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TITLE:

Elimination of Serotonergic Neurons by Stereotaxic Injection of 5,7-Dihydroxytryptamine in the Dorsal Raphe Nuclei of Mice

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KEYWORDS:

serotonergic neurons, stereotaxic injection, 5,7-dihydroxytryptamine, dorsal raphe nucleus, neurotoxin, anxiety, 5-hydroxytryptamine

SUMMARY:

This protocol describes a dorsal raphe nucleus (DRN)-lesioned mouse model (>90% survival rate in experimental mice) with stable loss of dorsal raphe serotonergic neurons by stereotaxic injection of 5,7-dihydroxytryptamine into the DRN using an angled approach to prevent injury to the superior sagittal sinus.

ABSTRACT:

Stereotaxic injection has been widely used for direct delivery of compounds or viruses to targeted brain areas in rodents. Direct targeting of serotonergic neurons in the dorsal raphe nucleus (DRN) can cause excessive bleeding and animal death, due to its location below the superior sagittal sinus (SSS). This protocol describes the generation of a DRN serotonergic neuron-lesioned mouse model (>90% survival rate) with stable loss of >70% 5-HT-positive cells

in the DRN. The lesion is induced by stereotaxic injection of a selective serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) into the DRN using an angled approach (30° in the anterior/posterior direction) to avoid injury to the SSS. DRN serotonergic neuron-lesioned mice display anxiety-associated behavior alterations, which helps to confirm success of the DRN lesion surgery. This method is used here for DRN lesions, but it can also be used for other stereotaxic injections that require angular injections to avoid midline structures. This DRN serotonergic neuron-lesioned mouse model provides a valuable tool for understanding the role of serotonergic neurons in the pathogenesis of psychiatric disorders, such as generalized anxiety disorder and major depressive disorder.

INTRODUCTION:

Serotonin, or 5-hydroxytryptamine (5-HT), is an important neurotransmitter mainly produced in the intestines and brain and impacts a variety of psychological functions. In the central nervous system (CNS), the serotonergic system plays a central role in the regulation of mood and social behavior, sleep and waking, appetite, memory, and sexual desire. In the CNS, serotonin is synthesized by serotonergic neurons, which can be separated into the following two groups: the rostral group, which has ascending projections innervating virtually the whole brain; and the caudal group, which mainly projects to the spinal cord[1]. The rostral group, which contains about 85% of serotonergic neurons in the brain, is composed of the caudal linear nucleus, median raphe nucleus, and DRN, in which the largest population of serotonergic neurons in the brain is located.

Dysregulation of the serotonergic system is generally believed to be linked with the pathogenesis of major depressive disorder (MDD) and generalized anxiety disorders (GAD)[2]. This is due to the fact that selective serotonin reuptake inhibitors (SSRIs) are effective pharmacological treatment for these psychiatric disorders[3,4]. In addition, accumulative evidences suggest that mania[5] and suicidal behavior[6] may be associated with lower levels of serotonin functioning in the DRN. It has also been reported that Pet1-Cre;Lmx1b$^{flox/flox}$ mice and hTM-DTA^{iPet1} mice (genetic mouse models lacking most central serotonergic neurons from late embryonic stage[7] and adulthood[8], respectively) display enhanced contextual fear memory. However, despite extensive research, the exact involvement of DRN serotonergic neurons in these psychiatric disorders remains to be elucidated.

In order to explore the mechanisms by which DRN serotonergic neurons regulate the pathogenesis of the serotonin-associated psychiatric disorders, animal models have been generated. Optogenetic tools have been applied to inhibit serotonergic neurons in rat DRN, and these animals display increased anxiety-like behaviors[9]. However, optogenetics has limitations. For example, a light-delivery device must be implanted into the targeted region deep within the brain, and the surrounding tissue may be injured during implantation surgery or by heat emitted from the light device. Even if temperature alteration may not cause detectable brain tissue damage, it can still induce remarkable physiological and behavioral effects[10].

Pharmacological manipulation may be an easier approach to create DRN serotonergic neuron-

lesioned animal models. Some groups have generated DRN serotonergic neuron-lesioned rats by stereotaxic microinjection of serotonin neurotoxin 5,7-DHT in the DRN. However, these rat models display different behavioral alterations, such as anxiolytic behavior[11], increased anxiety-like behavior[12], and impaired object memory[13]. Despite many studies in rats, fewer studies have been performed on the influences of 5,7-DHT on mice. One group reported excessive mortality (>50%) and limited serotonin depletion in experimental mice that received stereotaxic microinjections of 5,7-DHT in the DRN[14]. Another group reported that unpredictable chronic mild stress (UCMS) can induce significant attack latency alteration in 5,7-DHT-induced DRN-lesioned mice. However, no histological results were provided to confirm the exact serotonergic neuron loss in the DRN[15]. Stereotaxic injection in the DRN using standard procedures may lead to massive bleeding and high mortality to mice, given the fact that the anatomical location of DRN is below the SSS[16].

This protocol describes the protocol to generate a DRN serotonergic neuron-lesioned mouse model (>90% survival rate of the experimental mice) with stable loss of DRN serotonergic neurons by stereotaxic injection of 5,7-DHT. The injection in DRN uses an angled approach to prevent the injury to the SSS. This surgery consistently causes >70% loss of serotonergic neuron in the DRN of mice, and it produces anxiety-associated behavior alterations. The protocol used here is for inducing DRN lesions, but it can also be useful to researchers who want to perform stereotaxic injections in other midline structures. In addition, this DRN serotonergic neuron-lesioned mouse model provides a valuable tool for understanding the role of serotonergic neurons in psychiatric disorders (i.e., MDD and GAD) and assessing potential neuroprotective agents or therapeutic strategies for these conditions.

PROTOCOL:

All surgical interventions and animal care procedures have been approved by the Animal Committee of School of Life Sciences and Technology, Tongji University, Shanghai, China.

1. Housing of animals

1.1. Maintain male C57BL/6NCrl mice (10 weeks old, 25 g, n = 21) in standard conditions (24 °C temperature; 55% humidity) under a 12 h/12 h light/dark cycle.

1.2. Provide food and water *ad libitum*.

NOTE: Here, three of the mice are used for confirming the needle track.

2. Preparation of reagents

NOTE: All drug preparation steps must be performed in a laminar flow hood to avoid contamination. All prepared solutions are stored in a -80 °C freezer. It is recommended to use one aliquot at a time, thaw completely, mix well before use, discard the leftovers in the tube,

and avoid repeated freezing and thawing.

2.1. Dissolve 0.25 g of desipramine hydrochloride in 100 mL of 0.9% saline to yield a 2.5 mg/mL solution. Sterilize the solution using a syringe filter with 0.22 µm pore size hydrophilic PES membrane. Then, aliquot the solution (1.8 mL/tube) in 2 mL microcentrifuge (EP) tubes. Label, date, and store in a -80 °C freezer immediately. The recommended dosage for animal use is 25 mg/kg.

2.2. Dissolve 5 mg of 5,7-DHT in 1.67 mL of 0.9% saline containing 0.1% ascorbic acid to yield a 3 µg/µL solution. Vortex gently to mix, sterilize the solution using a syringe filter (0.22 µm pore size), and aliquot the solution (10 µL/tube) in 0.5 mL EP tubes. Label, date, and freeze at -80 °C immediately. The recommended dosage for animal use is 2 µL/mouse.

2.3. Dissolve ketoprofen (analgesic) following the previously described method[17]. Dissolve 250 mg of ketoprofen in 15 mL of water and 1 mL of 1 M NaOH, adjust the pH to 7.3 and make up the final volume to 125 mL, which will result in a final concentration of 2 mg/mL. Sterilize the solution using a 0.22 µm syringe filter and aliquot the solution (0.4 mL/tube) in 1.5 mL EP tubes. Label, date, and freeze at -80 °C until use. The recommended dosage for animal use is 5 mg/kg.

