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Non-invasive strategies for chronic manipulation of DREADD-controlled neuronal activity

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Corresponding Author:	Diego Carlos Fernandez National Institute of Mental Health Bethesda, MD UNITED STATES
Corresponding Author's Institution:	National Institute of Mental Health
Corresponding Author E-Mail:	diego.fernandez@nih.gov
Order of Authors:	Diego Carlos Fernandez
	Jesse Zhan
	Ruchi Komal
	William T. Keenan
	Samer Hattar
Additional Information:	
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TITLE:

Non-Invasive Strategies for Chronic Manipulation of DREADD-Controlled Neuronal Activity

AUTHORS AND AFFILIATIONS:

Jesse Zhan[1], Ruchi Komal[1], William T. Keenan[2], Samer Hattar[1], Diego C. Fernandez[1]

[1]Section on Light and Circadian Rhythms (SLCR), National Institute of Mental Health, Bethesda, MD, USA

[2]Howard Hughes Medical Institute, Department of Neuroscience, The Scripps Research Institute, La Jolla, CA, USA

Email Addresses of Co-authors:

Jesse Zhan (jesse.zhan@nih.gov)
Ruchi Komal (ruchi.komal@nih.gov)
William T. Keenan (keenanw27@gmail.com)
Samer Hattar (samer.hattar@nih.gov)

Corresponding Author:

Diego C. Fernandez (diego.fernandez@nih.gov)

KEYWORDS

non-invasive methods, chronic CNO, chemogenetic, DREADDs, remote neuronal control, eye-drops, drinking water, mice

SUMMARY

Here we describe two non-invasive methods to chronically control neuronal activity using chemogenetics in mice. Eye-drops were used to deliver clozapine-N-oxide (CNO) daily. We also describe two methods for prolonged administration of CNO in drinking water. These strategies for chronic neuronal control require minimal intervention reducing animals' stress.

ABSTRACT

Chemogenetic strategies have emerged as reliable tools for remote control of neuronal activity. Among these, designer receptors exclusively activated by designer drugs (DREADDs) have become the most popular chemogenetic approach used in modern neuroscience. Most studies deliver the ligand clozapine-N-oxide (CNO) using a single intraperitoneal injection, which is suitable for the acute activation/inhibition of the targeted neuronal population. There are, however, only a few examples of strategies for chronic modulation of DREADD-controlled neurons, the majority of which rely on the use of delivery systems that require surgical intervention. Here, we expand on two non-invasive strategies for delivering the ligand CNO to chronically manipulate neural population in mice. CNO was administered either by using repetitive (daily) eye-drops, or chronically through the animal's drinking water. These non-invasive paradigms result in robust activation of the designer receptors that persisted throughout the CNO treatments. The methods described here offer alternatives for the chronic DREADD-

mediated control of neuronal activity and may be useful for experiments designed to evaluate behavior in freely moving animals, focusing on less-invasive CNO delivery methods.

INTRODUCTION

Technical advances in the field of neuroscience have allowed scientists to precisely identify and control the activity of particular neuronal populations[1]. This has contributed to better understand the basis of neuronal circuits and their impact on animal behavior, as well as, revising established dogmas[2,3]. Among these novel tools, optogenetic and chemogenetic strategies have had a profound impact not only on the quality of discoveries but also on the way experiments are conceived and designed[4]. In the present manuscript, we focus on chemogenetic strategies for controlling the activation of neurons via engineered receptor–ligand strategies. Designer receptors exclusively activated by designer drugs (DREADDs) represent one of the most popular chemogenetic tools for the remote control of neuronal activity, as reviewed by Roth 2016[5]. DREADDs utilize modified muscarinic acetylcholine receptors that are specifically activated by an inert ligand, clozapine-N-oxide (CNO)[6].

Most studies use CNO administered by intraperitoneal (i.p.) injections, which effectively controls the dosage and timing of engineered receptors activation in an acute fashion. However, when repetitive or chronic DREADD activation is required, the use of multiple i.p. injections become unfeasible. To address this issue, different strategies for the chronic CNO delivery have been reported, including implanted minipumps[7] and intracranial cannulas[8,9]. To different extents, all these strategies cause the animals stress and pain[10], and require a surgical intervention that could also have a direct impact on the behavioral responses to be tested[11]. Here, we describe three non-invasive strategies for the chronic CNO delivery. For this purpose, mice were stereotaxically injected in the hippocampus with an adeno-associated virus (AAV) encoding an engineered version of the excitatory M3 muscarinic receptor (hM3Dq) that when activated by the ligand CNO leads to the burst-like firing of neurons[6]. It was previously shown that a single eye-drop containing CNO can effectively elicit a robust activation of DREADD-expressing neurons[12].

Here we describe a modified method for the repetitive delivery of eye drops. To achieve chronic and sustained control of the designer receptors, we next describe a non-invasive strategy to deliver CNO to mice through the drinking water. Finally, we describe an alternative paradigm for delivering CNO in drinking water during a restricted time window. Mice locomotor activity, as well as drinking behavior and the consumption of sweet caloric solutions, are mostly restricted to the dark portion of the light/dark cycle[13,14]. Therefore, we adopted a protocol based on the mouse's preference for sucrose. By measuring the induction of the immediate-early gene c-Fos in AAV-infected cells, as a readout for neuronal activation[12,15], we found that these CNO delivery strategies robustly activate DREADD-controlled neurons over extended durations.

PROTOCOL

All animals were handled in accordance with guidelines of the Animal Care and Use Committees of the National Institute of Mental Health (NIMH). All efforts were made to minimize the pain and the number of animals used.

