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## A method for remotely silencing neural activity in rats during discrete phases of learning --Manuscript Draft--

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<b>Corresponding Author:</b>	Siobhan Robinson, PhD Oberlin College Oberlin, OHIO UNITED STATES
<b>Corresponding Author Secondary Information:</b>	
<b>Corresponding Author E-Mail:</b>	srobinso@oberlin.edu
<b>Corresponding Author's Institution:</b>	Oberlin College
<b>Corresponding Author's Secondary Institution:</b>	
<b>First Author:</b>	Siobhan Robinson, PhD
<b>First Author Secondary Information:</b>	
<b>Other Authors:</b>	Julia S. Adelman
<b>Order of Authors Secondary Information:</b>	
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**TITLE:**

**A method for remotely silencing neural activity in rodents during discrete phases of learning**

**AUTHORS:**

**Siobhan Robinson**

Department of Neuroscience  
Oberlin College  
Oberlin, OH  
Siobhan.Robinson@oberlin.edu

**Julia S. Adelman**

Department of Neuroscience  
Oberlin College  
Oberlin, OH  
jadelman@oberlin.edu

**Corresponding Author:**

Name: Siobhan Robinson  
Phone: 440.775.6258  
Fax: 440.775.5397  
Siobhan.Robinson@oberlin.edu

**KEYWORDS:**

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**SHORT ABSTRACT:**

This protocol describes how to temporarily and remotely silence neuronal activity in discrete brain regions while rats are engaged in learning and memory tasks. The approach combines pharmacogenetics (Designer-Receptors-Exclusively-Activated-by-Designer-Drugs) with a behavioral paradigm (sensory preconditioning) that is designed to distinguish between different components of learning.

**LONG ABSTRACT:**

This protocol describes how to temporarily and remotely silence neuronal activity in discrete brain regions while animals are engaged in learning and memory tasks. The approach combines pharmacogenetics (Designer-Receptors-Exclusively-Activated-by-Designer-Drugs) with a behavioral paradigm (sensory preconditioning) that is designed to distinguish between different forms of learning. Specifically, viral-mediated delivery is used to express a genetically modified inhibitory G-protein coupled receptor (the Designer Receptor) into a discrete brain region in the rodent. Three weeks later, when designer receptor expression levels are high, a pharmacological agent (the Designer Drug) is administered systemically 30 min prior to a specific behavioral session. The drug has affinity for the designer receptor and thus results in inhibition of neurons that express the designer receptor, but is otherwise biologically inert. The

brain region remains silenced for ~2-5 h (depending on the dose and route of administration). Upon completion of the behavioral paradigm, brain tissue is assessed for correct placement and receptor expression. This approach is particularly useful for determining the contribution of individual brain regions to specific components of behavior and can be used across any number of behavioral paradigms.

## **INTRODUCTION:**

An exciting challenge within the field of behavioral neuroscience is to determine the neural substrates of complex behaviors. A number of techniques such as permanent lesions, temporary brain inactivation via cannulae implants and optogenetics have been employed to identify the contributions of discrete brain regions to subcomponents of complex behaviors. While these approaches inform our understanding of regional specificity during learning, each technique is not without limitations. Specifically, permanent lesions are typically conducted prior to behavioral testing, thus their effects are present throughout the duration of the paradigm. Cannulation studies that involve the presentation of a short-term neural inactivator (*e.g.*, tetrodotoxin) can produce substantial damage to brain tissue and can induce stress in subjects just prior to behavioral testing. Furthermore, inactivation through cannulation is limited to the region of tissue that surrounds the tip of the cannulae. Lastly, while optogenetics offers a range of flexibility for the temporal control of activity in specific brain regions, it is cost prohibitive and technically demanding.

These limitations can be surmounted using a pharmacogenetic approach (Designer-Receptors-Exclusively-Activated-by-Designer-Drugs, DREADDS)<sup>1,2</sup>. Importantly, while the concept of pharmacogenetics is sophisticated, the execution of the technique is straightforward. Similar to traditional stereotaxic surgical methods that involve infusion of toxin (*e.g.*, NMDA, ibotenic acid) into discrete brain regions, this technique involves infusing an adeno-associated virus (AAV) that contains a DNA fragment for a modified inhibitory G-protein coupled receptor (hM4Di; the designer receptor) into the region of interest of standard laboratory rodents (see Figure 1). The viral vector also contains a fluorescent reporter (mChitrine). Once incorporated into cells, the designer receptor (and reporter protein) are maximally expressed ~3 weeks post-infusion and can be selectively activated for ~2-5 h by systemic administration of the otherwise biologically inert designer drug, clozapine-N-oxide (CNO)<sup>1,3</sup>. Because the experimenter is endowed with precise, yet remote temporal control over neural activity in specific brain regions, pharmacogenetics combines particularly well with behavioral paradigms that are conducted in multiple phases. In this example, the contribution of the retrosplenial cortex (RSC) to stimulus-stimulus learning is compared to its role in Pavlovian learning, however this combination of approaches is well suited to any number of questions that seek to identify how specific brain regions contribute to complex behavior.

In addition, while not described in the present protocol, viral and transgenic approaches can be used to achieve cell type-specific DREADD expression<sup>2</sup>. As is inherent in behavioral paradigms that involve pharmacological and/or other types of experimental manipulations, careful consideration of experimental design and subsequent quantitative analysis is required when

employing the DREADD approach. Experimenters new to the DREADD approach are referred to a comprehensive review of current DREADD technology<sup>2</sup>.

Each day, organisms learn about new stimuli and events and their relationships to one another. Even in a familiar environment, such as home, one is quick to detect alterations in the relationships between stimuli because these changes may be predictive of meaningful events. Such stimulus-stimulus (*i.e.*, relational) learning involves the conjoining of multiple stimuli and has traditionally been associated with the hippocampus, which resides centrally within the medial temporal lobe<sup>4</sup>. However, the hippocampus does not exist nor act in isolation; cortical regions both within and outside of the medial temporal lobe provide critical sensory information to the hippocampal formation<sup>5-7</sup>. Traditional permanent lesion studies provide compelling evidence for the involvement of a number of cortical regions (*e.g.*, the retrosplenial, postrhinal and entorhinal cortices) in hippocampal-dependent learning but are limited in their ability to discern the role of a particular region during discrete phases of learning<sup>8-10</sup>.