3. Preparation of instruments and mice

3.1. Prepare a surgical pack (containing a scalpel, pair of tissue forceps, pair of scissors, needle holder, 3-0 sutures, ear tags, and ear tag applicator) previously sterilized in high temperature autoclaves. Place 75% ethanol, povidone-iodine, 3% hydrogen peroxide, vehicle solution (0.1% ascorbic acid in 0.9% saline), a cotton swab, ofloxacin eye ointment, and a mouse recovery cage on top of a heating pad, using a Hamilton syringe with a 32 G needle and 1 cc syringe.

3.2. 1 h prior to 5,7-DHT injection, weigh and record body weights of the mice, then subject them to intraperitoneal (i.p.) injections of desipramine (2.5 mg/mL, 10 µL/g weight).

NOTE: The purpose of desipramine administration 1 h prior to 5,7-DHT injection is to prevent catacholaminergic cell loss[18].

3.3. Anesthetize mice using isoflurane inhalation anesthesia (3% during induction, 1.5% during maintenance, flow rate = 2 L/min). Administer ketoprofen (2 mg/mL, 2.5 µL/g weight) via subcutaneous (s.c.) injection. Confirm adequate anesthesia depth by the absence of tail pinch response.

4. Stereotaxic injection

4.1. Place the anesthetized mouse on the stereotaxic platform, then fix its head with the ear bars and incisor bar of the stereotaxic apparatus.

4.2. Shave the mouse's head, then clean the exposed scalp with one scrub of 75% ethanol followed by one scrub of povidone-iodine.

4.3. Apply lidocaine ointment on the scalp using a cotton swab to provide local analgesia. Put ofloxacin eye ointment on the eyes to protect the cornea.

4.4. Make an incision on the scalp along the midline using a scalpel from 1 mm posterior to the eyes to the interaural line.

4.5. Use a 3% hydrogen peroxide-soaked swab to remove the periosteum, then dry the skull and expose the cranial sutures. Mark the location of the bregma and lambda.

4.6. Adjust the head position using the incisor bar until the bregma and lambda lay in the same horizontal plane. Adjust the needle tip to touch the bregma or lambda, record the medial/lateral (ML) and dorsal/ventral (DV) coordinates, and adjust the incisor bar so that the DV and ML coordinates of bregma and lambda are equal, respectively.

4.7. Unlock the perpendicular positioning button and lock screw, then set the manipulator arm (z-axis) to 30° in the anterior/posterior (AP) direction as shown in **Figure 1A**,**B**. Lock the button.

4.8. Fix the Hamilton syringe filled with 2 µL of 5,7-DHT (3 µg/µL) onto the holder (for the sham group, mice are injected with 0.9% saline containing 0.1% ascorbic acid). Adjust the needle tip to touch the bregma landmark, then zero the ML, AP, and DV values using the digital display module.

NOTE: The 5,7-DHT solution is brown colored liquid, making it easy to determine whether the Hamilton syringe is properly filled. If there is no access to a digital display module, record the number displayed on three axes when the needle tip touches the bregma, and the final coordinates represent “the recorded number plus the coordinate provided in this protocol”. For example, if the recorded number on the y-axis (A/P direction) manipulator arm is “a”, then the final coordinate in the A/P direction should be “a - 6.27”.

4.9. Move the manipulator arm to adjust the needle tip to the injection position (APa = -6.27, ML = 0; formula for calculating the final coordinates is listed in **Figure 1B**). Mark the target position using a marker pen.

4.10. Drill the burr hole using portable micromotor high-speed drill, then move the manipulator arm to lower the needle tip to the target (DVa = -4.04). Inject 2 µL of the solution into the brain slowly (0.5 µL/3 min), keeping the needle in situ for an additional 5 min to prevent solution leakage. Remove the needle gently after injection.

4.11. Apply interrupted sutures over the incision using 3-0 sutures. Wrap the sutured incision with a cotton swab soaked in povidone-iodine to avoid infection.

4.12. Clean the right ear with a povidone-iodine cotton swab, then apply the sterilized ear tag to the base of the ear for identification.

4.13. Remove experimental mice from the stereotaxic apparatus and place in a mice recovery cage on top of a heating pad until full recovery from anesthesia is observed.

5. Postoperative care of mice

5.1. Subject mice to s.c. injections of ketoprofen 1x/day up to 2 days after surgery.

5.2. Inspect mice daily up to 7 days after surgery.

6. Elevated T-maze test

NOTE: Perform the test as described previously[19,20].

6.1. Ensure that the elevated T-maze (ETM) test is comprised of two open arms (30 cm x 5 cm x 0.5 cm) perpendicular to two enclosed arms (30 cm x 5 cm x 16 cm x 0.5 cm) with a center platform (5.0 cm x 5.0 cm x 0.5 cm). One of the enclosed arms is blocked by an opaque plastic sheet to form a T-shape (**Figure 2A**).

6.2. Three days before the ETM test, habituate the animals by handling daily for 5 min.

6.3. On day 30 after surgery, perform the ETM test.

6.4. On the day of behavioral testing, expose mice to one of the open arms for 10 min.

6.5. For inhibitory avoidance testing, place each mouse in the distal end of the enclosed arm and record the time taken to leave this arm with four paws in three trials. The first trial is baseline avoidance, the second trial is avoidance 1, and the third trial is avoidance 2.

6.6. Keep mice in their cages for 30 s between trails and establish a cutoff time (here, 300 s is used for each trial).

7. Perfusion, fixation, immunohistochemical staining, and quantification

7.1. At the end of the study (35 days after surgery, after the behavioral test), perform whole body perfusion and fixation once the mouse is under general anesthesia.

7.1.1 Anesthetize the mouse as described in step 3.3. Place the deeply anesthetized mouse in dorsal recumbency.

7.1.2 Wet the fur with 75% alcohol over the entire ventral area.

7.1.3 Make a cut below the sternum followed by a V-shape incision in the rib cage. Grasp the rib cage using the clamp to expose the heart.

7.1.4 Insert the venous infusion needle into the left ventricle, then cut the atrial appendage immediately with scissors to let the blood flow out.

7.1.5 Turn on the peristaltic pump to perfuse saline into the whole body through the circulatory system. Switch from saline to 4% paraformaldehyde (PFA) when fluid exiting the right atrium becomes clear and when the liver changes from red to pale red in color. Perfuse another 5 mL of 4% PFA when the mouse tail moves, and the body is stiff.

NOTE: Swaying of the mouse tail is the sign of adequate perfusion.

7.2. Isolation of the brain after intracardiac perfusion with 4% PFA

7.2.1 Decapitate the mouse using large scissors, then cut the skin to expose the skull.

7.2.2 Make two cuts at the base of the skull (connected to the neck), make one cut along the line linking the eyes, then cut the skull along the sagittal suture. Grasp and peel the skull of each hemisphere outward to expose the brain using forceps.

NOTE: Cutting the skull along the sagittal suture must be performed carefully, otherwise the brain may be damaged and separate into parts.

7.2.3 Remove the brain and place it in 4% PFA at 4 °C overnight for post-fixation, then transfer the brain into 30% sucrose until it sinks to the bottom.

NOTE: Dehydration using 30% sucrose is not necessary if sections are cut with a vibratome.

7.3. Absorb any excess liquid around the tissue using a paper towel prior to embedding. Embed the brain tissue in OCT with the rostral face on the chuck.

NOTE: Orientation of the sample is important. The rostral face of the brain must be attached to the chuck to make sure that the cutting edge is the caudal portion.

7.4 Cut a coronal section of the DRN (4.0–4.8 mm posterior from bregma) at 30 µm thickness (four-section intervals) using a cryostat and collect the sections in PBS. Store in brain section cryopreservation solution (SCS) at -20 °C.

