Protocol
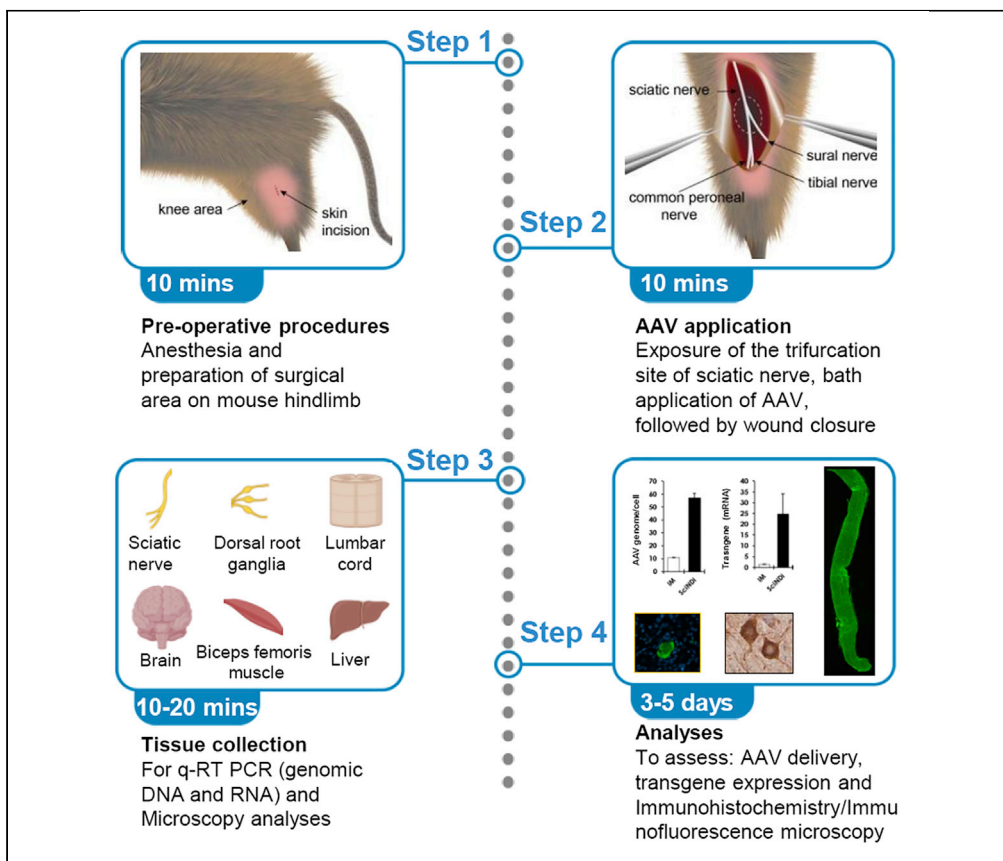
Recombinant adeno-associated virus mediated gene delivery in the extracranial nervous system of adult mice by direct nerve immersion

Step 1

10 mins

Pre-operative procedures
Anesthesia and preparation of surgical area on mouse hindlimb

Step 2

10 mins

AAV application
Exposure of the trifurcation site of sciatic nerve, bath application of AAV, followed by wound closure

Step 3

10-20 mins

Tissue collection
For q-RT PCR (genomic DNA and RNA) and Microscopy analyses

Step 4

3-5 days

Analyses
To assess: AAV delivery, transgene expression and Immunohistochemistry/Immunofluorescence microscopy

This protocol outlines a minimally invasive and quickly performed approach for transgene delivery in the extracranial nervous system of adult mice using recombinant adeno-associated virus (AAV). The technique, named Sciatic Nerve Direct Immersion (SciNDi), relies on the direct bilateral immersion of the exposed sciatic nerve with AAV. We show that in comparison with intramuscular AAV delivery, SciNDi results in widespread transduction in connected neuroanatomical tracts both in the sciatic nerve trunk and the lumbar spinal cord.

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Highlights

A facile approach for AAV delivery in the peripheral nervous system of adult mice

Transduction of sciatic nerve and modestly in spinal cord ventral horn neurons

Avoids tissue trauma associated with direct intraparenchymal injection of AAV

Protocol

Recombinant adeno-associated virus mediated gene delivery in the extracranial nervous system of adult mice by direct nerve immersion

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SUMMARY

This protocol outlines a minimally invasive and quickly performed approach for transgene delivery in the extracranial nervous system of adult mice using recombinant adeno-associated virus (AAV). The technique, named Sciatic Nerve Direct Immersion (SciNDi), relies on the direct bilateral immersion of the exposed sciatic nerve with AAV. We show that in comparison with intramuscular AAV delivery, SciNDi results in widespread transduction in connected neuroanatomical tracts both in the sciatic nerve trunk and the lumbar spinal cord.

For complete details on the use and execution of this protocol, please refer to Jan et al. (2019) and Richner et al. (2011, 2017).

BEFORE YOU BEGIN

AAV are engineered pseudotyped viral particles, derived from the native AAV (of the single-stranded DNA *Parvovirinae* family), and are widely used for gene delivery applications *in vivo* (Hocquemiller et al., 2016; Bedbrook et al., 2018; Wang et al., 2019). Based on their low immunogenicity profile and versatile capabilities for achieving transduction in several tissues, they have emerged as preferred vehicle for gene therapy applications (Bedbrook et al., 2018; Hudry and Vandenberghe, 2019; Wang et al., 2019). For this purpose, AAVs are delivered directly into the target tissue (i.e., intraparenchymal) or via peripheral routes (e.g., intramuscular, intravenous) to achieve local or more widespread transduction (Stoica et al., 2013). In this regard, direct intraparenchymal injection remains the preferred approach for AAV delivery into the nervous system of adult mice (e.g., brain, spinal cord, spinal nerves, DRG) (Naso et al., 2017; Bedbrook et al., 2018; Hocquemiller et al., 2016). The advantage of intraparenchymal delivery is that the transduction is predominantly localized at the site of injection, which limits potential undesirable effects in other tissues/regions that may present as confounding factors in data interpretation. However, this approach involves invasive procedures and is potentially limited in certain delivery aspects such that widespread transduction in peripheral nervous system is a significant challenge (Bedbrook et al., 2018; Hocquemiller et al., 2016; Tosolini and Sleigh, 2020; Yu et al., 2016). For example, direct nerve, DRG or intra-spinal injections are usually performed using 1–2 μL AAV load with the aim to achieve local transduction in neurons or glial cells (Homs et al., 2011), and specificity can be increased by AAV design incorporating cell-specific regulatory elements (Yu et al., 2016). In comparison, AAV mediated gene delivery in adult nervous