The present protocol tests the hypothesis that the RSC is necessary for stimulus-stimulus learning by silencing the RSC during a single phase of a 3-phase sensory preconditioning paradigm<sup>11,12</sup>. Briefly, rats receive infusions of an AAV that contains the designer receptor and ~3 weeks later are administered the designer drug (CNO) 30 min prior to the start of behavioral testing. In the present protocol, experimental rats receive CNO during the first phase of testing (when stimulus-stimulus learning occurs) and they receive vehicle during the next 2 phases of testing. To control for inadvertent effects of CNO on behavior, infuse rats with the designer receptor (hM4Di) and inject with vehicle instead of CNO. To account for general effects of viral infusion and receptor expression, infuse a control virus that does not contain the designer receptor and administer CNO.

A number of different serotypes of AAV are used to deliver genetic material. The current NIH Guidelines for Research Involving Recombinant or Synthetic Molecules maintains that AAV (all serotypes) and recombinant or synthetic AAV constructs, in which the transgene does not encode either a potentially tumorigenic gene product or a toxin molecule and are produced in the absence of a helper virus, require BSL-1 precautions (Appendix B-1. Risk Group 1 (RG1) Agents)<sup>13</sup>. A number of reviews pertaining to AAV structure, utility and safety are available<sup>14,15</sup>. Notably, though, due to concerns pertaining to possible reproductive<sup>16,17</sup> and potential carcinogenic mechanisms<sup>18,19,20</sup> in rodents, some institutions require the use of BSL-2 precautions when working with AAV. Verify the appropriate BSL prior to use by consulting with oversight committees at individual institutions where the research will be conducted, the Centers for Disease Control and the NIH Guidelines for Research Involving Recombinant DNA Molecules<sup>13</sup> when using viral vectors for gene manipulation in the United States. Personal protection, investigator training, vector containment, decontamination, disposal of decontaminated materials, and post-injection animal housing requirements are specified by these guidelines. In addition, consult and follow appropriate Institutional Animal Care and Use committee guidelines or equivalent institutional oversight committee guidelines to ensure the safe handling, administration and disposal of AAV.

## PROCEDURE:

The use of animals are approved by the Oberlin College Institutional Animal Care and Use committee and are in accordance with the Guide for the Care and Use of Laboratory Animals<sup>21</sup>.

### 1. Preparation for Viral Infusion

Note: When employing BSL-2 precautions, a disposable lab coat, gloves, shoe covers, eye protections and a particulate respirator (type N95) are required. All individuals handling BSL-2 compounds must be fit tested for a particulate respirator by a local public health agency. Refer to Lowery & Majewska (2010)<sup>22</sup> for additional details on handling and storage of viral vectors.

1.1. Upon first use, aliquot and store unused virus in 20  $\mu$ L microcentrifuge tubes to avoid repeated freezing and thawing.

1.2. Prepare the hood (Biosafety Cabinet Class II) by removing any unnecessary objects and sterilizing the surface with 70% ethanol. Prepare a 10% bleach solution for decontaminating AAV waste. Place a beaker full of the bleach solution and a sterile 10  $\mu$ L syringe in the hood. Place the microcentrifuge tube with the AAV in a container with crushed ice during set up.

1.3. Place the microcentrifuge tube with the virus into a standard bench vice. Load a 10  $\mu$ L syringe with at least 4  $\mu$ L of AAV. Take care not to bend the tip of the syringe against the bottom of the microcentrifuge tube during loading. Ensure that there are no air bubbles in the 4  $\mu$ L of AAV solution in the syringe.

1.4. Dispose of the empty virus tube in the beaker containing 10% bleach. Store unused portions of the virus as specified by the supplier.

1.5. Add additional 10% bleach to the waste container, and allow waste to sit for 30 min before disposing the decontaminated liquid waste.

1.6. Dispose of all protective equipment and plastic waste in a biohazard container as instructed by institutional guidelines. Sterilize any equipment or surfaces that came in contact with the virus using 10% bleach.

### 2. Surgery

2.1. Prepare the surgical area by placing absorbent bench paper under the stereotaxic apparatus and on an adjacent space designated as a dedicated virus handling area.

2.1.1. Place a 10% bleach waste container in the dedicated virus handling area near the surgical apparatus. Surgery can take place within or outside of a fume hood.

2.2. Induce a steady level of anesthesia and prepare the rat for surgery.

2.2.1 Place the rat into an induction chamber that contains a mixture of isoflurane gas (3%) and oxygen (~1 L/min) for 6 minutes. Maintain isoflurane anesthesia throughout the surgical procedure.

2.2.2 After 6 min, shave the rat from between the eyes to slightly behind the ears.

2.2.3 Place the rat back into the induction chamber for an additional 3 min.

2.2.4 Ensure that the isoflurane anesthesia system is connected to the nose cone of the stereotaxic surgery. Open the stopcock on the isoflurane tubing to begin the flow of isoflurane to the stereotax. Maintain the isoflurane and oxygen at the levels specified above.

2.2.5 Place the rat in a stereotaxic apparatus by securing the mouth onto the bite bar and securing the ear bars.

2.2.6 Apply eye lubricant to the eyes and betadine to the surgical site.

2.3 Make a 2.4 cm midline incision of the skin on the dorsal surface of the skull starting approximately 2 mm caudal to the eyes. Retract the skin to expose the skull.

2.4 Clear membranous tissue from the skull until bregma and lambda are clearly visible.

2.5 Ensure that the skull is level by measuring the dorsal-ventral coordinates at bregma and at lambda. Adjust the stereotaxic device accordingly until dorsal-ventral coordinates at bregma and lambda differ by no more than 0.04 mm.

2.6 At this time, reduce the isoflurane gas to between 2 and 2.5%.

2.7 Return the drill to bregma, rezero the coordinates and then move the drill to the desired medial-lateral and anterior-posterior coordinate.

2.8 Drill a hole at the coordinate using a 0.9 mm drill bit as it produces an appropriate size hole for a 28-gauge infusion syringe.

2.9 Set the infusion pump to deliver virus at a rate of 0.2  $\mu$ L/min. Place the syringe into the infusion arm of the stereotaxic device and lower the syringe into the desired coordinate. Deliver the desired amount of virus (range 0.1 to 0.8  $\mu$ L). For example, deliver 0.8  $\mu$ L of AAV over 4 min. Note: conduct pilot studies to determine the ideal infusion volume and the typical spread of virus in each region of interest.

2.9.1 After the delivery of virus is complete leave the syringe in place for 10 min to prevent backflow into the needle tract. Slowly raise the syringe at a rate of about 5 mm/min. Repeat until all coordinates have been infused with AAV.

2.10 Close the wound using surgical staples and coat the wound with topical antibiotic ointment.

2.11 Follow institutional guidelines for post-operative pain management.

2.12 Place the rat in a cage with bedding, a lid and proper signage and return the rat to the animal facility.

2.12.1 If working under BSL-2 precautions, collect bedding for the amount of time (typically 48 – 72 h) specified by institutional guidelines. Place bedding into a biohazard waste container.

