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Two Different Real-Time Place Preference Paradigms Using Optogenetics within the Ventral Tegmental Area of the Mouse

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Dear Editor,
Please find re-submitted our manuscript "*Step-by-step experimental protocol for two different real-time place preference paradigms using optogenetics within the Ventral tegmental area (VTA) in the mouse*" which we have modified in accordance with questions and comments raised by Editor and Reviewers 1-4. Point-by-point letters are also provided.

We hope with these changes that you will now find the manuscript acceptable for publication.

If you have any questions, please do not hesitate to contact me or Zisis Bimpisidis.

Sincerely,

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TITLE:

Two Different Real-Time Place Preference Paradigms Using Optogenetics within the Ventral Tegmental Area of the Mouse

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KEYWORDS:

aversion, behavior, conditioning, Cre-Lox, dopamine transporter (DAT), optogenetics, place preference, reward, transgenic mice, vesicular glutamate transporter (VGLUT2)

SUMMARY:

Here we present two easy-to-follow step-by-step protocols for place preference paradigms using optogenetics in mice. Using these two different setups, preference and avoidance behaviors can be solidly assessed within the same apparatus with high spatial and temporal selectivity, and in a straightforward manner.

ABSTRACT:

Understanding how neuronal activation leads to specific behavioral output is fundamental for modern neuroscience. Combining optogenetics in rodents with behavioral testing in validated paradigms allows the measurement of behavioral consequences upon stimulation of distinct neurons in real-time with high spatial and temporal selectivity, and thus the establishment of causal relationships between neuronal activation and behavior. Here, we describe a step-by-step protocol for a real-time place preference (RT-PP) paradigm, a modified version of the classical conditioned place preference (CPP) test. The RT-PP is performed in a three-compartment apparatus and can be utilized to answer if optogenetic stimulation of a specific neuronal population is rewarding or aversive. We also describe an alternative version of the RT-PP protocol, the so-called neutral compartment preference (NCP) protocol, which can be used to confirm aversion. The two approaches are based on extensions of classical methodology originating from behavioral pharmacology and recent implementation of optogenetics within the neuroscience field. Apart from measuring place preference in real time, these setups can also give information regarding conditioned behavior. We provide easy-to-follow step-by-step protocols alongside examples of our own data and discuss important aspects to consider when applying these types of experiments.

INTRODUCTION:

The implementation of optogenetics, a modern neuroscience experimental tool in which light is used to control neuronal activity, has in recent years led to major advancements in understanding how specific neuronal populations impact behavior[1–3]. The outstanding spatial and temporal selectivity of optogenetics allows the establishment of causal relationships between excitation or inhibition of cell groups of interest and behavioral output[2,3]. Spatial selectivity in optogenetics is commonly ensured through the Cre-Lox system in which the activity of Cre recombinase leads to recombination of any DNA sequences present between Lox sites, so called floxed alleles (flanked by lox sites)[4]. The goal with using the Cre-Lox system in optogenetics is to achieve expression of alleles encoding optogenetic opsins in specific neurons of interest while leaving surrounding neurons devoid of expression. Opsins are light-sensitive proteins that upon light-stimulation of specific wave-length allow ion flow that affects neural excitability or influence cellular functions by modulating downstream effector pathways. Novel variants of opsins that differ in action (excitatory, inhibitory, modulatory), mechanism, activation by light wavelength and kinetics properties[5] are continuously being developed to meet the needs of specific experimental approaches. Regarding excitability, using a depolarizing or hyperpolarizing opsin dictates the activity of the neurons (excitation or inhibition, respectively) upon light-stimulation at a specific wavelength delivered into the brain[3].

Selective promoter activity directs the expression of Cre recombinase to the neurons of interest. By implementing a floxed allele of the opsin of interest, Cre-mediated recombination will ensure that the opsin is selectively expressed in neurons that co-express Cre recombinase[3,6]. This use of double transgenics to direct spatial selectivity has proven very efficient in optogenetics. Thus, while light-stimulation to activate the opsins is broadly delivered through an intracerebrally implanted optic fiber connected to a light source (LED or laser)[3], only neurons expressing both Cre recombinase and the floxed opsin allele will respond to this stimulation. The Cre-Lox system in rodents can be achieved in different ways by using only transgenics (both Cre recombinase and the floxed opsin construct are encoded in transgenic animals), only viral injections (DNA constructs for Cre recombinase and the floxed opsin are both delivered via a viral carrier), or a combination of the two (for example, Cre recombinase is encoded by a transgenic animal which is injected with a virus carrying the floxed opsin construct)[5]. The floxed opsin DNA construct is usually cloned in frame with a reporter gene to enable visualization of Cre-mediated recombination in tissue sections. While optogenetics can also be performed in rats, the presented protocols have been generated for mice. For simplicity, mice carrying both Cre recombinase and the floxed opsin will be referred to as “optogenetics mice”. In the protocols described below, optogenetics mice have been generated by a mixed transgenic (Cre recombinase under control of two different promoters) and viral (using an adeno-associated virus, AAV, to deliver the floxed opsin DNA construct – in our case ChR2/H134R) approach. Obtaining and maintaining transgenic mouse lines is an essential part of the methodology. Cre-driver and floxed opsin transgenic mice can be produced for each purpose, or purchased if commercially available, as can a range of viruses carrying DNA sequences encoding Cre recombinase and floxed opsins in different forms.

Optogenetics coupled with behavioral testing has proven to be a valuable tool to study the role of distinct brain regions, or discrete neuronal populations, in particular types of behavior. In the context of reward-related behavior, optogenetics has enabled the verification of previous findings in the fields of behavioral pharmacology and experimental psychology, and also allowed a new level of spatio-temporally relevant dissection into how certain neurons affect behavior. One method which has been used in several studies to assess reward-related behavior is a modified version of the classical method known as Conditioned Place Preference (CPP). Classical CPP has been used to assess the rewarding or aversive properties of drugs of abuse through their ability to induce Pavlovian associations with cues of the environment[7,8]. In Pavlovian terms, the drug is an *unconditioned* stimulus since it can elicit approach or withdrawal if it is rewarding or aversive, respectively. Continuous pairing of the drug with various *neutral* stimuli, that themselves do not elicit any response, can lead to approach or withdrawal merely upon presentation of the previously neutral, but after pairing, so called *conditioned* stimuli[9]. CPP analysis is usually performed in an apparatus containing two compartments of the same size but where each compartment is defined by distinct characteristics, such as floor texture, wall patterns and illumination (neutral stimuli). The two compartments are connected either by a corridor or an opening between the compartments. During conditioning, the subject, usually a small rodent, receives passive injections of a drug while restricted to one of the two main compartments and saline while restricted to the other compartment. The rewarding effects of the drug are subsequently assessed in a drug-free session when the subject is allowed to freely explore the whole apparatus. The amount of time spent in the previously drug-paired compartment (the conditioned response) is considered to reflect Pavlovian learning mechanisms mediated between the rewarding effects of the drug and the cues of the compartment associated with its administration (*conditioned* stimuli). If the animal spends more time in the drug-paired compartment, the drug has induced a conditioned place preference which means that it has rewarding effects on behavior. On the other hand, if the drug is perceived as aversive, the animal will avoid the drug-paired compartment and spend more time in the saline-paired compartment, indicating conditioned place aversion (CPA)[8–11].

Since optogenetics can be implemented to control neuronal activity in “real-time”, the use of a behavioral paradigm similar to, but distinct from, the CPP setup allows for measurement of place preference upon direct neuronal activation. Optogenetics-driven analysis of place preference is therefore often referred to as a real-time place preference (RT-PP) analysis paradigm. In the RT-PP paradigm, optogenetic stimulation of distinct neurons via the Cre-Lox system replaces the systemic delivery of a drug performed in the classical CPP, so that the RT-PP paradigm instead measures if optogenetically induced neuronal stimulation is perceived as rewarding or aversive. The current description will focus on optogenetics mice, but also optogenetics rats can be tested using similar protocols.

Instead of conditioning to one compartment at a time as in the classical CPP paradigm, the optogenetics mouse in the RT-PP paradigm is allowed to move freely in the entire apparatus and behavior is recorded throughout the session. Entry into one of the compartments is paired with intracranial light-stimulation. Under appropriate light stimulation parameters, neurons that express an excitatory opsin will thereby be activated. If the light-stimulation is perceived as

rewarding, the optogenetics mouse will remain in the light-paired compartment, while if the light-stimulation is perceived as aversive, the mouse will exit the compartment to escape the stimulation. This type of analysis allows for assessing contingent learning: The subject can trigger light-stimulation and hence neuronal activation by entering a compartment, and stop the stimulation by exiting the compartment, similar to lever-pressing during an instrumental task. Furthermore, associative learning mechanisms can be assessed during subsequent sessions where time spent in each compartment is measured in the absence of stimulation. This way, the researcher can dissociate between the immediately rewarding effects upon stimulation of the neurons of interest and the associative memory formation related to it[12].

In the current study, we describe two step-by-step setup protocols for optogenetics-driven place preference behavior of freely-moving mice. The first protocol describes RT-PP within a three-compartment apparatus and has been outlined based on the protocols recently presented by Root and colleagues[13] and other authors[12,14–18]. The experiment consists of two phases comprising several daily sessions (shown in **Figure 1A**). Each session is designed for different purposes and the parameters of coupling stimulation with a compartment are changed accordingly. The first session, the "*Pretest*", is used to assess initial preference of the subject to either one of the compartments. While connected to the patch cord, the subject is allowed to freely explore the apparatus in the absence of stimulation for 15 min. If the initial preference to any one compartment is more than 80%, the mouse is excluded from the analysis since initial side bias might skew the analysis. After the *"Pretest"*, *"Phase 1"* begins. The first part consists of two consecutive, daily, 30 min sessions of *"RT-PP"*. During "*Phase 1"*, the optogenetics mouse is connected to the laser source through the patch cord and placed in the arena to freely explore it. The mouse receives intracranial laser stimulation upon entry into one of the main compartments. Pilot experiments can be performed to determine which compartment will be assigned as laser-paired and which as unpaired. If the stimulation is shown to be rewarding, the laser will be coupled to the least preferred compartment during the "*Pretest*" and to the most preferred if the stimulation is aversive. Thus, the presented RT-PP protocol follows a biased design in the sense that laser stimulation is not randomly assigned to any of the two main compartments (unbiased design), but is chosen to avoid any initial preference of the mouse. Entry into the other main compartment or the neutral compartment connecting the two main compartments does not give rise to intracranial light stimulation and are thus not light-paired. These sessions allow for real-time assessment of the rewarding or aversive properties of stimulation of specific neuronal populations. On the last day of *"Phase 1"*, a 15 min session without any stimulation takes place. This session serves to address conditioned responses ("*CR*") which result from associative learning between the stimulation and the environment where it was received. At least three days after *"Phase 1"*, the "*Reversal Phase"* takes place which follows the same structure as *"Phase 1"* but the previously non-paired main compartment is now paired with light stimulation. As in the case of *"Phase 1"*, the two stimulation sessions are followed by a "*CR*" session. The "*Reversal Phase"* is used to confirm that the behavior of the mouse is contingent upon optogenetic stimulation and not related to random parameters. Each session of the RT-PP experiment has to be separately programmed within the tracking software. The current protocol describes such settings within a specific software, but any other tracking software able to send transistor-transistor-logic (TTL)

modulation signals to the light source can be used.

The second protocol describes a novel setup termed the Neutral Compartment Preference (NCP) paradigm. This modified protocol of the RT-PP takes advantage of the small size and transparency of the connecting corridor which is naturally avoided by the mouse due to its narrow and transparent composition. By pairing both main compartments with light-stimulation and only leaving the corridor free of light-stimulation, the NCP setup can be used to test whether the optogenetic stimulation will force the mouse to spend more time in the corridor to avoid receiving optogenetic stimulation. By comparing the time spent in the two light-paired compartments with the time spent in the corridor, a verification of optogenetically-induced aversion can be made. The NCP experiment consists of two consecutive daily sessions where optogenetics mice receive stimulation (30 min each) to measure preference in real time, and one laser-free session (15 min) to assess conditioned responses similarly to the ones in the RT-PP protocol.

