Journal of Visualized Experiments

Intrathecal Delivery of Antisense Oligonucleotides in the Rat Central Nervous System

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60274R1
Full Title:	Intrathecal Delivery of Antisense Oligonucleotides in the Rat Central Nervous System
Keywords:	Antisense oligonucleotides Blood brain barrier Central nervous system Intrathecal delivery Rat RNA Spinal cord
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
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Cambridge, May 2 2019

To the editorial board of Jove Neuroscience,

I hope you will accept our submission for a manuscript called “Intrathecal delivery of antisense oligonucleotides in the rat CNS”.

In this paper, we have described a powerful method for delivering therapeutic agents (ASOs, but also other molecules), directly into the rat central nervous system. This procedure is easy to learn, and once seen the Jove video, the readers will be able to reproduce the method in their own lab.

We thank you for considering this manuscript for publication

Best regards,

Giulio Srubek Tomassy, PhD

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TITLE:

Intrathecal Delivery of Antisense Oligonucleotides in the Rat Central Nervous System

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KEYWORDS:

antisense oligonucleotides, blood brain barrier, central nervous system, intrathecal delivery, rat, RNA, spinal cord

SUMMARY:

Here, we describe a method for delivering drugs to the rat central nervous system by implanting a catheter into the lumbar intrathecal space of the spine. We focus on the delivery of antisense oligonucleotides, though this method is suitable for delivery of other therapeutic modalities as well.

ABSTRACT:

The blood brain barrier (BBB) is an important defense against the entrance of potentially toxic or pathogenic agents from the blood into the central nervous system (CNS). However, its existence also dramatically lowers the accessibility of systemically administered therapeutic agents to the CNS. One method to overcome this, is to inject those agents directly into the cerebrospinal fluid (CSF), thus bypassing the BBB. This can be done via implantation of a catheter for either continuous infusion using an osmotic pump, or for single bolus delivery. In this article, we describe a surgical protocol for delivery of CNS-targeting antisense oligonucleotides (ASOs) via a catheter implanted directly into the cauda equina space of the adult rat spine. As representative results, we show the efficacy of a single bolus ASO intrathecal (IT) injection via this catheterization system in knocking down the target RNA in different regions of the rat CNS. The procedure is safe, effective and does not require expensive equipment or surgical tools. The technique described here can be adapted to deliver drugs in other modalities as well.

INTRODUCTION:

The vascular system of the central nervous system (CNS) has evolved as a critical regulator of homeostasis, controlling traffic of molecules, supplying nutrients and getting rid of waste. This system is also the first line of defense from attacks of external pathogens, thanks to a dense distribution of tight junctions along the walls of the endothelial cells. These tight junctions make up one aspect of the blood brain barrier (BBB). While the BBB allows the transport of molecules required to fulfill nutrient and energy demands (e.g., ions, glucose), it also selectively limits the passage of pathogens as well as toxic chemicals[1-3].

Ironically, the same protective function of the BBB that limits passage of pathogens and toxic chemicals also is the major obstacle to our ability to easily access the CNS with therapeutic treatments after systemic administration to the organism[2,4,5]. This role of the BBB has prompted the development of a plethora of new drug distribution technologies and approaches[6].

One way to overcome this obstacle is to inject the drugs directly into the cerebrospinal fluid (CSF) that continuously perfuses both the brain and spinal cord[7-10]. In this article, we describe a method to successfully deliver agents into the lumbar intrathecal space by placing the internal end of the catheter completely in the cauda equina space of the rat spine. A description of this procedure was previously published by Mazur et al. elsewhere[11].

The protocol is very effective and produces a greater than 90% success rate of antisense oligonucleotide (ASO) delivery to the CNS when assessed by quantitative polymerase chain reaction (qPCR) analysis of target gene knockdown[8]. The procedure causes minimal discomfort to the animals, as 100% of the rats survive the surgery and show minimal swelling around the surgical wound and no signs of distress (e.g., hyperactivity, dehydration, circling, loss of balance, decreased food intake, and dehydration) during post-op observation. Another advantage of the method described here is that it does not require expensive equipment, nor any special tools.