2.12.2 Dispose of all AAV-contaminated materials as specified by guidelines for their safe disposal.

### **3. Behavioral Apparatus**

Note: The sensory preconditioning apparatus consists of a standard operant conditioning chamber (12" L x 9.5" W x 11.5" H) with a stainless steel grid floor, 2 Plexiglas sidewalls and 2 metal walls.

3.1 Mount a 2.8 W light on one of the aluminum walls to serve as the house light and as the visual stimulus when flashed at 2 Hz. Mount a speaker on the chamber to deliver the auditory stimuli (a 10 s, 1500 Hz, 78 dB pure tone and a 10 s 78 dB white noise).

3.1.1 Use a food hopper to deliver 45 mg food pellets into the food cup. Within the food cup, infrared photocells detect the total time spent in the food cup before, during and after presentations of the stimuli and food rewards.

3.1.2 House the chambers in sound-attenuating cabinets (22" W x 22" H x 16" D) outfitted with exhaust fans (~68 dB).

2.13 Use a PC computer with Med Associates software to control the operant chamber and acquire the data. During the Conditioning sessions, collect data on the total time spent with the head in the food cup during presentation of the light stimulus (5 s epoch) and during presentation of the food reward (5 s epoch). During the Test session, collect data on the total time spent in the food cup in response to presentations of the auditory stimuli (10 s epoch beginning when the auditory stimuli are terminated).

## **4 Overview of the Sensory Preconditioning Paradigm**

4.1 Following recovery from surgery, gradually food restrict the rats to 85% of their free feeding body weight. Consult veterinary staff for an appropriate food restriction paradigm.

Note: The 3-phase sensory preconditioning paradigm is illustrated in Figure 2.

4.2 Preconditioning Sessions (Phase 1; Daily 64 min training sessions conducted across 4 consecutive days)

4.2.1. Present rats with 12 intermixed trials that consist of delivery of auditory and visual stimuli. Include inter-trial intervals that average 4.5 min (range 4.0 to 5.0 min) after each auditory presentation.

4.2.2. On six of the trials, present a tone (preconditioned stimulus) for 10 s, followed immediately by a 5 s presentation of the flashing light stimulus.

4.2.3. On the other six trials, present the white noise (unpaired stimulus) alone for 10 s.

4.3. Conditioning sessions (Phase 2; Daily 64 min training sessions conducted across 5 consecutive days)

4.3.1. Present rats with 8 trials that consist of delivery of the visual stimulus (the flashing light stimulus) for 5 s followed immediately by delivery of two 45 mg food pellets. Include inter-trial intervals that average 7 min (range 6.0 to 8.0 min) after each trial. Occasionally rats require more than 5 conditioning sessions to learn the light → food association.

4.4. Test Session (Phase 3; a single 78-min session)

4.4.1. Present the rats with 12 intermixed trials that consist of delivery of the auditory stimuli alone. Include inter-trial intervals that average 4.5 min (range 4.0 to 5.0 min) after each auditory presentation.

4.4.2. On six of the trials, present the tone stimulus (the preconditioned stimulus) alone for 10 s.

4.4.3. On the other six trials, present the white noise (the unpaired stimulus) alone for 10 s.

4.5. Counterbalance the stimuli by completing the study with a second set of rats that receive the white noise as the preconditioned stimulus and the tone as the unpaired stimulus.

## 5. Pharmacological and Behavioral Procedure

5.1. Turn on the power to the behavioral apparatus and load the Med-PC code for the appropriate behavioral session. Wire and program the behavioral apparatus such that the fans that are mounted on the sound attenuating cabinets turn on when the power is switched on.

5.2. To prepare a 1 mg/mL solution of clozapine N-oxide (CNO), weigh 5.0 mg of CNO into a 15 mL conical tube and add 5 mL of sterile water or 5 mL of sterile 0.9% saline. Vortex until the solution is clear.

If CNO does not dissolve into solution or if more concentrated solutions of CNO are desired add a solubilizing agent, such as DMSO<sup>23</sup>. Specifically, to prepare a 1 mg/ml solution of CNO with 0.5% DMSO, weigh 5.0 mg of CNO and add 25 µL of DMSO to a 15 mL conical tube. Flick gently ensuring that the contents remain at the base of the tube. When the solution becomes clear, add 5 mL of sterile water or 5 mL of sterile 0.9% saline. Further instructions for preparing solutions of CNO are available on the DREADD wiki resource page<sup>25</sup>

5.2.1.

5.2.2. Prepare a new CNO solution each day that it is administered.

5.3. Inject the CNO intraperitoneally at a dose of 1 mg/kg approximately 30 min prior to behavioral testing. For example, inject a 200 g rat with 0.2 mL of the 1 mg/mL CNO solution. The dose and time course of CNO administration were selected based on previously published reports<sup>2, 12</sup>.

5.3.1. Inject CNO 30 min prior to each preconditioning session (Phase 1; total of 4 days of injections) but administer the vehicle prior to all subsequent behavioral sessions.

5.4. After 30 min, place the rats into the behavioral testing chambers and initiate the computer code for the appropriate behavioral session.

5.5. Upon completion of the behavioral task, immediately remove the rats from the chambers and return the rats to the animal colony.

## 6. Analyses of Behavioral Data

NOTE: The dependent variable for all behavioral sessions is the amount of time that the rat's head is inside the food cup as detected by interruption of the infrared photocells in the food cup. The data (in sec) are collected and recorded by the computer software.

6.1. Do not conduct analyses on data generated during the Preconditioning phase.

6.2. For the conditioning sessions: analyze each rats' ability to learn the light → food association by comparing food cup behavior during presentation of the visual stimulus across the 5 behavioral sessions. Specifically, calculate the average time spent in the food cup during the 5 s light epoch for each of 8 trials/session (*i.e.*, an average of 8 trials that are each 5 sec in duration/rat). Compare average control and experimental values for each of 5 daily sessions (see Figure 3A).

6.2.1. Analyze the rats' motivation to obtain food reward by comparing food cup behavior during delivery of the food reward (5 s epoch) across the 5 behavioral sessions using the same calculation as specified for the 5 s light epoch above. These values will be consistently higher than those acquired during presentation of the visual stimulus (see Figure 3B).

6.3. For the test session: calculate a discrimination ratio that describes the rats' food cup behavior in response to presentations of each auditory stimulus.

6.3.1. Specifically, for each rat, divide the average time spent in the food cup following each of 6 presentations of the sensory preconditioned stimulus (*i.e.*, the tone) by the sum of the average time spent in the food cup following each of 6 presentations of the sensory preconditioned stimulus (*i.e.*, the tone stimulus) plus the average of the total time spent in the food cup following each of 6 presentations of the unpaired stimulus (*i.e.*, the white noise stimulus). Each epoch is 10 s in duration.

