to stimuli (51). The arena used for this task was large and relatively open with the objects in the center. Given that rats do not naturally like to spend time in open spaces that can leave them vulnerable to predator attack, the activation of the PVT in this context are understandable. Cell count data can be found in Appendix Figures 1-3 below for PRH inputs.

Appendix A, Figure 1: Labeled Cell Counts in PVT



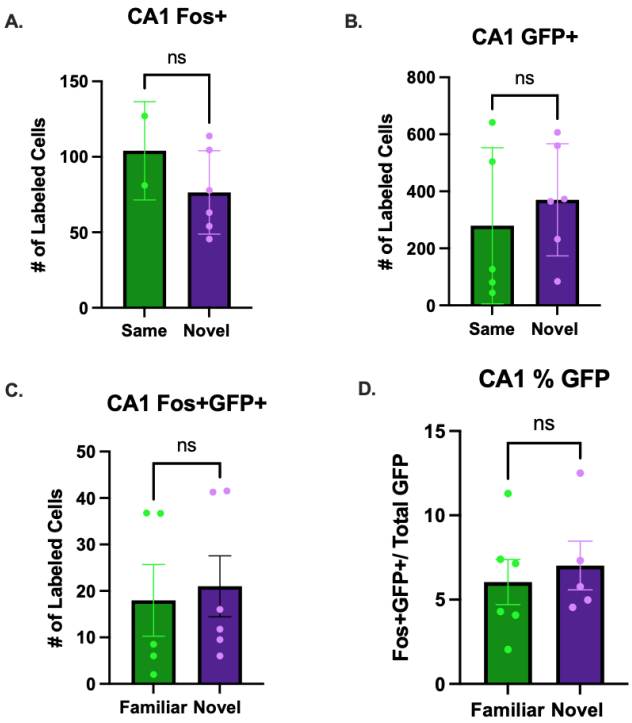
Supplemental Figure 1: Number of Cell Labeled with GFP Tracer and c-Fos activation in PVT A) Number of cells expressing c-Fos in PVT; two-tailed unpaired t-test, t(14)=0.6764, p=0.5098. (B) Number of cell expressing GFP in PVT; t(14)=1.628, p=0.1257. C) Number of cells expressing c-Fos and GFP in PVT; t(14)=2.091, p=0.0552. D) Number of cells expressing c-Fos and GFP as a percentage of the total GFP in PVT; t(14)=1.052, p=0.3104. Same: N=6. Novel: N= 10.

Appendix A, Figure 2: Labeled Cell Counts in vSub of Hippocampus



Supplemental Figure 2: Number of Cell Labeled with GFP Tracer and c-Fos activation in vSub A) Number of cells expressing c-Fos in vSub, two-tailed unpaired t-test, t(11)=1.577, p=0.1430. B) Number of cell expressing GFP in vSub, t(12)=1.482, p=0.1641. C) Number of cells expressing c-Fos and GFP in vSub; t(12)= 0.8832, p=0.3945. D) Number of cells expressing c-Fos and GFP as a percentage of the total GFP in vSub; t(12)=1.461, p=0.1697. Same: N=5. Novel: N=9.