The RT-PP and NCP protocols provided below were recently validated in our lab in the study of how different types of neurons located in the ventral tegmental area (VTA) are involved in various aspects of reward-related behavior[12]. Here, to exemplify the implementation of the RT-PP and NCP protocols, dopamine transporter (DAT)-Cre[19] and vesicular glutamate transporter 2 (VGLUT2)-Cre[20] transgenic mice were stereotactically injected with AAV carrying a floxed channelrhodopsin2 (ChR2) DNA construct into the VTA whereupon an optic fiber was implanted above the VTA. Behavioral responses obtained upon analysis of these mice using the provided RT-PP and NCP protocols shows how activation of dopaminergic and glutamatergic neurons within the VTA leads to different behavioral responses (**Figure 1**).

Step-by-step protocols for RT-PP and NCP paradigms are provided with information ranging from genotyping of transgenic mice, stereotaxic viral injections and fiberoptics placement, to programming of tracking software for laser-control and behavioral assessment. In addition, suggestions for modifications of the protocol in terms of stimulation parameters and experimental aspects that can affect the scientific outcome are discussed. While protocols are described in the context of the VTA, they can be applied to any brain area or neuronal population, provided the relevant optogenetics tools, such as relevant Cre-driver and floxed opsins, are available.

PROTOCOL:

This study has been carried out using heterozygous DAT-Cre[19] and VGLUT2-Cre[20] mice of both sexes, aged >8 weeks and weighing >20 g. All the experiments were conducted according to the Swedish (Animal Welfare Act SFS 1998:56) and European Union Legislation (Convention ETS 123 and Directive 2010/63/EU) with permission from the local Animal Ethical Committees.

1. Genotyping of mice

1.1. Take ear biopsies using an ear puncher to use for genotyping of the transgenic mice.

1.2. Prepare the ear punches to perform a polymerase chain reaction (PCR) reaction using purpose-made primers.

NOTE: In this protocol, Cre-directed primers were used.

1.2.1. Add 75 µL of lysis buffer (buffer 1: 250 mM NaOH, 2 mM EDTA) in each 1.5 mL tube containing an ear punch.

1.2.2. Incubate in a heating block at 96 °C for 30 min.

1.2.3. Let the samples cool down for 5 min and then add 75 µL of the neutralization buffer (buffer 2: 400 mM Tris-HCl pH 8.0).

1.3. Perform PCR according to standard procedures[12,21] using the appropriate primers (here: Cre FW 5'-ACGAGTGATGAGGTTCGCAAGA-3', Cre REV 5'-ACCGACGATGAAGCATGTTTAG-3').

CAUTION: Work on ice under a PCR hood and pay attention not to contaminate the reagents and the samples.

1.3.1. Prepare the PCR master mix. Multiply the following volumes according to how many samples are going to be analyzed, including appropriate control samples. Mix the reagents for a single 25 µL final volume reaction in the following order: distilled water (18.9 µL), 10x buffer with $MgCl_2$ (2.5 µL), 10 mM dNTP mix (0.5 µL), 10 µM forward primer (1 µL), 10 µM reverse primer (1 µL), 5 U/µL DNA polymerase (0.1 µL), and template DNA (1 µL; will be added in the next steps).

NOTE: Always add a negative, a positive, and an empty (without template DNA) control to ensure valid results.

1.3.2. Add 24 µL of master mix in PCR tubes.

1.3.3. Add 1 µL of template DNA (coming from the ear punch from each mouse) in each PCR tube.

1.3.4. Centrifuge the PCR tubes briefly to ensure the template DNA is inside the master mix.

1.3.5. Perform PCR with a thermal cycler using the cycling program in **Table 1**.

1.4. Prepare an agarose gel to run the samples using electrophoresis.

NOTE: The size will depend on the number of samples that need to be analyzed.

1.4.1. Add 1% w/v agarose powder in 1x Tris-acetate-EDTA (TAE) buffer in a glass bottle. Heat in

microwave until the agarose is fully dissolved, checking regularly that it does not boil over.

CAUTION: Take precautions to avoid burns.

1.4.2. Let the gel cool down to approximately 50 °C and add a nucleic acid gel stain (0.5 µL/50 mL of gel).

1.4.3. Pour the gel in the casting tray containing well combs and leave it in room temperature until it becomes completely solidified. Remove the combs gently.

1.4.4. Fill the electrophoresis tank with 1x TAE buffer and place the gel in the tank.

1.4.5. Add 2 µL of 1x DNA loading dye in each one of the DNA samples.

1.4.6. Load 4 µL of DNA ladder in the first well of the gel, then proceed to load the full volume of the samples in the remaining wells.

1.4.7. Set the power source of the electrophoresis to 140 V and run for 25−30 min.

1.4.8. Place the gel under a UV source and take a picture of the results.

2. Stereotaxic surgery

2.1. After genotyping, separate mice keeping the ones positive for Cre. Wait until they are at least 8 weeks old and weigh >20 g to perform surgery.

2.1.1. Sanitize the environment and surgical tools to perform surgery under aseptic conditions.

2.1.2. Inject the mice subcutaneously with analgesic 30 min before surgery.

2.1.3. Anesthetize the mice with isoflurane (2−3% in normal air for induction and 1.5−2.0% for maintenance of anesthesia). Ensure adequate anesthesia level is achieved by testing the absence of pain reflexes by gently pinching the toe of the mouse. Adjust the isoflurane delivery accordingly.

2.1.4. Place the mouse on the stereotaxic apparatus. Add eye lubricant to prevent eye lesioning due to dryness and shave the hair of the top of the skull. Use a heating pad to maintain the temperature of the mouse stable.

2.1.5. Inject 100 µL of local anesthetic under the skin of the skull and allow 5 min to take effect.

2.1.6. Apply iodine to the shaved skin using a cotton stick, gently lift the skin with forceps, and make an incision of ~1.5 cm along the rostrocaudal axis with surgical scissors to reveal the surface of the skull.

2.1.7. Using a cotton stick, apply H_2O_2 solution to remove the periosteum, then dry the skull and locate the bregma and lambda.

2.1.8. Ascertain flat skull alignment by positioning the tip of the injection needle, adjusted on the stereotaxic frame, on bregma and lambda. Measure the ventral coordinates for each position and compare. When the skull is flat the ventral coordinate for both bregma and lambda are identical. If not, adjust the head position and take the measurements again.

2.1.9. Find and mark the position (AP: -3.45 mm, ML: -0.2 mm from bregma according to Franklin and Paxinos[22]) where the injection of the Cre-dependent virus and the implantation of the optic fiber will take place and make a small hole using a micro drill.

2.1.10. Load 400 nL of virus in the 10 µL syringe mounted on the stereotaxic apparatus using a precision pump.

2.1.11. Lower the needle (34 G, beveled) carefully and inject 300 nL of Cre-dependent optogenetic virus (*AAV5-EF1a-DIO-ChR2(H134)-eYFP* [5.6 x 10^{12} vg/mL]) in the VTA (AP: -3.45 mm, ML: -0.2 mm from bregma and -4.4 mm from the surface of the skull, according to Franklin and Paxinos[22]) at 100 nL/min injection rate using the precision pump.

2.1.12. After injection, leave the needle in place for an additional 10 min to allow for diffusion of the virus (**Figure 2A**).

2.1.13. Retract the needle slowly from the injection site.

2.1.14. Make small holes using a microdrill to fit anchor screws that will stabilize the optic fiber and dental cement complex.

2.1.15. Take the bregma coordinates again and implant the optic fiber (200 µm diameter, 0.37 NA) at: AP: -3.45 mm, ML: -0.2 mm from bregma and -4.0 mm from the surface of the skull (**Figure 2B**) according to Franklin and Paxinos[22].

2.2.16. Secure the fiber on the skull using dental cement. Apply enough cement around the optic fiber ferule to secure it to the skull but pay attention to leave 3−4 mm of the top of the ferule free of cement to allow connection of the patch cord (**Figure 2C**).

NOTE: Pay attention not to fill the hole with cement as this can cause brain tissue damage. Hemostatic materials can be added in the hole to prevent this from happening.

2.1.17. Use tissue glue or absorbable sutures to close any open wound and leave the animal to recover for at least two weeks. Give an additional dose of analgesic 12−24 h after the surgery.

3. Setting up the control of the laser source

3.1. Use single board microcontrollers to control the laser source. Write a script using the appropriate software. Load the script on the microcontroller board using the appropriate connection cable to the computer.

NOTE: The script should include external modulation (input) coming from the tracking software through a TTL box, and an output to the laser to control stimulation parameters. For 10 ms pulse width at 20 Hz frequency, use the script found in the **Supplemental Coding File**.

3.2. Connect the board to the laser and the TTL box of the tracking hardware.

3.2.1. Use a network cable to connect the TTL box to the board (pin 5 for the provided script) (**Figure 3A,C**).

3.2.2. Ensure the laser is set to control by external modulation and connect the laser to the board using an FC/PC cable (pin 13 for the given script) (**Figure 3B,C**).

3.2.3. Connect the appropriate pins to ground parts of the board.

3.3. Connect the laser source to the optic fiber.

3.3.1. Connect the laser source to a rotary joint (**Figure 3D**).

3.3.2. Connect a patch cord (**Figure 3E**) to the rotary joint.

3.3.3. Stabilize the rotary joint above the apparatus but outside the recording area. Make sure the length of the fiber-optic patch cord is appropriate to allow the mouse to move without difficulties in the arena (**Figure 3F**).

4. Setting up the experiment for the RT-PP approach within the tracking software

4.1. Calibrate the arena setup. Use a ruler to measure a specific part of the physical apparatus, draw a line corresponding to the part measured on the image within the software under the **Draw Scale to Calibrate** tab and enter the already known value (step 1 in **Figure 4**).

4.2. Design the arena. Draw the area where the movement of the mice will be recorded (step 2 in **Figure 4**).

4.3. Create the zones. Draw the zones that will eventually be assigned as laser-paired, laser-unpaired and “neutral” (step 3 in **Figure 4**).

4.4. Validate the setup to confirm that there are no conflicting parameters, for example zones outside the arena (step 4 in **Figure 4**)

4.5. Set the experimental parameters under the tab **trial control settings** (step 5 in **Figure 4**).

4.5.1. Set the trial time as shown in step 1 in **Figure 5** for a 30 min RT-PP session.

4.5.2. Making sure "hardware control" is enabled, assign a compartment as laser-paired in which entry of the mouse will trigger a TTL signal through the tracking software to the microcontroller board. In **Figure 5** (step 2) the laser-paired compartment is compartment A. For the reversal phase, switch the compartments so compartment B will be laser paired and compartment A will be unpaired. Do so by replacing A with B and B with A in the software.

5. Modification of the setup to test the aversive properties of the stimulation using the NCP approach

5.1. Follow steps 4.1–4.4 as described previously.

5.2. Set the time of the experiment to 30 min through the "Repeat till" option in the "Reference" box settings (step 1 in **Figure 6**).

5.3. Assign both A and B zones as laser paired (step 2 in **Figure 6**) by adding "when center-point is in any of Zone A and Zone B" for the condition box related to the settings for A and B compartments. Note that the laser will turn off when the animal is located in the neutral compartment.

6. Performing an experiment using laser stimulation

6.1. Set up the detection settings.

6.1.1. Use a dummy to resemble the mouse in order to ensure appropriate detection settings.

6.1.2. Place the dummy in one compartment of the apparatus and use automated setup with dynamic subtraction.

6.1.3. Remove the dummy and place it to the opposite compartment. Make sure the dummy is fully detected and if not, adjust the settings via the software to achieve proper detection.

6.1.3.1. During this step also check if the stimulation works as it is supposed to. Start acquisition using the previously configured trial control settings and place the dummy in the laser paired compartment and see if the stimulation is triggered as it should. Then place the dummy in the unpaired and/or the neutral compartment and see if the stimulation is stopped.

6.2 Use a power meter with a sensor to set the laser power to 10 mW using the knob on the laser (**Figure 3B**). Perform this step each time laser stimulation is used.