Note: A discrimination score of 0.5 or greater demonstrates that rats learned the associations inherent in the sensory preconditioning paradigm.

## 7. Verification of AAV Placement and Expression

7.1. Anesthetize and perfuse the rats transcardially using 4% paraformaldehyde. Due to the use of fluorescent labels, do not exceed a post-fixation time of 2 h to minimize loss of antigenicity and to preserve the fluorescent signal.

7.2. Dehydrate perfused brains by transferring them into a brain jar containing 30 mL of a 20% sucrose solution in phosphate buffered saline (1X) for a minimum of 2 days or until the brain sinks to the bottom of the brain jar.

7.3. Use a freezing sliding microtome to make coronal brain sections (20 - 40  $\mu$ m) throughout the entire rostrocaudal extent of the region of interest.

7.4. Conduct immunohistochemistry directed against the tagged receptor or the reporter protein to ascertain the location and expression of the designer receptor and/or reporter<sup>12</sup>. An immunohistochemistry staining protocol is provided on the DREADD wiki resource page<sup>25</sup>

7.5. Mount the sections onto superfrost-plus slides and coverslip using 100 to 200  $\mu$ L of an aqueous mounting medium.

7.6. Perform fluorescence microscopy on brain tissue to verify AAV placement and protein expression<sup>12, 24</sup>.

## REPRESENTATIVE RESULTS:

### Behavioral Results

Upon completion of the experiment, the effectiveness of the region-specific temporary inactivation should be quantitatively and qualitatively assessed. The present example involves a 3-phase behavioral paradigm (sensory preconditioning), in which CNO was administered to attenuate neural activity in the RSC during the Preconditioning sessions to test the hypothesis

that the RSC is necessary for the formation of associations among neutral stimuli<sup>12</sup>. Importantly, experimenters are not limited to the behavioral paradigm or experimental design described herein as the pharmacogenetic approach can be coupled with most behavioral paradigms.

Whereas analyses are not typically performed on data generated during the Preconditioning sessions (Phase 1), it is important to quantify whether rats learned the light → food association during the Conditioning sessions (Phase 2). As shown in Figure 3A, both experimental (Expt) and Control (Ctrl) rats demonstrate increasing food cup behavior during presentations of the light stimulus (Figure 3A) indicating that rats acquired a Pavlovian association between the visual stimulus (light) and the food reward. In addition, both groups demonstrate increasing food cup behavior during presentation of the food reward (Figure 3B) indicating equivalent motivation to obtain reward. During the critical Test session, when the auditory stimuli are presented in the absence of other stimuli, control rats have a discrimination score that is significantly different from experimental rats (Figure 3C). Visual inspection of the graph reveals that the average discrimination ratio of control rats is greater than 0.5, indicative of greater food cup behavior in response to presentations of the auditory stimulus that was paired with the light (during Preconditioning) compared to their food cup behavior in response to presentations of the auditory stimulus that was unpaired (during Preconditioning). In contrast, experimental rats fail to demonstrate a difference score that is above chance. Thus, Figure 3C demonstrates that control but not experimental rats showed the sensory preconditioning effect.

### Verification of AAV Placement

At the completion of behavioral testing, analyze rat brain tissue for correct placement and expression of the designer receptor. Conduct immunohistochemistry<sup>12, 25</sup> using primary antibodies directed against a receptor tag (*e.g.*, an anti-HA primary antibody) or the fluorescent reporter (in this example, mCitrine which is detected by an antibody to Green Fluorescent Protein (GFP)). A schematic of a coronal section through the rat brain is shown in Figure 4A. Figure 4B illustrates robust fluorescent immunodetection of the reporter protein in the RSC in a representative experimental rat with no fluorescent label detected in surrounding regions. Figures 4C and 4D illustrate representative fluorescent labeling of reporter proteins in experimental (Expt; rats were infused with an AAV construct containing the designer receptor gene and the mCitrine gene) and control (Ctrl; rats were infused with a similar AAV construct containing the GFP gene but no sequence for the designer receptor) rats, respectively. Receptor levels can also be displayed as minimum, representative and maximal expression<sup>24</sup>. If labeling is detected in regions outside of the area of interest, exclude those data from the behavioral analyses.

**Figure 1. Schematic diagram of the AAV construct.** A diagram of the *hSyn-HA-hM4D(Gi)-IRES-mCitrine* AAV used to express the inhibitory designer receptor (hM4Di) under a neuronal specific synapsin promoter (hSyn) in RSC neurons. Immunofluorescent reporters (HA-tag and/or mCitrine) can be used to visualize expression of the protein and reporter, respectively. hSyn, human synapsin promoter; specifies expression in neurons. HA, hemagglutinin tag; this tag is fused to the designer receptor and serves as an epitope tag enabling detection of receptor expression. hM4Di, the gene for the designer inhibitory G-protein coupled receptor. IRES,

internal ribosome entry site; allows the reporter (mCitrine) to be translated. mCitrine, a fluorescent reporter that is a variant of GFP but is more resistant to photobleaching.

**Figure 2. Schematic diagram of the sensory preconditioning paradigm.** (A) During the Preconditioning sessions, 12 intermixed trials are presented. During 6 of the trials, an auditory stimulus is presented for 10 sec followed immediately by a flashing house light stimulus (2Hz, for 5 sec). During the other 6 trials, a second auditory stimulus is presented without a visual stimulus. (B) During the Conditioning sessions, the previously paired visual stimulus is paired with food reward. (C) During the Test session, there are 12 intermixed presentations of the two auditory stimuli in the absence of visual stimuli or food.

**Figure 3. Behavioral results.** Average food cup responding during the (A) Light and (B) Food epochs during the Conditioning sessions (Phase 2). Data were analyzed using repeated measures analysis of variance. (C) Discrimination ratios during the Test session (Phase 3). The dotted line indicates equal amounts of conditioned food cup responding to each auditory stimulus (*i.e.*, no sensory preconditioning) Data were analyzed using independent samples t-test. Expt; experimental rats (n = 17) infused with an AAV construct containing the DNA sequence for the inhibitory G-protein coupled designer receptor (hM4Di) and the DNA sequence for a fluorescent reporter (mCitrine). Ctrl; control rats (n = 6) infused with hM4Di and administered vehicle combined with control rats (n = 4) infused with an AAV virus that does not contain the designer receptor and that were administered CNO. Control groups did not significantly differ from each other ( $p > 0.05$ ). Error bars denote  $\pm$  SEM.