NOTE: Identification of the DRN is very important. The consecutive anatomical structures are shown as **Figure 3A**–**H**. The characteristic structures are as follows: lateral recess of the fourth

ventricle (LR4V, red dash line in **Figure 3A,E**), fourth ventricle (4V, red dash triangle in **Figure 3B,F**), second cerebellar lobule (2Cb, red dash diamond-shape structure in **Figure 3C/G**), and aqueduct (Aq, red dash hole in **Figure 3D,H**). Start to collect the tissues when structures are observed as shown in **Figure 3D,H**, then cut in 30 µm thick sections (four-section intervals) to yield 24 total coronal sections. H&E staining of the sections is shown in (**Figure 3E–H**). The fourth section of each of the four sections will be selected to perform immunofluorescent staining. The remaining sections are stored in the SCS. If it is difficult to recognize the structures, mounting one piece of the sections on the slide is helpful.

7.5 Perform immunohistochemical assay as described previously[21,22].

7.6. Count 5-HT-positive cells at different section levels throughout the entire DRN using ImageJ.

8. Statistical analysis

8.1. Use statistical software for data analysis. Express the results as means ± SEM.

8.2. Process the values for statistical analysis by two-way ANOVA or unpaired *t*-test and consider the differences significant at $^{**}p < 0.01$.

REPRESENTATIVE RESULTS:

In a coronal section, the location of the DRN is just below the SSS and aqueduct (**Figure 1B,C**); thus, targeting the DRN using standard procedures can lead to massive bleeding and high mortality in mice[16]. Therefore, stereotaxic injections were performed here using an angled approach instead of the standard vertical approach to avoid damage to the SSS (**Figure 1A,B**). To confirm the location of the needle entering the brain, mice that received stereotaxic injections were sacrificed immediately after surgery, and the perfused brains were isolated for histological examination. As shown in **Figure 1C**, the lesion (the needle track) was localized just to the DRN, below the aqueduct.

It has been reported that inhibitory avoidance in the ETM may be a suitable behavioral task to assess anxiety[19]. Mice tend to avoid the open arms of an elevated maze and prefer enclosed arms; thus, mice spending less time in enclosed arms demonstrate anxiolytic behavior[20]. Results from this protocol showed that 5,7-DHT-lesioned mice displayed remarkable lower avoidance latencies than the sham group (**Figure 2B**, $p < 0.05$), suggesting that the treatment with 2 µL of 5,7-DHT (3 µg/µL) may have impaired inhibitory avoidance.

Thirty-five days after surgery, the effects of 5,7-DHT on DRN serotonergic neurons were histologically analyzed (**Figure 4A,B**), and quantification of 5-HT-positive cells in DRN were performed (**Figure 4C**). Serotonergic neurons were stained positively with anti-5-HT antibody. Remarkable loss of >70% 5-HT-positive cells was observed in the DRN of 5,7-DHT-lesioned mice

(N_{lesion}= 7, cell number = 305 ± 32) compared to the sham group (N_{sham}= 5, cell number = 1164 ± 95) (**Figure 4C**), suggesting that stereotaxic injection of 5,7-DHT in the DRN destroyed >70% of serotonergic neurons without inducing animal death, since no animal was found dead until perfusion.

FIGURE AND TABLE LEGENDS:

Figure 1: Stereotaxic injection using an angled approach and verification of the target area in the DRN. (**A**) Mouse placed in the stereotaxic apparatus with the z-axis manipulator arm set to 30°. (**B**) Schematic illustration of the injection using an angled approach and the formula needed to calculate the target coordinates (SSS = superior sagittal sinus). (**C**) The remaining needle track in the coronal section of the brain at the level of -4.6 mm from the bregma. (n = 3, scale bar = 1 mm, APa = adjusted AP, DVa = adjusted DV).

Figure 2: Behavioral assessment of 5,7-DHT-lesioned mice. (**A**) Schematic representation of the experimental procedures measuring inhibitory avoidance in the elevated T-maze (ETM). (**B**) Effects of 5,7-DHT lesion on inhibitory avoidance latencies as measured by the ETM. (n = 9 per strain, **p < 0.01, two-way ANOVA).

Figure 3: Identification of DRN during sectioning. (**A,B,C,D**) Brain structures shown in the caudal to rostral direction. (**E,F,G,H**) Brain sections of the structures shown in **(A–D**), respectively. (**I,J,K,L**) H&E staining of the sections shown in (**E–H**), respectively. Scale bar = 1 mm (LR4V = lateral recess of the fourth ventricle, 4V = fourth ventricle, 2Cb = second cerebellar lobule, Aq = aqueduct).

Figure 4: Loss of serotonergic neurons in the DRN after 5,7-DHT lesion. Immunofluorescence images showing 5-HT-positive cells in the (**A**) lesion and (**B**) sham groups at different levels of the entire DRN from the rostral to caudal regions (left to right, 4.0**–**4.8 mm posterior from the bregma). Scale bar = 100 µm. (B) Quantification of serotonergic neurons in the DRN (values are presented as mean ± SEM, N_{lesion} = 7, N_{sham} = 5, **p < 0.01, unpaired *t*-test).

DISCUSSION:

This protocol successfully describes production of a reliable DRN serotonergic neuron-lesioned mouse model with high lesion reproducibility and low mortality rate. Targeting the DRN is a complex task, since it can damage the SSS located just above the DRN[16] and lead to excessive bleeding and even death[14]. Therefore, stereotaxic injections were performed by setting the manipulation arm at 30° in the AP direction to avoid injury to the SSS (**Figure 1A,B**). The needle track shown below the aqueduct (**Figure 1C**) confirms accurate targeting of the DRN. One month after surgery, the DRN serotonergic neuron-lesioned mice display significantly lower latencies in ETM test, indicative of anxiolytic behavior (**Figure 2B**), with loss of >70% serotonergic neurons in the lesioned DRN (**Figure 4**).

Although an angled approach while performing stereotaxic injections helps to avoid midline structures, there are still some limitations to this technique. Some factors (i.e., anatomical variability, localization of the bregma and lambda, size of the mice, etc.) may affect the targeting accuracy and lead to unsuccessful damage to DRN serotonergic neurons. Increasing the injection volume of the neurotoxin (5,7-DHT) may help overcome the problem. Indeed, some mice that received 1 µL of 5,7-DHT (3 µg/µL) in a preliminary study showed insufficient DRN serotonergic neurons loss (data not shown), which may have been caused by inaccurate targeting due to variable factors. In contrast, 2 µL of 5,7-DHT (applied in this study) stably induced more than 70% loss of serotonergic neurons in the DRN.

It has been reported that 5,7-DHT is a selective serotonergic neurotoxin[23]. In the present study, 5,7-DHT-lesioned mice display anxiolytic behavior (**Figure 2B**). Consistent with these results, Sena et al. found that rats treated with 5,7-DHT in DRN showed impaired inhibitory avoidance indicative of anxiolytic behavior[11]. Jia et al. also reported that the central 5-HT-deficient (conditional deletion of Tph2 or Lmx1b) mice showed reduced anxiety-like behavior[24]. However, contradictory results have reported that 5,7-DHT-induced DRN lesion led to increased anxiety or other behavioral alterations in rats. In the same study, intracerebroventricular (i.c.v.) injection of 5,7-DHT led to increased anxiety in rats[12]. Lieben et al. reported that 5,7-DHT lesions in the DRN of rats impaired object memory instead of anxiety-associated behavior[13]. Optogenetic inhibition of serotonergic neurons in DRN have also been shown to increase anxiety-like behaviors in rats[9].

Despite many studies on rats, few articles reported the influences of 5,7-DHT on mice. Martin et al. reported >50% excessive mortality of experimental mice and limited serotonin depletion (15%) occurring in 5,7-DHT-induced DRN-lesioned mice[14]. Yalcin et al. reported that UCMS could induce significant attack latency alteration in 5,7-DHT-induced DRN-lesioned mice[15]. In fact, variances in the route of 5,7-DHT administration, the target of 5,7-DHT delivered to, and the type of animal used for research can all lead to different behavioral alterations[13]. Different volumes of 5,7-DHT can also lead to behavioral alterations. For example, moderate deletion of serotonergic neurons may lead to increased or normal extracellular serotonin levels because of the compensatory function of remaining neurons[12].