CAUTION: Use protective eye equipment as direct exposure to laser light can cause permanent

eye damage.

6.3. Place the mouse in the apparatus.

6.3.1. Gently take the mouse out of its cage and connect the fiber-optic implant to the fiber-optic patch cord using a ceramic sleeve.

6.3.2. Place the mouse gently in the neutral compartment of the three-compartment apparatus.

6.3.3. Wait until the mouse is detected by the software.

6.3.4. Remove the vertical sliding doors restricting the animal from entering the main compartments.

6.3.5. Allow the animal to explore freely without any disturbances.

NOTE: The same procedure is followed when the animal is not receiving stimulation with the exception that step 6.2 is not needed and that the laser is off the whole time.

REPRESENTATIVE RESULTS:

The three-compartment apparatus (**Figure 3F**) is suitable to address the rewarding effects of drugs and to assess in real time the rewarding or aversive properties of direct stimulation of neurons using optogenetics. It consists of two main compartments (20 cm [W] x 18 cm [L] x 25 cm [H]) and one smaller connecting compartment (20 cm [W] x 7 cm [L] x 25 cm [H]). The main compartments have distinct wall and floor texture and patterns in order to facilitate associative learning, while the connecting/neutral compartment is narrow and transparent so the mice spend naturally less time in it. As described above, the tracking software can be used to record several behavioral parameters of the mice including movement and time spent in each compartment, and to control the laser stimulation. The entire RT-PP experiment takes place throughout 8 sessions (**Figure 1A**) and allows both the assessment of the rewarding or aversive properties of the direct stimulation (days 3, 4, 6, and 7) and the formation of associations, positive or negative, in response to previous experience (days 5 and 8, "*CR*").

Firstly, we tested DAT-Cre mice injected with *AAV-ChR2-eYFP* virus in the VTA to target dopaminergic neurons. In accordance with the literature, we observed that the mice preferred to spend time in the compartment paired with the stimulation (**Figure 1B**, phase 1, days 3 and 4, blue circles, two-way repeated measures [RM] analysis of variance [ANOVA], effect of compartment $F_{(2,18)} = 141$, $p < 0.001$; effect of session x compartment $F_{(12, 108)} = 42.1$, $p < 0.001$; Tukey's post hoc test paired vs unpaired $p < 0.001$). The reversal phase, where the pairing of the compartments to laser stimulation was switched, confirmed these observations (**Figure 1B**, days 6 and 7, blue circles, Tukey's post hoc test paired vs unpaired compartment $p < 0.001$) thus excluding the possibility that the results obtained from phase 1 were related to side biases or random parameters. Averaging the time spent in each compartment during the four days of RT-

PP confirmed that the mice spent on average about 70% of their time in the laser paired compartment as opposed to the unpaired (~20%) and the neutral (~10%) compartments (**Figure 1B** bar graph, one-way RM ANOVA, effect of stimulation $F_{(2,6)} = 139$, $p < 0.001$, Tukey's post how paired vs unpaired and neutral compartments $p < 0.001$). Furthermore, in the absence of stimulation, on days 5 and 8, the mice spent significantly more time in the compartment previously paired with laser stimulation (Tukey's post hoc $p < 0.001$), indicating that the previous experience was sufficient to induce associative learning behaviors reflected as "seeking" of the stimulation. These data are in accordance with the literature and demonstrate that the current method can be used reliably to investigate the rewarding effects of optogenetic stimulation of specific neuronal populations in the VTA.

We then tested VGLUT2-Cre mice injected with *AAV-ChR2-eYFP* in the VTA as above, to target glutamatergic neurons of the VTA. In this experiment, we observed an opposite behavioral phenotype from the one demonstrated by the DAT-Cre mice. Thus, the mice avoided the compartment paired to the stimulation and spent more time in the unpaired during all RT-PP days (**Figure 1C** left, two-way RM ANOVA, effect of compartment $F_{(2,12)} = 40.9$, $p < 0.001$; effect of session x compartment $F_{(12, 72)} = 16.1$, $p < 0.001$; Tukey's post hoc test paired vs unpaired $p < 0.001$; **Figure 1C** right, one-way RM ANOVA effect of stimulation $F_{(2,6)} = 162$, $p < 0.001$, Tukey's post hoc paired vs unpaired and neutral compartments $p < 0.001$). Interestingly, during the "*CR*" days 5 and 8, the mice did not show a clear avoidance of the previously paired compartment (no differences between paired and unpaired compartments). It is possible that the lack of conditioned response is due to inadequate time spent in the laser paired compartment, which prevented the formation of associations between laser activation and the particular environment where that took place. To further explore this avoidance phenotype, we used a modified protocol which we named "neutral compartment preference", abbreviated NCP. In this experiment, both main compartments were paired to stimulation and the neutral compartment remained stimulation-free (**Figure 7A**). We hypothesized that, if the stimulation has aversive properties, then the mouse will be forced to spend time in the smaller, neutral compartment, to avoid it. Indeed, in both days of stimulation (Stim1 and Stim2) the mice spent the majority of the time in the neutral compartment (about 80%) compared to the paired compartments (**Figure 7B,C**; left: two-way RM ANOVA effect of compartment $F_{(2,8)} = 70.9$, $p < 0.001$; effect of session x compartment $F_{(4,16)} = 6.9$, $p = 0.002$, Tukey's post hoc "Stimulation 1" neutral compartment vs compartment 1 and 2 $p < 0.01$, "Stimulation 2" neutral compartment vs compartment 1 and 2 $p < 0.001$; right: one-way RM ANOVA, effect of stimulation $F_{(2,2)} = 54.2$, $p = 0.018$, Tukey's post hoc test paired 1 and 2 vs neutral $p < 0.05$). As in the case of "*CR*" days during the RT-PP test, the mice did not seem to form negative associations between the compartments and the stimulation; that is, in the absence of stimulation (CR), they explored all the compartments to the same degree (**Figure 7B**, no differences between time spent in paired compartments and neutral compartment). The results of these experiments confirmed the behavioral phenotype observed during the RT-PP setup and thereby support the combinatorial implementation of both the RT-PP and NCP paradigms.

FIGURE AND TABLE LEGENDS:

Figure 1: Behavioral testing using optogenetics in the RT-PP paradigm. (**A**) Schematic

representation of the timeline of the experiments. (**B,C**) Top left: graph representing the percentage of time spent in each compartment throughout the RT-PP experiment for DAT-Cre (N = 10) and VGLUT2-Cre (N = 7) mice injected with *AAV-ChR2-eYFP*. Blue circles: laser-paired compartment; white, black circles: main compartments; gray circles: neutral compartment. Top right: average percentage of time spent in each compartment during days 3, 4, 6, and 7 (RT-PP). Bottom: representative heatmaps of time spent in each compartment for a DAT-Cre and a VGLUT2-Cre mouse. All data were normally distributed (Shapiro-Wilk test). Results are presented as mean ± SEM. ***$p < 0.001$ paired vs unpaired; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ paired vs neutral compartment; §§$p < 0.01$, §§§$p < 0.001$ unpaired vs neutral compartment. This figure has been modified from Bimpisidis et al.[12].

Figure 2: Surgical procedure for optogenetic experiments. (**A**) Injection of Cre-dependent viral vector in the VTA. (**B**) Implantation of the optic fiber above the injection site. Note the anchor screws used for stabilization. (**C**) Permanent anchoring of the fiber on the skull using dental cement.

Figure 3: Equipment used in the optogenetics experiments. (**A**) The TTL box used in the studies. It receives input from the tracking software and sends TTL signals to the microcontroller board. (**B**) Front (top) and rear view (bottom) of the laser source used for the experiments. (**C**) The microcontroller board used to control the laser stimulation. Note the connections from the TTL box and to the laser source. (**D**) Rotary joint. (**E**) Fiber-optic patch chord used in the experiments. (**F**) The three-compartment apparatus used for RT-PP and NCP experiments.

Figure 4: Designing arena and zones within the tracking software. Step 1: Calibration of the setup. Step 2: Drawing of the whole arena. Step 3: Drawing zones within the arena. Step 4: Setup validation. Step 5: Trial Control Settings tab for setting up time and stimulation parameters.

Figure 5: Setting up time and stimulation parameters of an RT-PP experiment within the tracking software. Adding specific rules for the duration (step 1) and the conditions for light stimulation (step 2). The conditions can be easily changed to fit the requirements for the reversal phase.

Figure 6: Setting up time and stimulation parameters of NCP experiments within the tracking software. The duration of the stimulation sessions (step 1) is similar to the ones for RT-PP but the conditions for light stimulation activation (step 2) are different. Entry to either main compartment (here named zone A and zone B) results in optogenetic stimulation that is terminated only when the mouse enters the neutral compartment.

Figure 7: Behavioral testing using optogenetics in the NCP paradigm. (**A**) Schematic representation of the experimental setup. (**B**) Left: graph representing the percentage of time spent in each compartment during the two days of stimulation (Stim1 and Stim 2) and during the conditioned response (CR) session for VGLUT2-Cre mice injected with *AAV-ChR2* in the VTA

(N = 5). Right: average percentage of time spent in each compartment during the two days of stimulation of the NCP. (**C**) Representative heatmap of time spent in each compartment for a VGLUT2-Cre mouse during one of the stimulation days. Data were normally distributed (Shapiro-Wilk test). Results are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ unpaired vs paired 1 and paired 2 compartments. This figure has been modified from Bimpisidis et al.[12].

Table 1: The PCR cycling program.

DISCUSSION:

In the current study, we present two step-by step protocols of how to perform different types of place preference analyses using optogenetics in mice. The protocols outlined were used to assess the rewarding or aversive behavioral phenotypes of VTA neurons (**Figure 1** and **Figure 6**)[12], but can be utilized to explore the behavioral role of neurons in other brain regions as well.

Several recent studies have described RT-PP paradigms in two-compartment[23,24] and three-compartment apparatuses[13–18]. The current protocols describe detailed setups for the RT-PP and NCP protocols in a three-compartment apparatus resembling those traditionally used in CPP experiments to assess behavioral effects upon administration of drugs of abuse. While the results are only presented here as the percentage of time the mouse spent in each compartment, the tracking software does allow for analysis of several other behavioral parameters, such as transitions to zones, velocity, time spent immobile and more. Analysis of different parameters can be of importance to the interpretation of data.

The current RT-PP protocols are flexible and can be modified to test if different types of stimulation patterns have rewarding effects. The parameters of laser control can be easily changed either through the script of the microcontroller board or within the tracking software, demonstrating the versatility of the setup. We suggest a 20 Hz stimulation frequency which is within the range, and sometimes lower, of frequencies applied in previous studies using the same opsin variant (ChR2/H134R) to study dopaminergic and glutamatergic neurons and their terminals[13,14,16–18,23–27]. Recent studies have demonstrated that higher stimulation frequencies can have the opposite effects on behavior than lower ones, and that these effects are mediated through a depolarization block caused by higher frequencies[28]. Similarly, differences in behavioral output have been shown when stimulating glutamatergic and GABAergic neurons in the lateral preoptic area[15]. These studies examined neurons of different areas than the VTA and the largest effects were observed on high frequencies of non-glutamatergic neurons[15,28]. Our choice on 20 Hz is based on previous studies of glutamatergic and dopaminergic VTA neurons demonstrating that by varying stimulation frequencies, reward-related behavioral output is not significantly altered[24,26].

An additional parameter that can be adjusted and which may influence the experimental outcome is the power of the light source. Higher laser power can increase the size of the light-stimulated area, which may be beneficial in some types of experiments but with the drawback of an increase in temperature[5]. Indeed, a recent study has demonstrated that laser-induced

increases in temperature can alter brain physiology and affect behavioral measurements[29]. These observations highlight the importance of including opsin-negative controls in the experimental design. In the current protocol, we used 10 mW laser power that is similar and has been previously shown to be effective in stimulating dopaminergic and glutamatergic neurons in the VTA[16,24,26]. When setting up experiments, it is important to pay attention to the size of the area in which the cells of interest are located and the fiber-optics and patch cord properties (numerical aperture, core diameter). These parameters are essential to take into consideration when performing calculations related to laser power. For details, the calculator developed by Karl Deisseroth's lab (http://web.stanford.edu/group/dlab/cgi-bin/graph/chart.php) can be used.

