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TITLE:

Targeting the Corticospinal Tract in Neonatal Rats with a Double-viral Vector Using Combined Brain and Spine Surgery

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KEYWORDS:

Neuroscience, cervical spine injury, neonate, axon regeneration, sprouting, gene therapy, Designer Receptors Exclusively Activated by Designer Drugs (DREADDs)

SUMMARY:

This protocol demonstrates a novel method for applying gene therapies to subpopulations of cells in neonatal rats at postnatal ages 5–10 days by injecting an anterograde chemogenetic modifier into the somatomotor cortex and a retrogradely transportable Cre recombinase into the cervical spinal cord.

ABSTRACT:

Successfully tackling the obstacles that constrain research on neonatal rats is important for studying the differences in outcomes seen in pediatric spinal cord injuries (SCIs) compared to adult SCIs. In addition, reliably introducing therapies into the target cells of the central nervous system (CNS) can be challenging, and inaccuracies can compromise the efficacy of the study or therapy. This protocol combines viral vector technology with a novel surgical methodology to accurately introduce gene therapies into neonatal rats at postnatal day 5. Here, a virus engineered for retrograde transport (retroAAV2) of Cre is introduced at the axon terminals of corticospinal neurons in the spinal cord, where it is subsequently transported to the cell bodies. A double-floxed, inverted-orientation designer receptor exclusively activated by designer drug(s) (DREADD) virus is then injected into the somatomotor cortex of the brain. This double-infection technique promotes the expression of the DREADDs only in the co-infected corticospinal tract (CST) neurons. This is the first protocol demonstrating successful co-infection of the CST in neonatal rats. Thus, the simultaneous co-injection of the somatomotor cortex and cervical CST terminals is a valid method for studying the chemogenetic modulation of recovery following cervical SCI models in neonatal rats.

INTRODUCTION:

While SCI is a relatively rare occurrence in the pediatric population, it is particularly traumatic and causes a permanent disability requiring immense logistical foresight. Furthermore, a higher proportion of pediatric SCIs is classified as cervical and complete compared to the adult population[1,2]. A hallmark across mammalian species is that neonates recover notably better from SCI than adults, and this offers an opportunity to assess the driving mechanisms for recovery in younger populations[3-5]. Despite this, there are fewer multimodal studies tackling neonate and infant rodent research, partly due to the added difficulty of accurately targeting select populations of neurons in the much tighter anatomical landmarks of younger animals[6]. This article focuses on the direct injection of highly efficient anterograde and retrograde adeno-associated vectors into the rat spinal cord to modulate major motor pathways with the application of Cre-dependent-DREADDs, expanding the reach of multimodal regeneration studies.

Viral vectors are important biological tools with a breadth of applications, including the introduction of genetic material to substitute for target genes, upregulate growth proteins, and trace the anatomical landscape of the CNS[7-9]. Many of the anatomical details of spinal motor pathways have been studied using classical tracers, i.e., biotinylated dextran amine. While traditional tracers have been instrumental in unearthing neuroanatomy, they are not without their disadvantages: they indiscriminately label pathways even if correctly injected, and studies have found that they are taken up by damaged axons[10-12]. Consequently, this could lead to incorrect interpretations in regeneration studies where severed axons could be mistaken for regenerating fibers.

The following method utilizes the two-viral vector system recently popularized in modulation studies, with two different viral vectors in two separate areas of the same neuron[13,14]. The first is a vector that locally infects the cell bodies of projection neurons. The other is a retrograde vector being transported from the axon terminals of the projection neurons (**Figure 1**). The retrograde vector carries Cre recombinase, and the local vector incorporates the “Cre-On” double-floxed sequence in which a fluorescent protein (mCherry) is encoded. The native transgene expressing both hM3Dq and mCherry is inverted relative to the promoter and is flanked by two LoxP sites (**Figure 2**).

Thus, mCherry is only expressed in the doubly transduced projection neurons where Cre recombinase induces a recombination event between the LoxP sites, flipping the orientation of the transgene into the appropriate reading frame and allowing the expression of both DREADD and the fluorescent protein. Once the viral transgene is in the correct orientation, and when applicable, the DREADDs can transiently induce neuromodulation through a separately injected ligand, i.e., clozapine-N-oxide. The protocol was designed to authenticate inducible neuromodulation research in neonates, wherein DREADDS are injected to modulate the CSTs selectively. The two-viral system acts as an insurance policy, ensuring that every DREADD-permeable cell is traceable under fluorescence with high fidelity to validate the injections.

This method also helps to bridge the gap in neonatal research. Pediatric SCI presents its challenges, and research analyzing regeneration, sprouting, or plasticity should emphasize

the differences between neonates and adults[3,15-17]. By optimizing the surgical procedure and performing prior anatomical studies with Nissl staining, the coordinates for both the cranial and spinal injections were validated. The aim was to provide a method for dual injections into a neonatal rat with increased fidelity and survivability.

For the current model, the anterograde vector was injected into the cell bodies of the somatomotor cortex using bregma as reference[18,19]. In terms of the spinal injections, the retrograde vector was injected into laminae V–VII, where the CST axon terminals reside[20,21]. There are many fundamental questions underlying how certain lesion models affect younger animals differently, and how the subsequent recovery diverges from an older animal. This study demonstrates a robust means of studying cervical injuries and the recoverability of forelimb function in neonatal rodents. In contrast, the majority of previous studies have addressed recovery locomotion following lumbar or thoracic injuries[5,22-24]. By pairing the double-viral vector with the novel injection technique described here, this protocol helps mitigate certain issues (i.e., survivability) that may plague neonatal rodent investigations. This method is robust, practical, and versatile: slight variations in the technique will allow for targeting different pathways, i.e., ventral CST, dorsal CST, and the ascending dorsal pathways.