system using peripheral routes remains an evolving field (Bedbrook et al., 2018; Hoyng et al., 2015). Since the focus of this manuscript is an experimental protocol, we are unable to provide a detailed discussion on advantages and efficacy of different peripheral routes of AAV delivery into rodents-e.g., intramuscular (Towne et al., 2009), intraperitoneal (Dane et al., 2013), intravenous (Gessler et al., 2019), intra-nerve (Homs et al., 2011) and into dorsal root ganglia (DRG) (Yu et al., 2016), and the reader is encouraged to consult some recent review articles elsewhere for in-depth discussion and specific research studies (Tosolini and Sleigh, 2020; Hoyng et al., 2015; Wang et al., 2019; Gessler et al., 2019; Naso et al., 2017; Mason et al., 2011; Bedbrook et al., 2018; Hocquemiller et al., 2016; Samulski and Muzyczka, 2014). In summary, the above cited examples show that peripheral routes of AAV delivery can be utilized for achieving optimal levels of transgene expression in the nervous system, either within the local neuroanatomical structures (e.g., in peripheral nerve terminals and neurons within the DRG or spinal cord following intramuscular injection (Jan et al., 2019; Towne et al., 2009; Tosolini and Sleigh, 2020)) or more distally (e.g., spinal cord or brain following intravenous (Gessler et al., 2019; Stoica et al., 2013)).

Several laboratories, including our own, have attempted to refine the AAV delivery for targeting structures in the rodent nervous system using peripheral routes capitalizing on the retrograde transduction capability of AAV, both using conventional AAV serotypes as well as newly engineered retro-AAV (Chen et al., 2020; Chan et al., 2017; Jan et al., 2019; Tervo et al., 2016). Previously, we have studied transgene (i.e., enhanced green fluorescent protein-eGFP) delivery into the extracranial nervous system of adult mice by intramuscular (IM) injection, and compared three commonly used serotypes (AAV2/6, AAV2/8 and AAV2/9) with eGFP expression driven by either the ubiquitous cytomegalovirus (CMV) promoter or neuron-specific synapsin (hSyn) promoter (Jan et al., 2019). Here, we present a significant refinement in our attempts at peripheral AAV delivery for targeting structures in the nervous system via direct immersion of a peripheral nerve, using sciatic nerve as the proof-of-concept site- hence the acronym Sciatic Nerve Direct Immersion (SciNDi) (see Figure 1). Using a AAV2/6 (CMV:eGFP) vector, our data show that bilateral SciNDi led to significantly improved AAV delivery in the sciatic nerve, in comparison with bilateral hindlimb IM delivery. Furthermore, enhanced transgene (eGFP) expression was observed in sciatic nerve, and discrete instances of eGFP positive neurons and neuronal processes were detected in the lumbar spinal cord (See Figures 2A–2I; also see expected outcomes). Based on these observations, we consider that the procedure is of potential interest to researchers exploring the transgene delivery and/or manipulation of gene expression in local structures (i.e., sciatic nerve, DRGs, spinal cord- ventral horn), especially in situations where a minimum procedure-induced trauma in nervous structures is desired. However, it is worthwhile mentioning that the described protocol intends to provide a technique for modestly improved transgene delivery into the sciatic nerve and connected neuroanatomical structures, including muscle tissue, relying on the transgene (i.e., eGFP) expression driven by the ubiquitous CMV promoter. Nonetheless, for the reader's benefit we have alluded to examples from published literature throughout the text below that can serve as a guide in situations where specificity for targeting discrete cell populations is desired.

The protocol below primarily describes the specific steps for using AAV2/6 application in adult mice. Nevertheless, the protocol could also potentially be adapted for application in other rodents, as well as in rodents of various ages. In these experiments, bilateral SciNDi or hindlimb IM injections were performed in 2–3 months old mice using 2.8^{e10} AAV particles (*serotype, AAV2/6; promoter:transgene insert, CMV:eGFP; volume, 10 μL*) and were euthanized 4 weeks post-procedure for tissue collection and further analyses.

> **Note:** Throughout this manuscript, AAV refers to recombinant AAV and is not to be confused with retro-AAV and/or retrograde transduction.

INSTITUTIONAL AUTHORIZATION. All animals were handled in full compliance with Danish and European regulations, under the authorization 2017-15-0201-01203 (issued to PHJ, co-author).

Preparation of equipment

🕐 Timing: 60 min

1. Sterilize the surgical equipment by autoclave.
2. Secure sterile 2–10 μL filtered pipette tips.
3. Transfer an aliquot of AAV particles (stored at −80°C) and thaw on ice. Consult the guidelines from AAV production facility for handling and use. In this protocol, we have used AAV2/6 by the Iowa Viral Vector Core and the relevant guidelines are listed on the website https://medicine.uiowa.edu/vectorcore/technical-resourcesfaq. In the current example, the aliquots were 25 μL/tube and thawed on ice approximately for 10–15 min until a liquid suspension was clearly visible.

⚠ CRITICAL: Ensure aseptic equipment and a clean work area in a surgical facility for the animals throughout the procedure. Keep the thawed AAV suspension on ice throughout the procedure. Desired titers of AAV particles should be adjusted by dilution in sterile phosphate-buffered saline (PBS). Also see troubleshooting, problem 1.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
eGFP, Rabbit polyclonal Working dilution, 1:500	Cell Signaling Technologies	Cat# 25555
eGFP, Chicken polyclonal Working dilution, 1:200	Abcam	Cat# ab13970
Neurofilament light chain (NF-L), Mouse monoclonal Working dilution, 1:200	Cell Signaling Technologies	Cat# 2835
S100 (Schwann cell marker), Rabbit polyclonal Working dilution, 1:200	DAKO	Cat# Z0311
CD68 (Monocyte macrophage marker), rat monoclonal Working dilution, 1:200	Novus Biologicals	Cat# NBP2-33337
Bacterial and virus strains		
Recombinant adeno-associated vector (rAAV)	Iowa Vector Core, Iowa University	Iowa-547
Chemicals, peptides, and recombinant proteins		
Meloxicam (analgesic)	Boehringer Ingelheim	Cat# EU2/97/004/006; CAS:71125-38-7
Temgesic (analgesic)	Indivior Europe Limited	Cat# 521634; CAS:52485-79-7
Xylocaine 2% (analgesic)	Aspen Pharma Ireland Limited	Cat# 453053; CAS: 137-58-6
Isoflurane (furane) IsoFlo Vet 100%	Abbott	Cat# 002185; CAS: 26675-46-7
Betadine	Avrio Health	Cat# NDC 67618-154-16
3,3′-diaminobenzidine (DAB)	Electron Microscopy Sciences	Cat# 13080
Haematoxylin (Mayer)	Sigma-Aldrich	Cat# 51275
Ophthalmic ointment	Ophta	Cat# 53 96 68
Sterile PBS	GIBCO	Cat# 10010-015
Sterile saline	Fresenius Kabi AB	Cat# 141856
Sterile cotton-tipped applicator	E-vet A/S	Cat# 140041
Experimental models: Organisms/strains		
Mice: Wild type C57bl6/J (2–3 months old, both sexes)	Janvier, France	C57bl6/J
Other supplies		
Isoflurane vaporizer	E-vet A/S	Cat# 905050