Histological verification of the Cre-Lox recombination is another critical aspect when applying optogenetics experiments. Validation of the recombination efficiency should always take place in a pilot cohort before the initiation of any behavioral experiments in a large group of animals. This is important for ethical reasons but also for optimized experimental output. Each viral construct might show variable specificity for distinct neuronal types and in different regions[5], a parameter which can affect the experiments in unpredictable and even misleading ways. For instance, we have previously validated the Cre-Lox recombination pattern of *AAV5* viruses in the VTA of DAT-Cre mice and found that unilateral injections were sufficient to target the majority of the area of interest. When we then studied spatially restricted subpopulations within the VTA, such as one characterized by NeuroD6 expression, we observed that bilateral viral injections were more efficient to target larger number of neurons giving more pronounced behavioral effects upon optogenetic light-stimulation[12]. Furthermore, the time from surgery to initiation of behavioral experiments has to be addressed carefully. Two weeks is enough time for a ChR2 DNA construct to be expressed in cell bodies as we show here, but longer waiting times (~8 weeks) might be needed if the investigator is testing the effect of stimulation in projection areas[13–15,17].

It worth noting that the volume of virus injected (in our case 300 nL) might be suitable when studying neurons in the VTA, but volume and titer must be adjusted depending on efficiency of transduction and the size of the structure studied. Additionally, for bilateral structures located at a distance from the mediolateral axis, it might be necessary to perform bilateral injections, and to also implant fiberoptics bilaterally to ensure activation/inhibition in both hemispheres.

Finally, it is always necessary to perform post-mortem histological analysis to validate and confirm the efficiency of the Cre-Lox recombination and to verify the correct implantation site of the optic fiber at the intended location. Unexpected, over-restricted or excessive Cre-Lox recombination might occur due to unknown distribution of neurons expressing Cre outside the borders of the intended area, or due to differences in the virus serotype, poor handling of the virus, clogging of the syringe for virus delivery or other surgery-related problems. Verification of satisfactory Cre-Lox recombination and correct fiberoptics-implantation has to be performed to confirm any statistical results of the behavioral assessments in order to draw safe conclusions.

In terms of the data provided here as examples of how the two behavioral paradigms can be

used, the significant preference to the light-paired side obtained by optogenetic stimulation of dopaminergic neurons in the VTA by analyzing DAT-Cre mice in the RT-PP paradigm was expected based on previous findings[23–27] while the avoidance of this side shown by the VGLUT2-Cre mice was not anticipated. VGLUT2 neurons of the VTA and their projections have been shown to be involved in both reward and aversion[16,17,24,30,31], and we therefore performed the NCP analysis to assess the apparent avoidance behavior observed in the current RT-PP setup in more detail. By using the narrow, transparent corridor as the only non-light paired compartment to confirm the aversive properties of stimulation of VTA glutamatergic neurons, it is evident that in this particular three-compartment setup, optogenetic activation of these neurons causes an aversive response. These experiments, which were shown here to exemplify situations that might benefit from using both the RT-PP and NCP protocols, were part of a recently published study, and the complete data set as well as discussions regarding these findings can be found in this publication[12].

In addition to the NCP, alternative ways to confirm aversion include the strong illumination of an area within an open field area while pairing the rest of the arena to laser activation, or perform an active avoidance task in which the mouse has to perform a specific pattern of behavior to terminate the laser stimulation[15].

To summarize, the protocols described provide critical information of how to successfully perform RT-PP and NCP analysis in the most efficient way in order to unravel the role of neuronal activation in reward and aversion. Depending on the scientific hypothesis, a range of parameters can be analyzed using these protocols, and each protocol can also be combined with other validated paradigms for optimized behavioral analyses implementing optogenetics to address specific brain areas and neurons of interest.

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DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Fenno, L., Yizhar, O., Deisseroth, K. The Development and Application of Optogenetics. *Annual Review of Neuroscience*. **34** (1), 389–412 (2011).
2. Kim, C.K., Adhikari, A., Deisseroth, K. Integration of optogenetics with complementary methodologies in systems neuroscience. *Nature Reviews Neuroscience*. **18** (4), 222–235 (2017).
3. Deisseroth, K. Optogenetics: 10 years of microbial opsins in neuroscience. *Nature Neuroscience*. **18** (9), 1213–1225 (2015).
4. Nagy, A. Cre recombinase: The universal reagent for genome tailoring. *Genesis*. **26** (2), 99–109 (2000).

5. Yizhar, O., Fenno, L.E., Davidson, T.J., Mogri, M., Deisseroth, K. Optogenetics in Neural Systems. *Neuron*. **71** (1), 9–34 (2011).
6. Pupe, S., Wallén-Mackenzie, Å. Cre-driven optogenetics in the heterogeneous genetic panorama of the VTA. *Trends in Neurosciences*. **38** (6), 375–386 (2015).
7. Spanagel, R. Animal models of addiction. *Dialogues in Clinical Neuroscience*. **19** (3), 247–258 (2017).
8. Tzschentke, T.M. Measuring reward with the conditioned place preference (CPP) paradigm: Update of the last decade. *Addiction Biology*. **12** (3–4), 227–462 (2007).
9. Hoffman, D.C. The use of place conditioning in studying the neuropharmacology of drug reinforcement. *Brain Research Bulletin*. **23** (4–5), 373–387 (1989).
10. Huston, J.P., Silva, M.A.D.S., Topic, B., Müller, C.P. What's conditioned in conditioned place preference? *Trends in Pharmacological Sciences*. **34** (3), 162–166 (2013).
11. Bardo, M.T., Bevins, R.A. Conditioned place preference: What does it add to our preclinical understanding of drug reward? *Psychopharmacology*. **153** (1), 31–43 (2000).
12. Bimpisidis, Z. et al. The NeuroD6 subtype of VTA neurons contributes to psychostimulant sensitization and behavioral reinforcement. *eNeuro*. **6** (3), e0066-19.2019 (2019).
13. Root, D.H., Mejias-Aponte, C.A., Qi, J., Morales, M. Role of Glutamatergic Projections from Ventral Tegmental Area to Lateral Habenula in Aversive Conditioning. *Journal of Neuroscience*. **34** (42), 13906–13910 (2014).
14. Steidl, S., Wang, H., Ordonez, M., Zhang, S., Morales, M. Optogenetic excitation in the ventral tegmental area of glutamatergic or cholinergic inputs from the laterodorsal tegmental area drives reward. *European Journal of Neuroscience*. **45** (4), 559–571 (2017).
15. Barker, D.J. et al. Lateral Preoptic Control of the Lateral Habenula through Convergent Glutamate and GABA Transmission. *Cell Reports*. **21** (7), 1757–1769 (2017).
16. Wang, H.-L., Qi, J., Zhang, S., Wang, H., Morales, M. Rewarding Effects of Optical Stimulation of Ventral Tegmental Area Glutamatergic Neurons. *The Journal of Neuroscience*. **35** (48), 15948–54 (2015).
17. Qi, J. et al. VTA glutamatergic inputs to nucleus accumbens drive aversion by acting on GABAergic interneurons. *Nature Neuroscience*. **19**, 725–733 (2016).
18. Qi, J. et al. A glutamatergic reward input from the dorsal raphe to ventral tegmental area dopamine neurons. *Nature Communications*. **5**, 5390, (2014).
19. Ekstrand, M.I. et al. Progressive parkinsonism in mice with respiratory-chain-deficient dopamine neurons. *Proceedings of the National Academy of Sciences of the United States of America*. **104** (4), 1325–1330 (2007).
20. Borgius, L., Restrepo, C.E., Leao, R.N., Saleh, N., Kiehn, O. A transgenic mouse line for molecular genetic analysis of excitatory glutamatergic neurons. *Molecular and Cellular Neuroscience*. **45** (3), 245–257 (2010).
21. Papathanou, M. et al. Targeting VGLUT2 in Mature Dopamine Neurons Decreases Mesoaccumbal Glutamatergic Transmission and Identifies a Role for Glutamate Co-release in Synaptic Plasticity by Increasing Baseline AMPA/NMDA Ratio. *Frontiers in Neural Circuits*. **12**, 64 (2018).
22. Franklin, K.B.J., Paxinos, G. *The mouse brain in stereotaxic coordinates*. Academic Press. Boston, MA (2008).
23. Tsai, H.-C. et al. Phasic firing in dopaminergic neurons is sufficient for behavioral

749 conditioning. *Science*. **324** (5930), 1080–4 (2009).
750 24. Yoo, J.H. et al. Ventral tegmental area glutamate neurons co-release GABA and promote
751 positive reinforcement. *Nature Communications*. **7**, 1–13 (2016).
752 25. Pascoli, V., Terrier, J., Hiver, A., Lüscher, C. Sufficiency of Mesolimbic Dopamine Neuron
753 Stimulation for the Progression to Addiction. *Neuron*. **88** (5), 1054–1066 (2015).
754 26. Ilango, A., Kesner, A.J., Broker, C.J., Wang, D. V., Ikemoto, S. Phasic excitation of ventral
755 tegmental dopamine neurons potentiates the initiation of conditioned approach behavior:
756 parametric and reinforcement-schedule analyses. *Frontiers in Behavioral Neuroscience*. **8**, 155
757 (2014).
758 27. Kim, K.M. et al. Optogenetic mimicry of the transient activation of dopamine neurons by
759 natural reward is sufficient for operant reinforcement. *PLoS ONE*. **7** (4), 1–8 (2012).
760 28. Kroeger, D. et al. Galanin neurons in the ventrolateral preoptic area promote sleep and heat
761 loss in mice. *Nature Communications*. **9**, 4129 (2018).
762 29. Owen, S.F., Liu, M.H., Kreitzer, A.C. Thermal constraints on in vivo optogenetic
763 manipulations. *Nature Neuroscience*. **22** (7), 1061–1065 (2019).
764 30. Root, D.H., Estrin, D.J., Morales, M. Aversion or Salience Signaling by Ventral Tegmental
765 Area Glutamate Neurons. *iScience*. **2**, 51–62 (2018).
766 31. Lammel, S. et al. Diversity of transgenic mouse models for selective targeting of midbrain
767 dopamine neurons. *Neuron*. **85** (2), 429–438 (2015).
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Figure 1