Appendix A, Figure 3: Labeled Cell Count in CA1 of Hippocampus

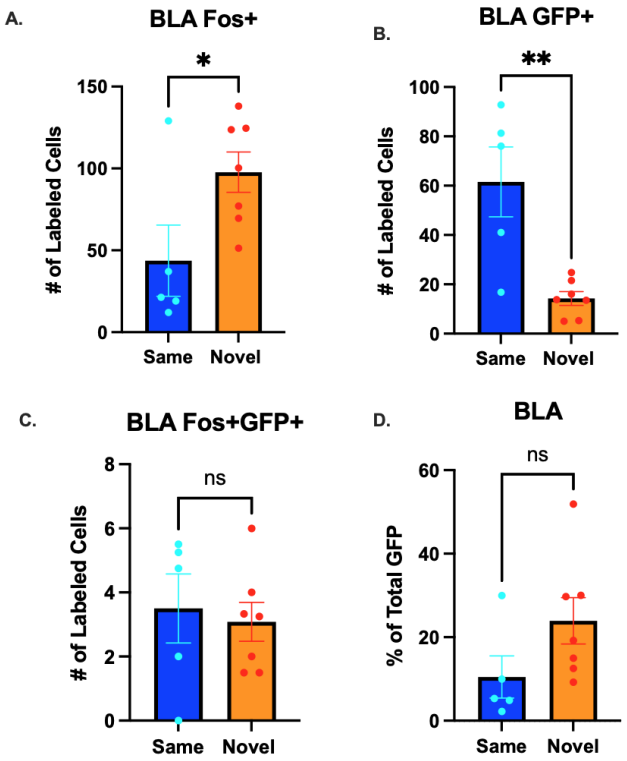


Supplemental Figure 3: Number of Cell Labeled with GFP Tracer and c-Fos activation in CA1 A) Number of cells expressing c-Fos in CA1; two-tailed unpaired t-test, t(6)=1.184, p=0.2810. B) Number of cell expressing GFP in CA1; t(9)=0.6408, p=0.5376. C) Number of cells expressing c-Fos and GFP in CA1; t(9)=0.2996, p=0.7713. D) Number of cells expressing c-Fos and GFP as a percentage of the total GFP in CA1; t(9)=0.4974, p=0.6308. Same: N=6. Novel N=6

Appendix B: Brain Regions Projecting to PFC

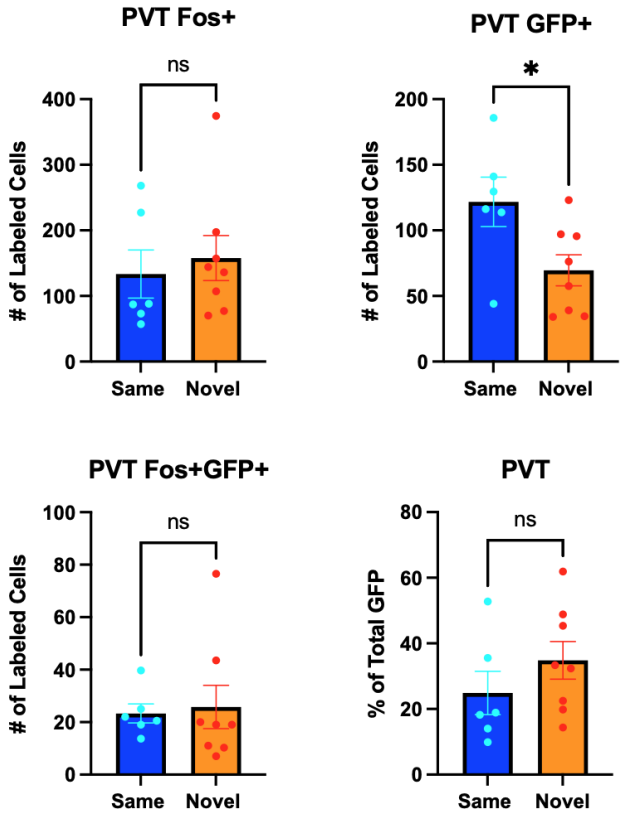
Other regions of activation following NOR task with projections to the PFC were investigated. These regions were found to include the BLA, PVT, and vSub of the HPC. The BLA was activated in animals that saw the novel object, with a greater number of c-Fos+ cells than those that saw the familiar object (Appendix B, Figure 1). The PVT acts as a relay station for rewarding and threatening responses to stimuli (44) while the amygdala is the central substrate to process fearful stimuli (45). The arena used for this task was large and relatively open with the objects in the center. Given that rats do not naturally like to spend time in open spaces that can leave them vulnerable to predator attack, the activation of the PVT and the BLA in this context are understandable. Because both regions project to the PFC, the final determination to investigate the object could be processed here. Each region was imaged for GFP expression and c-FOS though none showed significant differences among the groups following normalization. Cell count data can be found in Appendix B Figures 1-3 for the PFC inputs.