For this system, one locally acting virus (e.g., AAV2) is injected in the region of the neuronal cell bodies of interest. In contrast, a second retrogradely transported virus that controls the expression of the local virus is injected at the axon terminals for that neuronal population. Thus, by definition, only corticospinal neurons are labeled. The retroAAV-Cre virus was chosen with a constitutively active CMV promoter as the shuttle plasmid is used to generate several AAV serotypes for Cre-dependent expression in several cell types. For cortical injections, AAV2 was chosen with the transgene driven by the synapsin-1 promotor to limit any expression to neurons. Because the 2-viral system relies more on the origin and termination of the neuronal population of interest, several different promoters could be used, if they can drive the expression of the genes of interest within the neuronal population of interest. For example, the excitatory promoter, CamKII, could be substituted for the synapsin-1. In addition to the use of these AAV serotypes, retrograde transport into immature, and to a much lesser extent, adult corticospinal motor neurons can also be achieved using the high retrograde transportable lentivirus (HiRet)[25]. HiRet lentiviruses use a chimeric Rabies/VSV glycoprotein to target uptake at the synapse for retrograde transport. Combined with a Tet-On promoter, this 2-viral system supports inducible expression in a retrograde-dependent fashion[26,27].

Retrograde viruses insert vectors into the synaptic space of a target neuron, allowing it to be taken up by that cell's axon and transported to the cell body. While lentiviral vectors have previously had tremendous success, providing long-term expression in gene therapy studies, this method pivoted towards adeno-associated viral vectors for a few simple reasons[26,28]: AAV is more economical, similarly effective, and presents less of a logistical burden, given that it has a lower biosafety level designation[29-32]. While AAV2, the most used serotype, demonstrates robust transfection of CST axons, future researchers may note that AAV1 offers some versatility as it labels transynaptically, thus putting forth several possible iterations in future studies[33]. The final adaptation is to encode the retrograde virus with Cre-recombinase so that multiple anterograde vectors can be introduced simultaneously, thereby reducing

unnecessary in-house virus waste and maximizing the likelihood of the DREADDs expressing in the correct orientation.

Ultimately, this protocol demonstrates simultaneous injection into the cortex and cervical spine, specifically targeting the cell bodies and the axon terminals of the corticospinal tract, respectively. High-fidelity transfection is seen in the cerebral cortex and spinal cord. While the protocol described was perfected for Sprague Dawley rats 5 days of age, it is suitable for postnatal days 4–10 with minor adjustments to stereotactic coordinates and anesthesia.

PROTOCOL:

All of the following surgical and animal care procedures have been approved by the Animal Care and Use Committee of Temple University. The protocol described is a survival surgery, and the animals were eventually euthanized by intraperitoneal injection of 100 mg/kg sodium phenobarbital at the completion of their time points.

1. Pre-surgical preparation

1.1. Prepare at least two pulled glass needles for viral injection using 3.5 nL glass capillary pipettes; one needle for the DREADD and one needle for the rCre. As a precautionary measure, prepare 4–5 needles in case they break intraoperatively.

1.2. Using microscissors, cut off 1–2 mm of excess glass from the needle.

1.3. For each needle, position it at 30°, use a micropipette beveller to create a tip with a 30–40 µm aperture and a 45° beveled angle.

1.4. Store the needles in a covered Petri dish and then sterilize them by placing the Petri dish in a biosafety hood under UV light for 15 min.

1.5. Prepare the necessary viruses by removing a suitable volume from the -80 °C freezer before the procedure, bearing in mind that each animal will require 3 µL of each virus.

NOTE: Transport and store the virus on ice when not in use. This protocol was developed using AAV2-hM3Dq-mCherry and AAV2-retroCre to inject 3 µL of each virus per animal. The DREADD plasmid, pAAV-hSyn-DIO-hM3Dq-mCherry (see the **Table of Materials**), was used to make the anterograde AAV2 with a viral titer of 1.54×10^{12} genome copies (GC)/mL. The Cre plasmid, pAAV-CMV-scCre, was used to make the retrograde AAV2 with a viral titer of 4.27×10^{12} GC/mL.

1.6. Plug the injector into the micropump and place it in a micromanipulator with a Vernier scale.

1.7. To help visually confirm the presence or absence of virus in the needle, load colored dye, i.e., red oil, into the needle. Avoid bubbles in the needle.

1.8. Insert the glass needle into the injector, ensuring that the needle fits correctly.

1.9. If available, repeat the entire process with a separate injecting pump for each virus. If there is only one available injecting pump, prepare two separate needles and replace the used needle when it is time to swap the viruses.

2. Anesthesia and surgical site preparation

2.1. Weigh the animal on a digital scale. Record the preoperative weight to determine the volume of anesthetic required.

NOTE: This protocol found that the most reliable anesthetic technique to ensure a satisfactory anesthetic plane throughout the operative time is a combination of ketamine and hypothermia. Ketamine is insufficient to ensure anesthesia on its own, and supplementing it with xylazine has a narrow threshold before increasing intraoperative mortality. Hypothermia on its own is not sufficient for prolonged surgeries (dual spine and brain surgery likely require >1 h of steady anesthesia).

2.2. Anesthetize the pup by injecting diluted ketamine (10 mg/mL) subcutaneously so that the animal receives a 100 mg/kg dose of ketamine; wait for 5 min.

NOTE: For example, at postnatal day 5, a rat pup weighs ± 10 g and receives 0.1 mL of the diluted ketamine solution (10 mg/mL).

2.3. Place the pup on crushed ice for 6–8 min. Protect it against frostbite by placing the animal in a latex glove or parafilm to avoid direct contact with ice.

2.4. Pinch the foot firmly using forceps to confirm an appropriate anesthetic plane. If reflexive withdrawal occurs, leave for an additional 2 min on ice before proceeding.

2.5. Broadly apply antiseptic to the animal's head and back area using sterile gauze soaked with a 5% iodine solution. Then, sterilize with gauze soaked in 70% ethanol. Apply the antiseptic wipes three times each, alternating between iodine and ethanol to soak the gauze.

NOTE: There is no need for eye care as young pups only open their eyes at ± 14 days of age.

2.6. If an animal is to receive dual surgery (brain + spine), provide a normal saline bolus before surgery (0.02 mL/g) subcutaneously.

NOTE: if the animal becomes responsive during the surgery and further anesthesia is required, then replace the animal onto ice for 5 min.

3. Surgical field and instrument preparation

3.1. Autoclave the surgical tools that include a scalpel holder, rongeurs, hemostats, medium point curved forceps, and retractors

3.2. Ready the microscope and install the neonatal rat stereotaxic adaptor to firmly position it within the adult stereotaxic holder.