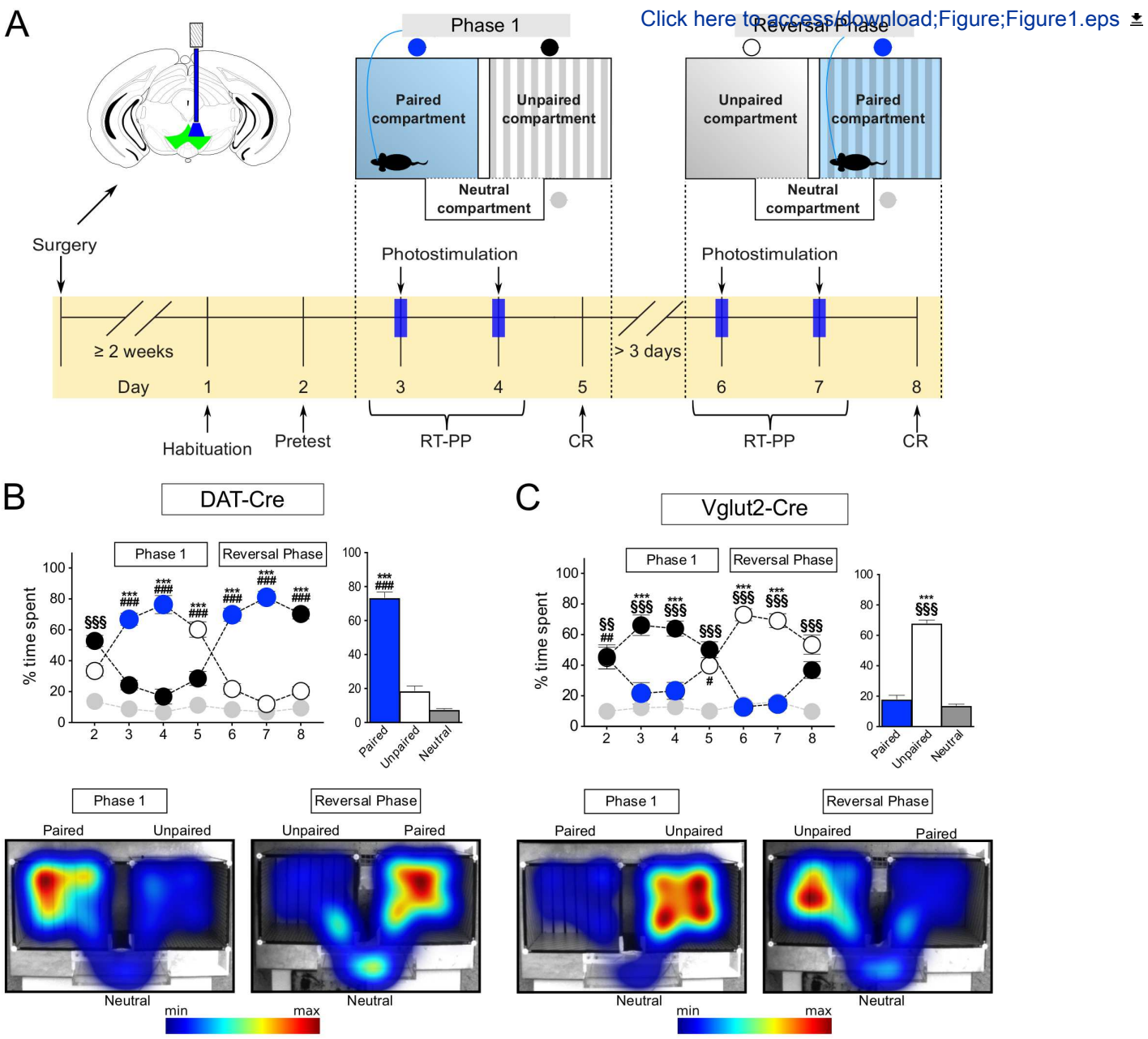

Figure 2

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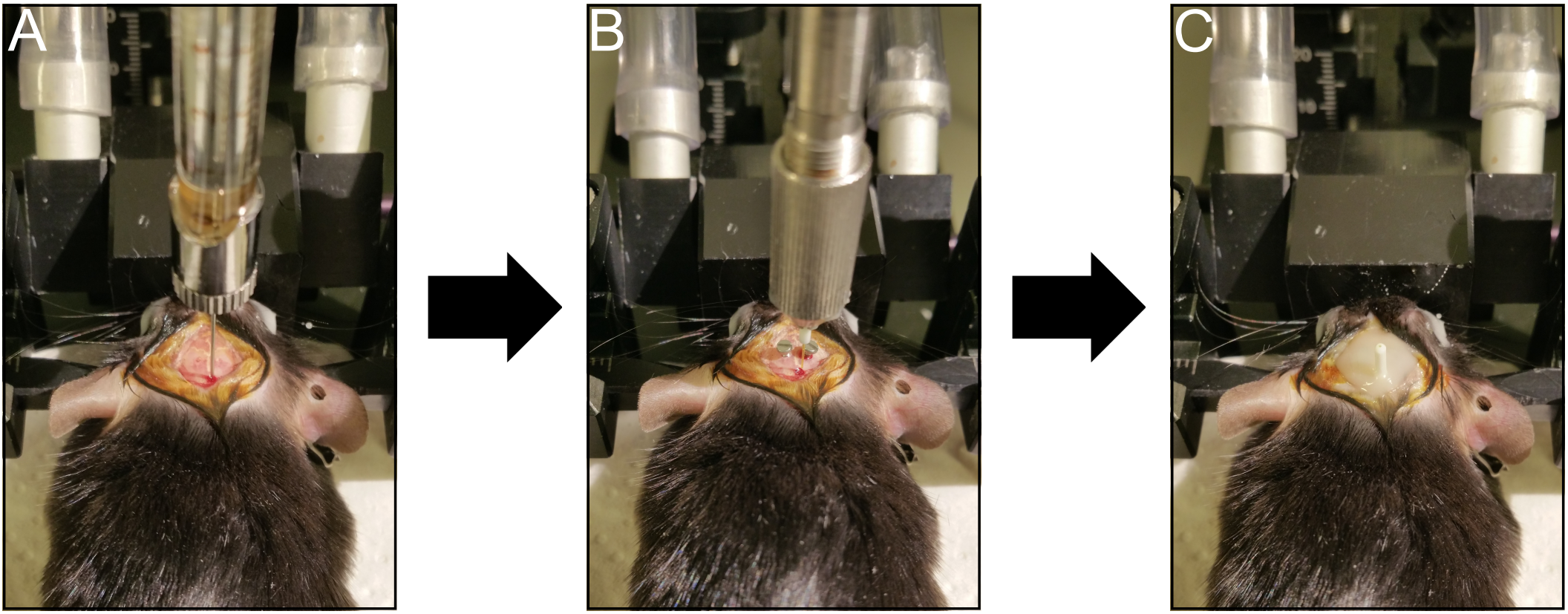

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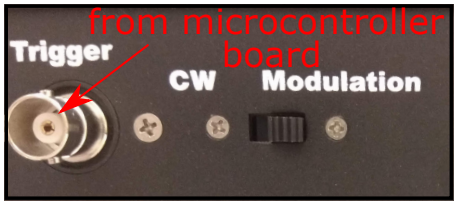

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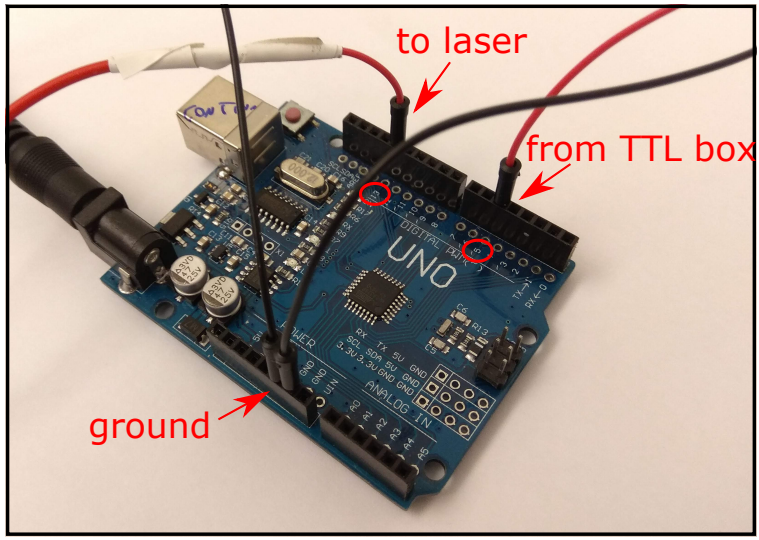

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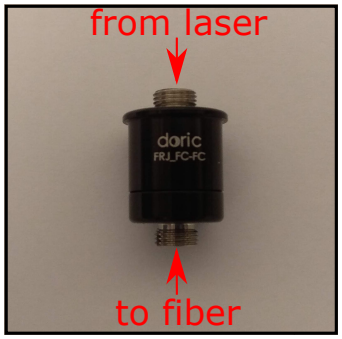

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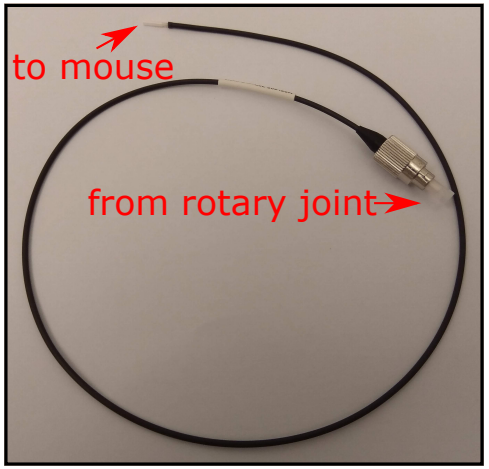

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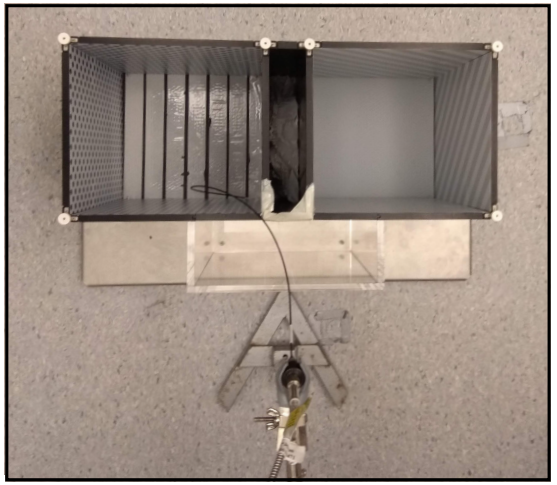

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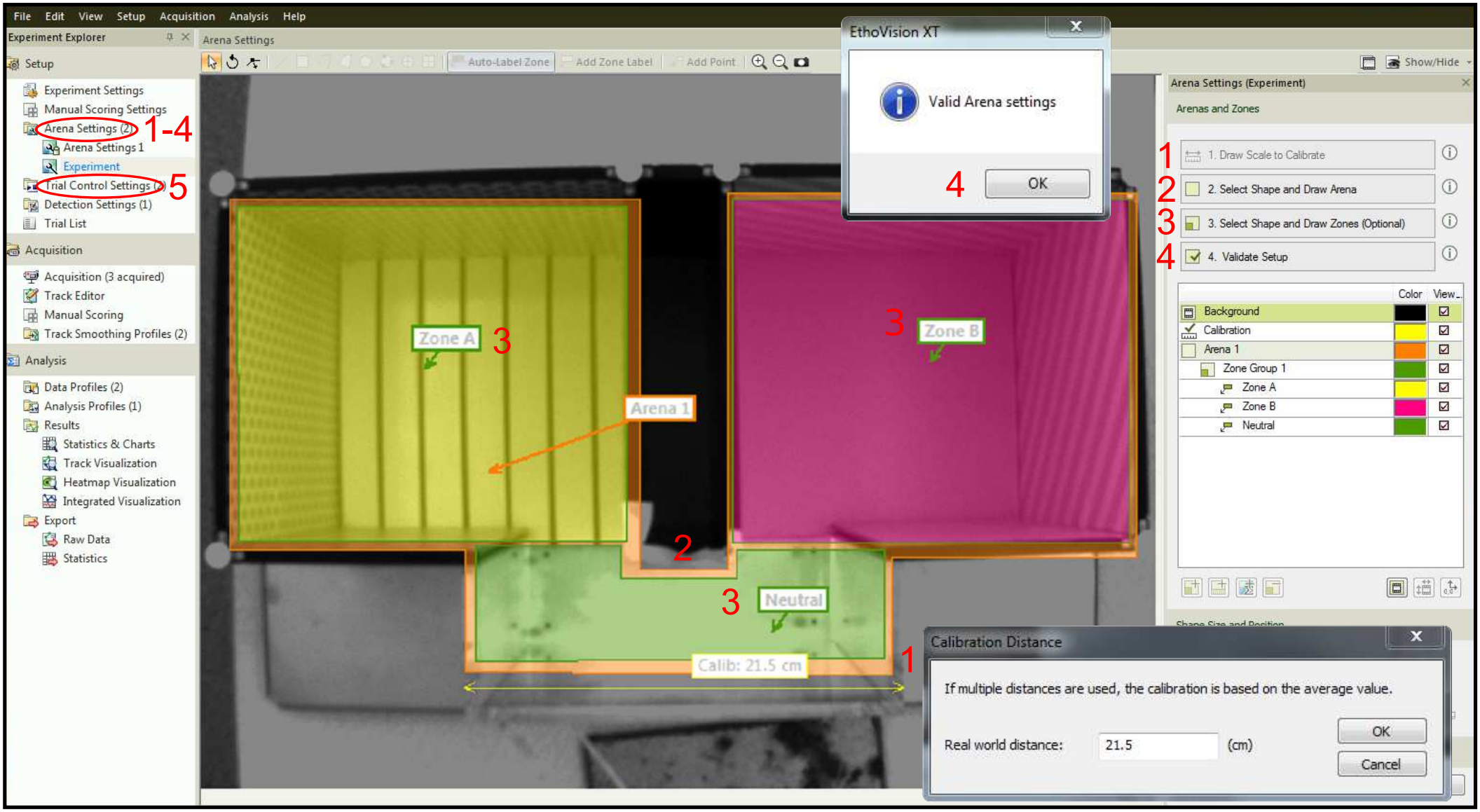