Another reason for these contradictory results may be due to the elongated structure of the DRN and its extensive projections. Marcinkiewcz et al. reported that stimulation of mouse BNST-projecting DRN serotonergic neurons is anxiogenic[25], while Ohmura et al.[26], Nishitani et al.[9], and Correia et al.[27] showed that activation of DRN serotonergic neurons has no effect on anxiety-associated behavior. DRN serotonergic neurons in different projected areas may encode anxiogenic or anxiolytic signals; thus, manipulation of DRN serotonergic neurons in different projected areas can induce different behavioral alterations. The mechanisms underlying animal behaviors caused by the loss of DRN serotonergic neurons remains enigmatic, and further research is needed.

Despite many DRN lesion studies using rat models have been published, fewer are available

that involve mice. This protocol contributes to the field by providing histological and behavioral data from a 5,7-DHT-induced DRN lesion mouse model. Great success in animal survival rates as well as the elimination of serotonergic neurons in the DRN could is achievable using a 30° angle in the A/P direction. It may be helpful to distinguish some of the contradictory results found in the existing literature by using a reliable method for administering 5,7-DHT in the DRN.

Furthermore, this method is used here for DRN lesions, but it may also provide useful data for the researchers who want to perform stereotaxic injections in other midline structures of the brain. Given that this protocol generates a DRN serotonergic neuron-lesioned mouse model that displays decreased anxiety-like behavior, it may be a valuable tool to delineate the role of serotonergic neurons in psychiatric disorders (i.e., MDD and GAD) and assess new therapeutic approach to treat these conditions.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Figure1.psd