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Unserrated Forceps, Dumont, # 3	F.S.C. (Fine Science Tools)	Cat# 11231-30
Pipette, 1–10 µL volume	Thermo Fisher Scientific	Cat# 4642030
Pipette tip, filtered, 10 µL	Thermo Fisher Scientific	Cat# 94052000
Scalpel; Swann-Morton surgical blade no. 11	Swann-Morton	Cat# REF0203
Scissors, straight, type 3, 25 mm cutting edge	Bochem	Cat# 4070
Standard syringes, 1 mL	Terumo	Cat# MDSS01SE
Syringe needle 27 G	Terumo	Cat# AN*2716R1
Tissue adhesive	Klinibond	Cat# 115 500
Software and algorithms		
Prism	GraphPad Software	https://www.graphpad.com/scientific-software/prism/

STEP-BY-STEP METHOD DETAILS

Anesthesia

⏱ Timing: 5 min

This step ensures deep anesthesia of the mouse, which is required for the surgical procedure.

1. Anesthetize the adult mouse (2–3 months old; male or female) using an anesthetic vaporizer to administer an inhalation agent (e.g., isoflurane), preferably in a ventilated fume hood. Alternatively, use a small induction chamber/container for induction in accordance with institution guidelines, and minimize human exposure.
2. Confirm surgical level of anesthesia by gently pinching the tail or paw with unserrated forceps. Do not proceed until the mouse is unresponsive to the pinch.
3. Using a cotton-tipped applicator, generously apply ophthalmic ointment onto both eyes.

ALTERNATIVE TO ISOFLURANE ANESTHESIA: Injectable regimens, e.g., 75 mg/kg ketamine plus 1 mg/kg medetomidine can also be used as outlined previously (Stoica et al., 2013). Consult the anesthesia dosage according to veterinary guidelines for species, size and age. In comparison with the inhalation agents, the injectable regimens are associated with much longer duration of anesthesia, hence sufficient ophthalmic ointment and postoperative care are required, i.e., subcutaneous fluid administration, heating pad under the cage.

Preparation of surgical area on upper hindlimb

⏱ Timing: 2 min

This step prepares the surgical area on the upper hindlimb of the mouse for surgery. Initially, the fur is removed using either a scalpel or an electrical rodent shaver, based on the user's preference. If using an electrical rodent shaver, the user can skip step 4 below.

4. Spray the fur along both hindlimbs with 70% ethanol to control the fur.
5. Using a scalpel or an electrical rodent shaver, remove the fur on the hindlimb in an area extending from slightly below the knee up to the hip region, as illustrated in the Figure 1A.
6. Disinfect the surgical area with an iodine containing solution (e.g., betadine). Wait for 15 s so that the applied betadine is dried on the skin.

ALTERNATIVE TO BETADINE: If betadine is not accessible, 70% ethanol can alternatively be used in a similar fashion.
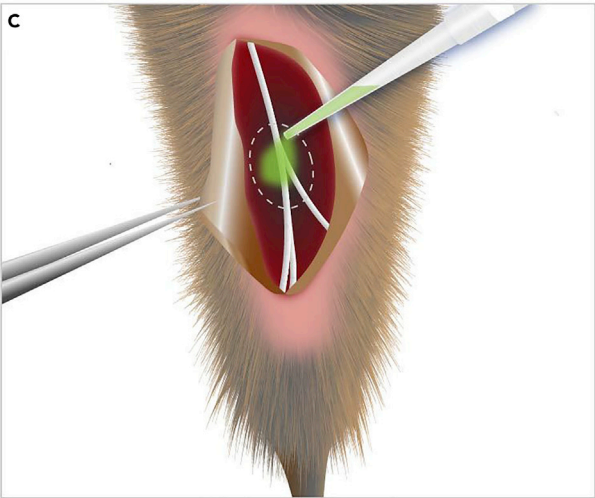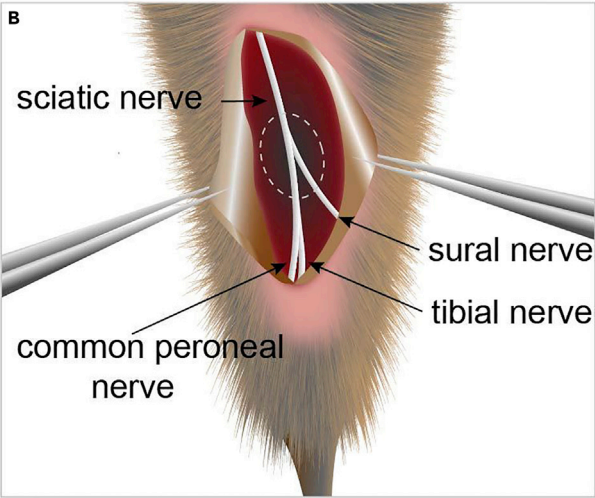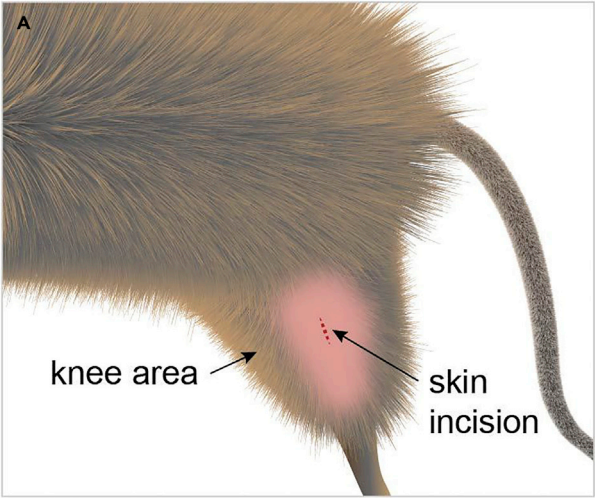
Figure 1. SciNDi procedure at a glance