1. Adeno-associated virus injections in the hippocampus

NOTE: Wild type male mice of mixed background (B6/129 F1 hybrid, 3 months old) were for stereotaxic injection of an AAV encoding the M3 muscarinic receptor (hM3Dq) into the hippocampus. During the entire experiment, mice were single-housed, under a regular 12 h light: 12 h dark (T24) cycle, with access to food and water *ad libitum*.

1.1. Before performing stereotaxic surgeries, clean and sterilize the stereotaxic frame and all needed instruments.

1.2. Deeply anesthetize the mouse using isoflurane. To do this, first adjust the oxygen flow meter to approximately 1.5 L/ min, and then adjust the isoflurane vaporizer to approximately 3-5% for induction and approximately 1-3% for maintenance.

1.2.1. To ensure that the animal is fully unconscious, pinch the mouse's paw; the animal is properly anesthetized when the flinching response to pinch is absent.

1.3. Place the mouse on a heating pad to maintain the stability of the mouse's body temperature.

1.4. Fix the head of the mouse to the stereotaxic frame, shave the top of the head, clean the surface by scrubbing with povidone-iodine and 70% ethanol, and expose the skull using a sterile scalpel.

1.5. Calibrate the frame to bregma point, then drill at a medial-lateral coordinate of 2.9 mm and an anterior-posterior coordinate -2.7 mm to target the hippocampus.

NOTE: If other brain target needs to be injected, determine the desired coordinates for injection using the Paxinos and Franklin mouse atlas[16].

1.6. Once the brain is exposed, unilaterally inject 90 nL of the AAV at the dorsal-ventral depth of -3.0 mm in the hippocampus using a microinjector and pulled microcapillary pipettes (**Figure 1A**).

NOTE: See **Table of Materials** for the titer of AAV used in this experiment. For other brain areas, adjust the AAV volume of injection as needed.

1.7. At the end of the surgical procedure, close the incision with nylon sutures and apply topical antibiotics to the wound site.

1.8. Administer analgesics (buprenorphine, 0.1 mg/kg) systemically before and after surgery.

1.9. Beginning 4 weeks post-injection, subject mice to any of the paradigms described in the following section to chronically control neurons expressing the designer excitatory receptor.

2. Repetitive CNO delivery using eye-drops

2.1. Acclimate the animals to handling by scruffing each mouse 3 min daily for 3-4 days prior to the administration of eye-drops.

2.2. Dissolve Clozapine-N-oxide (CNO, 5 mg) in 1 mL of sterile 0.9% saline solution (stock solution: 5 mg CNO/ mL). Keep the solution refrigerated at 4 °C.

2.3. Weigh each mouse before starting the experiment to determine the amount of CNO to be delivered. Use 1-3 µL drop (per eye) to achieve 1.0 mg CNO/ kg body weight.

NOTE: As an example, a 20 g mouse should receive bilateral (2 µL each) eye-drops.

2.4. Deliver the eye-drops during the inactive (light) phase of mice, 2 h before lights turn off (*zeitgeber time* (ZT) 10). In cases where CNO needs to be delivered during the active (dark) phase of mice, ensure the presence of dim red light for proper animal handling.

NOTE: Precautions should be taken to avoid disrupting the circadian (and light/dark) cycle of experimental animals.

2.4.1. Using a P10 micropipette, load the required amount (1-3 µL) of CNO solution to achieve 1.0 mg CNO/ kg.

NOTE: Use a new and sterile pipette tip for each eye-drop. In this set of experiments, bilateral eye-drops of CNO were performed; however, if a lower CNO concentration is required, unilateral eye-drops could be also applied.

2.4.2. Immobilize the mouse via scruff.

2.4.3. Slowly expel the solution until a stable droplet forms on the pipette tip.

2.4.4. Carefully bring the droplet close to the cornea of the mouse's eye until the solution is delivered. The pipette tip should never contact the mouse's eye.

2.4.5. Release the mouse, placing it back in its home cage.

2.5. Repeat this procedure every day for 5 days.

NOTE: This duration can be adjusted as per the experimental requirements.

2.6. For control experiments, use AAV/DREADD-injected mice subjected to sham treatment (eye-drops containing only saline solution), and mice injected with an empty vector (e.g., AAV/mCherry) exposed to the described CNO eye-drops protocol.

3. Chronic CNO treatment delivered through drinking water

3.1. Make small bottles using 50 mL (plastic) conical tubes and rubber stopper spouts; cover with aluminum foil to avoid any light-mediated effects on CNO stability.

3.2. Three days before starting with the CNO treatment, replace regular water bottles with small bottles, containing 10 mL of regular water, to allow mice to acclimate to them. Secure the bottles to the cages using tape.

3.3. Measure the daily water consumption for each mouse.

3.4. Weigh each mouse before starting the experiment.

3.5. Dissolve Clozapine-N-oxide (CNO, 5 mg) in 1 mL of 0.9% sterile saline solution. Refrigerate the stock solution at 4 °C.

3.6. Use the body weight and the average amount of water consumed to define the concentration of CNO solution to achieve 1.0 mg CNO/ kg (body weight).

NOTE: Adult male mice (~20 g body weight) consume ~5 mL of water per day (**Figure 2A**). Therefore, to achieve a CNO concentration of 1 mg CNO/ kg, 6.4 µL of CNO stock solution should be added to a final volume of 8 mL of water (final concentration: 4 µg CNO/ mL). Thus, the dose of CNO for a 20 g animal that drinks 5 mL water per day results in 1 mg CNO/ kg.