Figure 5

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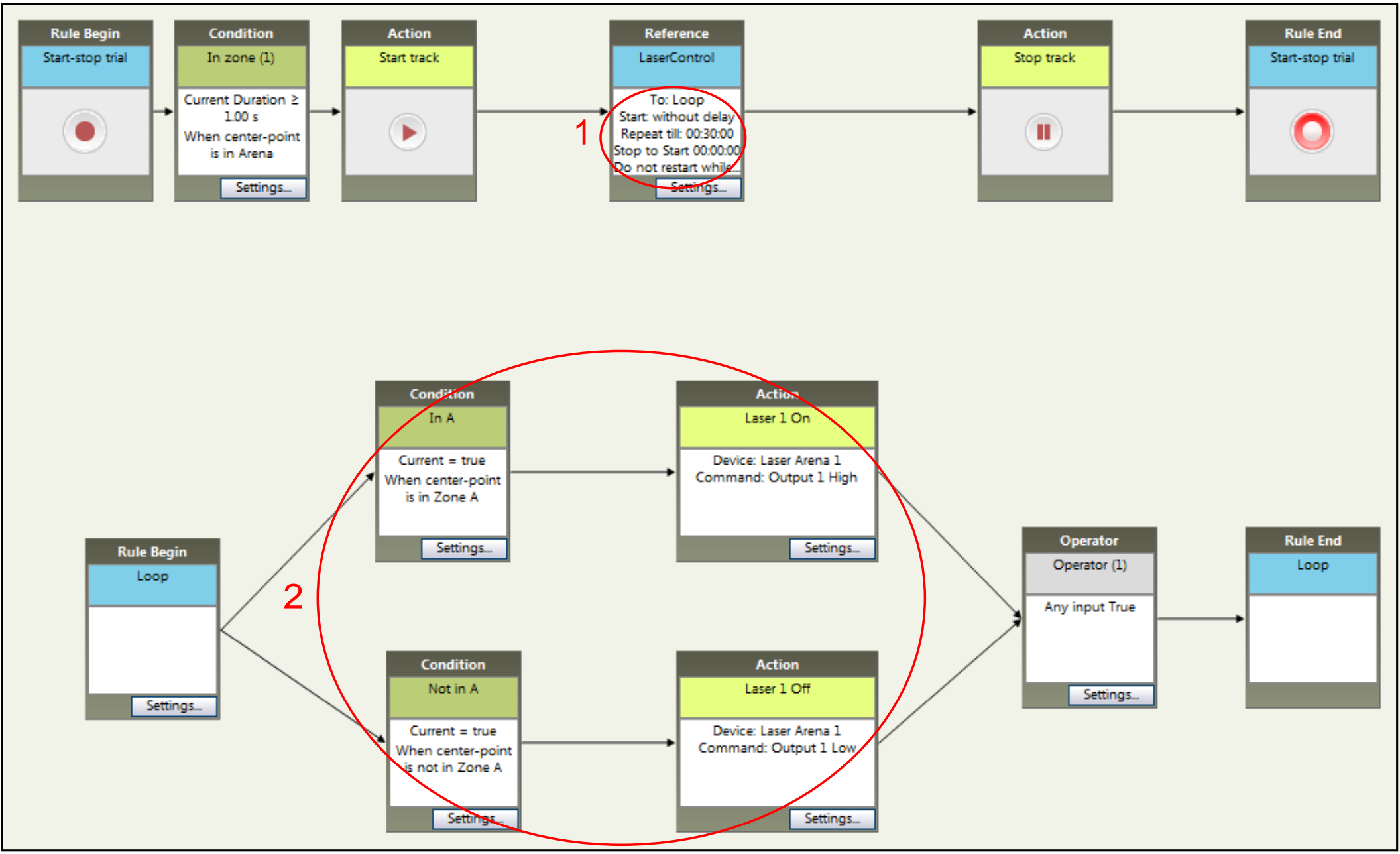

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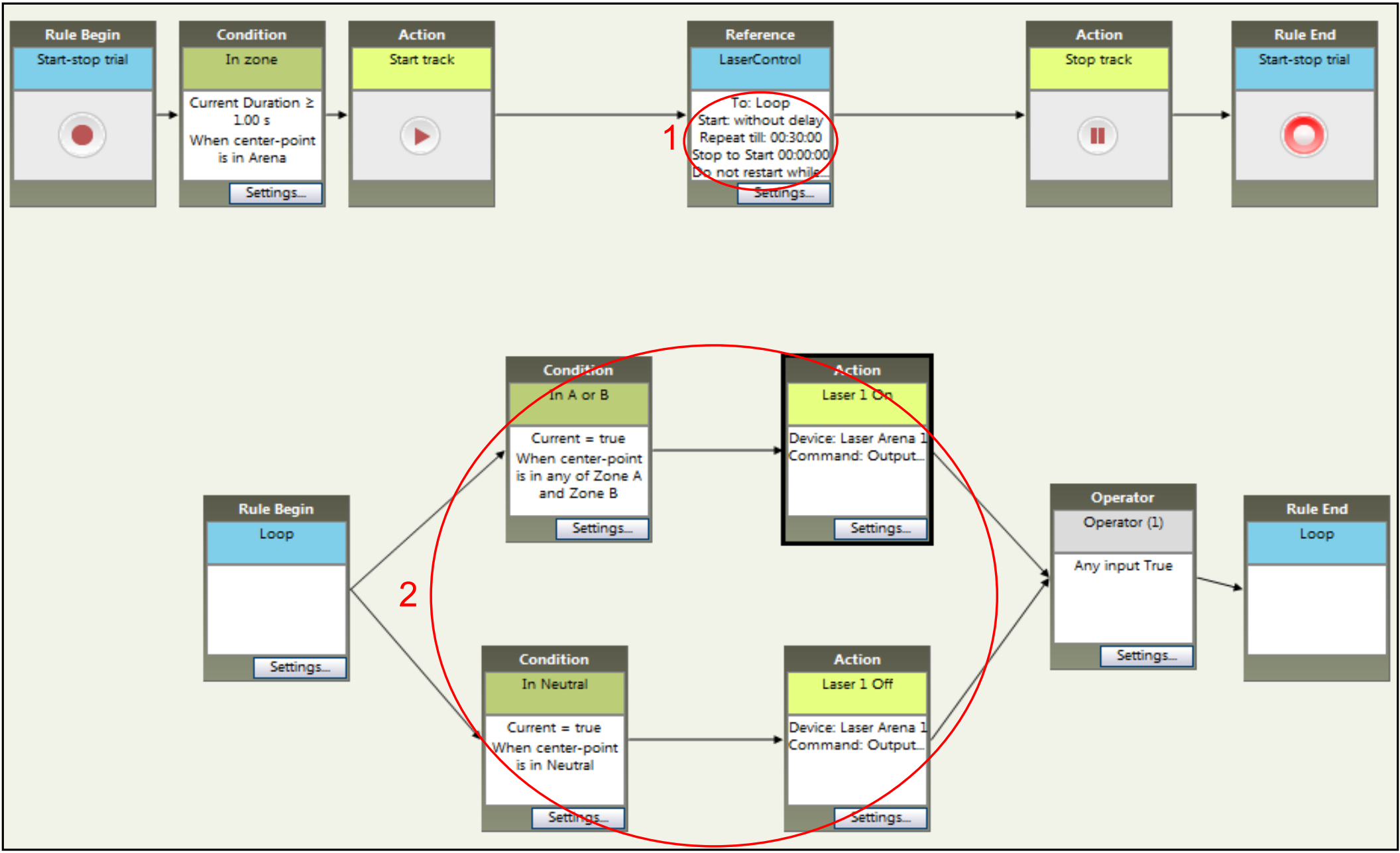

Figure 7

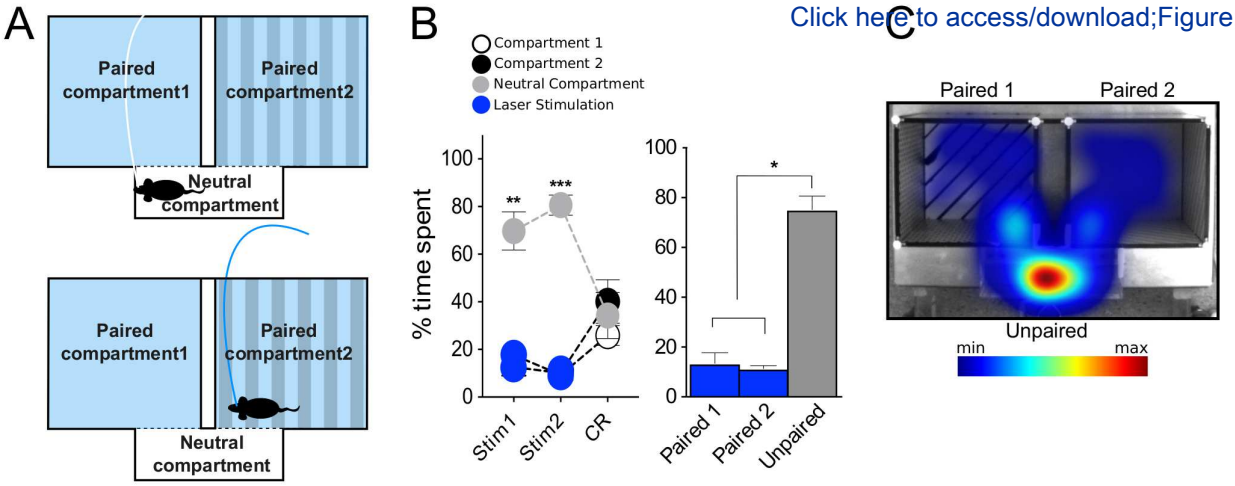

Table 1

Step	Temperature	Duration	Cycles
1. Initial denaturation	95 °C	4 min	1
2. Denaturation	95 °C	30 s	
3. Annealing	55 °C	30 s	30
4. Extension	72 °C	40 s	
5. Final extension	72 °C	6 min	1
6. Hold	4 °C	Until stopped by the experimenter	1