Graphical illustration of AAV delivery using direct immersion of sciatic nerve in mice (SciNDi):

(A) Area on the hindlimb prepared for surgery. The skin incision site is marked (red dashed line with pointing arrow) and also knee area is indicated (arrow),

(B and C) Sciatic nerve trifurcation site marked by dashed circle (arrows indicate: sciatic nerve as well as the nerve branches common peroneal nerve, tibial nerve and sural nerve in the left hindlimb, visible upon separation of skin and muscle layers, and (C) application of AAV particles on the sciatic trifurcation site (marked by dashed circle) using a 10 μL filtered pipette tip. For simplicity, the procedure is depicted only on the left hindlimb. Structures are not drawn to scale.

Exposure of the trifurcation site of sciatic nerve

⏱ Timing: 5 min

This step is performed to surgically expose the sciatic nerve such that the sciatic nerve trifurcation site lies in the center of the surgical field (highlighted with the dashed circle in Figures 1B and 1C), ensuring a consistent anatomical reference point (spot) for the application of AAV particles (Richner et al., 2011; Bala et al., 2014; Savastano et al., 2014).

7. Orient the hindlimb in a slightly elevated position and secure with surgical tape onto underlying surface for optimal nerve exposure (Richner et al., 2011). OPTIONAL: Readers may consider the injection of a local anesthetic (e.g., lidocaine) into the biceps femoris muscle according to the dosage advised by the local veterinarian.
8. Having located the knee joint and thigh bone (femur), make a small (approx. 3–5 mm) longitudinal incision in the dermal layer proximal to the knee joint with a scalpel.
9. Further open the skin by blunt dissection using the tip of a pair of scissors.
10. Now, carefully separate the muscle layer close to femur by blunt dissection with a pair of scissors, revealing the trifurcation site of the sciatic nerve, exposing approx. 5 mm of the nerve structure above and approx. 5 mm below the trifurcation site (Richner et al., 2011).

See troubleshooting, problem 2 and problem 3.

AAV application

⏱ Timing: 30 s

With this step, AAV particles are applied directly onto the sciatic nerve trifurcation site exposed in step 10.

11. Briefly vortex (approx 3–5 s) the freshly thawed aliquot of AAV particles and avoid repetitive pipetting.
12. Keep the thawed AAV suspension on ice throughout the procedure.
13. Aspirate desired volume ((in the range of 2–10 μL; (Jan et al., 2019; Towne et al., 2009)) with a filtered pipette tip.
14. Using an unserrated forceps, gently open the separated muscles (Figure 1B), then drop-wise (e.g., 1 drop every 2 s) apply the AAV suspension onto the sciatic nerve trifurcation site without touching the nerve (Figure 1C).
15. A co-worker may facilitate the rapid application of AAV, i.e., by loading the pipette with AAV suspension while the surgeon performs nerve exposure (Figures 1B and 1C), and immediate muscle closure post-application, so that the risk of the exposed nerve becoming dry is minimized. In this manner, the step 14 of the procedure usually takes 5–10 s.
16. Avoid repetitive touching of the nerve and do not insert any foreign material into the incision, e.g., under the nerve to restrict the diffusion of AAV solution as it may damage the delicate nerve tissue. See troubleshooting, problem 4.

Closing of the surgical wound

⊙ Timing: 5 min

This step describes closing of the surgical wound.

17. Gently appose the muscle layers for approximately 10 s with the tip of an unserrated forceps. This allows for smooth diffusion of AAV particles around the nerve trunk.
18. Close the skin wound using a veterinarian approved tissue adhesive (alternatively sutures).

Post-operative analgesic treatment and monitoring

⊙ Timing: 30 s

This step describes post-operative care and monitoring.

19. Inject an analgesic solution subcutaneously (e.g., non-steroidal anti-inflammatory drug, NSAID), and apply a local analgesic (e.g., lidocaine) according to national and institutional guidelines.
20. After returning the mouse into its home cage, monitor for approximately 2 h for any signs of unexpected discomfort and pain (restricted mobility, shivering, and vocalizations, biting the closed wound). See troubleshooting, problem 3.

   ⯈⯇ Pause point: House the mice in your animal facility for 3–6 weeks post-procedure, and then proceed to assess the AAV delivery and transgene expression in the tissue(s) of interest (see below).

Tissue isolation for AAV delivery and gene expression analyses

⊙ Timing: 10–20 min

This step describes isolation of tissues for further analyses. Duration of the isolation process varies according to the desired tissues, including sciatic nerve, DRG, lumbar spinal cord and local muscle tissue (Jan et al., 2019; Richner et al., 2017).

21. Euthanize the mouse according to institutional guidelines.
22. Create a similar intramuscular space by blunt dissection (step 10 above) and collect the trunk of sciatic nerve (extending from its emergence at the sciatic notch to the trifurcation in the popliteal fossa behind the knee joint (Jan et al., 2019; Bala et al., 2014; Richner et al., 2011; Savastano et al., 2014).
23. Dissect and collect a piece of biceps femoris muscle (50–100 mg), using a forceps and a small scissor. A small piece (50–100 mg) of liver may be dissected and collected (using a forceps and small scissor) as a control tissue for gene and/or and protein expression studies.
24. Isolate DRG and spinal cord tissue as illustrated previously (see accompanied video demonstration in (Richner et al., 2017).
25. Process the collected tissue(s) for gene and/or protein expression, microscopy and desired assays relevant to interest (Benkhelifa-Ziyyat et al., 2013; Chen et al., 2020; Dane et al., 2013; Jan et al., 2019).

EXPECTED OUTCOMES

In this manuscript, we have outlined a step-by-step procedure of AAV delivery in rodents using direct immersion of sciatic nerve with AAV2/6, for targeting of local neuroanatomically connected structures (i.e., muscle, nerve, DRG and spinal cord). Several research laboratories, including our own, have previously exploited the IM route of AAV delivery for this purpose. Extending these

efforts, we illustrate the efficacy of AAV2/6 mediated gene delivery by SciNDi or IM route in adult mice. Accordingly, we compared AAV2/6 delivery and transgene (eGFP) expression following intramuscular (IM) AAV injection in hindlimb biceps femoris (Jan et al., 2019) and SciNDi in adult- wild type, male- C57BL6/J mice. Briefly, 2–3 months old mice were bilaterally inoculated with 2.8^{e10} AAV particles (*serotype, AAV2/6; promoter, CMV:eGFP: volume, 10 μL*) and were euthanized 4 weeks post-procedure for tissue collection and further analyses (examples in Figures 2 and 3).