3.7. Determine the optimal CNO dose that displays the maximum effectiveness with minimal CNO concentration by testing a range of concentrations. Perform a dose response analysis to determine the optimal CNO dose for the drinking water method.

NOTE: The following CNO doses were tested for this experiment: 1.0 mg/ mL, 0.5 mg/ mL, 0.25 mg/ mL, 0.1 mg/ mL, and saline. 1.0 mg CNO/ kg was first tested, based on i.p. and eye-drops protocols.

3.8. On day 1, fill the bottle with 8 mL of regular water and add the required amount of CNO.

NOTE: This amount of water is enough for 24 h of *ad libitum* water access for an adult male mouse. In case other rodent species are used, first measure the amount of water consumed daily to determine the volume needed.

3.9. Monitor the health of the animals throughout the protocol to ensure that there are no adverse side effects caused by water + CNO consumption.

3.10. After 24 h, replace the bottles with fresh water + CNO solution. Record the volume consumed during the previous day.

3.11. Dispose of the water + CNO solution that was not consumed in waste containers. Discard plastic bottles and replace the rubber stoppers every day, after sanitizing them according to the animal facility guidelines.

NOTE: Do not mix aqueous wastes with organic solvents. Contact the Chemical Disposal Service for instructions for storage and pick-up.

3.12. Replace the bottles at the same time every day for 5 days.

NOTE: This duration can be adjusted as per the experimental requirements.

3.13. Include control groups, as described in step 2.6.

4. Restricted CNO treatment using mice's preference for sucrose

4.1. 3 days before starting with the CNO treatment, place a small bottle containing 10 mL of water + 1% sucrose on the cage, preferably away from the original water bottle.

NOTE: Use the same small bottles described in Step 3.1.

4.2. Expose animals to water + 1% sucrose during the last portion of their active phase (ZT 18 – 24). After this exposure, remove the bottle with water + sucrose from the cage.

NOTE: Different time windows of CNO delivery could be used. Additionally, mice could be placed under an inverted light/dark cycle, where the onset of light occurs in the evening hours to facilitate the CNO delivery.

4.3. Measure the daily water + 1% sucrose consumption for each mouse.

4.4. Weigh each mouse before starting the experiment.

4.5. Use the body weight and the average amount of water + 1% sucrose consumed to determine the dose of CNO solution to achieve 1.0 mg CNO/ kg (body weight).

NOTE: The optimal CNO dose that displays the maximum effectiveness with minimal CNO concentration should be tested, as explained in step 3.6.

4.6. On day 1, fill bottles with 5 mL of water + 1% sucrose + CNO (1 mg CNO/ Kg) and place them on the cage (always at the same location) during the determined time window.

4.7. At the end of the restricted time window, remove the bottles and measure the amount of water + sucrose + CNO consumed.

NOTE: Materials and solutions are sanitized or discarded as previously described in step 3.11.

4.8. Repeat this procedure every day for 5 days.

NOTE: This duration can be adjusted as per the experimental requirements.

4.9. Include a control group, as described in step 2.6.

5. Data analysis

5.1. Perfuse mice intracardially with 4% paraformaldehyde (PFA) either 2 or 6 h after receiving the last repetitive (5th day) CNO eye-drop. When CNO is delivered through drinking water, replace CNO + water with water at the end of the mouse's active phase, then perfuse the mouse after either 2 or 6 h post-CNO access.

NOTE: If light exposure could affect the c-Fos induction in the area of interest, keep mice in constant darkness during the last day of the experiments, and before the perfusion.

5.2. Carefully dissect the brain out and submerge in 4% PFA solution for 9-12h.

5.3. After PFA fixation, cryoprotect the brain tissue using a 30% sucrose solution (wait until the brain sinks), then section the brain using a cryostat.

5.4. Transfer the coronal brain sections (35 μm) into a solution containing 1x PBS, 10% bovine serum albumin, and 0.3% Triton X-100 for 1 h at room temperature.

5.5. Incubate the brain sections with an anti-c-Fos (1:2500) antibody solution at 4 °C overnight with constant agitation.

5.6. After 3 washes of 5 min each with a solution containing 1x PBS and 0.3% Triton X-100, incubate the samples with an Alexa-conjugated secondary antibody (1:500) solution for 1 h at room temperature away from light and with constant agitation.

5.7. Obtain digital images using a confocal microscope. Assemble and process captured images with a photo editing and analysis software (e.g., Adobe Photoshop).

5.8. For data analysis, outline and measure the AAV-infected area (mCherry(+) cells) using ImageJ software, and quantify the number of c-Fos(+) cells within this region to obtain the number of activated cells per area. Combine the results obtained from 3 separate sections per animal.

REPRESENTATIVE RESULTS

We observed that repetitive CNO delivery using eye-drops elicited a robust induction of c-Fos expression in most infected neurons (**Figure 1C**), showing that the effectiveness of CNO delivery is sustained during the repetitive exposure. Furthermore, a significant induction of c-Fos was

observed in samples collected 2 h after CNO treatment, compared to samples obtained 6 h after CNO exposure (**Figures 1D-E**), demonstrating that changes induced by CNO are time-dependent.

We then measured the effectiveness of the chronic CNO treatment delivered through drinking water. We observed that the daily consumption of water + CNO was not significantly different compared with the total volume of regular water consumed (**Figure 2A**). Similarly, the amount of water + 1% sucrose consumed during the night (6 h time window) was not affected by the addition of CNO (**Figure 2B**). Further, no differences in the daily consumption (5 days) of both water + CNO (**Figure 2C**) and water + sucrose + CNO (**Figure 2D**) were found throughout the experiment for all the animals.