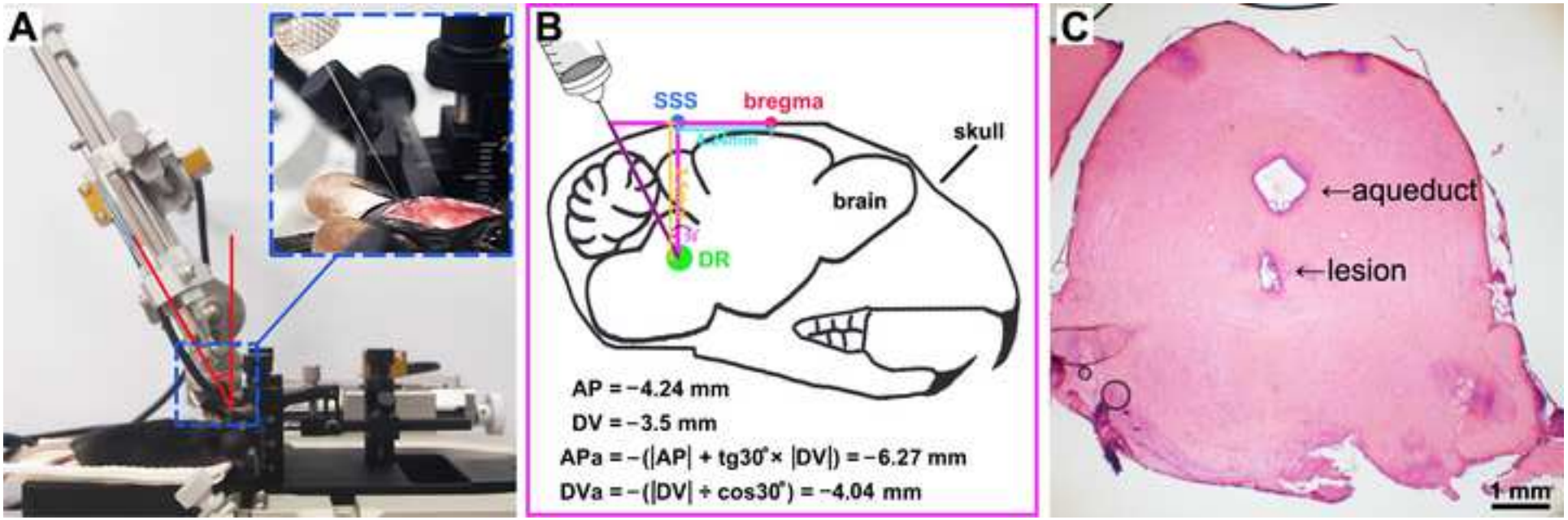

Figure 2

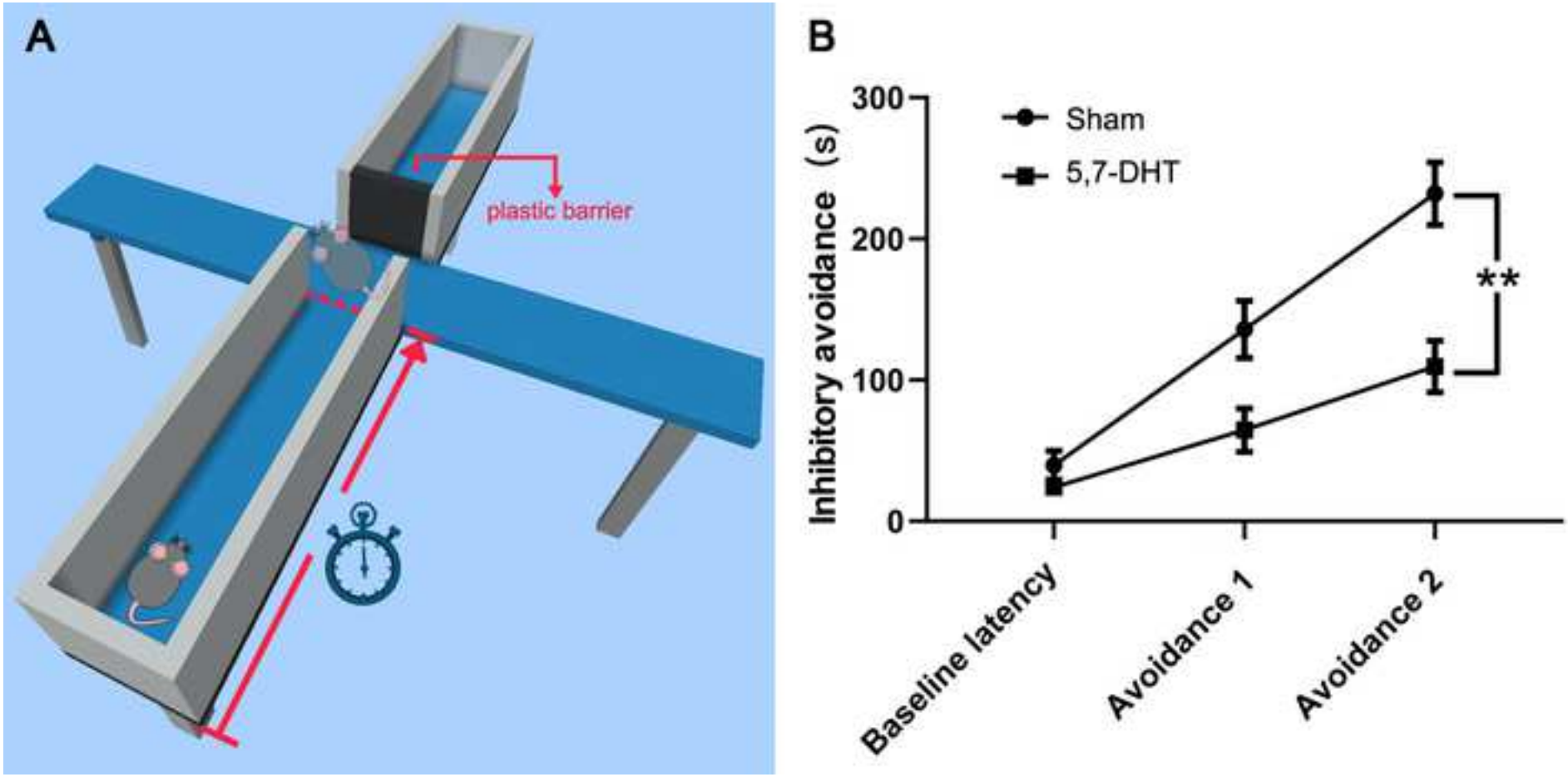

Figure 3

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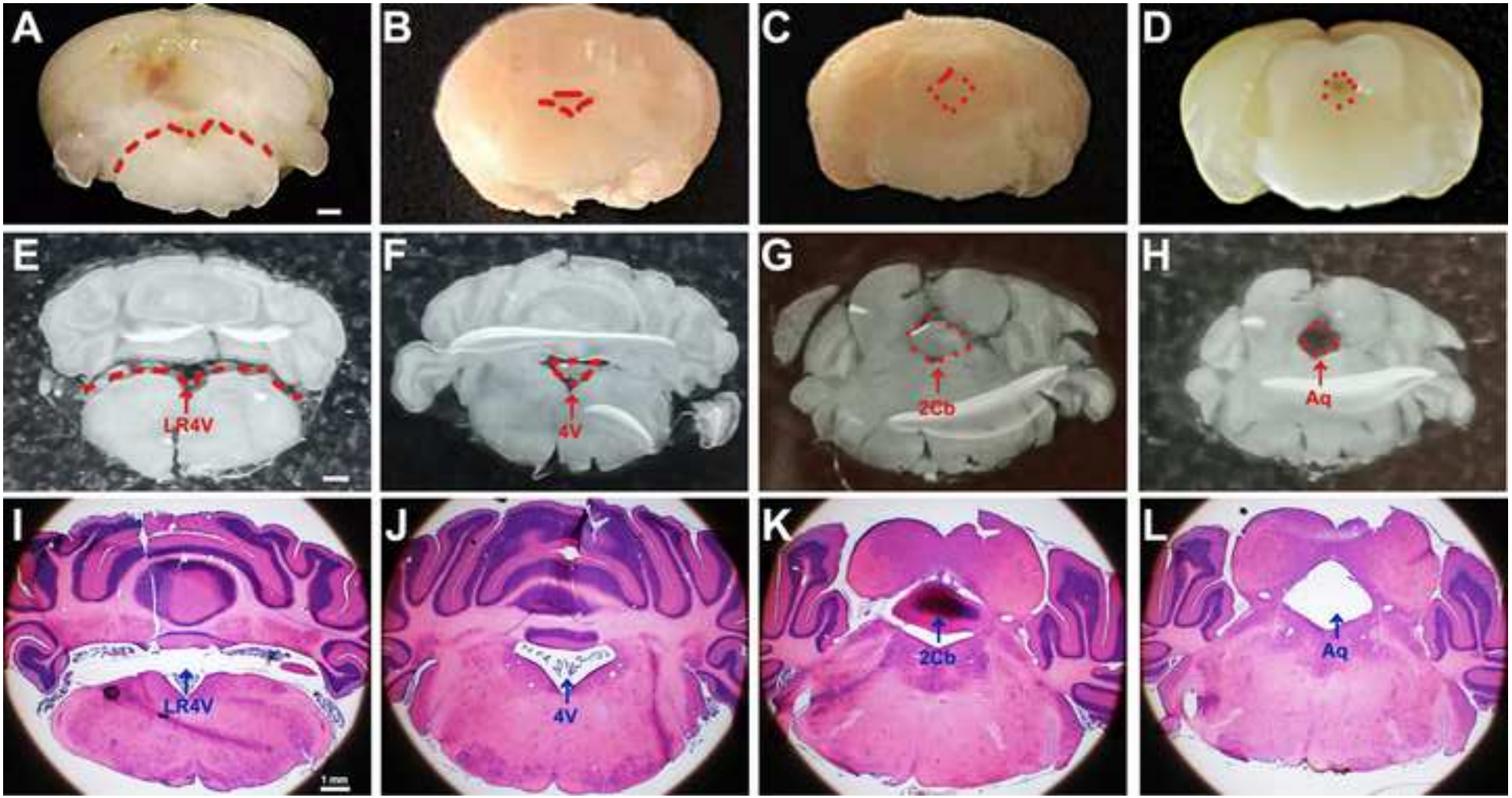


Figure 4

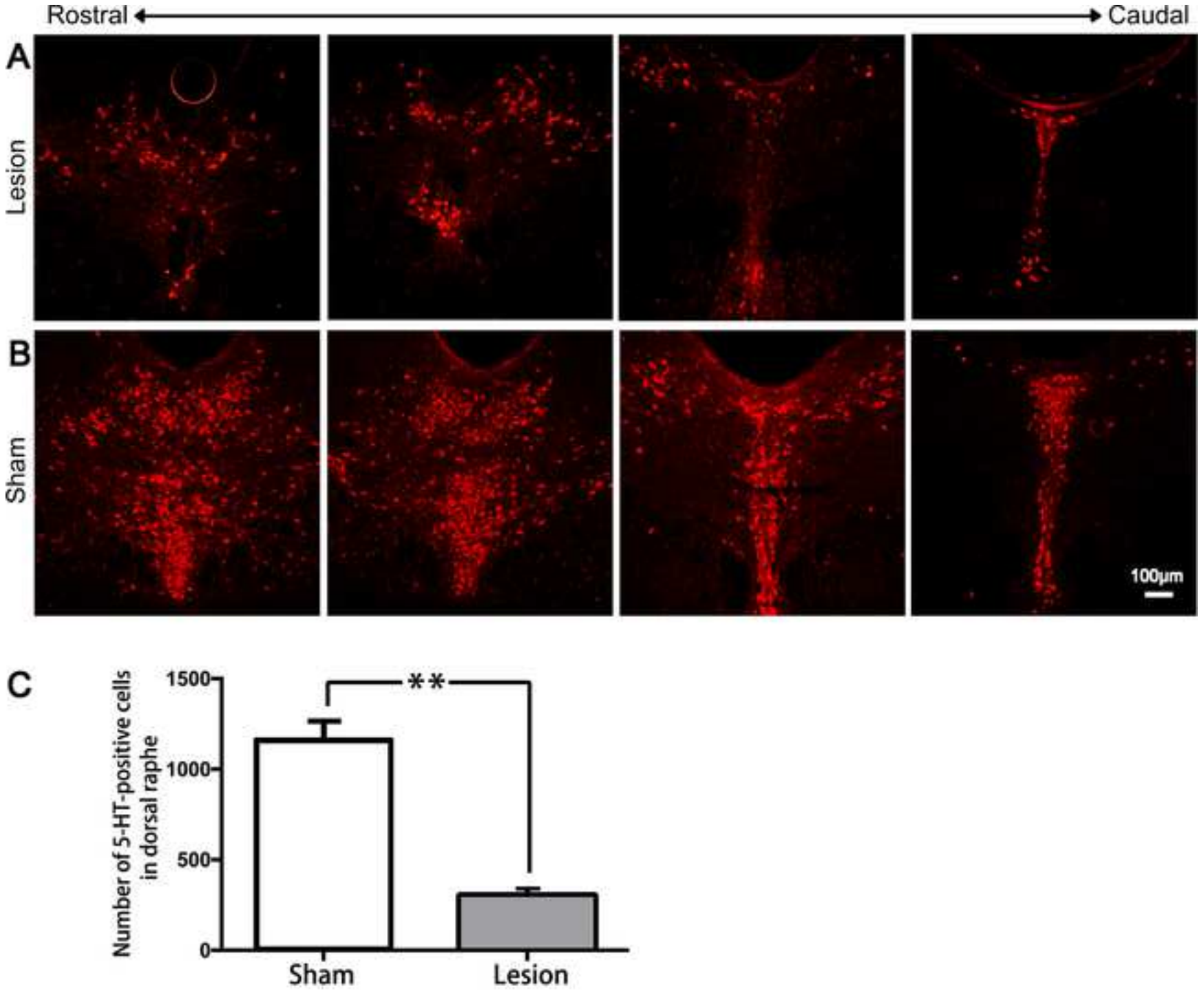


Table of Materials

Click here to access/download;Table of Materials;Table of materials_JoVE_Lu revise.xlsx