We observed pronounced green coloration, indicative of eGFP expression in muscle tissue around the application site, both by IM or SciNDi (Figure 2A). In contrast, robust GFP expression in the lower hindlimb musculature was also observed following SciNDi, suggesting either widespread transduction in the region of sciatic nerve innervation or possibly that the AAV particles reach lower leg region through an undefined route, (Figure 2A; compare IM and SciNDi). One possibility is that the AAV that was not absorbed by the sciatic nerve could diffuse into lower hindlimb after the muscle has been closed (step 17). Then, we assessed viral genome (AAV2-ITR) and transgene (eGFP) expression in select tissues (i.e., muscle, sciatic nerve, DRG, spinal cord, brain and liver). By qRT-PCR analyses, we detected slightly higher AAV2-ITR (Figure 2B, ∼ 1.2-fold higher AAV2-ITR in SciNDi) and eGFP mRNA expression (Figure 2C, ∼ 1.5-fold eGFP mRNA) in the biceps femoris muscle in the SciNDi cohort. This is not surprising as IM injection leads to localized transduction in the vicinity of the injection site, while SciNDi allows the diffusion of applied AAV particles over relatively larger surface area as indicated by expression of eGFP in the muscle tissue (Figure 2A, notice the difference between IM and SciNDi). qRT-PCR analyses of the sciatic nerve, in contrast, showed considerably higher (mean, >5-fold) levels of AAV genome (Figure 2B, AAV2-ITR; also compare Muscle and Sc. nerve) and eGFP mRNA expression (mean, >16-fold) in the trunk of sciatic nerve with SciNDi compared to the IM injection (Figure 2C, eGFP mRNA; also compare Muscle and Sc. nerve).

We also examined eGFP epifluorescence in L4 DRGs, which give rise to sensory fibers in sciatic nerve, and observed scant eGFP immunodetection, either by SCiNDi or IM injection (Figure 2D; only L4 DRGs from the left side are shown). This is not surprising as peripheral AAV injections are less efficient means of AAV delivery into the DRGs compared with intra-nerve or intra-DRG injections (Homs et al., 2011; Jan et al., 2019; Towne et al., 2009; Yu et al., 2016). Microscopic analyses also revealed enhanced eGFP expression, reflected by eGFP epifluorescence along the length of sciatic nerve in the hindlimb (i.e., between the hip joint and the knee) (Compare Figure 2E- IM injection in biceps femoris with Figure 2F-SciNDi); Also compare Figure 3A- phosphate-buffered saline, PBS cohort). Furthermore, we also detected discrete instances of eGFP immunopositivity within neuronal cell bodies and intra-grey matter nerve cell processes in the lumbar ventral horn following AAV delivery via SciNDi, which was more pronounced compared with IM delivery (Figures 2G–2I; bilateral SciNDi AAV2/6 application; only left ventral horn L4/L5 is shown; also see Figure 3B, showing right ventral horn). Lastly, we did not observe eGFP immunostaining in the spinal lumbar dorsal horn (Figure 3B) using SciNDi, suggesting either lower AAV delivery and/or different transduction efficiency in the sensory interneuron populations.

These observations highlighted above (Figure 2) support the effects of AAV particles in the immediate vicinity of sciatic nerve, since we found negligible systemic AAV dissemination in qRT-PCR analyses of lumbar spinal cord, whole brain and liver (Figures 3C and 3D). Double immunofluorescence studies showed that, while eGFP was clearly detected along substantial proportion of neurofilaments in sciatic nerve, eGFP detection in Schwann cells was negligible (Compare Figure 2F- NF-L co-detection, with Figure 3E- S100 Schwann cell marker co-detection). Lastly, we did not observe inflammatory response following AAV application using SciNDi, as assessed by the co-detection of phagocyte marker CD68, either in sciatic nerve (Figure 3F), or the muscle tissue in upper hindlimb (Figures 3G–3I), indicating significant lack of tissue damage/inflammation using this technique.
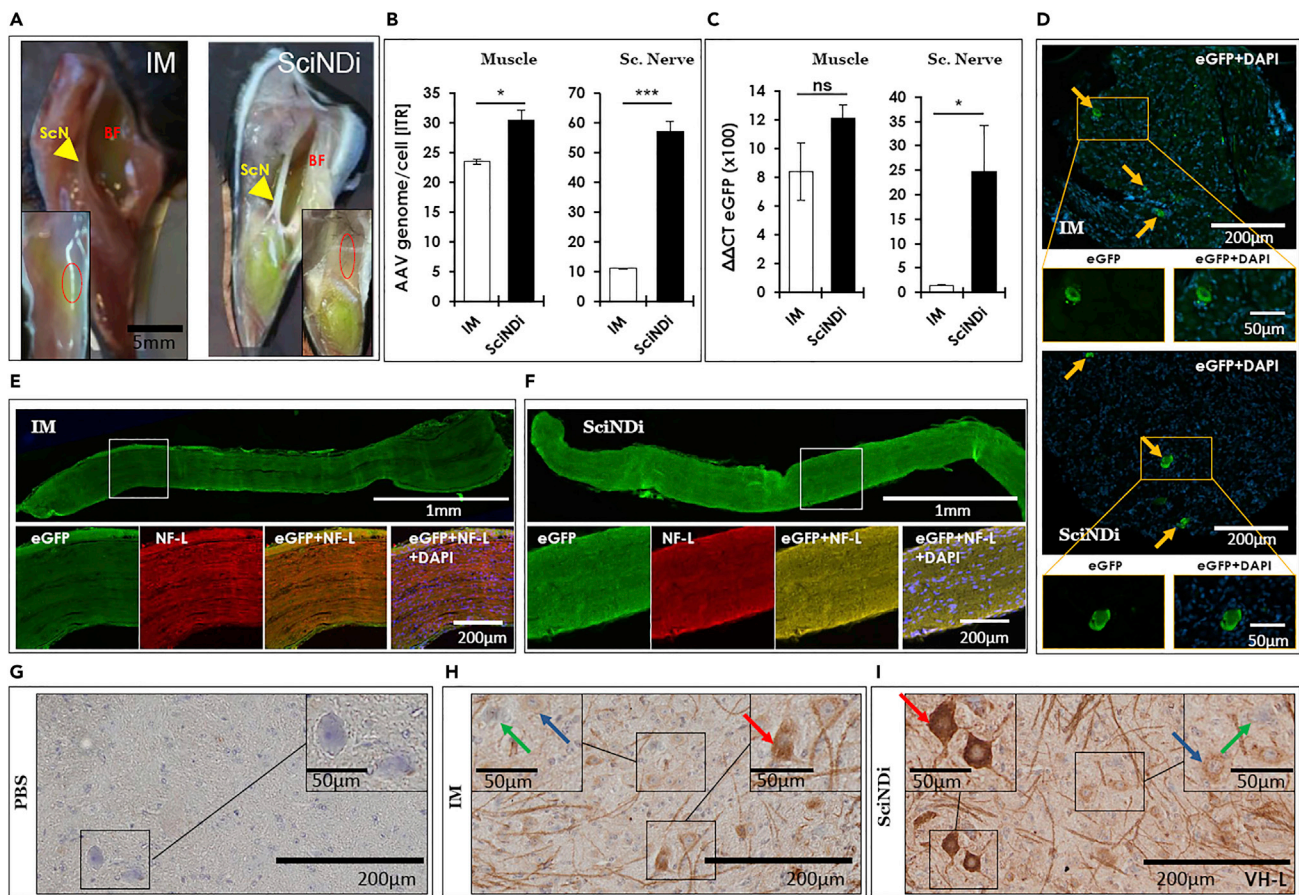
Figure 2. AAV delivery via bilateral sciatic nerve direct immersion (SciNDi) in adult mice