Similar to what we found using CNO eye-drops, robust induction of c-Fos was observed after 2 h but not 6 h upon CNO access (**Figures 2E-F**).

Finally, we measured the dose response of CNO added to drinking water. To do this, mice were exposed to the following CNO doses: 1.0 mg/ mL, 0.5 mg/ mL, 0.25 mg/ mL, 0.1 mg/ mL, saline. In all cases, animals were perfused 2 h after CNO exposure. We found that there is a clear threshold of effectiveness for CNO, where a low CNO dose (0.1 mg/ mL) does not elicit c-Fos activation compared to saline control, whereas higher doses (0.25 mg/ mL, 0.5 mg/mL and 1.0 mg/ mL) induced robust and similar c-Fos induction (**Figure 2G**).

FIGURE LEGENDS

Figure 1: Repetitive CNO delivery using eye-drops. (**A**) AAV/hM3Dq-mCherry was stereotaxically injected in the hippocampus of adult (3 months old) male mice. (**B**) Four weeks post-injection, CNO was administered using eye-drops once daily for 5 consecutive days. A dose of 1.0 mg CNO/ kg was used. (**C**) Finally, mice were sacrificed, and brain tissue was tested for c-Fos (green) immunoreactivity in the AAV-infected area (mCherry-positive cells, red). A representative coronal section of the injection site and the CNO-mediated c-Fos activation is shown. (**D**) The number of c-Fos positive cells in the AAV-infected area was measured in mice that were perfused 2 or 6 h after the last CNO administration. Data are mean ± SEM. ****p < 0.001*; by Student's t-test (n = 2-3 mice). (**E**) Representative images for the two groups are shown. Scale bar: 100 μm

Figure 2: Chronic CNO treatment delivered through drinking water. (**A**) No differences in the total liquid consumption were observed between control (water) or treated (water + CNO, dose: 1.0 mg CNO/ kg) animals. Data are mean ± SEM (n = 13-14 mice). (**B**) Similarly, no significant differences were observed in the volume of water + 1 % sucrose consumed (during a 6 h time window), after adding CNO (1.0 mg/ Kg). Data are mean ± SEM (n = 5 mice). (**C**) Daily consumption of water + CNO (1.0 mg/ kg) for individual mice is shown. No differences in the daily consumption were observed. Data are mean ± SEM (n = 5 mice). (**D**) Daily liquid consumption (during a 6 h time window) of 1% sucrose + CNO (1.0 mg/ kg) for individual mice is shown. No differences in the daily consumption were observed. Data are mean ± SEM (n = 3 mice). (**E**) 2 or 6 h after the last CNO administration, mice were sacrificed, and the number of c-Fos positive cells was quantified in the AAV-infected area. Data are mean ± SEM. ****p < 0.001*; by Student's t-test (n = 5 mice). (**F**) Brain coronal sections were tested for c-Fos (green) immunoreactivity in the AAV-infected

(mCherry-positive cells, red) region. Representative images are shown. (**G**) Four CNO doses were administered (0.1, 0.25, 0.5, and 1.0 mg CNO/ kg), and the c-Fos induction was measured. Data are mean ± SEM. ****p < 0.001*; by ANOVA, followed by Tukey's test (n = 2 mice). Scale bar: 100 μm

DISCUSSION

DREADDs have emerged as a popular and effective approach to remotely manipulate neuronal activity[17]. The design of alternative strategies for CNO delivery will broadly increase the spectrum of options available for specific experimental settings. In addition, non-invasive strategies for the delivery of CNO minimize any potential misinterpretation of results by reducing adverse side effects that can directly impact the animal's health. Here, we described two non-invasive strategies for CNO delivery that confer a robust activation of DREADDs (hM3Dq) and offer a wide spectrum of possibilities. Further, we believe that the protocols described here might also be useful for different DREADD variants for neuronal manipulation, including genetically engineered muscarinic or opioid receptors.

CNO delivery using repetitive eye-drops represents a painless alternative to repetitive intraperitoneal CNO injections while preserving the power to precisely control dosage and timing of CNO delivery. Therefore, we recommend using this protocol when repetitive DREADD activation is required. Eye-drops are also the least expensive option for CNO delivery, particularly compared with the protocol using CNO added to the drinking water. CNO delivered through drinking water, on the other hand, confers a chronic and sustained activation of DREADDS, avoiding any mouse handling. It is important to mention that this protocol lacks precise control over the timing of CNO delivery. A third alternative, time-restricted access to a sucrose solution containing CNO, combines advantages of both protocols previously discussed. This strategy is at the same time non-invasive, repetitive and easy to perform. Additionally, it offers a better control of the timing of CNO delivery compared with the 24 h access to water with CNO. A caveat of this approach is that it can only be used during the active phase of animals. We recommend using both strategies involving CNO in drinking water in combination with infrared cameras or a lick-o-meter system to obtain precise temporal information about CNO consumption and, therefore, DREADD activation.