3.3. Conduct the surgery using sterilized gloves. Open a pack of prepackaged sterile surgical gloves and place the sterile glove wrap on the table, using the wrapper as an additional area to place used tools.

NOTE: It is important to follow good surgical practice and maintain sterility throughout the procedure.

3.4. Secure a 11- blade in the scalpel holder. Position sterile saline, 4.0 chromic catgut suture, 4.0 silk suture, and materials to control bleeding, e.g., sterile gauze, sterile cotton-tipped applicators, and triangles.

3.5. Set up two surgical fields as described above, with one site assigned for craniotomy and the other site assigned for cervical laminectomy.

3.6. Retrieve the animal and secure it in the stereotaxic adaptor: as the neonates are very cartilaginous, fix them gently by directing the earbars broadly towards the mandibular joints. Once horizontally stabilized, gently introduce the front mouthpiece.

NOTE: A nonbinding rule used to provide a "flat" surgical area is to fix the earbars at the same height and the mouthpiece at approximately 2–3 levels lower.

4. Performing the craniotomy and exposing the somatomotor cortex

4.1. Identify the area where the incision will be made by pressing the forceps in the midline on the top of the scalp, feeling for the sagittal suture. Make a 2 cm incision along the sagittal suture plane, starting immediately above the eyeline. Hold the skin taut to ensure a clean, straight, and precise incision.

4.2. Identify bregma (the convergence of the coronal and sagittal sutures) by gently probing the forceps along the surface of the skull and paying close attention to the suture lines as well as any indentation caused by the forceps running along the parietal and frontal bones.

4.3. Maintain the opening with the application of weighted hooks on the side of interest. Note that the spinal injection is made on the right side, the cranial injection is on the left side.

4.4. Clear the aponeurosis with a combination of the cotton tips and microscissors to maximize the exposure of bregma for increased accuracy. Ensure that the coronal suture is in clear view far enough laterally to provide a rough template for subsequent injections.

4.5. Using the microscissors, cut out approximately a 3 x 2 mm section of the left frontal skull bone immediately adjacent to bregma.

NOTE: Once the bone flap has been carefully removed, there should be a clear window with

exposed brain matter ready for injection. The remaining suture lines on the contralateral side of the brain will act as a visual guide for maintaining accuracy along the anterior-posterior (AP) axis.

4.6. Clear up any debris, cerebrospinal fluid (CSF), and blood with cotton tips.

NOTE: It is normal for blood or CSF to slightly obscure the visual line, so it is advisable to clear the area with cotton tips regularly.

Figure 3

5. Loading the virus and positioning the injector

5.1. Load the virus into the injector by pipetting ~5 µL onto a piece of parafilm, and position the needle so that the tip is resting atop the droplet of the virus.

5.2. Ensure that extra virus is loaded into the injector to ensure smooth injection. For example, when injecting a total of 3 µL (1 µL for each injection site), withdraw 4 µL of the virus at a rate of 250 nL/s using the micropump.

5.3. Remove the excess virus with a laboratory wipe.

5.4. Position the micromanipulator so that the Vernier scale is visible, and position the needle above the sagittal suture.

5.5. Lower the needle to just above the area representing bregma (**Figure 3**), and note down the AP and medial–lateral (ML) coordinates.

5.6. Given that the injections will be on the left-side, generate target coordinates by subtracting 1, 1.5, and 2.0 mm (ML: -1.0, -1.5, -2.0) from the ML coordinates for bregma.

NOTE: The AP position will be +0.5mm from bregma for all three injections.

5.7. Move the needle into position for the first injection. AP: +0.5, ML: -1.0

5.8. Lower the needle to the exposed brain until it is indenting the outer cortex. Note down the height of the needle and bring the needle down into position by subtracting 0.6 mm from the height of the surface of the brain. Depth of injection: cortical surface -0.6 mm.

NOTE: It is important to note the depth of the surface for each injection as slight disruptions to the rat's positioning may occur.

6. Injecting virus into the somatomotor cortex

6.1. Once the needle is in place, program the injector to inject 1 µL at a rate of 250 nL/min.

6.2. Once the injection is completed, allow the needle to rest in the cortex for 3 min.

6.3. Repeat the injections for the other coordinates: a total of 3 injections along the cortex in relation to bregma, all at a depth of -0.6 mm: 1) AP: +0.5 mm, ML: -1.0 mm 2) AP: +0.5 mm, ML: -1.5 mm 3) AP: +0.5 mm, ML: -2.0 mm.

6.4. After completion of the injections, refer to section 9 to suture, and transfer the animal to the other operating table for cervical laminectomy.

7. Creating a spinal window for precise spinal cord injections

7.1. Identify the incision site by using fingers to palpate the base of the skull. At midline, begin the incision 1–2 mm posterior to the skull base using a #11 blade and extend the incision 1 cm posterior to expose the superficial muscle. Hold the skin taut by applying tension with the thumb and index finger to ensure a clean incision.

7.2. Using the sharp point of the blade, gently make a series of cuts along the midline of the superficial muscle to expose the spinal cavity and deep spinal muscles. Use a pair of forceps to spread open the muscle and visualize the surgical window.

NOTE: The superficial muscle will appear light pink, while the deep spinal muscles will appear a light whitish-grey.

7.3. Once the deep spinal muscles have been exposed, insert retractors into the surgical window. If necessary, use forceps to grab onto the lateral skin and muscles to stretch them around the teeth of the retractors. Retract the surgical window to 7–8 mm in width, allowing an unobstructed view of the spine.

7.4. Identify the second cervical (C2 or axis) vertebra by its prominent spinous process that projects dorsally and encapsulation in a large dome-shaped muscle. Use forceps or a blunt probe to feel for and identify this process, as this will be the guiding landmark.

NOTE: The adjacent C3 vertebra is often slightly occluded by the large C2 muscle; therefore, gentle dissection of muscle at C3 with forceps or a bone scraper helps to define the vertebra and aid in vertebral counting.