Name	company	Catalog number	comments
0.22micron syringe filter	Millipore	SLGPRB	
3% hydrogen peroxide	Caoshanhu Co.,Ltd, Jiangxi, China		
5,7-Dihydroxytryptamine	Sigma-Aldrich	SML2058	3ug/ul, 2ul
Compact small animal anesthesia machine	RWD Life Science Co., Ltd	R500 series	
Cryostat	Leica Biosystems, Wetzlar, Germany	CM1950	
Cy 3 AffiniPure Donkey Anti-Goat IgG (H+L)	Jackson ImmunoResearch	705-165-003	1:2,000
dapi	Sigma-Aldrich	D8417	
desipramine hydrochloride	Sigma-Aldrich	PHR1723	25mg/kg
Eppendorf tube	Quality Scientific Plastics	509-GRD-Q	
goat anti-5-HT antibody	Abcam	ab66047	1:800
GraphPad Prism	Graphpad Software Inc, CA, US		
Hamilton Microliter syringe	Hamilton	87943	
Ketoprofen	Sigma-Aldrich	K1751-1G	5mg/kg
L-ascorbic acid	BBI Life Sciences	A610021-0500	0.10%
lidocaine ointment	Tsinghua Tongfang Pharmaceutical Co. Ltd	H20063466	
ofloxacin eye ointment	Shenyang Xingqi Pharmaceutical Co.Ltd, China	H10940177	
peristaltic pump	Huxi Analytical Instrument Factory Co., Ltd, Shanghai, China	HL-1D	
stereotaxic apparatus	RWD Life Science Co., Ltd	68018	
ultra-low temperature freezer	Haier	DW-86L388	
Vortex	Kylin-bell	VORTEX-5	

Responses to 2nd version of Editorial comments:

Editorial comments:

1. The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.

Response:

We have done this accordingly.

2. Please address all the minor specific comments marked in the manuscript.

Response:

We have addressed all the minor specific comments marked in the manuscript.

3. Once done please ensure that the highlighted section is no more than 2.75 pages including headings and spacings.

Response:

We have revised the manuscript accordingly.

Note: Videos and supplementary figures mentioned in the rebuttal letter are not for publication.

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.
   Response:
   Spelling and grammar of the manuscript has been checked.

2. Please make the title concise by removing words like efficient and effective.
   Response:
   The words “efficient and effective” in the title have been removed.

3. Please provide an email address for each author.
   Response:
   Lining Cao: crystalvivining@aliyun.com, 19619@tongji.edu.cn
   Zhiqiang Zhang: zzq695265428@163.com
   Xinyu Lu: leilaluxinyu@163.com
   Guanhao Wang: wangguanhao@tongji.edu.cn
   Du Meng: nagemengdu@163.com
   Chang Liu: lclchaha@163.com
   Ji Yun: 2162828059@qq.com
   Ting Xu: 1632745@tongji.edu.cn
   Chun Zhao: chunour@163.com
   Jianfeng Lu*: lu.jianfeng@tongji.edu.cn (corresponding author)

4. Please also mark the corresponding author
   Response:
   The corresponding author is Prof. Jianfeng Lu, the name is marked by asterisk (*).

5. 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 and Reagents.
   For example: Eppendorf, Graphpad, etc.
   Response:
   We have checked the manuscript with this requirement.

6. Please ensure that all steps in the protocol are numbered action steps written in imperative tense.

   Response:

   We have checked the manuscript with this requirement.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

   Response:

   We have checked the manuscript with this requirement.

8. Please ensure you answer the “how” question, i.e., how is the step performed?

   Response:

   We have checked the manuscript with this requirement.

9. 3.1: Is there any age, sex specific bias?

   Response:

   We performed the experiments described in the manuscript with mice whose age were about 10 weeks (weight: around 25 grams) to pursue the highest survival rate. Male instead of female C57BL/6NCrl mice were used in the experiments since the variability of female sex hormones (menstrual cycle) might affect the test results. Given the fact that many women experience premenstrual syndrome (PMS), anxiety (one of the symptoms of PMS) might be affected by the fluctuating levels of estrogen and progesterone[1]. Considering that female sex hormones might lead to incorrect results by influencing anxiety-associated behavior, we did not use female mice in this research.

10. 7.1: How many days after surgery, do you perform this?

    Response:

    We performed the whole-body perfusion 35 days after surgery. This information has been mentioned in the 7.1 part of the revised manuscript.

11. 7.2: Please detail the steps involving intracardiac perfusion. How do you perform the brain removal?

    Response:

    The details of intracardiac perfusion have been described in the 7.1 part of the revised manuscript. The procedures of brain isolation have been described in the 7.2 part of the revised manuscript. (Videos are also attached with the rebuttal letter to show how to perform brain isolation and a sign of adequate perfusion)

12. 7.3: Do you wash the brain tissue prior to embedding? Please include all the details.

How do you visually identify DRN? Please include a citation for immune histochemistry.

Response:

The brain tissue was not washed prior to embedding with OCT. 30% sucrose around the tissue was absorbed with paper towel prior to embedding. This step has been described in the 7.3 part of the revised manuscript. The procedures of brain embedding have been described in the 7.4 part of the revised manuscript. The procedures of identification of DRN have been described in the Note of the 7.4 part of the revised manuscript. Immunohistochemistry was performed as described previously[2,3].

13. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Response:

We have done this in the revised manuscript.

14. 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].”

Response:

No figure is reused from a previous publication.

15. 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

Response:

(a) The critical steps have been described in the part 4 of the manuscript;

(b, c) The limitations, drawbacks and troubleshooting of the technique have been mentioned in the 2nd paragraph of the discussion section;

(d, e) The significance and future application of the method have been listed in the last paragraph of the discussion section.

16. Please do not abbreviate the journal titles in the references section.

Response:

We use the JOVE style downloaded from JOVE website and edit with EndNote.

17. Please sort the table of materials in alphabetical order. Please remove any trademark or copyright symbol.
    Response: We have revised this in the manuscript.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript suggests a full protocol for lesioning serotonergic neurons in the dorsal raphe nucleus. The authors focus on the SSS deviation as the main contribution of this protocol, which is confirmed by the behavioural tests used - apart from histological analysis.
I have some concerns that lead me to the decision of not recommending this manuscript for publication.

1. The histological brain sites analysed lacks resolution, thus making difficult to confirm the effects of 5,7-DHT lesions.
   Response:
   Thanks for the comments. We have revised the images of H&E staining to make the brain sites/structure clear (Figure 1C and supplementary Figure 1).

2. The behavioural tests took place in an opened area, with other environmental cues to the animals - it is recommended that behavioural tests like this should take place in sound attenuated rooms, for example. In this case, the videos show that the experiments took place in a room where other cages and equipments can be found. It is important to address that ultrasoun vocalization has been registered during behavioural test and may influence the behaviour of animals in this situation.
   Response:
   Thanks for the comments. The behavioral tests were carried out in a room of a Specific Pathogen Free (SPF) facility, since some of the animals need to be maintained after the behavioral tests for a longer term for other purposes. The mouse cages on the shelves in the video were empty (there were no name tags on these cages in the videos). The machine in the video (with 2 blue lines on it) is a part of the Individually ventilated cage (IVC) system (produced by Suzhou Fengshi laboratory animal equipment co. LTD) shown as supplementary Figure 2A, which helps to maintain low ammonia and CO_2 concentrations, to support a low relative humidity, and to reduce the spread of infective agents and allergenic contaminants. Such equipment is required for a SPF facility. A white folding screen was used to isolate the animals from the researcher. Both the control group (animals received the sham surgery) and the experimental group (animals received 5,7-DHT injection) took the behavioral tests under the same environment and conditions.