Long-lasting effects conferred by CNO delivered through drinking water were previously reported. We have successfully applied a chronic CNO (5 μg/ mL) treatment during 14 consecutive days[15] to evaluate the behavioral consequences of tonic activation of a thalamo-cortical circuit involved in mood control. Alternatively, CNO provided in the drinking water at a concentration of 40 mg/L has been used to chronically modulate the activity of serotonergic neurons of the dorsal raphe nucleus[18], whereas the function of pancreatic β-cells was controlled using CNO at a concentration of 0.25 mg/mL water[19]. Combined, these results suggest that different CNO concentrations can be tuned to effectively control DREADDs. Here, we found that different doses of CNO added to drinking water elicited similar c-Fos activation, suggesting that a dose-response analysis should be performed to define the lowest and effective CNO dose required. Recent studies have shown that CNO is not entirely pharmacologically inert[20]; in addition, it was also demonstrated that the in vivo activation of DREADDs is mediated by the CNO metabolite clozapine, which has several

endogenous targets[21]. Therefore, the authors suggest using subthreshold doses of clozapine, instead of high CNO doses. Although we have not evaluated the effectiveness of clozapine in the methods described, we found that CNO concentration could be reduced without significantly reducing neuronal activation, and therefore, minimizing side effects caused by the CNO-to-clozapine conversion.

In summary, the strategies presented here represent potential schemes for CNO delivery that can be easily adapted to a variety of experimental designs. They were conceived as non-invasive strategies that may be useful for repetitive or chronic CNO-mediated activation of DREADD-controlled neurons, reducing the impact of CNO delivery on animal behavior.

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DISCLOSURES

The authors have nothing to disclose.

REFERENCES

1. Park, H.G., Carmel, J.B. Selective Manipulation of Neural Circuits. *Neurotherapeutics*. **13** (2), 311–324, doi: 10.1007/s13311-016-0425-7 (2016).
2. Muir, J., Lopez, J., Bagot, R.C. Wiring the depressed brain: optogenetic and chemogenetic circuit interrogation in animal models of depression. *Neuropsychopharmacology*. 1, doi: 10.1038/s41386-018-0291-6 (2018).
3. Wiegert, J.S., Mahn, M., Prigge, M., Printz, Y., Yizhar, O. Review Silencing Neurons: Tools, Applications, and Experimental Constraints. doi: 10.1016/j.neuron.2017.06.050 (2017).
4. Urban, D.J., Roth, B.L. DREADDs (Designer Receptors Exclusively Activated by Designer Drugs): Chemogenetic Tools with Therapeutic Utility. *Annual Review of Pharmacology and Toxicology*. **55** (1), 399–417, doi: 10.1146/annurev-pharmtox-010814-124803 (2015).
5. Roth, B.L. DREADDs for Neuroscientists. *Neuron*. **89** (4), 683–694, doi: 10.1016/j.neuron.2016.01.040 (2016).
6. Armbruster, B.N., Li, X., Pausch, M.H., Herlitze, S., Roth, B.L. Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proceedings of the National Academy of Sciences*. **104** (12), 5163–5168, doi: 10.1073/pnas.0700293104 (2007).
7. Donato, F., Jacobsen, R.I., Moser, M.-B., Moser, E.I. Stellate cells drive maturation of the entorhinal-hippocampal circuit. *Science*. **355** (6330), eaai8178, doi: 10.1126/science.aai8178 (2017).
8. Mahler, S. V et al. Designer receptors show role for ventral pallidum input to ventral tegmental area in cocaine seeking. *Nature Neuroscience*. **17** (4), 577–85, doi: 10.1038/nn.3664 (2014).
9. Lichtenberg, N.T. et al. Basolateral Amygdala to Orbitofrontal Cortex Projections Enable Cue-Triggered Reward Expectations. *The Journal of Neuroscience*. **37** (35), 8374–8384, doi: 10.1523/JNEUROSCI.0486-17.2017 (2017).
10. Schotman, P., Reith, M.E.A., Gispen, W.H. Effects of stressful procedures as ether

anesthesia and intracranial injections on amino acid incorporation into brain protein. *Brain Research Bulletin*. doi: 10.1016/0361-9230(77)90002-8 (1977).
11. Frumberg, D.B., Fernando, M.S., Lee, D.E., Biegon, A., Schiffer, W.K. Metabolic and behavioral deficits following a routine surgical procedure in rats. *Brain Research*. doi: 10.1016/j.brainres.2007.01.134 (2007).
12. Keenan, W.T., Fernandez, D.C., Shumway, L.J., Zhao, H., Hattar, S. Eye-Drops for Activation of DREADDs. *Frontiers in Neural Circuits*. **11**, 93, doi: 10.3389/fncir.2017.00093 (2017).
13. LeGates, T.A., Altimus, C.M. Measuring circadian and acute light responses in mice using wheel running activity. *Journal of Visualized Experiments*. (**48**), doi: 10.3791/2463 (2011).
14. Bainier, C., Mateo, M., Felder-Schmittbuhl, M.-P., Mendoza, J. Circadian rhythms of hedonic drinking behavior in mice. *Neuroscience*. **349**, 229–238, doi: 10.1016/J.NEUROSCIENCE.2017.03.002 (2017).
15. Fernandez, D.C. et al. Light Affects Mood and Learning through Distinct Retina-Brain Pathways. *Cell*. **175** (1), 71–84.e18, doi: 10.1016/j.cell.2018.08.004 (2018).
16. Franklin, K.B.J., Paxinos, G. *Paxinos and Franklin's The mouse brain in stereotaxic coordinates*.
17. Urban, D.J., Roth, B.L. DREADDs (designer receptors exclusively activated by designer drugs): chemogenetic tools with therapeutic utility. *Annual Review of Pharmacology and Toxicology*. **55**, 399–417, doi: 10.1146/annurev-pharmtox-010814-124803 (2015).
18. Urban, D.J. et al. Elucidation of The Behavioral Program and Neuronal Network Encoded by Dorsal Raphe Serotonergic Neurons. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*. **41** (5), 1404–15, doi: 10.1038/npp.2015.293 (2016).
19. Jain, S., Ruiz De Azua, I., Lu, H., White, M.F., Guettier, J.-M., Wess, J. Chronic activation of a designer G q-coupled receptor improves β cell function. *The Journal of Clinical Investigation*. **123**, doi: 10.1172/JCI66432 (2013).
20. MacLaren, D.A.A. et al. Clozapine N-Oxide Administration Produces Behavioral Effects in Long-Evans Rats: Implications for Designing DREADD Experiments. *eNeuro*. **3** (5), doi: 10.1523/ENEURO.0219-16.2016 (2016).
21. Gomez, J.L. et al. Chemogenetics revealed: DREADD occupancy and activation via converted clozapine. *Science*. **357** (6350), 503–507, doi: 10.1126/science.aan2475 (2017).