Table of materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
AAV-Cre dependent virus	UNC Vector Core	-	a great variety of viruses to suit any project's needs
Agarose	VWR Life Science	443666A	
Buffer for PCR	KAPA BIOSYSTEMS	KB1003	10x, contains 1.5mM MgCl2 at 1x
Bupivacaine (Marcain)	Aspen	N01BB01	local anesthetic, 5 mg/ml solution, requires prescription
Carprofen (Norocarp)	N-Vet	27636	anti-inflammatory, analgesic; 50 mg/ml solution, requires prescription
dNTP set	ThermoFischer Scientific	R0181	100mM, have to be dilluted to 10mM and mixed
DNA ladder	ThermoFischer Scientific	SM0243	100 bp, 50µg Gene Ruler
DNA loading dye	ThermoFischer Scientific	R0611	6x, dilute to 1x before using
Ear puncher	AgnThos	AT7000	ear puncher to take tissue samples for PCR or to mark animals
Fiberoptic patchcords	Doric Lenses	ZF1.25	
Implantable fiberoptics	Doric Lenses	MFC_200/245-0.37_5mm_ZF1.25_FLT	the properties of the fibers might change depending on the experiment
Infusion pump for virus	AgnThos	Legato 130	contains remote pump to secure the syringe directly on the stereotexi frame
Isoflurane (Forane)	Baxter	N01AB06	Volatile anesthetic, requires prescription
Jewelry screws	AgnThos	MCS1x2	
Laser source	Marwell Laser Systems	CNI MBL-III-473-100mW	
Microcontroller board	Arduino	"Uno" board	can be ordered from several companies
Microdrill	AgnThos	1474	could be ordered with or without stereotaxic holder
Needle (34G)	World Precision Instruments	NF36BV	
Nucleic Acid gel stain - GelRed	Biotium	41003-T	
PCR tubes	Axygen	PCR-0208-CP-C	
Power meter	Thorlabs	PM100D	
Place Preference Apparatus	Panlab	76-0278	
Rotary joint	Doric Lenses	FRJ_1x1_FC-FC	
Sleeves	Doric Lenses	SLEEVE_ZR_1-25	mating sleeve to connect the patchcord with the implanted optic fiber
Stabilization cement	Ivoclar Vivadent	Tetric EvoFlow	
Syringe (10ul)	World Precision Instruments	NanoFil	
Taq polymerase	KAPA BIOSYSTEMS	KE1000	500U
TAE buffer	omega BIO-TEK	SKU:AC10089	50x concentration, has to be dilluted before use
Thermal cycler	BIO-RAD S1000	1852148	necessary to perfrom PCR reactions
Tissue glue	AgnThos	Vetbond	
Tracking software	Noldus	Ethovision XT	
TTL box	Noldus	Noldus USB-IO box	
UV transilluminator	azure biosystems	c200 model	

Reviewer's comments JoVe

Dear Dr. Bimpisidis,

Your manuscript, JoVE60867 "Step-by-step experimental protocol for two different real-time place preference paradigms using optogenetics within the Ventral tegmental area (VTA).," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Your revision is due by Nov 06, 2019.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision". Please note that the corresponding author in Editorial Manager refers to the point of contact during the review and production of the video article.

Best,

Peer Review,
Peer Review
JoVE

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Answer: We have proofread the manuscript and we are confident that there are no spelling or grammar issues.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Answer:
Figures have been reprinted from eNEURO in which we have published in the open access track. We have now added the appropriate citation to each figure.

3. Please revise the Protocol text to avoid the use of personal pronouns (e.g., I, you, your, we, our) or colloquial phrases.

Answer: The personal pronouns have been removed from the protocol.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Noldus, Marcain, EthoVision XT, Marwell, etc.

Answer:
All the commercial names of products have been removed from the main text and the figure legends.

5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Answer: More details and new parts have been added to the protocol.

6. 1.1: Please specify the age and gender of the mice used.

Answer: The age and gender of the mice have been specified.

7. 1.2: What samples? It is unclear.

Answer: “Samples” has been replaced by “ear punches”.

8. 1.2.1: Add to what?

Answer: Corrected.

9. 1.3: Please specify PCR conditions.

Answer: The protocol for PCR has been added to the manuscript.

10. 2.1.3: Please mention how adequate anesthetization is confirmed.

Answer: Added in the section.

11. Please specify all surgical tools used throughout the protocol.

Answer: The surgical tools have been specified.

12. 2.1.5: How large is the incision?

Answer: The approximate size of the incision has been added in the text.

13. Lines 230-258, 280-288: Everything in the protocol (except for the introductory ethics statement) should be in a numbered step (in the imperative tense and with no more than 4 sentences), numbered header, or a “NOTE”. Please move these paragraphs to the Introduction, Results, or Discussion (as appropriate) or break into steps.

Answer: The suggested parts have been moved to the introduction.

14. 5.1.2 and 5.1.3: Please describe how these are actually done.

Answer: This has now been added in the text.

15. Table of Materials: Please sort the materials alphabetically by material name.

Answer: The materials in the table have been sorted alphabetically.

Reviewer #1:

Manuscript Summary:
Bimpisidis and colleagues provide a very thorough protocol for a real time place preference and neutral compartment preference protocol. The protocol is thoroughly written and provides ample description of the procedures. The only comments I have relate to either small omissions from the surgical procedures or potential problems with the generalizability of the current protocol based on the use of very specific or proprietary devices.

Major Concerns:

1. The use of an eye ointment/lubricant should be included in the stereotaxic protocol to prevent lesions of the eye.

Answer: The use of an eye lubricant has been added in section 2.1.4.

2. The use of iodine is indicated at the incision site, but most standard surgical procedures include 3x alternations with iodine and alcohol.

Answer: Thank you for your comment. We have used iodine in the experiments described in the protocol and we did not observe any major inflammatory responses at the incision site. We will indeed consider adopting your approach on our future surgeries.

3. The authors suggest a biased design for their Phase 1 RT-PP experiment, stating that laser stimulation occurs in the "least-preferred compartment". This makes sense for when the stimulation is rewarding, but not if the stimulation ends up being aversive. The authors should consider promoting an unbiased design or discussing the use of pilot studies to determine the proper bias to choose.

Answer: Indeed, that was the case and the text has been modified accordingly to include the mentioning of a pilot study for this purpose.

4. The clear chamber is very specific to the apparatus used by the authors. It would be valuable to discuss some alternative to this, such as using a bright light over the open field to create a similar conflict.

Answer: We agree and this has been added in the text (Discussion).

5. The authors should discuss the selection of stimulation frequency and its importance for running this task. Recent work from Kroeger et al., (2018) demonstrated that certain stimulation frequencies can produce opposing effects using ChR2 through depolarization block and a recent paper from Barker et al (2017) also shows differences across frequencies in their supplemental data. The task in that paper is a nice variation on what is presented here, as mice are required to actively shuttle in these same CPP-type chambers to earn or avoid stimulation.

Answer: These references and the reviewer's concerns have been added in the Discussion.

Minor Concerns:

1. It would be helpful for the authors to describe how to flatten the skull.

Answer: A section describing how to flatten the skull has been added in the surgery description.

2. The authors may want to discuss a range of viable injection volumes instead of a single value. The literature ranges as wide 100-500nl in mice, from my experience.

Answer: Agreed and we have added relevant information in the Discussion.

3. It may help to change step 2.2.10 to state "Put enough cement to cover all but 3-4 mm of the ferrule with cement". If individuals follow this as a protocol, they might first cover 'most' of the ferrule, only to realize they have covered too much.

Answer: Agreed and we have rephrased this sentence.

4. The authors may want to indicate that a second, optional conditioned response test can be conducted after reversal.

Answer: The possibility of adding a second CR session during the Reversal Phase has now been made clearer. Note that the description of the RT-PP and NCP has now been moved to Introduction.

Reviewer #2:

Manuscript Summary:
This review nicely describes the methodological and technological components for running a Real Time Place Preference Paradigm (RT-PP). The authors provide sufficient rationale for when this paradigm should be implemented and detail the CRE-LOX system employed for cellular specificity during this task. Additionally the authors indicate certain limitations (heat induced changes) and outline considerations for researchers to account for when using this procedure (viral transfection differences). Lastly, the authors provide a new technique for directly assessing aversion using a Neutral Compartment Preference test. Holistically, this article systematically details how to measure the behaviorally rewarding or aversive effects of neuronal manipulation in distinct cell types in awake behaving animals.

Major Concerns:

1. "Place preference paradigms" is used often however, it is really place conditioning because a preference or aversion could be detected

Answer: We have followed standard nomenclature in the field and with detailed descriptions provided, we think the reader will be able to understand the concept of preference and aversion.

2. discussion of testing different frequencies, intensities, etc would be useful and how that is incorporated here

Answer: We agree and a section describing different frequencies and intensities of different stimulations has been added in the discussion.

3. additional discussion of drawbacks is warranted, see below

a. in classic CPP/A the subject spends equal time in each compartment but this does not have to be the case in the real time method. This is the likely reason that vglut2 experiments failed to show a conditioned response, there wasnt enough time to form an association.

Answer: This possibility has been added in the results section.

b. disadvantage of the 2 compartment mazes is that the subject can see the other side of the maze while being stimulated in the other compartment. this is confounding because it isnt clear which environmental stimuli are being conditioned (like used by Yoo). the 3 chamber maze used here does not have this concern. of course the narrow corridor is also problematic because rodents tend to prefer these small enclosures. Relatedly, the authors spend time explaining differences between the current study and those by Yoo. However, the aversive properties of vglut2 cells shown here is consistent with a number of studies showing projections of these cells as aversive in addition to firing patterns of these neurons. Some discussion of these issues might be useful.

Answer: We agree and this part of the Discussion has been modified with addition of this particular point. Further, the focus on the findings by Yoo et al has been removed as this has been discussed in our previous paper (Bimpisidis et al, eNEURO, 2019) which we refer to here, and hence the focus in the current Discussion is on methodology and not particular findings with the Cre-drivers used here as examples of how to use the methods.

4. To our knowledge, much of the rtPP paradigm shown here was first devised by Root et al 2014 JNeurosci, but it was not mentioned

Answer: We agree and apologize for not making this fact clearer. This has now been added in the text.

Minor Concerns:

Line 50- omit "on"

Answer: Corrected

Line 56/57-authors provide two examples of opsins for background but only use ChR2 in their VTA experiments. Understanding more about how stimulation would differ across opsins and whether there are other opsins to consider would clarify this topic.

Answer: A short part regarding different opsins has now been included in the introduction.

Line 61-63- redundant information from the sentences above. The only new information that should be emphasized is the use of the promoter for this specificity.

Answer: Corrected

Line 81-omit "originally derived for the field of experimental psychology"...the sentiment is addressed in line 77

Answer: Omitted.

Line 82-83-clarifying unconditioned and conditioned stimuli for non reinforcement learners will benefit a broader audience of scientists

Answer: The terms have now been clarified within the text.

line 83 - i believe the authors mean conditioned stimuli

Answer: Indeed, corrected.

Line 87- unclear (addressed later) whether they receive the injections at the same time

Answer: The sentence has been rephrased to make this statement clear.

Line 92- similar to comment 5 but expanding on "conditioned stimuli" terminology

Answer: Corrected.

Line 95- change "spent" to "spend"

Answer: Corrected

Line 96- change "thus inducing" to "indicating"

Answer: Corrected.

Line 132-133- consideration for the Neutral Compartment Preference would be to establish a baseline for each animal to ensure that they naturally avoid the smaller compartment instead of assuming this for each animal

Answer: The Pretest can also provide information regarding the time spent in the neutral compartment. This has been made clearer in the discussion.

Line 236- justification for the 80% cut off criterion would be helpful (if you observe a natural preference but stimulate on the opposite side and reverse this preference this would suggest an even more robustly rewarding outcome)

Answer: The justification has now been added in the introduction.

line 241 - stimulating in the place that was least preferred in the pre-test would not be optimal for stimulation that is aversive

Answer: Indeed, that is the case here and the biased design has now been mentioned in the introduction.

How might the optical implants change when the structure is bi-lateral

Answer: Agree, and this has now been added in the discussion.

Reviewer #3:

This paper, entitled "Step-by step experimental protocol for two different real-time place preference paradigms using optogenetics within the Ventral tegmental area (VTA)," is a follow-up to a recent study published by this group in the Journal of eNeuro. Combining optogenetics with two behavioral paradigms, the paper describes in detail the protocol for performing a Time Place Preference test (RT-PP) and a modified version of the Conditioned Place Preference (CPP). Although the behavioral paradigms are well described, I have some concerns regarding the specificity of the cre-lox recombination in the VGluT2-cre VTA and optogenetic stimulations.

Major comments:

The abstract and the discussion claim that locomotor parameters (speed and acceleration) can be monitored in response to photostimulation, but there are no data to support this conclusion.
This statement should either be removed or data should be added to support it.

Answer: This statement has been removed from the abstract.

The authors should correct their use of the term "spatial and temporal resolution" in the abstract and discussion. A volume of 300nL is a lot and does not comply with the statement regarding spatial specificity. It may be correct in the case of dopaminergic neurons, because dopaminergic neurons are well delineated and circumscribed in nuclei, but it is unlikely correct for glutamatergic neurons which are spread all over the brain.
Even by adding some IHC to support this claim, the volume is so high that the authors have likely induced a cre-lox recombination outside of the VTA in VGluT2-cre mice. Furthermore, not all dopaminergic neurons express DAT in some dopaminergic groups. What about VTA neurons?