7.5. Using the flat edge of a bone scraper, expose C3–C7 vertebrae by gently scraping away the deep spinal muscle to the sides. Start medial and scrape laterally along the direction parallel to the vertebral laminae to ensure proper exposure, which will allow clear distinction of vertebral laminae. Control any bleeding with cotton tips.

7.6. Using a pair of microscissors, carefully cut the lateral edges of the cartilaginous laminae at C6 and C7. Use C2 as a guide when counting vertebral levels.

7.7. Using a pair of fine forceps, carefully remove the dissected portion of the lamina to expose the spinal cord. Make sure the spinal window is large enough to accommodate the desired injection site. Remove any sharp or jagged parts of bone that may puncture the spinal cord with microscissors and forceps as described above.

7.8. Set the animal up in the stereotaxic cranial holder as described in section 3.6. In addition, place a rolled-up piece of gauze under the animal's trunk to elevate its hindquarters.

NOTE: Elevation of the animal's hindquarters effectively lifts the thorax off the surface of the holder to prevent respiratory movements from affecting the position of the needle during injection.

7.9. Before beginning injections, clear the spinal window of any blood or cerebrospinal fluid by gently applying cotton tips and Sugi triangles to the area without insulting the spinal cord. Further, create a barrier around the perimeter of the window to prevent occlusion of the injection site by continuous bleeding or CSF leakage. To do this, place a small piece of absorbable cotton in the lateral portions of the spinal window.

8. Direct injections into the spinal cord targeting axon terminals

8.1. Using the tip of the glass injection needle, approximate the midline of the spinal cord by identifying the spinal artery. However, if the spinal artery is noticeably off-center or deviates in any way, approximate the midline by using the position of the C2 spinous process and extrapolating this down the length of the spine.

NOTE: Refer to section 5.1 for instructions on loading the virus.

8.2. Once identified, situate the needle just posterior to the C5 lamina at the approximate midline and use this as the reference point. Then, using the micromanipulator, move the needle laterally to the right by 0.3 mm. Lower the needle until it just touches the surface of the spinal cord; from this depth, plunge the needle 0.6 mm into the spinal cord. If necessary, continue to plunge the needle until it punctures the spinal cord; then, retract or lower the needle to the appropriate depth.

8.3. Inject 1 µL of retroAAV2-scCre at 250 nL/min. After the injection is complete, wait 2 min for the virus to diffuse into the spinal cord before slowly withdrawing the needle. Repeat the injections using the same lateral and depth coordinates at two more sites within the spinal window, one at the midpoint and the final just anterior to the T1 lamina.

9. Wound closure and postoperative care

9.1. Remove the animal from the stereotaxic holder and take out the retractors or hooks. Clear out the wound area with a few drops of sterile normal saline.

9.2. Suture the scalp with 4.0 silk suture, two or 3 sutures in total.

9.3. When suturing the cervical opening, use 4.0 chromic gut to tightly reattach the muscle layers (2 sutures should suffice). Suture the cervical skin opening with 4.0 silk (4 sutures expected).

9.4. Once the animal is closed up, judiciously apply liquid bandage across the sutures.

9.5. Place the animal under a heating lamp and monitor it closely until entirely re-awakened. Once the animal is awake, moving, and dry, gently clean the wounds with sterile gauze. Do not leave the animal unattended until it has regained sufficient consciousness to maintain sternal recumbency.

9.6. Return the animal to the homecage with its mother. Take care to prevent neglect and infant cannibalization from the mother towards the pups:

9.6.1. Familiarize the investigators with the mother in anticipation of surgery through gentle handling (5–10 min) twice daily, beginning one week before surgery.

9.6.2. Return the pups to the homecage together to limit disruption to the mother.

9.6.3. Inject the mother with acepromazine 1.5 mg/kg q12 h subcutaneously on the day of surgery.

NOTE: Pain management serves the dual purpose of providing analgesia for the pups and encouraging earlier return to activity, thus promoting reintegration with the mother.

9.6.4. Inject buprenorphine 0.05 mg/kg subcutaneously q8 h starting after surgery for 3 days total (postoperative days 0, 1, 2).

REPRESENTATIVE RESULTS:

Successful injection and transport of the viral vector should result in the transduction of unilateral neurons in the spinal cord and the motor cortex. **Figure 4** demonstrates the labeling of layer V CST neurons in the motor cortex of a brain coronal section expressing Cre-dependent-DREADDs-mCherry co-injected with a contralateral spine injection of rCre. The sections were stained with dsRed antibody.

Figure 4.

FIGURE AND TABLE LEGENDS:

Figure 1: An illustration demonstrating the two-viral injection methods as used in this protocol.

Figure 2: Illustration of the viral constructs used in this protocol, along with the double-floxed inverted orientation being corrected by the retroAAV2-scCRE.

Figure 3: A schematic illustration of the cranial injection coordinates (mm) relative to bregma.

Figure 4: Transduction of neurons in the motor cortex. (**A**) Magnification of 5x. dsRed immunohistochemistry demonstrating mCherry expression in layer V neurons of the animal's left motor cortex. Scale bar = 500 μm. (**B**) Magnification of 10x. dsRed immunohistochemistry demonstrating mCherry expression in layer V neurons of the animal's left motor cortex. Scale bar = 200 μm.

DISCUSSION:

Inducible genetic modulation of brain activity with injectable chemogenetic modifiers is a powerful tool in studying the various mechanisms that underlie the recovery from SCI. The accuracy of the targeting for the inducible G-protein-coupled receptors (DREADDs) is further increased when considering that fluorescence tracing validates the anatomical precision in histology. This paper discusses a reliable method for exploring whether or not inhibiting or stimulating select neuronal pathways (with either excitatory or inhibitory DREADDS) results in enhanced axon regeneration or sprouting[34,35]. Injection of a retrogradely transportable vector into the spinal cord can bring attention to select neuronal populations, either directly or via their synaptic connection, making this method an excellent choice for assessing the neurobiological response to injury.

As illustrated, studying SCI in neonatal models to elucidate any previously unknown discrepancies between pediatric populations and adult populations only serves to complicate the research further. As such, the study designs are narrower. The consensus in neonatal research is that the improved outcomes seen following SCI depend on increased axonal regeneration and increased sprouting until around postnatal day 7 in rodents[3-5,36]. However, this amplified plasticity is not seen beyond postnatal day 10 and recovery plateaus until it represents adult rodent injury models too[37-40]. The underlying mechanisms for the enhanced plasticity in neonates are elusive and remain debated, highlighting the importance of developing easily replicated, survivable, and efficient surgical models to facilitate focused research on neonatal SCI.