(A) Gross inspection of the left hindlimb musculature and exposed trunk of the sciatic nerve. Notice the iridescent (green) coloration, which is discreetly localized in the case of intramuscular (IM) injection, and is widespread following SciNDi. In the dissected views, sciatic nerve (ScN) is indicated by a yellow arrow head, while biceps femoris muscle (BF) is also marked. The insets show the un-dissected views and the area of the dissected muscle tissue is highlighted with a red oval.

(B) qRT-PCR determination of vector genome copy numbers (AAV2-ITR/2N*β-tubulin) comparing AAV delivery using bilateral IM injection or SciNDi.

(C) qRT-PCR determination of eGFP (transgene) expression (ΔΔCT, relative to phosphate buffered saline (PBS) injected cohorts). Statistics in (C and D) (Mean ± s.e.m.; muscle, n = 6; sciatic nerve, n = 4; Mann-Whitney test, *= p ≤ 0.05, ***= p ≤ 0.005, ns = not significant).

(D) Representative immunofluorescence (IF) images showing eGFP epifluorescence in the left L4 dorsal root ganglia following AAV delivery using bilateral IM injection (top panels) or SciNDi (bottom panels). Instances of eGFP positive cells are indicated by yellow arrows. Insets show 40× magnified views of eGFP alone (green) and eGFP with nuclear marker DAPI (blue). Antibody: eGFP (Cell Signaling Technologies #2555, Polyclonal rabbit, 1:500). High resolution IF images were acquired using a Zeiss observer inverted microscope equipped with colibri 7 LED illumination.

(E and F) Dual-IF co-detection of eGFP and neurofilament light chain (NF-L) in the sciatic nerve following AAV delivery using bilateral IM injection (in E) or SciNDi (in F). Representative images (above: nerve trunk; below: 10× magnified views) showing eGFP alone (green), NF-L (red), merge of eGFP and NF-L and nuclear marker DAPI (blue). Antibodies: eGFP (Cell Signaling Technologies #2555, Polyclonal rabbit, 1:500) and NF-L (Cell Signaling Technologies #2835, mouse IgG1, 1:200). High resolution IF images were acquired using Olympus VS120 digital slide scanner equipped for brightfield scanning and fluorescence single-band emitters for Hoechst, FITC, Cy3, Cy5 and Cy7. See also Figure 3A (PBS). (G–I) Immunohistochemical (IHC) detection of eGFP in the left lumbar L4/L5 spinal ventral horn (VH-L) after IM application of PBS (in G), AAV delivery using IM injection (in H), or AAV delivery using SciNDi (in I). In H-I, select instances of eGFP immunostaining are highlighted in the cell bodies (red arrows: intense; blue arrows: clearly visible green arrows: faint/non-detected), and in intra-grey matter nerve cell processes (yellow stars). IHC was performed using an eGFP antibody (Cell Signaling Technologies #2555, Polyclonal rabbit, 1:500) and detected using DAB (3,3′-diaminobenzidine) chromogen. Haematoxylin was used as counterstain (20× magnified views, obtained using a Nikon brightfield microscope).