**Figure 4. Histological verification of protein expression.** (A) A schematic illustrating the location of the RSC in a coronal section through the rat brain. (B) Representative images of immunohistochemically labeled rat brain tissue from an experimental rat (Expt) that was infused with an AAV construct containing the DNA sequence for the inhibitory G-protein coupled designer receptor (hM4Di) and the DNA sequence for a fluorescent reporter (mCitrine). mCitrine is a highly fade-resistant variant of green fluorescent protein. (C) Representative image from an experimental rat illustrating the location of the fluorescent reporter label (mCitrine). (D) Representative image from a control rat (Ctrl) infused with an AAV construct containing the DNA sequence for a fluorescent reporter (enhanced GFP). Scale bars: B, 500  $\mu$ m; C and D, 100  $\mu$ m.

## DISCUSSION:

This protocol describes how to apply a pharmacogenetic approach (DREADD) to investigate how a specific brain region contributes to a multi-phase complex learning task. With the ability to temporarily and remotely silence neural activity in discrete brain regions across phases of learning, this combination of approaches provides a platform to investigate a wide range of behaviors, including more nuanced or masked forms of learning. In the example described in this protocol, control rats and rats that express the designer receptor in the retrosplenial cortex (RSC) were tested in a 3-phase behavioral task<sup>12</sup>. The first phase of the task involved presentation of multiple neutral stimuli with the assumption that control rats would acquire a stimulus-stimulus association between two of the stimuli. The working hypothesis is that RSC is

necessary for stimulus-stimulus learning, thus, on each of 4 conditioning days, 30 min prior to the start of behavioral testing, control and experimental rats were given systemic administration of either the designer drug (CNO) or vehicle. When bound to the designer receptor, CNO reduces the activity of neurons in which that receptor is expressed. During the remaining phases of behavioral conditioning and testing, when the RSC is not hypothesized to influence learning, CNO was not administered and thus neural activity was not disturbed.

### **Critical Steps within the Protocol**

*Safe handling of AAV compounds:* The surgical procedures involved in the pharmacogenetic approach are no more technically demanding than a simple stereotaxic infusion, however, the use of AAV at some institutions requires that experimenters adhere to BSL-2 precautions. It is critical that investigators follow the guidelines established by the Centers for Disease Control, funding agencies, home institutions and other oversight committees specific to their research program. Information on the safe handling of AAVs is readily available<sup>13</sup>.

*Preparation and administration of the designer drug:* CNO, the designer drug, binds to the designer receptor and silences neural activity but is otherwise biologically inert<sup>1,3</sup>. Shipments of CNO from suppliers can vary in consistency. The compound should arrive as a powder that is not adhered to the sides of the container.

*Important control groups to consider:* To control for non-specific effects of the designer drug, prior to behavioral testing, infuse a different set of experimental rats (*i.e.*, those that express the designer receptor) with vehicle injections instead of CNO. In addition, to control for non-specific effects of the designer receptor include a group of rats that are infused with a control virus that contains a fluorescent reporter but not the modified designer receptor and inject these rats with the designer drug (*i.e.*, CNO). Ensure adequate experimental design by counterbalancing across groups.

*Verifying expression of the construct:* There are a number of ways to maximize the expression and detection of the designer receptor. Prior to infusion, verify that the viral titre is near  $10^{12}$  particles/ml. Often visualization of the fluorescent reporter is low and thus, it is recommended that immunohistochemistry be performed on the region of interest using antibodies directed against the reporter(s) included in the viral construct<sup>25</sup>. In this example, anti-GFP immunoreactivity provides a robust signal (Figures 4B-D). Importantly, because of the nature of fluorescent labels, limit the length of fixation time to 2 h.

### **Advantages of the Techniques**

Once the viral construct has been delivered to the brain via stereotaxic surgery, the pharmacogenetic approach allows for the temporary inactivation of brain activity in discrete regions by means of a minimally invasive systemic injection of the designer drug. Drug administration can occur repeatedly, which is advantageous for behavioral tasks that occur across successive days or weeks<sup>12, 24, 26</sup>. Furthermore, evidence indicates that the designer drug (CNO) does not interfere with locomotor behavior or appetite<sup>27, 28</sup>. Thus, the method provides the opportunity to attenuate neural activity for a short period of time (2 – 5 h), while avoiding

the stressors inherent in other methods of temporary inactivation. Specifically, in cannulation studies, temporary inactivation is achieved by delivery of neurotoxins through cannulae that are permanently affixed to the skull. This approach is limited in that keeping the cannula clear of debris and protected is challenging. Furthermore, to induce inactivity, animals are handled extensively (to administer the toxin through the cannulae) just prior to behavioral testing, which imposes stress on the animal and also increases the likelihood that cannulae may dislodge from the skull. Because the effects of CNO are relatively short term, the possibility of long term compensatory mechanisms (such as those observed following permanent lesions or in genetically engineered mice) are minimized<sup>29, 30</sup>.

### **Limitations of the Techniques**

DREADD is a relatively new method of non-invasively attenuating neuronal activity. As such experimenters may be inclined to independently verify that neuronal silencing occurs in their preparation. Verification can be performed using electrophysiological approaches, but these experiments are time consuming, costly and require specific expertise. In addition, while chemogenetic techniques are less costly than optogenetics, the approach is more expensive than traditional inactivation with pharmacological agents such as TTX. Another limitation of the DREADD approach is that infusion of the viral constructs does not result in 100% infection of neurons in the region of interest, nor does inactivation via CNO lead to 100% reduction of neuronal activity. Lastly, some AAV serotypes can be retrogradely transported which can complicate interpretation of experimental results<sup>2</sup>.

### **DISCLOSURES:**

The authors declare that they have no competing financial interests.

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Figure 2 - Sensory Preconditioning Paradigm  
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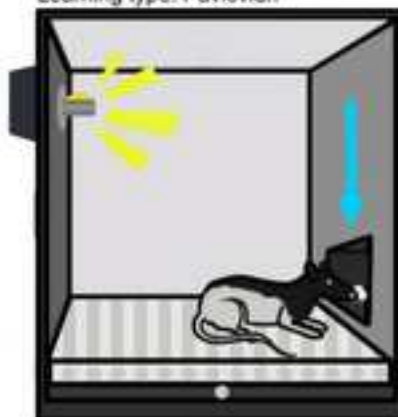
**A Phase 1: Preconditioning**  
12 trials, 4 days

Learning type: Stimulus-Stimulus Association  
CNO is administered during this phase.