For example, some have posited that the disproportionate level of sprouting seen in younger animals negates certain aspects of functional recovery from CST insult[19]. Ultimately, questions remain whether the spontaneous recovery is due to organic regeneration or simply increased sprouting of aberrant pathways that conveniently bypass the lesion. The protocol described here is relatively straightforward and is modular and tunable to study corticospinal tract regeneration or sprouting and artificially inhibiting or stimulating recovery processes with the advent of inducible vectors.

The viruses used in this study were chosen to provide a blueprint for future research investigating the plausibility of influencing recovery from SCI in younger rodents. By design, the transgene expressing mCherry would only label positively under fluorescence if the DREADD (hM3Dq) were expressing simultaneously, as they are both present on the same transgene. While the current protocol does not include behavioral and phenotypic assessments, it has laid the groundwork for successfully investigating DREADD activity in future studies.

The most critical elements to successful injections of viral vectors are knowing the correct anatomy and ensuring sufficient diffusion of virus. With regards to injecting the anterograde vector into the sensorimotor cortex, there are numerous methods described in the literature, including three injections in the vicinity of bregma at a shallow depth (0.5–0.7 mm). Targeting the correct area for the retrograde injection of a vector into the spinal cord requires precision and a deft understanding of the neurobiology of the neurons of interest. The majority of the CST terminals are localized to laminae V–VII throughout the spinal cord grey matter, and

successful transfection may require injections at multiple cervical levels[41]. For instance, segments C4–C7 can be located with distinct landmarks, thus priming the surgical window for routine laminectomies and subsequent injections. Ensuring adequate diffusion of the injected material is dependent on the properties of the vector, volume of the injection, and the density of the neuronal tissue.

Fortunately, the neonatal brain is very receptive to transfection as the lower density allows for a more rapid and disseminated spread of the injected material. The spinal component is more complex, with a significantly tighter injection window. Nevertheless, retroAAV is efficient at transducing the target synapses. It is important to note that the retrograde vector takes time to reach the cell body and flip the DREADD vector into the correct sequence, so the recommended time before conducting behavioral assessments or histological studies is ± 4 weeks from the date of injection. In summary, the retrograde AAV is appropriate, given that it has several immediate advantages, and given that the double-floxed system only fluorescently expresses target neuronal populations. Despite the narrow operative window, the neonatal spinal cord is similarly receptive to transfection and demonstrates florid expression[42].

Pediatric spinal cord research is a niche within a field that has many barriers to multilayered investigations. With each new iteration and surgical breakthrough in methodology, the research itself becomes easier, and in turn, the likelihood of uncovering deterministic outcomes increases. Accurately injecting a viral vector into a spinal cord pathway is useful for a host of investigational reasons, and expanding the viral vector technology to include DREADDs is useful for selectively enhancing or attenuating target proteins. The hope is that the improved accuracy from the double-floxed injection system paired with the novel surgical protocol will foster a multitude of similar research goals.

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The authors have nothing to disclose.

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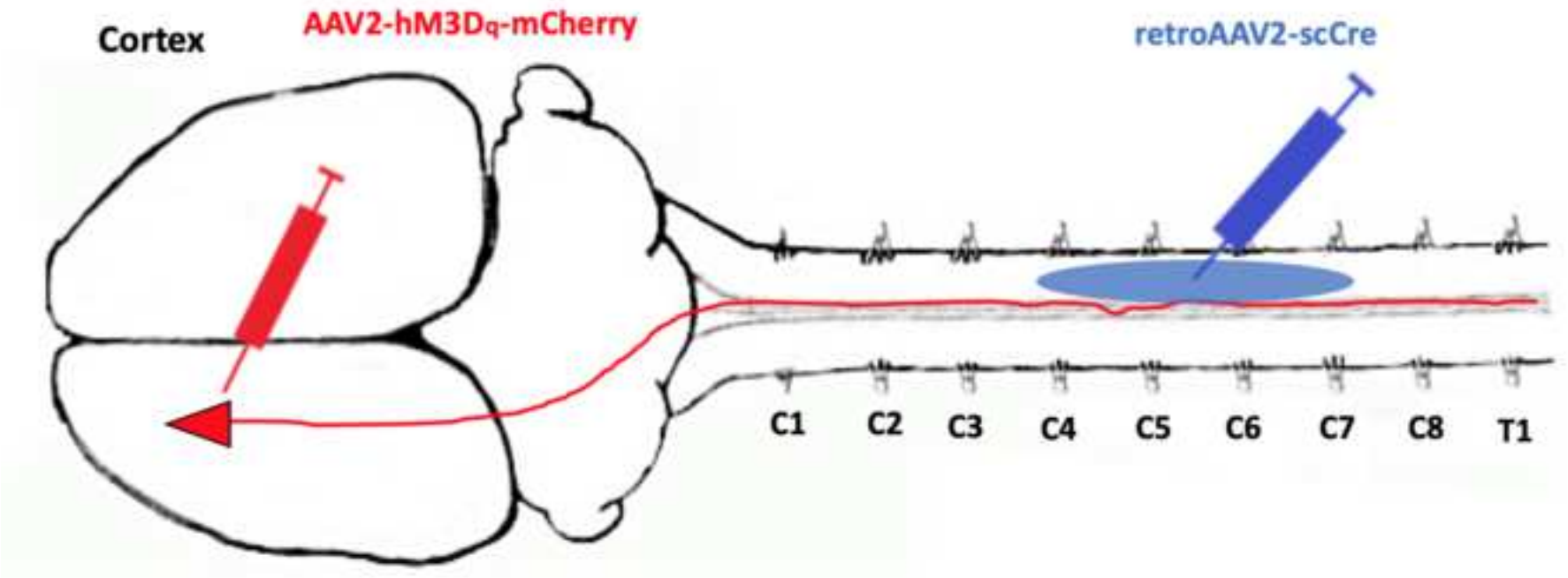
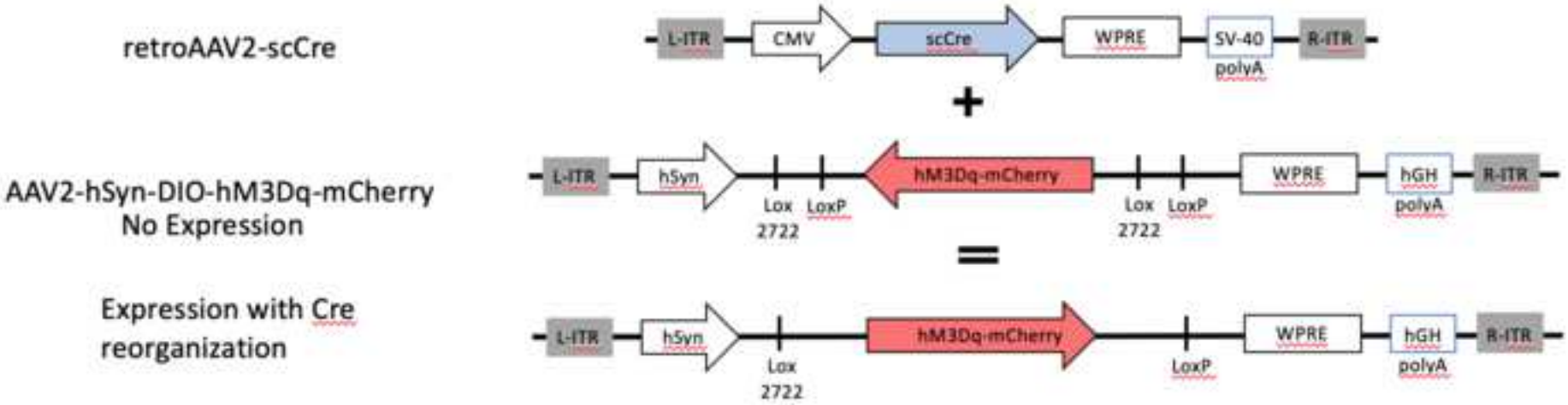
Illustration of DIO