QUANTIFICATION AND STATISTICAL ANALYSIS

The data were statistically analyzed in GraphPad Prism software (version 9), and the final graphs were prepared in Microsoft Excel. Statistical significance was calculated by the analysis of variance (ANOVA) or pair-wise comparisons using Mann–Whitney U test, as indicated in the figure legends
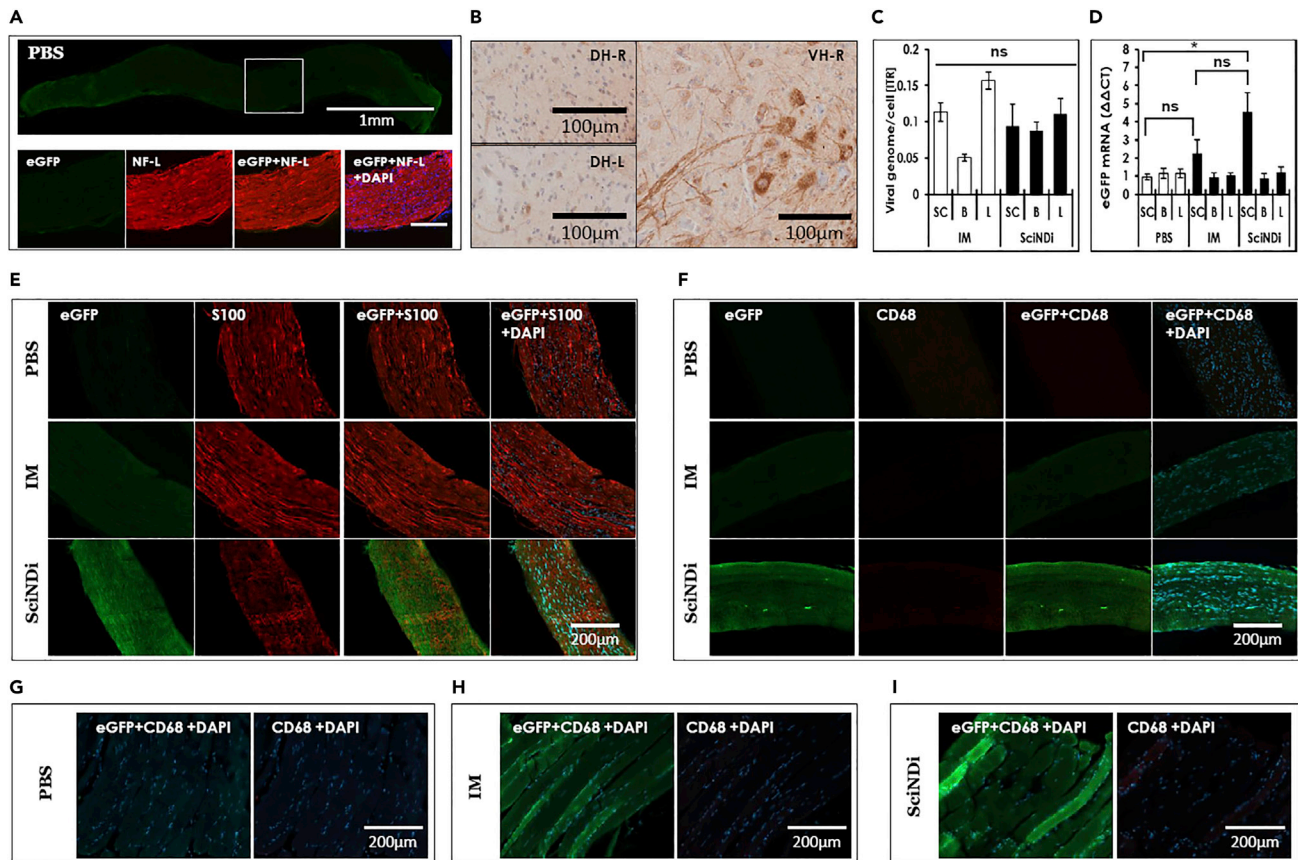
Figure 3. Systemic AAV dissemination, eGFP expression in Schwann cells and inflammation at the site of AAV delivery using SciNDi

(A) Dual-IF detection of eGFP and neurofilament light chain (NF-L) in the sciatic nerve trunk following bilateral IM application of PBS (vehicle). Representative images showing eGFP alone (green), NF-L (red), the merge images of eGFP and NF-L, and nuclear marker DAPI (blue). IF procedures and image acquisition were performed as described under Figures 2E and 2F (nerve trunk and 10× magnified views).

(B) Immunohistochemical (IHC) detection of eGFP in the right lumbar L4/L5 spinal ventral horn (VH-R) or both dorsal horns (DH-R and DH-L) after AAV delivery using SciNDi. IHC procedures and image acquisition were performed as described under Figures 2G–2I.

(C) qRT-PCR determination of vector genome copy numbers (AAV2-ITR/2N*β- tubulin) comparing AAV delivery via IM route or SciNDi (Mean ± s.e.m.; SC: lumbar spinal cord, n = 6; B: whole brain, n = 4–6; and L: liver, n = 6; One- way ANOVA; ns, not significant).

(D) qRT-PCR determination of eGFP (transgene) expression (ΔΔCT, relative to PBS injected cohorts). Statistics in A-B (Mean ± s.e.m.; SC: lumbar spinal cord, n = 6; B: whole brain, n = 4–6; and L: liver, n = 6; Mann-Whitney test: *= p ≤ 0.05, ns = not significant).

(E and F) Dual-IF co-detection of eGFP in sciatic nerve along with either the Schwann cell marker (S100, in E), or monocyte/macrophage marker (CD68, in F) following bilateral IM application of PBS, and AAV delivery using IM injection or SciNDi. Representative images (20× views) showing eGFP alone (green), S100 alone (red) (in E) or CD68 alone (red) (in F), and the respective merge images along with the nuclear marker DAPI (blue). Antibodies: eGFP (abcam #13970ab, Polyclonal chicken, 1:200), S100 (DAKO #Z0311, rabbit polyclonal, 1:200), CD68 (Novus Biologicals #NBP2-33337, rat mAb FA-11, 1:200). High resolution IF images were acquired using a Zeiss observer inverted microscope equipped with colibri 7 LED illumination. (G–I) Dual-IF co-detection of eGFP and and monocyte/macrophage marker (CD68) in hindlimb femoris muscle following PBS application using SciNDi (in G), and AAV delivery using IM injection (in H) or SciNDi (in I). Representative images (20× view, merge with DAPI, IF procedures and image acquisition were as described under Figure 3F).

LIMITATIONS

Development and validation of SciNDi for AAV delivery in rodent is a very recent refinement, and several aspects regarding the limitations and utility of the approach remain to be explored: 1) Targeted delivery of a functional transgene or gene-silencing constructs selectively in neuronal or glial populations, such as by using neuron-specific promoters and or selective targeting of Schwann cells; 2) Utility of SciNDi in achieving targeted gene delivery in brain structures, potentially using a large cranial nerve such as vagus with possibility of modulating visceral physiology and autonomic dysfunction in relevant models; and 3) Cross comparison of commonly used neurotropic AAV

serotypes with AAV serotypes showing superior capability for retrograde transduction (Tervo et al., 2016; Chen et al., 2020; Cushnie et al., 2020; Samulski and Muzyczka, 2014); and 4) Lastly, longitudinal analyses of stability and/or retrograde propagation of transgene expression over protracted durations (i.e., 2, 3 and 4 months), for instance, in higher brain regions with descending projections to spinal cord (i.e., brainstem, motor cortex).

TROUBLESHOOTING

Problem 1
The study involves a different AAV construct (serotype, promoter, transgene), and not AAV2/6 (CMV:eGFP) (step 11).

Potential solution
Pilot experiments for determining the efficacy of other AAV constructs for specific experimental readouts may be needed. For example: i) choosing a neuronal (e.g., synapsin) or glial specific promoter (e.g., GFAP) (Gessler et al., 2019), ii) optimal titer and iii) time of tissue collection post-procedure. To demonstrate AAV delivery using SciNDi technique, we used tissue collected four weeks post-procedure to provide a comparison with our IM delivery data, collected in a similar fashion in the current manuscript and in our previously published data on AAV2/6, AAV2/8 and AAV2/9 (Jan et al., 2019). As mentioned under problem 1, the readers are also encouraged to consult relevant literature on factors affecting AAV tropism and recent developments in the refinements in vector design for applications in nervous system (Bedbrook et al., 2018; Hudry and Vandenberghe, 2019; Hocquemiller et al., 2016).