**B Phase 2: Conditioning**  
8 trials, 5 days

Learning type: Pavlovian



**C Phase 3: Testing**  
12 trials, 1 day

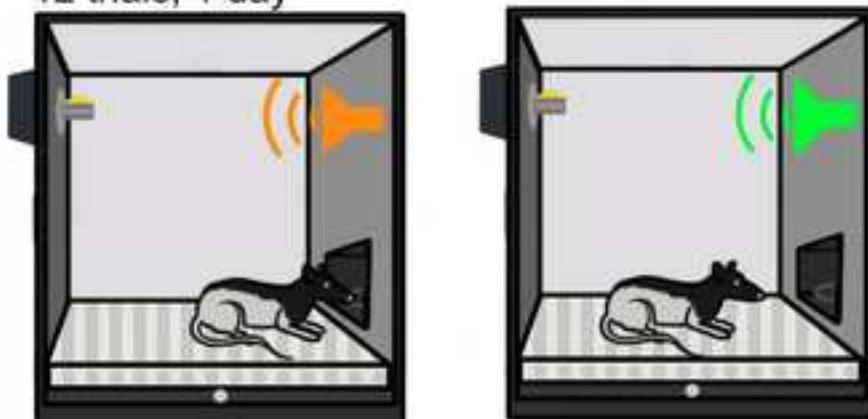
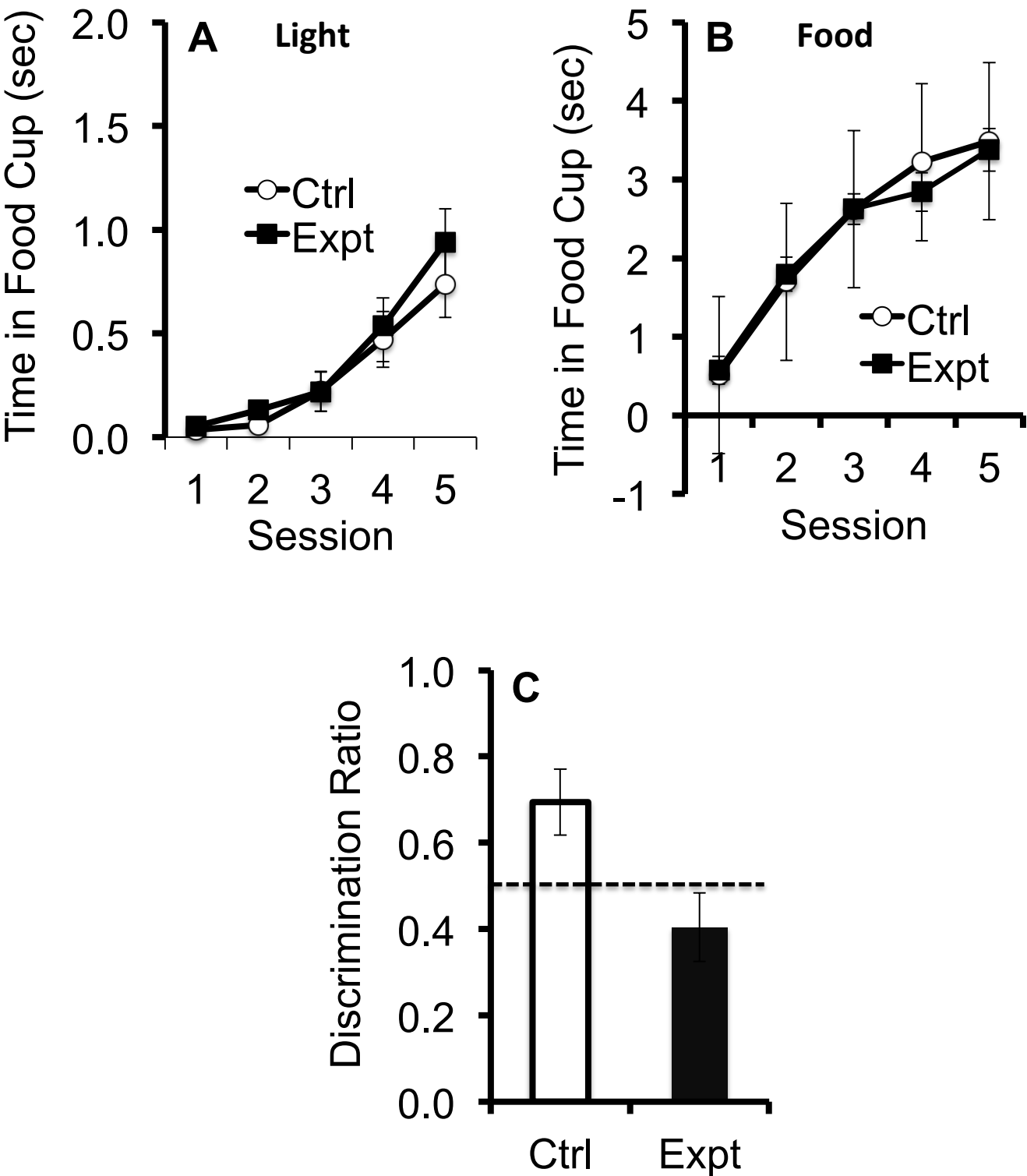
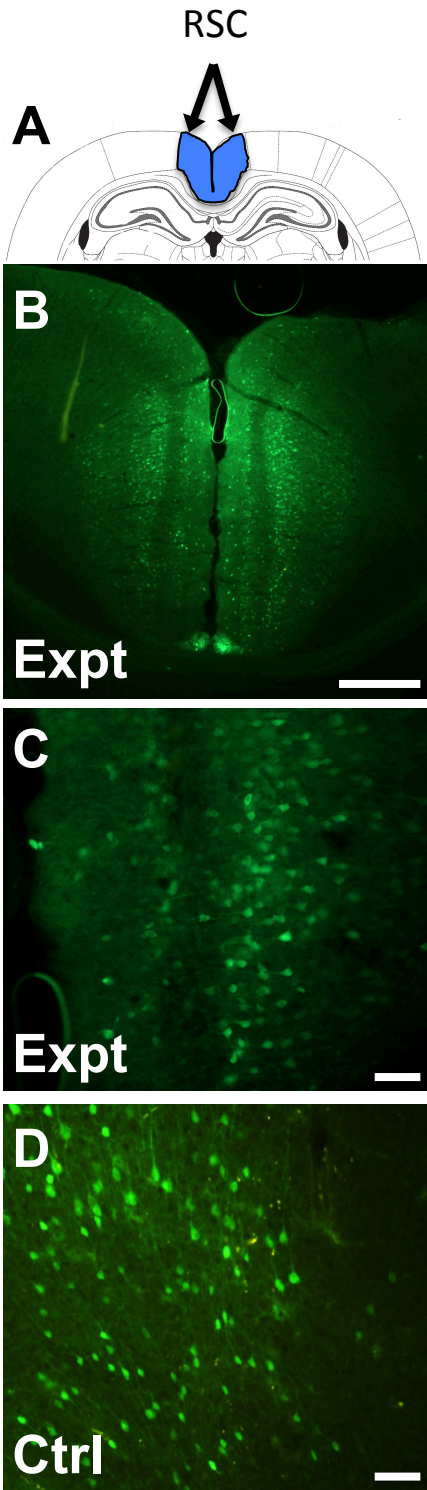