3. The Elevated T/Plus maze has a removable wall that does not fit properly the space.

Response:

Thanks for the comments. Although the removable wall does not fit perfectly, indeed it blocks the arm and make the Plus-shaped maze a T-shape as used by others[4]. We will make it fit the space as perfect as possible in our future research.

4. At least, the researcher is not properly dressed (shoes???) - consider this point as it may be an educational video.

Response:

The slippers (shoes) are provided by the SPF animal facility; and putting on slippers is required when entering SPF rodent colonies (supplementary Figure 2B). The seamless protective coat covers the whole body of the researcher, including the feet (supplementary Figure 2B). Slippers are recyclable compared to shoe covers; and all the slippers sterilized by water, chlorine-containing disinfectant (20 min) and UV light (20 min) can be transferred into the SPF facility with standard operating procedure (SOP). The slippers are with soft tread, which minimizes the sound of steps.

Reviewer #2:

Manuscript Summary:

The authors are demonstrating an improved method of injecting 5,7-DHT (a serotonergic neurotoxin) into the dorsal raphe nucleus (DRN) of mice. This is a particularly tricky and risky surgery due to the location of the DRN with regard to the superior sagittal sinus, and many mice and rats bleed out or suffer consequences due to brain injury during surgery. While many studies using rat models for this procedure have been published, there is little research available on mice, in part due to the difficulty of the surgery. It is important to add and complement existing research on the DRN and serotonergic function by easing the process in mice. Intracerebroventricular administration of 5,7-DHT seems to have bipolar effects on anxiety in animals, while the explicit destruction of 5-HT neurons in the DRN has produced less reliable information due to the challenges of the surgery and elongated structure of the DRN. The authors have had great success in animal survival rates as well as elimination of 5-HT neurons in the DRN using a caudal approach at a 30 degree angle. Having a reliable method for administering 5.7-DHT in particular may help tease apart some of the contradictory results found in the literature. This manuscript and accompanying videos demonstrate this technique. This can also be applied for injection of other substances into the DRN, and thus offers valuable approaches for modifying 5-HT output from the DRN.

Major Concerns:

The manuscript is well written and concise. The authors make a good case that this a technique that can be very useful for further investigating the DRN, as well as demonstrating the breadth of contradictory results available for the effects of 5,7-DHT

lesions. The figures are well laid out and informative. **I am not sure of the purpose of the videos.** I understand that the videos are data examples, and this is good to see. But I was under the impression that the purpose of JOVE was to show surgical techniques, etc. however perhaps that is done at a later time and is my misunderstanding. **The authors should consider addressing some of the discrepancies in 5,7-DHT results in the literature with a consideration of the length and specificity of the DRN itself.** Subregions within the DRN have very specific inputs and terminal fields. Incomplete destruction of the entire DRN can result in conflicting outcomes for measures of anxiety due to 5-HT cells remaining in some subregions but not others. This is a major issue in trying to target the entire DRN with any substance, as it is almost impossible to eliminate the entire nucleus or to control what subregions remain more intact than others. While the technique demonstrated here is clearly needed and important, the previously mentioned roadblock is something that DRN researchers have been trying to address for years. Future directions here may involve procedures for more even 5-HT depletion across the DRN, however the practicality and need for something this extensive is small.

Response:

We thank the reviewer for the professional comments with a broad vision. As mentioned by the reviewer, the videos provided in the first submission were used as data examples. Since the focus of the manuscript is to introduce an efficient and effective way to eliminate DRN serotonergic neurons with the stereotaxic injection, JOVE will film the surgery procedures if the manuscript is accepted. We are also conducting experiments to test the discrepancies in 5,7-DHT-induced lesion. Hopefully, with the surgical method mentioned in this manuscript, it will be helpful for uncovering the relationship and mechanism between the degree of DRN serotonergic neuron loss and the different behavior phenotypes. And furthermore, we would like this method to be applied by other researchers, which may help the field answer scientific questions not only related to the DRN serotonergic neurons, but also those related to other brain structures in the midline.

Minor Concerns:

There are a few grammatical errors here and there.

Response:

Thanks for the careful reading. Grammatical errors have been corrected in the revised manuscript.

Reviewer #3:

Manuscript Summary:

This manuscript by Cao et al describes the method to delete 5-HT neurons in the DRN by using 5,7-DHT microinjection and examined anxiety-like behavior in male mouse.

Major Concerns:

1) DRN surgery with angled injection (from caudal to rostral) was already conducted by other groups (e.g. Li et al 2016 Nature Communications). Although the angle was different, I do not think the method reported in this manuscript has any novelty.

Response：

We thank the reviewer for the comments. The purpose of this manuscript is to introduce an efficient and effective way to eliminate DRN serotonergic neurons with the stereotaxic injection in details, since currently there is no detailed protocol showing such an approach. Although some researchers have tried DRN surgery with different angled injection (from caudal to rostral)[5] (15 degree, not for damaging DRN serotonergic neurons), detailed protocol is still limited; and different surgical degrees may make totally different pathological and behavior results. To our knowledge, the method of stereotaxic injection of DRN with a 30-degree angle to eliminate serotonergic neurons has not been published yet. Here we try to provide a detailed protocol with exact lesion dosage and direction of angel adjustment, pathological data and related behavior change.

2) Also, behavior test (elevated T maze) is not new and has been reported by other group (Graeff et al 1998).

Response：

The behavior test (elevated T maze) provided here was used to show the effect of loss of DRN serotonergic neurons, which is the result of the surgery and confirms the loss of DRN serotonergic neurons. The videos of the behavior test in the first submission were used as data examples. Since the focus of the manuscript is to introduce an efficient and effective way to eliminate DRN serotonergic neurons with the stereotaxic injection, JOVE will film the surgery procedures once the manuscript is accepted.

3) Reference is quite limited and many of sentences in this introduction is one-sided. Following is some examples.
Line 62. "Optogenetic tool was applied to inhibit SN in rat DRN and those animals displayed increase anxiety-like behavior (Nishitani et al 2019)." This example is very limited and also only in the rat. At least include findings form the following papers in addition to Nishitani's study (Mishitani et al also used mouse in their study. Since current paper uses mouse, the authors should mention their findings with mouse as well).
Ohmura et al (2014) Optogenetic activation of serotonergic neurons enhances anxiety-like behaviour in mice. Int J Neuropsychopharmacol
Marcinkiewcz et al (2016) Serotonin engages an anxiety and fear-promoting circuit in the extended amygdala. Nature
Correia et al (2017) Transient inhibition and long-term facilitation of locomotion by phasic optogenetic activation of serotonin neurons. eLife.
Line 68. The reference does not support their opinion (findings from songbird).
Response:

Thanks for the suggestions. The articles from Nishitani, Ohmura, Marcinkiewcz and Correia have been cited in the discussion part of the revised manuscript. The reference (findings from songbird)[6] suggests that temperature could regulate biophysical dynamics in the brain and birdsong (which is a motor behavior or an oscillatory behavior). This reference might support our opinion that local temperature alteration in the brain might induce physiological and behavioral effects. We have revised the sentence as “even if temperature alteration may not cause detectable brain tissue damage, it can still induce remarkable physiological and behavioral effects”.

Minor Concerns:

4) SN is not typical abbreviation for serotonergic neurons. Either use as it is ("serotonergic neurons") or 5-HT as the abbreviation of serotonin ("5-HT neuron" or "5-HTergic neuron").

Response:

Thanks for the suggestion. We have replaced all SN with serotonergic neuron(s) in the revised manuscript.

Reviewer #4:

Manuscript Summary:

This manuscript reports an effective methodology to selectively lesion 5-HT neurons in the DRN without disrupting the central sinus. More generally, the methody they present is a useful technique for all stereotaxic injections that require use of an angle to avoid midline structures.