PROTOCOL:

All in vivo procedures were performed under Biogen Institutional Animal Use and Care Committee (IACUC) approved protocols which follow the guidelines set forth by the United States National Institutes of Health guide for the care and use of laboratory animals.

1. Material and instrument preparation

1.1. Prepare the special guide cannulas.

1.1.1. Use a rotary tool with cut-off wheel (or a sharp saw) to cut off the two ends of a 19 G needle, resulting in a ~1.5–2 cm long guide cannula (**Figure 1Aiii**). Use the grinding wheel of the rotary tool to smooth the two ends.

NOTE: Alternatively, premade and sterile guide cannulas can be purchased from a commercial vendor (**Table of Materials**).

1.2. Prepare the catheter/wire assembly.

1.2.1. Cut an 8 cm long piece of PE-10 tubing (polyethylene tubing, diameter 0.011 inch) to serve as the intrathecal catheter. Make a mark 2 cm from one end with an ethanol resistant marker pen. Cut an 11 cm long stylet wire from polytetrafluoroethylene coated stainless steel wire. Insert the stylet wire (**Figure 1Aii**) into the lumen of the PE-10 catheter (**Figure 1Ai**).

NOTE: One catheter/wire assembly set (**Figure 1Av**) is needed for each animal. Alternatively, catheters and stylet wires can be purchased from a commercial vendor (**Table of Materials**).

1.3. Prepare delivery catheter assembly.

1.3.1. Cut a piece of PE-50 catheter (5−10 cm, polyethylene tubing, diameter 0.023 inch) (**Figure 1Bi**). To one end of the PE50 catheter insert a 23 G tubing adaptor (**Figure 1Biv**). This end will be connected to a 100 µL syringe (**Table of Materials**) during surgery.

1.3.2. To the other end, connect a small piece of PE-10 tubing (~1 cm, **Figure 1Bii**) and then insert a modified 30 G needle with the hub cut off into the PE-10 tubing (**Figure 1Biii**). This end will be connected to the distal end of the implanted PE-10 catheter in the rat during surgery.

NOTE: Alternatively, the delivery catheter assembly (**Figure 1Bv**) can be purchased from a commercial vendor (**Table of Materials**).

1.4. Sterilize all surgical instruments including the guide cannula and the wire/catheter sets using an ethylene oxide sterilizer for 12 h.

NOTE: All surgical instruments except the catheter can be autoclaved; catheter will melt at high temperature.

2. Surgery preparation

NOTE: This procedure is routinely performed on male and female Sprague Dawley rats with body weights between 200 g and 400 g. Two rats are housed per cage under a 12 h light/dark cycle with free access to food and water.

2.1. Place a rat in an isoflurane chamber to induce anesthesia (1−5% isoflurane in O_2, titrated to effect).

NOTE: The rat is then continuously anesthetized with isoflurane to maintain deep anesthesia via a nose cone throughout the procedure. An alternative anesthesia method (e.g., administration of ketamine 100 mg/kg and xylazine 10 mg/kg) can be used as approved by the IACUC.

2.2. When the rat fails to respond to a toe pinch, place the rat on a heating blanket to maintain normal body temperature, apply ophthalmic ointment on the eyes and shave its back from the tail to the caudal thoracic spine.

2.3. Inject sustained release buprenorphine (1.0 mg/kg; **Table of Materials**) subcutaneously in the rat.

NOTE: An alternative pain relief can be used as approved by the IACUC protocol.

2.4. Move the rat into the sterile field and clean the shaved lower back with povidone scrubs and alcohol scrub. Place a 50 mL conical centrifuge tube under the abdomen of the animal in order to flex the spine in the lumbar region (**Figure 2A**). Drape the animal with a sterile transparent drape that has been fenestrated over the surgical site.