Problem 2
Bleeding occurs during the blunt dissection of hindlimb muscle layers (step 10).

Potential solution
Whenever possible, perform blunt dissection through the muscular layer instead of cutting through the muscle, as it allows faster wound healing (Fossum, 2019) and easy identification of anatomical structures. The reader is also encouraged to watch a video demonstration of this procedure (Richner et al., 2011). Do not use excessive force as that may tear blood vessels in the vicinity. If bleeding occurs, apply cotton-tipped applicators or pieces of gauze to absorb the blood. Press gently until the bleeding stops. Ensure maintained anesthesia if using anesthetic vaporizer.

Problem 3
The animal exhibits signs of discomfort post-procedure (step 20).

Potential solution
The mice should recover fully within 10–15 min once removed from the anesthesia and transferred to their house cages. If any signs of discomfort (mentioned above) persist two hours after NSAID administration, subcutaneously inject a stronger analgesic, e.g., opioids, and continue to observe the animal by direct inspection, according to national and institutional guidelines. This can be the case if the operating person has inadvertently induced muscle damage while dissection or tearing of the blood vessels in the vicinity.

If performed correctly, the procedure (i.e., opening the muscle layer by blunt dissection) is not associated with muscle damage or reduced mobility. However, we advise the readers as the following: i) watch the video demonstration for the procedure of exposing sciatic nerve using blunt dissection carefully (Richner et al., 2011) and ii) perform a trial SciNDi procedure in 4–6 mice and observe for signs of muscle damage post-procedure in close consultation with your veterinarian, i.e., inflammation and delayed wound healing or reduced mobility. If such training trials are not possible, we advise the reader to exclude animals from the experiment that show signs of discomfort and pain (restricted mobility, shivering, and vocalizations, biting the closed wound) despite NSAID

administration and treatment advised by the local veterinarian. This is partly because local bleeding, inflammation, tissue edema may induce variability in the spreading of AAV in the vicinity of the sciatic nerve, e.g., AAV diffusion into circulation due to the damaged vessels.

Problem 4
AAV diffusion leads to the transduction of non-neural tissues.

Potential solution
This issue is not specific to SciNDi as other common peripheral of AAV delivery routes (for example IM, intravenous or intraperitoneal) are also associated with such off-target effects in peripheral tissues (Towne et al., 2009; Gessler et al., 2019; Ai et al., 2017). This can be circumvented by using AAV constructs in which the transgene expression is driven by neuron-specific promoters (e.g., synapsin) instead of a ubiquitous promoter such as CMV used in this protocol (see also (Jan et al., 2019; Gessler et al., 2019)). We have outlined SciNDi primarily as a procedure for modestly increased transgene delivery in adult mice (as compared to the IM route- Figures 2 and 3), thus the readers need to optimize the efficiency of their AAV constructs (if not using AAV2/6; CMV:eGFP) and specificity of transgene expression in the desired tissues.

Problem 5
Expected gene expression is not observed (step 25).

Potential solution
Several factors are known to affect the transduction efficiency by AAV in a given tissue and/or cell populations, including: i) the AAV preparation (e.g., titer and purity), ii) AAV tropism (promoter, size and nature of the transgene) and iii) specific factors related to the procedure (e.g., AAV accessibility to the nerve tissue, duration of experiment etc.). For optimal results, obtain high purity, high titer AAV particles in small aliquots (e.g., 25 μL), avoid repetitive freeze-thaw cycles, and always keep on ice (for thawing and throughout the procedure). To ensure optimal immersion of sciatic nerve, the ideal volumes for SciNDi are 8–10 μL and should be no less than 2 μL.

The readers are also encouraged to consult relevant literature on factors affecting AAV tropism and challenges on using the peripheral routes of AAV delivery, several of which are discussed in the review articles cited in Bibliography (Tosolini and Sleigh, 2020; Hoyng et al., 2015; Wang et al., 2019; Gessler et al., 2019; Naso et al., 2017; Mason et al., 2011; Bedbrook et al., 2018; Hocquemiller et al., 2016; Samulski and Muzyczka, 2014). Lastly, the readers are also encouraged to watch our video demonstration of the sciatic nerve exposure and familiarize themselves with local anatomical structures in the hindlimb (Bala et al., 2014; Richner et al., 2011; Savastano et al., 2014).

Problem 6
Bilateral AAV application does not generate intra-individual control tissue (step 25).

Potential solution
SciNDi can be performed unilaterally, especially if the target tissue is ipsilateral sciatic nerve and/or DRGs. In these scenarios, contralateral neuromuscular tissues (i.e., hindlimb muscles, sciatic nerve, DRG) can be collected as the corresponding controls. However, when applied unilaterally, caution should be exercised in data interpretation from the contralateral side, especially spinal cord tissue, as common AAV serotypes have been found to exhibit variable degrees of hematogenous spreading (Towne et al., 2009; Benkhelifa-Ziyyat et al., 2013), or even transduction of the contralateral spinal ventral horn as an epiphenomenon of potent retrograde transduction (Chen et al., 2020).

Hence, we recommend bilateral application of AAV using SciNDi, if optimal targeting of spinal cord is desired. This is to avoid not only the potential confounding aspects of contralateral spreading (if any) indicated above, but also that bilateral AAV delivery enables administration of twice as much

AAV particles compared with unilateral delivery. In this regard, it is also worthwhile to mention that the AAV2/6 has been shown to exhibit some degree of retrograde transduction, hence in our opinion, the reader should incorporate these factors in decision-making regarding the choice of vector. Additional controls may include the tissues dissected from animals injected bilaterally with vehicle.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Asad Jan (ajan@aias.au.dk).

Materials availability
This study did not generate any new unique reagents.

Data and code availability
All of the data generated and analyzed during this study are included in the main manuscript and the associated figures (Figures 2 and 3).

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AUTHOR CONTRIBUTIONS

M.R. and A.J. designed and performed research; C.B.V., P.H.J., and J.R.N. contributed with infrastructural support and data evaluation; M.R., N.P.G., and A.J. wrote the manuscript; M.R. and A.J. drafted the graphical abstract using BioRender, under license to C.V. (co-author). All authors read and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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