Click here to access/download;Figure;Figure 1.png





Bregma schematic

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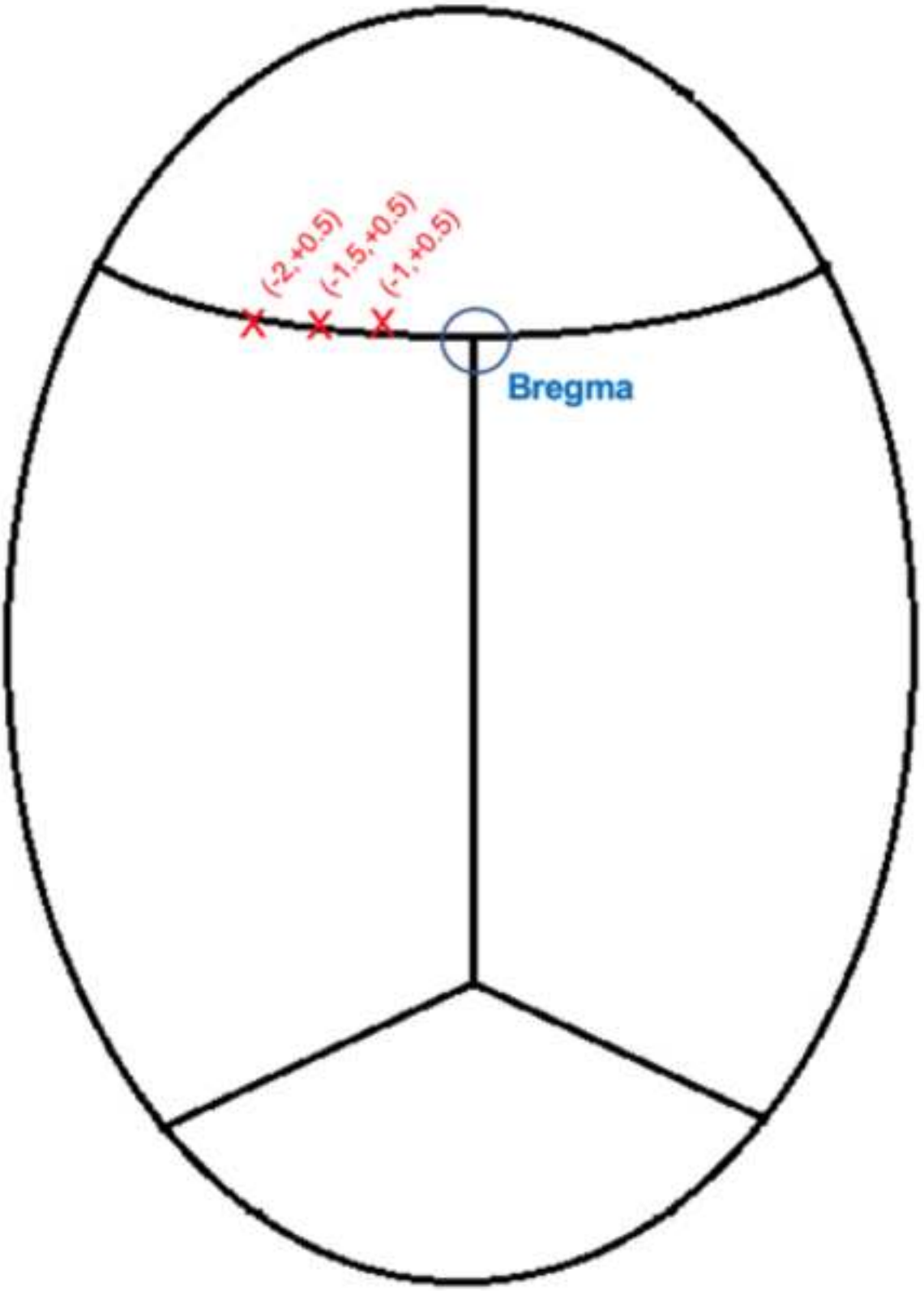