2.5. Prepare the guide cannula-needle assembly (**Figure 1Avi**) by placing a guide cannula (**Figure 1Aiii**) over the end of a 23 G needle.

3. Surgery

3.1. With the rat supported by the 50 mL conical tube, identify the two natural pits between muscles above the pelvis (arrows in **Figure 2A**). With one hand holding those pits, use the other hand to gently press and feel the spine from caudal to rostral direction and find the first major indentation between vertebrae and this is the intervertebral space between S1 and L6 vertebrae (**Figure 2B**).

3.2. Move slightly rostrally to identify the next indentation, the intervertebral space between the L5 and L6 vertebrae and the injection site (* in **Figure 2A**). Use a scalpel to make an incision no more than 2 cm long in the skin along the midline from rostral to caudal so that the injection site is at the center of the incision (dotted line in **Figure 2A**).

3.3. Use dissection scissors to dissect away the connective tissue in order to visualize the muscle layer. Then make a 1 cm incision in the muscle capsule immediately lateral to the dorsal spinal process of the L6 lumbar vertebra.

NOTE: The bones of the L6 lumbar vertebra could be visualized at this point.

3.4. Position the guide cannula-needle assembly near the anterior aspect of the 6th lumbar vertebra and push it into the intervertebral space so that the end of the needle penetrates the spinal canal. Remove the 23 G needle leaving only the guide cannula in place.

NOTE: Generally, CSF fluid can be seen entering the needle hub (this fluid may be tinged with a hint of blood, but this does not indicate that harm has been done or that the needle is not placed correctly). The authors have not seen large amount of blood or severe bleeding during this

procedure. If either occurs, a veterinarian should be contacted to determine the appropriate treatment and if animals should be euthanized.

3.5. Insert the catheter-wire assembly into the guide cannula. Remove the guide cannula, leaving the catheter with the stylet wire in place.

3.6. Angle down the catheter at approximately a 45° angle to the spinal canal and force the end approximately 0.3 cm into the spinal canal. Remove the stylet wire approximately 2.5 cm from the intrathecal tip of the catheter and advance the catheter into the spinal canal until the 2 cm mark is at the entrance of the canal (just visible below the muscle) as shown in **Figure 1Bvi**.

NOTE: The inserted catheter should extend rostrally into the subarachnoid space. Successful placement should allow for free movement of the catheter in that space.

3.7. Completely withdraw the stylet wire, and CSF should be seen entering the implanted catheter. Suture the implanted catheter in place for better stability.

3.8. Connect the delivery catheter assembly to the distal end of the implanted catheter via the 30 G needle end (**Figure 1Bv,vi**).

3.9. Load 60 µL of sterile saline into a 100 µL syringe (flushing syringe). Load a bolus of 30 µL of the test compound into a second syringe (injection syringe).

3.10. Connect the flushing syringe (loaded with saline) to the tubing adaptor end of the delivery catheter assembly (**Figure 1Bv**). Inject 20 µL of sterile saline into the intrathecal space (pre-injection flushing).

3.11. Connect the injection syringe (loaded with test compound) to the tubing adaptor end of the delivery catheter assembly (**Figure 1Bv**). Inject 30 µL of test compound into the intrathecal space over 30 s.

NOTE: The routine injection volume of ASO is 30 µL to achieve good knockdown in the spinal cord and in the cortex. It has been reported that the injection volumes may impact compound distribution[12], though different injection volumes have not been tested. If other compound or volume is used, the safety and effectiveness need to be empirically determined.

3.12. Repeat step 3.10 and flush the catheter with another 40 µL of sterile saline (post-injection flushing). Then detach the delivery catheter assembly from the implanted catheter.

NOTE: The pre and post-injection flush is thought to reduce local sequestration of the compounds and improve their distribution to the rostral structures[12].

3.13. Aseptically cut and heat seal the implanted catheter: Place a pair of sterile dissection forceps in a bead sterilizer until they are very hot, then clamp down on the tubing with the hot end of the forceps.