Figure 3 - Behavioral data  
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<b>Name of Material/ Equipment</b>	<b>Company</b>
Male, Long Evans Rats, 55-60 d	Hilltop Lab Animals Inc
rAAV8/hSyn-HA-hM4D(Gi)-IRES-mCitrine	Virus Vector Core
rAAV8/hSyn-GFP	Virus Vector Core
Clozapine-N-Oxide	R&D Systems
Disposable Polypropylene shoe covers	Sara Glove Company
Disposable Polypropylene lab coats	Sara Glove Company
Particulate Respirator N95	3M
Rat Cage lid (Polycarbonate)	Alternative Design
Filer Paper (Replacement)	Alternative Design
Table Top Vise	JETS
Medline Biohazard liners	Staples
United Solutions 34 gallon rectangular wheeled trashcan with hook and lock handle	Amazon.com
Isoflurane, 100 mL	Patterson Veterinary Supply Inc.
Dual Small Animal Stereotaxic with Digital Display Readout Console	David Kopf
Non-rupture Ear Bars, set of 2 (Rat)	David Kopf
Anesthesia Mask (Rat)	David Kopf
High speed Stereotaxic drill includes table top motor controller, foot pedal, handpiece, stereotaxic handpiece holder	David Kopf
Microdrill burrs, 0.009 mm	Fine Science Tools Inc
Automated Syringe pump with Micro4 Controller	David Kopf
Pro-animal detachable Ceramic Blade Clipper Kit	Ahdis
Betadine Skin Cleanser	Perdue Products L.P
Triple Antibiotic Ointment	Medline Supply
Puralube Vet Ointment	Only Veterinary Supply
Dino-lite	Microscope
Dino-lite Rigid TableTop Boom Stand	Microscope
28 Gauge 10uL Syringe	Hamilton
Extra Tall MDF Sound attenuating Cubicle	Med Associates, Inc
Extra Tall Modular Test Chamber	Med Associates, Inc
Stainless Steel Grid Floor	Med Associates, Inc
House Light	Med Associates, Inc
Modular Pellet Dispenser	Med Associates, Inc
Pellet Recetacle, Cup Type	Med Associates, Inc
Head Entry Detector for Rat	Med Associates, Inc
Dustless precision food pellets, 45 mg	Bio-Serv
Cage Speaker for Rat Chamber	Med Associates, Inc

Programmable Audio Generator  
Smart Ctrl 8 Input/16 output Package  
Large Table Top Cabinet and Power Supply  
PCI Interface Package  
MED Intel core Computer Pkg with X Pro 19" Monitor  
Paraformaldehyde (grannular), 1 kg  
Rabbit Monoclonal antibody (HA-Tag)  
XP Rabbit monoclonal antibody (GFP)  
Anti-Rabbit IgG  
Superfrost Plus slides

Med Associates, Inc  
Med Associates, Inc  
Med Associates, Inc  
Med Associates, Inc  
Med Associates, Inc  
Electron Microscopy Sciences  
Cell Signaling Technologies  
Cell Signaling Technologies  
Cell Signaling Technologies  
VWR international

Catalog Number	Comments/Description
4936-10	Caution: This is a BSL-2 compound Caution: This is a BSL-2 compound Designer Drug Personal Protection Equipment Personal Protection Equipment
8210	Personal Protection Equipment
FT 8XL-PC	Used to cover animal cages 48-72 hours post infusion
FP-R-1018XAD	Filter paper that goes with cage lids
2201-265	For holding microcentrifuge tubes containing AAV in the hood
113444	Biohazard Trash Bags  Biohazard Trash Can
07-890-8540	Anesthetic
Model 942	Surgical equipment
Model 955	Surgical equipment
Model 906	Surgical equipment
Model 1474	Surgical equipment
19007-09	Surgical supply
Model UMP3-1	Surgical equipment
21420	Surgical supply
67618-149-04	Surgical supply
53329-087-01	Surgical supply
17033-211-38	Surgical supply
AD7013MTL	An alternative to the traditional dissection scope
MS36B	Surgical equipment
80308-701SN	Surgical equipment
ENV-018MD	22'Wx22"Hx16"D
ENV-007	Behavioral equipment
ENV-005	Behavioral equipment
ENV-215M	Used as the house light and stimulus light
ENV-203M-45	Behavioral equipment
ENV-200R1M	Behavioral equipment
ENV-254-CB	Behavioral equipment
F0165	Behavioral supply
ENV-224AM	Behavioral equipment

ANL-926	Behavioral equipment
DIG-716P2	Behavioral equipment
SG-6510D	Behavioral equipment
DIG-700P2-R2	Behavioral equipment
COM-103V	Behavioral equipment
19210	Hazard: carcinogen, weigh in hood
3724S	Histology reagent
2956S	Histology reagent
4412S	Histology supplies
483111-703	Histology supplies



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Siobhan Robinson

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Department of Neuroscience

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## Oberlin College

Department of Neuroscience  
119 Woodland Street  
Oberlin • Ohio • 44074

## Siobhan Robinson, Ph.D.

Assistant Professor of Neuroscience  
Telephone: (440) 775-6258  
Fax: (440) 775-5397  
E-mail: [Siobhan.Robinson@Oberlin.edu](mailto:Siobhan.Robinson@Oberlin.edu)

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1. Proofread

**Response:** The document has been thoroughly copy edited. Several changes were made throughout and are indicated in red font.

2. Include DOIs when available

**Response:** DOIs have been added.

## **REVIEWER 1**

**Major concerns:** None

**Minor concerns:**

1. Modify line 100 pertaining to “neural activation in RSC is attenuated...”

**Response:** Reviewer 1 is correct in that this manuscript does not show attenuation of neuronal activity. The attenuation effect has been omitted from **line 110** and a discussion of verification of attenuation of electrophysiological activity has been added to the section of the Discussion pertaining to Limitations of the techniques (**line 619**).

2. Modify Figure 1 so that the viral construct does not look like a plasmid.

**Response:** Figure one has been modified; the viral vector is now displayed as linear.

3. Modify the legend for Figure 3 by including the number of replicates per group and including the meaning of the error bars (STDEV or SEM).