Answer: These concerns have now been addressed in the Discussion. However, the use of DAT-Cre and VGLUT2-Cre mice was shown as examples and the data *per se* has been published elsewhere (Bimpisidis et al, eNEURO, 2019), and we now refer to this previous publication for discussion around specificity and implications of findings.

Regarding temporal resolution, in the absence of neural or motor recordings there is no information about the efficacy of dopaminergic or glutamatergic neurons of the VTA to fire action potentials upon photostimulation. Although the stimulation parameters (10 ms pulse width at 20 Hz) appear to be in the physiological range according to other optogenetic studies, there is no information about the normal firing pattern of dopaminergic and glutamatergic neurons. If the temporal specificity can apply in several other optogenetic studies, it does not in the current one.
Please remove the term "spatial and temporal selectivity" or rephrase your statement.

Answer: See response above. Further, in the above-mentioned publication, electrophysiological recordings were provided. We apologize for not making the reference to the previous publication sufficiently clear, but this has now been corrected.
As the focus of the current study is on the methodological protocol, we have reduced the discussion around the previous findings and instead opted to refer to the previously published study for discussion about the findings.
“Spatial and temporal resolution” and “Spatial and temporal selectivity” has been used in the context of optogenetics, we are not sure how this should be changed.

Stimulation parameters: In the absence of neuronal readouts there is no evidence that the authors are activating or over-activating these neurons. If the light transmission of optical probes is not tested prior to implantation, there is no guarantee that setting up the laser power at 10mW at the output of the laser will deliver the same power through the neural tissue.
The authors need to clarify this point.

Answer: This is a good point. The purpose of this protocol was the behavioral recording and assessment but we agree that this is important and have added a section in the Discussion about stimulation parameters.

Minor comments:

Abstract and introduction

Line 51: The establishment of a causal relationship between neuronal firing and behavioral output. Yes and no, as the stimulation can activate neurons but also block them if they are overstimulated.

Answer: This is true and this point has been added.

Line 76: "behavioral optogenetics" is awkward. This is not a behavioral (optogenetic) stimulation, but a neuronal (or brain) stimulation evoking a behavioral response.

Answer: We have removed “behavioral” in front of the word “optogenetics” throughout.

Line 110: Remove "intracranial."

Answer: Corrected.

Line 127 and 129: Need to add references to support these statements.

Answer: References have been added in the text.

Protocol

Line 153: Are these Jackson mice? The stock# and the correct reference should be mentioned.

Answer: References to the papers describing the generation of these mice have been added.

Line 176: Need to add a local block prior to cutting the skin over the skull.

Answer: Corrected in the text.

Line 179: I am not sure I understand the purpose of adding a local anesthetic over the skull after the skin incision?

Answer: The sequence of events has been corrected in the protocol.

Line 187: 300nL is a lot, but this will not be the first study using such a large volume. As noted above in the comments above, the use of spatial specificity can raise some questions in VGluT2-cre mice.

Answer: We have used 300nL of virus which is within the volume range used in previous studies on VTA glutamate neurons (e.g. Root et al., 2014 *J Neurosc* and Wang et al., 2015 *J Neurosc* used 350nL; Yoo, Zell et al., 2016 Nat Com used 300nL) and the data we provide as examples have been published elsewhere (Bimpisidis et al *eNEURO*, 2019). A Discussion regarding the virus volume has been added in the text.

Line 196: Usually, hemostatic materials are placed in the opening to protect the brain and several thin layers of dental acrylic are added several times to avoid burning the brain.

Answer: We added a note regarding this concern in the protocol.

Line 199: Why use tissue glue? Stitches or (even better) absorbable sutures should be sufficient.

Answer: Rephrased.

Line 213: I don't understand why the authors need to turn the switch to external modulation to control the laser while the animal is not yet plugged. Perhaps the authors should describe how to plug the animal, then how to set up the parameters for the equipment.

Answer: The external modulation refers to the TTL signal coming from the tracking software. In the absence of signal the laser will not be turned on. Setting the laser to external modulation does not affect laser functionality. If it was not switched to this option the laser would be constantly on.
A description of such an experiment has been added in the protocol.

Results
Add a statement regarding the normality of the data distribution

Answer: A statement has been added in the figure legends.

Discussion
Line 407 to 417: This is speculation. There are no data supporting these statements. Remove the statements or add some data.

Answer: We have rephrased this sentence, added references and more information after suggestion by reviewers incl. yourself.

Line 429 and other sections in the text: Correct "viral injection" by "cre-lox recombination." One cannot verify the viral injection; the virus is not coupled to a fluorescent tag, but one can assess the extent of the cre-lox recombination by fluorescence.

Answer: “viral injection” was replaced with “Cre-Lox recombination” throughout.

Line 442: Correct "transfection" by "cre-lox recombination."

Answer: Corrected.

Reviewer #4:

Manuscript Summary:

Bimpisidis and colleagues provide a detailed protocol for the real-time place preference (RT-PP) paradigm. This paradigm has become common among investigators who utilize optogenetic techniques to study the role of cell groups and the projections they give rise to in motivated behavior. The protocol they describe utilizes a 3-chamber conditioning apparatus in combination with video tracking of the animal's position within the apparatus.

The RT-PP paradigm has become popular so this protocol paper seems timely and will provide investigators, novice and experienced, some guidelines. It may thus be useful to many and I think it is worthy of publication. The manuscript is well written, the figures are appropriate and I expect the resulting video file will provide additional benefit. My comments are minor, with the exception of (18), and addressing them will improve the quality of the manuscript.

Minor Concerns:

1) Introduction, lines 50-52: The sentence "The outstanding spatial and temporal selectivity of optogenetics allows the establishment of causal relationships between neuronal firing and behavioral output" is a misleading. The use of "neuronal firing" clearly suggests recording/quantification of neuron firing rates. While it is possible to combine optogenetics with recordings (i.e. optrodes) this does appear to be what the authors have in mind here as the paper is exclusively on using optogenetics to modulate excitability of neurons. Perhaps replace with " …. causal relationships between excitation or inhibition of cell groups of interest and behavioral output" or explain in more detail the reference to "neuronal firing".

Answer: Corrected.

2) Introduction, lines 71-73: The explanation of the Cre-Lox system can be improved, especially given that this an instructional paper. The Lox part of Cre-Lox is explained but not the Cre part. The authors should make it clear that the investigator needs to obtain a Cre transgenic animal (usually mouse) and what that is (Cre recombinase constitutively expressed in the cell population of interest). They need to purchase them (Jackson Labs is common), maintain a breeding facility, etc.

Answer: The suggested information has been added in the text.

3) Introduction, line 83: the authors in parenthesis refer to cues of the environment as unconditioned stimuli …. must be conditioned stimuli?

Answer: Indeed, that was a typo and we meant conditioned stimuli. Corrected.

4) Introduction, line 88: rewarding effects of the drug are assessed is likely a better explanation of what happens with the traditional Pavlovian version of CPP. The term reinforcing is more appropriate for drug self-administration.

Answer: “Reinforcing” was replaced with “rewarding” throughout.

5) I think the authors are making too much of the NCP approach. First what is the justification that the "neutral compartment" is naturally avoided? Why can't aversion just as easily be seen/obtained by pairing one of the conditioning chambers with stimulation and observing avoidance of that chamber?

Answer: As can be seen from the results of the pre-test, mice spent a small proportion of the session time in the corridor (~15%) – they prefer to explore the bigger, main compartments. While the first protocol can be readily used to assess the aversive properties of the stimulation, the NCP described here is used to confirm these findings. We have reduced the text mass about the NCP but maintained it as a separate protocol.

6) I think the authors need to include some discussion of biased vs unbiased chamber assignment for RT-PP. Anybody who has run CPP or CPA experiments will know it is rarely ever the case that mice/rats have 50/50 baseline preferences for conditioning chambers. Relevant to (5), the authors' NCP approach is a biased conditioning procedure (i.e. pairing the initially preferred chamber[s] with hypothesized aversive stimulation). Biased conditioning procedures (which also include pairing the initially less preferred chamber with hypothesized rewarding stimulation) are common and maximize the effect. The reader needs to be made aware of this.

Answer: Agree and we have added a discussion paragraph concerning biased and un-biased design in the Introduction.

7) Introduction, line 145: replace "providing" with "provided".

Answer: Corrected.

8) Protocol, section 2.1.: If homozygous Cre mice are bred why is it necessary to geneotype?

Answer: We use heterozygous Cre-mice and this has now been clarified in the text.

9) Protocol, section 2.1.1.: I am fairly certain is will be impossible to sterilize the "environment" … perhaps the authors mean "sanitize".

Answer: Indeed, that was the case. Corrected

10) Throughout the manuscript the word "skull" is misspelled as "scull".

Answer: Corrected

11) Protocol, section 2.1.8: Since this should serve as an instructional paper for investigators who want to run these types of experiments it should be made clear that an automated infusion pump that can be mounted on the manipulator arm of a stereotaxic apparatus is needed. Also 300 nl of viral solution may work in some caseds but may not be appropriate for other brain targets.

Answer: The use of an automated infusion pump has been added in the protocol. Discussion regarding the volume of viral solution has been added in the discussion.

12) Protocol, sections 3-5: unclear why these sections need to be highlighted in yellow.

Answer: The yellow highlight indicates what section will be filmed and it was a requirement from the journal.

13) Protocol, section 3: it is fine that the authors are describing their specific set-up but I think it would help if the user is made aware of alternatives. For example, any electrical pulse generator (AMPI Master 8 or Master 9 are common) will work ¬¬- Arduino may not be

available easily to everyone, Noldus Ethovison is great but there are also other videotracking systems (e.g. Stoelting ANYmaze).

Answer: We agree and we have removed the statements for specific equipment, as also suggested by the editor. We have only included specific company names in the table of materials.

14) Protocol, section 3.4.: 10 mW output from the laser has to take into consideration the quality of the patchcord and the implanted probe. The reader should be made aware that the thing to be concerned about is the light power in the brain site being manipulated. That will be a combination of mW coming out of the laser, the patch cord used, and the quality of the implanted probe.

Answer: We have added a section regarding these factors in the Discussion.

15) Protocol, section 4: more detail is definitely required: are the two RT-PP session on the same day or on consecutive days and why? What stimulation parameters are used and why? Do they differ for cell body vs terminal manipulations?

Answer: As suggested by the editor, the description of the test has been moved to the Introduction and outlined in more detail. Information regarding the stimulation parameters and cell body vs terminal manipulation has been added in the Discussion.

16) Protocol, section 4: relevant to (6) above, here the authors do discuss to some extend what amounts to a biased conditioning procedure but the opposite would be required then for aversive effects.

Answer: Indeed, that was the case and this has been clarified in the RT-PP description.

17) The time after surgery, before behavioral experiments are initiated, is a variable that needs to be discussed. This will differ depending on whether the target is a cell body or a distant projection target of that cell body.

Answer: This information has been added in the Discussion.

18) Discussion: The authors should convey an accurate understanding of the available literature that uses mouse RT-PP in combination with optogenetic manipulations rather than selectively including only certain papers. Effects on the frequency of chamber entries, independent from chamber times, have clearly been demonstrated with a 3-chamber apparatus and glutamatergic optogenetci stimulation (see Steidl et al [2017] EJN 45: 559-571). In no way is this unique to a two chamber apparatus.

Answer: The text has been modified and additional references were added.

19) Figure 6 panels B and C: Not clear what the gray circles in the line graphs represent.

Answer: The symbols have been clarified in the figure legend.

20) Figure 6C heatmap: The graph above the heat map indicated that mice spend little time in the paired chamber during the reversal test but the heat map suggests exactly the opposite? Was the wrong image inserted here?

Answer: This is indeed correct, but the mistake was in the figure labeling. The paired-unpaired sequence was meant to be the same as in the case of the heatmaps in Figure 6B. The mistake has now been corrected.