mCherry brain

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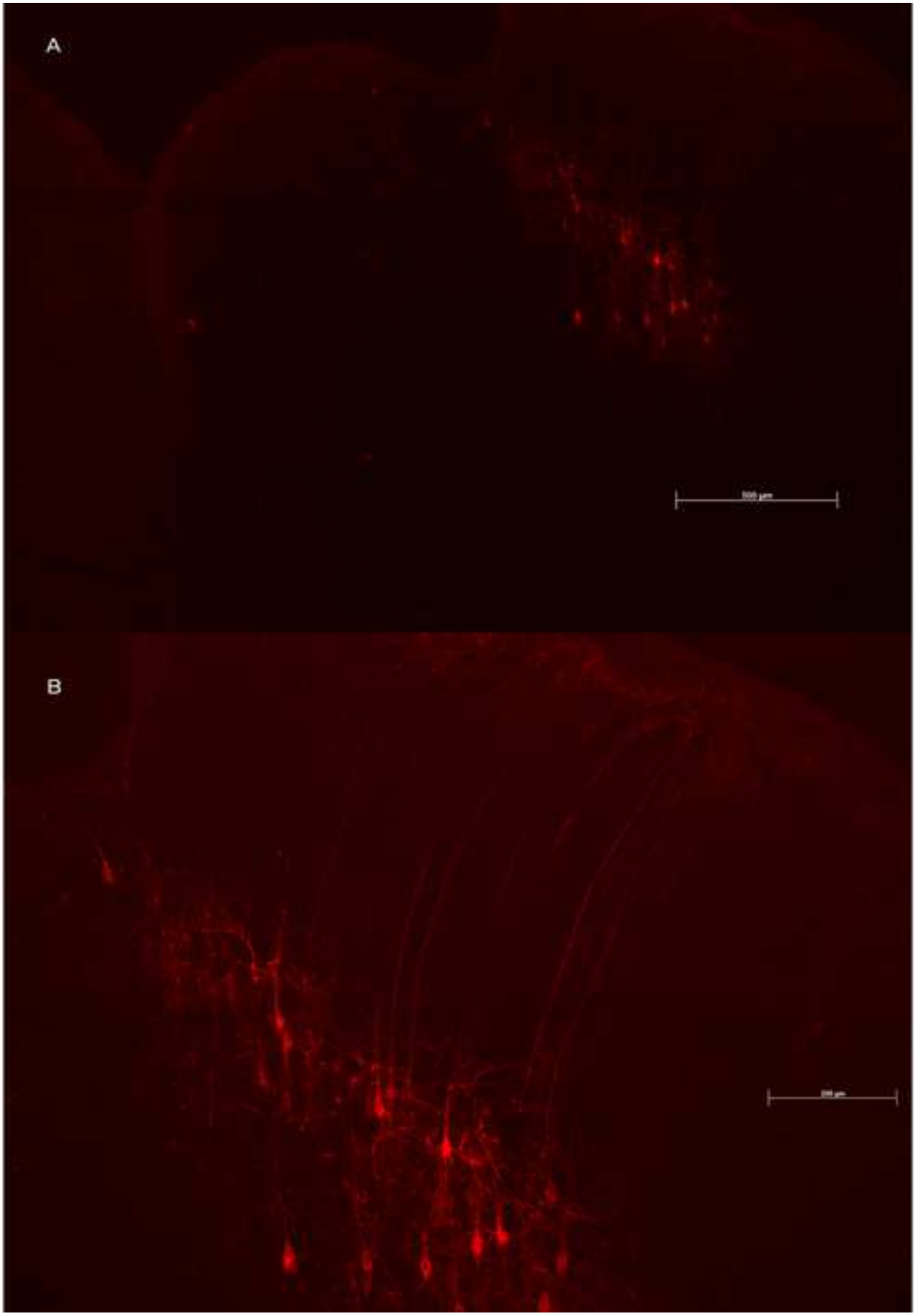

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
#11 scalpel blades	Roboz	RS-9801-11	For use with the scalpel.
#10 Scalpel Blades	Roboz	RS-9801-10	For use with the scalpel.
1 mL Syringes	Becton, Dick	309659	For anesthetic SC injection and fluid bolus
4.0 silk suture	Ethicon	771-683G	For skin closure
4.0 Chromic Catgut Suture	DemeTECH	NN374-16	To re-bind muscle during closing.
48000 Micropipette Beveler	World Precis	32416	Used to bevel the tips of the pulled glass capillary tubes to form functional gl
5% Iodine Solution	Purdue Prod	L01020-08	For use in sterilzation of the surgical site.
70% Ethanol	N/A	N/A	For sterilization of newly prepared glass needles, animal models during surg
Ketamine (Ketaset)	Zoetis	240048	For keeping the animal in the correct plane of consciousness during surgery
Bead Sterilizer	CellPoint	5-1450	To heat sterilize surgical instruments.
Digital Scale	Okaus	REV.005	For weighing the animal during surgical preparation.
Flexible Needle Attachment	World Precis	MF34G-5	For cleaning glass needles and loading red oil into glass needles.
Glass Capillary Tubes	World Precis	4878	For pulled glass needles - should be designed for nanoliter injectors.
Hemostats	Roboz	RS-7231	For general use in surgery.
Medium Point Curved Forceps	Roboz	RS-5136	For general use in surgery.
Micromanipulator with a Vernier Scale	Kanetec	N/A	For precise targeting during surgery.
Microscissors	Roboz	RS-5621	For cutting glass whisps off of freshly pulled glass capillary tubes.
Lab Standard Stereotaxic Instrument	Stoelting	51600	To hold the neonatal sterotaxic holder in place
Lab Standard with Mouse & Neonates Adaptor		51615	For neonatal skull fixation during cranial surgery and spinal injections
Microscope with Light and Vernier Scal	Leitz Wetzla	N/A	Used to visualize and measure beveling of pulled glass capillary tubes into f
MicroSyringe Pump Controller	World Precis	62403	To control the rate of injection.
Nanoliter 2000 Pump Head Injector	World Precis	500150	To load and inject virus in a controlled fashion.
Needle Puller	Narishige	PC-100	To heat and pull apart glass capillary tubes to form glass needles.
pAAV-CMV-scCre	Wu lab		Cre plasmid
pAAV-hSyn-DIO-hM3Dq-mCherry (plasmid #44361)	Bryan Roth's lab through Addgene		DREADD plasmid
Parafilm	Bemis	PM-996	To assist with loading virus into the nanoinjector.
PrecisionGlide Needles (25G x 5/8)	Becton, Dick	305122	For use with the 1mL and 10 mL syringes to allow injection of the animal mc
Rat Tooth Forceps	Roboz	RS-5152	For griping spinous processes.