**Response:** The Figure 3 legend has been adjusted as specified (**line 517**).

## **REVIEWER 2**

**Major concerns:**

1. Reviewer 2 commented on the selection of antibodies used to detect mchtrine, which is the fluorescent reporter that is contained in the viral construct used in this protocol. Reviewer 2 stated that mchtrine is a

derivative of DsRed, not GFP. They continue by including that GFP antibodies should not be used to detect mchtrine protein expression.

**Response:** While we appreciate the efforts of Reviewer 2 to improve the immunohistochemical section of this manuscript, we respectfully disagree with the comments. In fact, mchtrine is a derivative of YFP, not DsRed. The bioluminescent protein, GFP was originally obtained from the Aequorea Victoria jellyfish. Because GFP, YFP and mchtrine are modifications of the same protein, GFP antibodies are well suited for their detection. In contrast, fluorescent proteins in the red family (i.e., DsRed) were derived from tropical coral reefs. Reviewer 2 is referred to the Abcam antibody website that verifies that GFP antibodies detect mchtrine: [http://www.abcam.com/gfp-antibody-ab5450.html#description\\_images\\_2](http://www.abcam.com/gfp-antibody-ab5450.html#description_images_2). Furthermore, in my personal correspondence with Bryan Roth's lab, (Dr. Roth is one of the pioneers that established the DREADD technology) they recommend the GFP antibody for the detection of mchtrine expression. This information can also be verified by viewing the August 20 entry on the chemogenetic users blogspot at <http://chemogenetic.blogspot.com/> and on the official DREADD wiki site: <http://pdspit3.mml.unc.edu/projects/dreadd/wiki/WikiStart>. These sites are maintained by the group (Roth lab) that made the viral construct used in this protocol.

2. Reviewer 2 suggested that sections of the manuscript pertaining to experimental design and the consideration of controls be expanded. They suggested that a discussion of the types of data analysis that can be coupled with chemogenetic experiments be included.

**Response:** We enthusiastically agree with the Reviewer's comments that experimental design and subsequent quantitative analyses of behavioral paradigms that involve pharmacological and/or other types of experimental manipulations is essential. Text pertaining to the importance of experimental design and analyses as well as references to publications that use DREADD in combination with a variety of behavioral designs and statistical approaches are now included in the introduction (line 84). Also included is reference to a comprehensive review of the current uses of DREADD, including a chart with 29 publications that use the many variations of the DREADD approach. In addition, a more detailed description of the controls used in the present study has been included in the Introduction (line 112). Lastly, a description of the statistical methods used to analyze the data in the present manuscript is included (line 514, 516). However, it is our strong conviction that due to the wide variety of manipulations, experimental groups and test phases that may or may not be included in any particular experiment, it would be inappropriate to include instructions as to how future experiments conducted by other investigators should be designed or quantified.

3. Reviewer 2 suggested including a description of the limitations of the chemogenetic approach.

**Response:** A paragraph describing some limitations of the chemogenetic approach was added to the discussion section (line 619).

#### **Minor concerns:**

4. Reviewer 2 requested clarification on which AAV serotypes require the use of Biosafety Level 2 precautions.

**Response:** Text describing the difference has been added to complement the existing text pertaining to safety precautions (line 117 and 162).

5. The reviewer suggested an expanded discussion of the types of DREADDs available. The reviewer requested information about why a construct with the hSyn promotor was used and where we obtained our viral vectors.

**Response:** A section has been added to the discussion describing the types of DREADDs that are available, a comprehensive review is identified and a reference to the DREADD wiki, which lists many available options for DREADDs was included (line 84). Text pertaining to the function of the hSyn promotor was included in the legend for Figure 1 (line 492). In keeping with the guidelines established by the JoVE

editorial office, the source of the DREADDs used in this protocol has been listed in the reagents section of the manuscript.

6. Minor misc. comments were addressed

**Response:** The range of the titre of the virus has been changed from  $4.2 \times 10^{12}$  to  $10^{12}$  (line 582).

**Response:** The word “isoflurane” is now spelled correctly and m-citrine was changed to mcitrine throughout the text.

**Response:** Additional advantages of the DREADD approach have been added to the introduction (line 84).

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### REVIEWER 3

**Major concerns:** None

**Minor concerns:**

**Response:** The time course for CNO treatment has been changed from 2-4 hours to 2-5 hours (line 45).

Introduction

**Response:** Additional limitations of traditional cannulation methods have been added (line 61).

**Response:** the word “that” was deleted (line 96).

**Response:** Information about the subjects that comprise the control group was added (line 112).

Procedure

**Response:** Clarification was provided about the use of a fume hood (line 199).

**Response:** A sentence describing that isoflurane anesthesia is used throughout the surgery was included (line 208).

**Response:** The word “redact” was replaced with the word “retract” (line 225).

**Response:** Because spread of the virus can vary from surgery to surgery and it may differ depending on which brain regions are targeted, an exact amount of spread of virus was not provided in this revision. However, to address the reviewers important point, text instruction experimenters to conduct pilot studies to determine the spread of the viral construct in their preparation was included (line 244).

**Response:** The word “triple” was replaced with the word “topical” (line 258).

**Response:** The metal walls of the operant chamber were described (line 275).

**Response:** Clarification about the time that the brain should be soaked in paraformaldehyde was added (line 420 & 587).

**Response:** The published immunohistochemistry protocol that is supplied by the company that produces the DREADD was referenced (line 432 & 585).

**Response:** In keeping with the guidelines established by the JoVE editorial office, a specific brand name of mounting media was not specified in the text of the manuscript, but remains included in the reagents section of the manuscript.

**Response:** We politely disagree with the reviewers suggestion to include information about the filters used for the fluorescent imaging of DREADDS because it is our contention that this information is beyond the scope of the present protocol. Furthermore, investigators can choose from a number of fluorescent secondary antibodies, which would influence the filters that would be appropriate.

### REVIEWER 4

**Major concerns:** None

**Minor concerns:**

1. The reviewer requested a rationale for the time parameter for CNO.

**Response:** A justification for the time course of CNO injection and bioactivity has been provided (line 369).

2. Similarly to Reviewer 2, Reviewer 4 suggested including a description of the limitations of the chemogenetic approach.

**Response:** A paragraph describing some limitations of the chemogenetic approach was added to the discussion section **(line 619)**.

3. Similarly to Reviewer 2, Reviewer 4 suggested including an expanded discussion of the types of DREADDs available.

**Response:** A section has been added to the discussion describing the types of DREADDs that are available and a reference to a comprehensive DREADD review, which lists many available options, was added **(line 84)**.

We look forward to hearing from you.

Respectfully,

*Siobhan Robinson*