Major Concerns:

While I have no major concerns with the scientific approach, I would not call this a "newly-developed" technique, unless there is a unique detail I am missing. An angled approach to targeting the DRN/PAG (midline structures) has been employed for decades. I recommend modifying the language in this manuscript to be more general to reach a broader audience? The technique described here could be useful to many scientists seeking to inject viral vectors, DREADDS etc into the DRN or other midline structures. You could add a paragraph in the intro saying that as an example, 5-HT lesions will be conducted but this approach could be used for the following experimental purposes.....

Response:

We thank the reviewer for the suggestions. The phrase “newly developed” has been deleted from the revised manuscript. We also revised the manuscript according to the reviewer’s suggestion in the manuscript (line 399-400: “Furthermore, this method is not only for DRN lesion, it might also provide some useful data for the researchers who want to perform stereotaxic injections on other midline structures of brain.”).

Minor Concerns:
This is an old technique (unless I misunderstand a novel detail) - please revise the manuscript language accordingly.
Response:
We thank the reviewer for the suggestions. The phrase “newly developed” has been deleted from the revised manuscript.

Section 2.
- Add preparation of Hydrogen Peroxide. I'm assuming it is diluted from 30% to 3%?
Response:
The original concentration of Hydrogen Peroxide is 3% as purchased.

Section 3.
- Swap steps 3.2 and 3.1. I think autoclaving and collecting supplies should occur prior to injection of desipramine. Also, when listing supplies, add hydrogen peroxide here.
Response:
We thank the reviewer for the suggestion. Steps 3.2 and 3.1 have been swapped; hydrogen peroxide has been listed in supplies.

- Is desipramine injected 1 hr prior to start of surgery or 1 hr prior to injection of 5,7-DHT?
Response:
Desipramine is injected 1 hr prior to 5,7-DHT injection. (3.2 in the manuscript)

- 3.3 - what is flow rate for vaporizer? Approximately how long are mice in the induction box prior to establishing a full plane of anesthesia?
Response:
Flow rate is 2 L/min. This information has been added in the 3.3 part of the manuscript. It takes about 5 min to induce a complete anesthesia in the induction chamber.

Section 4
- 4.8 - add additional notes for those who may not have access to a digital stereotaxic arm. Add notes on how to ensure hamilton syringed is properly filled.
Response:
We have added the notes the reviewer mentioned in the revised manuscript (Line 177-182).

Section 5
- After recovery, are mice singly housed are group housed?
- I recommend weighing the mice for several days after surgery to monitor health.
Response:
Mice are group housed after recovery since isolation might affect animal behavior.
We will monitor health (including animal weight) in our future study, as serotonergic system may affect appetite. Thanks for the suggestion.

Section 7

- More details about how to conduct an intracardial perfusion are needed
- Add a notation that if the user is slicing on a vibratome, dropping into sucrose is not necessary.

Response:

Details of an intracardial perfusion are added at the 7.1 part of the revised manuscript. We thank for the suggestion. The notation that “dehydrating using 30% sucrose is not necessary if sections are cut with a vibratome” has been added at Line 261.

Reference

1 Nillni, Y. I., Toufexis, D. J. & Rohan, K. J. Anxiety sensitivity, the menstrual cycle, and panic disorder: a putative neuroendocrine and psychological interaction. *Clin Psychol Rev.* **31** (7), 1183-1191, (2011).

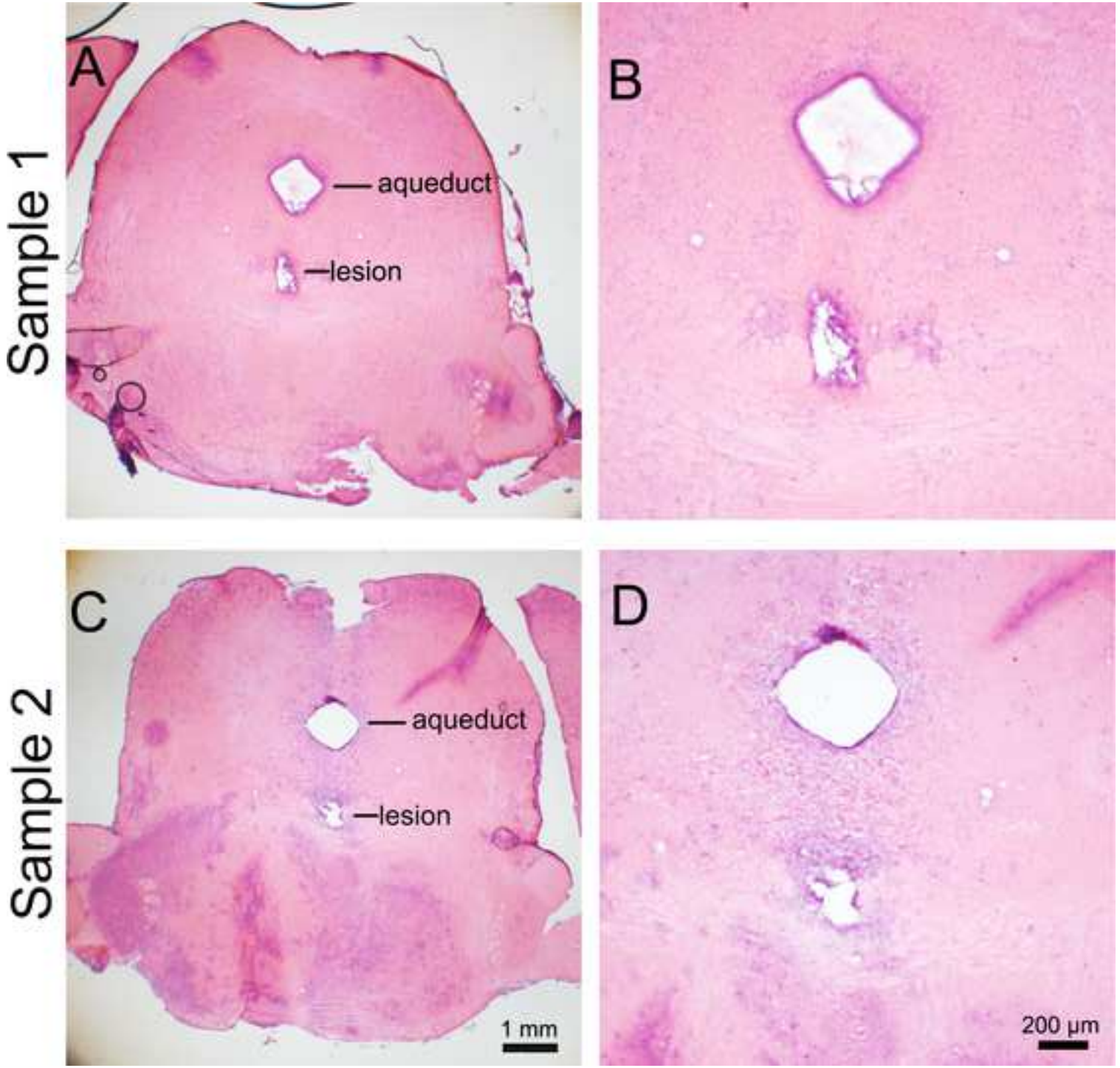
2 Lu, J. *et al.* Generation of integration-free and region-specific neural progenitors from primate fibroblasts. *Cell Rep.* **3** (5), 1580-1591, (2013).

3 Cao, L. *et al.* Characterization of Induced Pluripotent Stem Cell-derived Human Serotonergic Neurons. *Front Cell Neurosci.* **11** 131, (2017).

4 de Medeiros, G. F., Pereira, E., Granzotto, N. & Ramos, A. Low-anxiety rat phenotypes can be further reduced through genetic intervention. *PLoS One.* **8** (12), e83666, (2013).

5 Li, Y. *et al.* Serotonin neurons in the dorsal raphe nucleus encode reward signals. *Nat Commun.* **7** 10503, (2016).

6 Long, M. A. & Fee, M. S. Using temperature to analyse temporal dynamics in the songbird motor pathway. *Nature.* **456** (7219), 189-194, (2008).



retuttal sup_figure2

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retuttal video_perfusion_sign