Red Oil	N/A	N/A		To provide a front for visualization of virus entering tissue during injection.
Retractors	Roboz	RS-6510		To hold open the surgical wound.
Rongeurs	Roboz	RS-8300		To remove muscle from the spinal column during surgery.
Scalpel Blade Handle	Roboz	RS-9843		To slice open skin and fat pad of animal model during surgery.
Scissors	Roboz	RS-5980		For general use in surgery.
Staple Removing Forceps	Kent Scientif	INS750347		To remove the staples, should they be applied incorrectly.
Sterile Cloth	Phenix Rese	BP-989		To provide a sterile surface for the operation.
Sterile Cotton-Tipped Applicators	Puritan	806-WC		To soak up blood in the surgical wound while maintaining sterility.
Sterile Gauze	Covidien		2146	To clean the surgical area and surgical tools while maintaining sterility.
Sterile Saline	Baxter Healt		281324	For use in blood clearing, and for replacing fluids post-surgery.
Surgical Gloves	N/A	N/A		For use by the surgeon to maintain sterile field during surgery.
Surgical Heating Pad	N/A	N/A		For maintaining the body temperature of the animal model during surgery.
Surgical Microscope	N/A	N/A		For enhanced visualization of the surgical wound.
Surgical Stapler	Kent Scientif	INS750546		To apply the staples.
Water Convection Warming Pad	Baxter Healt	L1K018		For use in the post-operational recovery area to maintain the body temperat
Weighted Hooks	N/A	N/A		To hold open the surgical wound.
Liquid bandage	NewSkin		985838	To apply along sutures following surgery and encourage wound heal
Wire Cage Lamp	ZooMed	LF10EC		To help animals recover from anesthesia and retain warm body tem

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Rebuttal letter

We would like to take this time to thank all the reviewers for providing such constructive and insightful feedback that has helped shape the paper and will make it significantly stronger.

Editorial comments:
We carefully reviewed all the wording to make sure that pronouns were replaced. I believe the paper now better demonstrates "how" each step is performed. Certain wording was re-arranged, and numbers were standardized. With regards to the lines that needed revision to avoid plagiarism, I believe we did so sufficiently but it is important to note that our lab has previously published a similar Jove protocol and we conduct surgeries with a routine that is owed to years of cumulative experience. scale bars have been included in the images.

Reviewer 1:
The bulk of first point raised by first author relate to the details of the viruses we used. There were changes made to introduction and protocol where more specifics were discussed. The details on titer, plasmid, and reference number from Addgene were included in the protocol section of the paper to help clarify for reviewer 1. We believe what has been added is sufficient, as the main goal of this method was to reliably and safely develop a technique to accurately inject into neonates. The proof of concept was then going to be carried forward for future projects working with neonates. In that sense, the exact type of virus used only needed to be a double-floxed virus that would only work in the case of co-infection. Our lab has previously had success with AAV2 and so we used it for familiarity. We may conduct injections of a negative control to demonstrate that in the absence of the double infection, the fluorescence does not stain, but it would mean a new batch of injections and waiting on pups to be born in the near future, which could slow down the finalization of the paper.
Because this is a paper with surgeries on P5 animals, the timeline is optional and user-dependent beyond injections.
We changed the rAAV to retroAAV.
A rough diagram illustrating the coordinates for injection relative to bregma has been included.
We have merged the two images into A and B.
The red dye is not a requirement but is recommended for all injection protocols in our lab, this has been reflected in the paper.
In terms of the question on AAV1 we have observed it labelling transynaptically as DREADD mCherry construct, but we have recently designed a AAV1-GFP and we are hoping to use it soon.

Reviewer 2:
We have included more information on the specifics of the viruses used. We have not included a diagram of the constructs, but I would like to stress that the purpose of the protocol is to highlight the methodology for successfully targeting the anatomy of the younger animals. The hope is that researchers will use the broad concepts to study various models with various constructs. We were originally going to use AAV2-mCherry but opted

for the DREADDs as this is the construct used in the lab to actually study neuromodulation, and that way we had some consistency between projects.
The reviewer raised a good question pertaining to the relevance of this protocol across age groups. We have not confirmed the coordinates in ages other than p4,p5,p6, but we have experience with sprague dawley's p10 and believe that minor changes would guarantee equally strong outcomes.
The most important point of the paper is to help researchers produce strong expression in the CST at younger ages, so that the therapeutic action of proposed interventions can take place earlier. At the time of submission, we had to euthanize the rats earlier than intended because there was a mycoplasma scare in the animal housing facilities. We found that the strong DREADD mCherry expression in the brain took place as early as p14. There is currently a cohort of injected animals that we are using to assess the day at which the DREADD mCherry begin expressing in the spinal cord, but that data will take another 3 weeks to gather.
dsRed primary antibody was used for the immunohistochemistry, this change has been amended.
The DREADDs used in this study have previously demonstrated strong abilities to modulate functionality, and we are currently testing out the abilities to neuromodulate in neonates in a separate study. We chose to use the DREADDs to remain consistent for the proof of concept. Once the ongoing studies have developed further, there will be more information to work with on neuromodulation.

Reviewer 3:
The in house virus waste statement was anecdotal and something we noticed across experiments. We have found that the larger quantity of active ingredient from DREADDs should be directed at the larger surface area of the brain where there is more likelihood of it reaching the targeted tracts.
We have included the statement on two separate needles, where two pumps are not available.
The age range for the surgery has been included.
The necessary precautions to prevent infanticide have been added.
The anesthesia method was developed as we anecdotally ran into issues and modified it until it worked. We have added more explanation on this matter in the protocol.
The minor changes recommended by reviewer 3 have been adopted.

Reviewer 4:
The minor changes suggested by the reviewer were adopted.