Figure 1

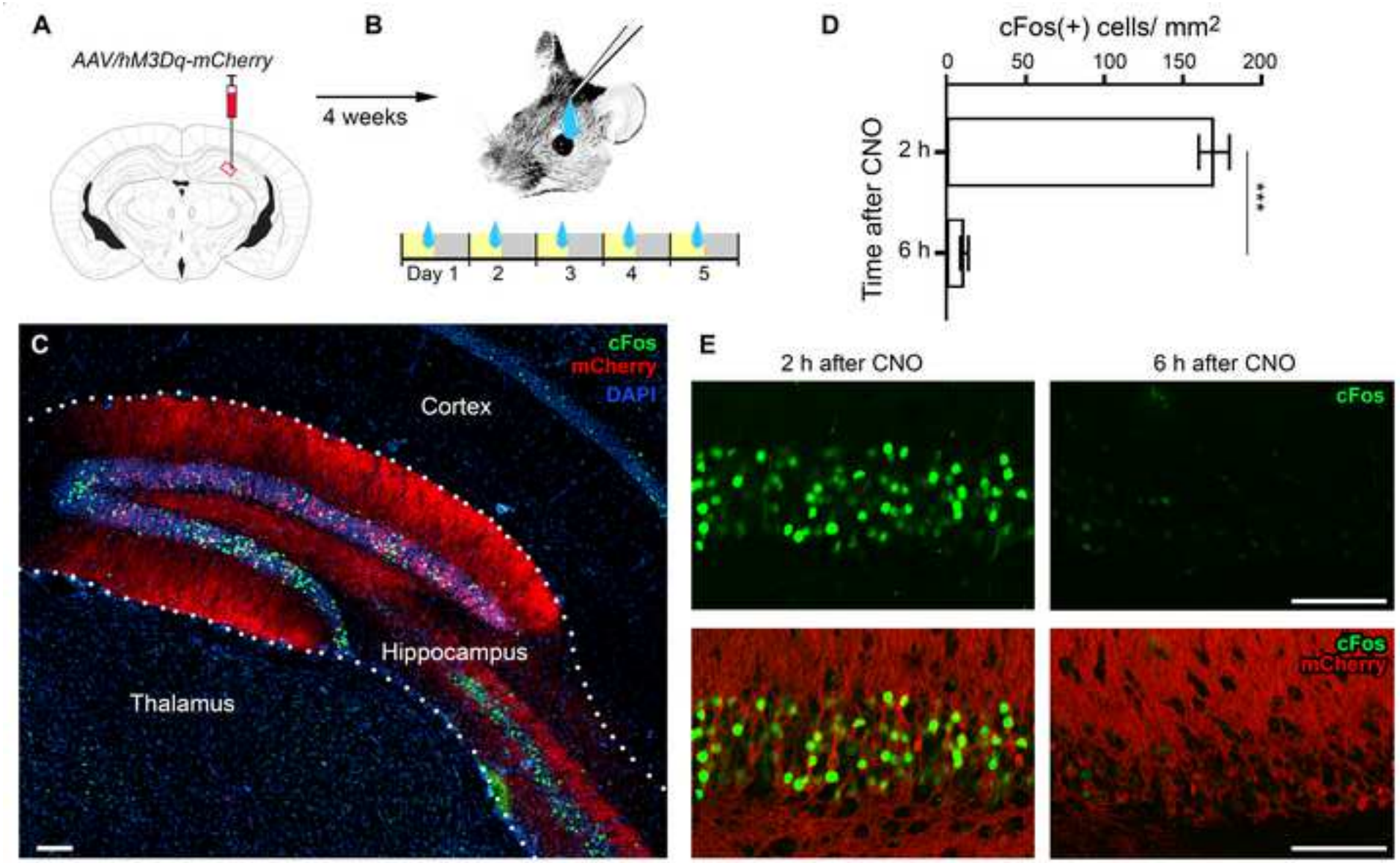

Figure 2

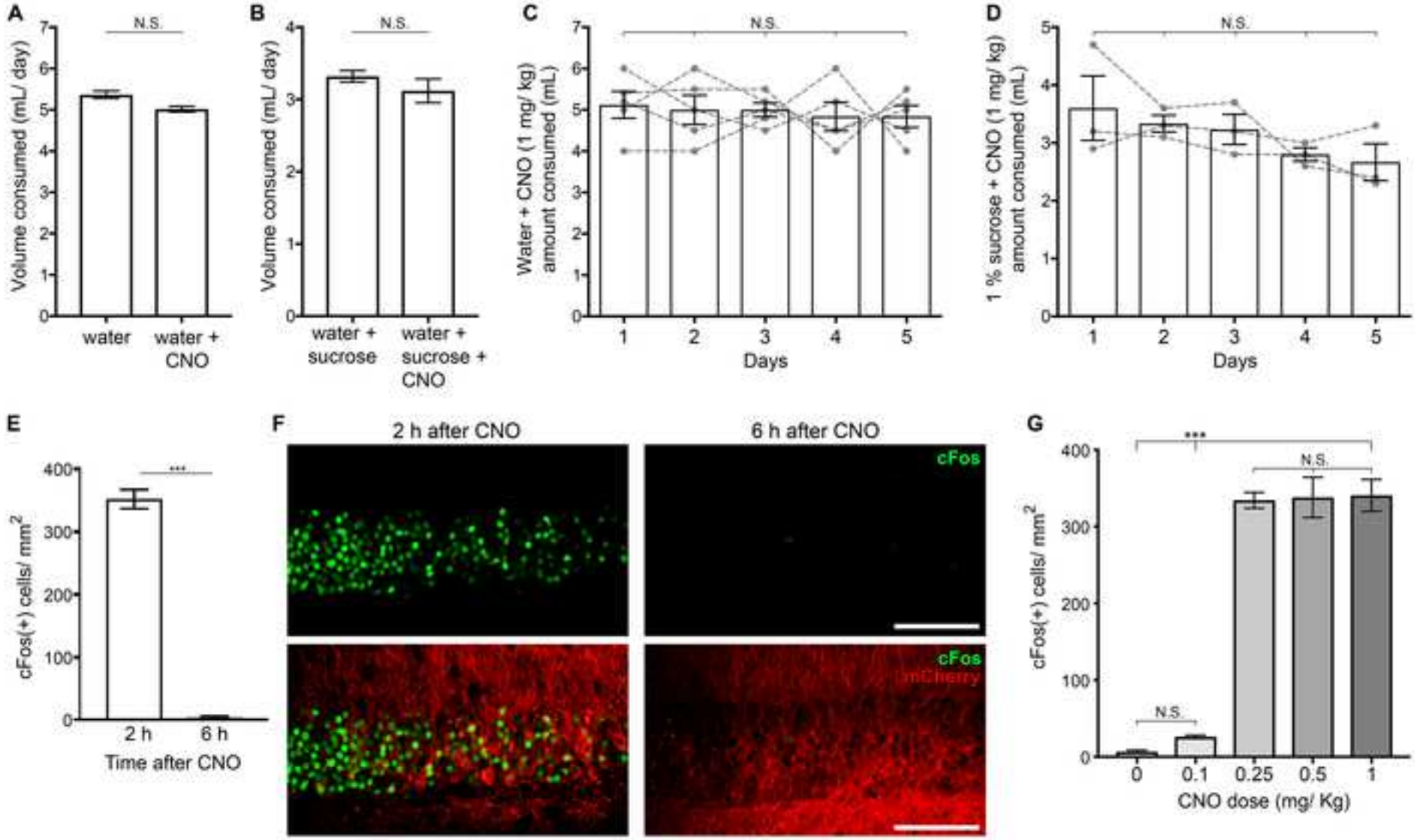

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
BSA	Sigma life science	#A2153-100G
C57BL/6J mice	The Jackson laboratory	#000664
Capillaries	Drummond Scientific Company	#3-000-203-G/X
Clozapine-N-oxide	Sigma	#C0832
Forane	Baxter	#NDC 10019-360-60
Microinjector III	Drummond Scientific Company	#3-000-207
Mounting media	Invitrogen	#P36930
Paraformaldehyde	Electron Microscopy Sciences	#15710
Primary c-Fos Antibody	Cell signaling technology	#2250S
rAAV5/hSyn-hm3D-mCherry	UNC Vector Core	
rAAV5/hSyn-mCherry	UNC Vector Core	
Secondary Antibody	Invitrogen	#A21206
Triton X-100	americanbio.com	#AB02025-00100

Comments/Description
Lyophilized powder ≥96% (agarose gel electrophoresis)
male mice, 3 months old
Outer diameter: 1.14 in.
5mg
Isoflurane, USP
Nanoject III - Programmable Nanoliter Injector
Prolong Gold antifade reagent
16% aqueous solution (methanol free), 10 ml
c-Fos (9F6) Rabbit mAb (100µl)
Titer: ~3x10e12 vg/mL
Titer: ~3x10e12 vg/mL
Alexa Fluor TM 488 Donkey anti-rabbit IgG(H+L), 2mg/ml

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Author(s):	Jesse Zhan, Ruchi Komal, William T. Keenan, Samer Hattar, Diego C. Fernandez

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CORRESPONDING AUTHOR

Name:	Diego Carlos Fernandez
Department:	Section on Light and Circadian Rhythms (SLCR)
Institution:	National Institute of Mental Health (NIMH)
Title:	Staff Scientist
Signature:	
Date:	10/26/2018

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Comment 1

Goal of the protocol can be briefly summarized here as well. AAV encoding hM3Dq details, significance, why this was used, etc. What kind of ligand CNO?

We modified the introduction to address the significance and purpose of our protocols.

Comment 2

We cannot have paragraph of texts in the protocol section. Please ensure that all the text in the protocol is a numbered action step and describe how is the step performed.
Please detail the steps exactly how you perform your experimental steps with all specific details associated with the step. Please ensure all notes, caution etc start from a new line.
Please do not use personal pronouns in the protocol section.