Appendix 2, Figure 1: Labeled Cell Count in BLA



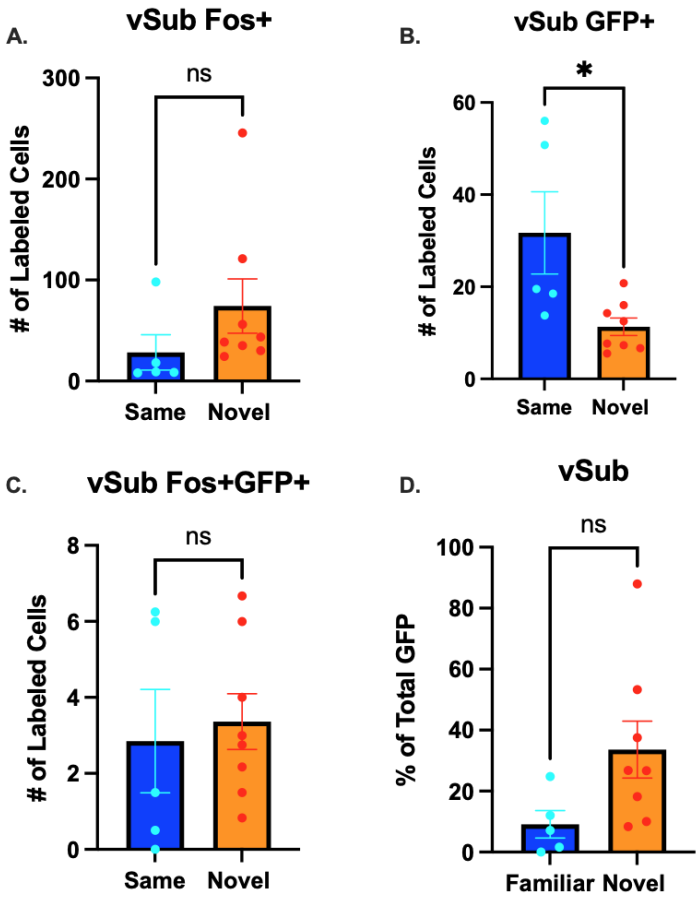
Supplemental Figure 4: Number of Cell Labeled with GFP Tracer and c-Fos activation in BLA A) Number of cells expressing c-Fos in BLA; two-tailed unpaired t-test, t(10)=2.321, p=0.0427. B) Number of cell expressing GFP in BLA; t(10)=3.880, p=0.0031. C) Number of cells expressing c-Fos and GFP in BLA; t(10)=0.3615, p=0.7253. D) Number of cells expressing c-Fos and GFP as a percentage of the total GFP in BLA; t(10)=1.710, p=0.1181. * Indicates significant p-value less or equal to 0.05. ** Indicates signitficant p-value less or equal to 0.01.

Appendix 2, Figure 2: Labeled Cell Counts in PVT



Supplemental Figure 5: Number of Cell Labeled with GFP Tracer and c-Fos activation in PVT
A) Number of cells expressing c-Fos in PVT; two-tailed unpaired t-test, t(12)=0.4802, p=0.6397. B) Number of cell expressing GFP in PVT; t(12)=2.453, p=0.0304.
C) Number of cells expressing c-Fos and GFP in PVT; t(12)=0.2457, p=0.8101. D) Number of cells expressing c-Fos and GFP as a percentage of the total GFP in PVT; t(12)=1.133, p=0.2794. * Indicates significant p-value less or equal to 0.05.

Appendix 2, Figure 3: Labeled Cell Counts in vSub of Hippocampus



Supplemental Figure 6: Number of Cell Labeled with GFP Tracer and c-Fos activation in vSub A) Number of cells expressing c-Fos in vSub; two-tailed, unpaired t-test, t(11)=1.243, p=0.2397. B) Number of cell expressing GFP in vSub; t(11)=2.790, p=0.0176. C) Number of cells expressing c-Fos and GFP in vSub; t(11)=0.3657, p=0.7216. D) Number of cells expressing c-Fos and GFP as a percentage of the total GFP in vSub; t(11)=1.959, p=0.0759. * Indicates significant p-value less or equal to 0.05.