We have modified the protocol section accordingly.

Comment 3

Please consider moving this to the table of materials.

It was moved to table of materials.

Comment 4

This can be moved to the discussion section as future applications.

We moved this sentence to the discussion.

Comment 5

Do you check the depth of anesthesia? If yes how?

A note to step 1.2 was added to check depth of anesthesia.
"NOTE: To ensure animal is fully unconscious, pinch the mouse's paw. Animal is properly anesthetized when flinching response to pinch is absent".

Comment 6

What is the desired coordinate with respect to your experiment and why?

Coordinates used in our experiments are now specified in steps 1.5 and 1.6.

Comment 7

Do you shave the animal's fur at the site of surgery? Do you clean with iodine-based scrub, do you first perform an incision in the muscle and skin before drilling? What is the region of interest in your experiment and why?

More specific instructions are now provided in steps 1.4 to 1.6.

Comment 8

Concentration of the injected AAV? MOI of injection used in your experiment? Citations if any?

We have included the titer of the AAVs used in the table of materials.

After this step please include how do you close the drilled area -dental cement, etc.? Do you close the skin and the muscle with suture etc.?

Sutures were used to close incision after injection, as described in step 1.7 of the updated protocol.

Comment 9

Please make this as an action step, the way you performed your experiment. Please use imperative tense throughout. So, in this case eye drops were deliver twice a day?

Text is now rewritten in the imperative tense. We also clarified that eye-drops were delivered once a day.

Since steps 2.4.1-2.4.5 describe how this is done, it has been converted to sub steps. Please check.

We agree with the sub-steps modification. Step 2.5 was adjusted accordingly as well.

Comment 10

Again what is the concentration used?

The amount of CNO to be delivered per mouse was described in step 2.3. To avoid any confusion, we have also specified the CNO concentration in step 2.4.1.

Comment 11

Bring out clarity to perform step either step 2 or step 3 after AAV injection and not both. Also, after how many days is this step performed? Please be crisp and write the steps with respect to the experiment performed in your laboratory.

We have modified the step 1.9 to direct users to use either step 2, 3, or 4. Step 1.9 also indicates that the next step should be taken 4 weeks after injection.

"1.9. Beginning 4 weeks post-injection, subject mice to any of the paradigms described in the following section to chronically control neurons expressing the excitatory receptor".

Comment 12

This is not needed. The protocol should describe action steps which should state how the experiment is performed in imperative tense only. Describe all the action in stepwise manner.

It was moved to the introduction.

Comment 13

We cannot have two notes following one step.

Only one note follows now.

Comment 14

This can be converted to a step and can be reworded to be a discrete step. e.g.,Monitor the health of the animal and do not include animals in the study showing more than xx% weight loss

We agree with this suggestion, and have added a new step to the protocol.

Comment 15

Please describe what is proper this case?

We removed the word *proper* from step 3.10, which could lead to confusion. As suggested in the note, different facilities may have particular rules regarding waste solutions. Therefore, we recommend users to contact the Chemical Disposal Service for specific instructions.

Comment 16

This is how every step should be. Please write crisp/discrete steps with respect to your experiment and other details can be provided as note. However, discussion about the step should be present in the discussion section only.

We have modified the text accordingly.

Comment 17

Include a step to show how controls are treated in this case?

Description for experimental controls are now detailed in steps 2.6, 3.12, and 4.9.

Comment 18

Introduction of each step/section can be moved to the introduction.

We moved this section to the introduction

Comment 19

Please rewrite the step to show how you performed your experiment in imperative tense, providing all specific detail. The generalization can be included as a note.

The step has been broken down into concise steps written in the imperative tense. Notes have been added for generalization.

Comment 20

How many days?

We have now added step 4.8 to indicate number of days to repeat exposure. Generalization is included as a note.

Comment 21

We cannot have paragraph of text in the protocol section. Please make substeps and use imperative tense throughout. Please start with sacrificing the animal after xx days. How is this done?

We have modified the protocol section accordingly.

Then immunostaining, then imaging, then processing the image using imageJ etc. For software step, please provide button clicks in the software. Open the image by clicking ***Open****, then click* ***Analyze****.*

Quantification of c-Fos induction was used to measure effectiveness of DREADD activation. Details for staining and data analysis are now included. This is a standard method that can be manually performed, and therefore, we do not include explicit instruction for analysis because we believe it may distract from the main purpose of the protocols described.

Comment 22

Why c-fos staining?

We have now explained in the introduction that c-Fos induction in response to CNO delivery is a robust read-out of DREADD-mediated neuronal activation.

Comment 23

Please include one figure to show the adeno viral delivery M3 muscarinic receptor (hM3Dq).

In panel 1A we describe the AAV injection. The AAV construct information was included in the table of materials.

Also need negative control imaging results as well.

Quantification of c-Fos induction for negative control (saline solution) was included in the original Figure 2G. Additionally, data obtained from mice that were perfused 6 h after CNO exposure showed no substantial c-Fos induction, similar to negative controls.

Please also include the number of mice tested for each case.

Number of mice tested was included in the figure legend of the original submission.

Comment 24

Bring out clarity by stating what is referred to as the first set of experiment.

We modified the text to eliminate any confusion.

Comment 25

Please bring out the relationship of DREADD, M3 muscarinic receptor (hM3Dq) and reduction in c-fos somewhere in the introduction, before stating this result to bring out clarity.

We discuss this point in the introduction.

Comment 26

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All figures and images are originals.

Comment 27

As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

All these points are addressed in our discussion.