NOTE: This action melts the catheter. Thus, the hole in the tube is collapsed and all sides stick to each other, sealing the tubing in an aseptic fashion and then it is placed into the subcutaneous space.

3.14. Use absorbable monofilament sutures to close the skin over the remaining heat-sealed catheter.

NOTE: Wound clips can also be used as approved by the IACUC protocol. The technique is compatible with repeated injections though it has only been used for one-time injections. The feasibility of repeated injections should be empirically evaluated with the approval of the IACUC.

3.15. Allow the animal to recover from the anesthesia in a heated incubator until mobile, at which point it is returned to its home cage (two rats per cage).

NOTE: When performing surgeries on multiple rats on the same day, clean tools using water to remove blood and re-sterilize using a heated dry bead sterilizer (for at least 20 s, with time to cool) between animals. A new set of instruments is used every 5 animals.

3.16. Monitor the animals daily for at least 3 days after surgery and continue to monitor the animals weekly after recovering from surgery according to the IACUC protocol.

NOTE: If any complications occur (incision infections, neurological disorder such as seizure or paralysis), a veterinarian should be contacted to determine the appropriate treatment and if animals should be euthanized. If sustained release buprenorphine is not used, pain relief should be given daily after surgery according to the IACUC protocol.

4. Evaluation of tissue specific knockdown after IT injection

4.1. Two weeks after IT bolus injection of ASOs, collect different regions of the brain (i.e., cerebral cortex, striatum and cerebellum) as well as different segments of the spinal cord (i.e., cervical, thoracic, and lumbar). Extract total tissue RNA using a commercial RNA extraction kit and perform cDNA synthesis reaction as described previously[13].

NOTE: Standard reagents were used for qPCR with the following assays: rat Malat1 and rat GAPDH. The relative transcript levels were calculated using the $2^{-\Delta\Delta CT}$ method (C_T = threshold cycle).

REPRESENTATIVE RESULTS:

Using the method described here, we injected two groups of adult female rats (250−300 g; n = 10/group) with either a single bolus of phosphate-buffered saline PBS or 300 µg of ASO targeting

the long non-coding (linc) RNA Malat1; in our lab we routinely use the Malat1 ASO as a tool compound, because Malat1 is expressed ubiquitously and at high levels in all tissues[14], including brain and spinal cord. The Malat1 ASO works via an RNaseH1-mediated mechanism[15] that degrades the RNA, leading to knockdown (KD). In the experiment described here, we collected different regions of the brain (i.e., cerebral cortex, striatum and cerebellum) as well as the lumbar segment of the spinal cord, two weeks after delivery of the ASO. RNA from each of the collected region was then extracted and analyzed via qPCR, to assess the levels of expression of the Malat1 RNA.

When the tested agent is an ASO, we recommend to: 1) always collect multiple regions of the CNS, in order to compare ASO efficacy; 2) given the technical complexity of the surgical method, we recommend to include a positive control group, where a compound with well-established pharmacokinetic and pharmacodynamic properties (i.e., Malat1 ASO in our lab) is tested in parallel to the test agent; this will provide information on the effectiveness of the surgeries, should unexpected or unexplainable results be obtained (e.g., lack of or insufficient RNA regulation).

In the experiment described here, we obtained very good KD in all regions collected, as shown in **Figure 3**. However, we did observe some degree of regional variability with the spinal cord showing the highest percentage of KD (cerebral cortex = 87% KD, striatum = 77% KD, cerebellum = 74% KD, spinal cord = 94% KD). We have not accessed in vivo knockdown efficiency earlier than 2 weeks post-surgery. In our experiences with several ASOs, we detected significant knockdown of the target genes up to 6−8 weeks post-surgery (data not shown). A time-course study should be carried out if the precise time-dependent knockdown efficiency of a given ASO is of interest.

FIGURE LEGENDS:

Figure 1: Customized material and catheter sets used in intrathecal injections. (**A**) The catheter/wire assembly (v) is made by inserting stylet wire (ii) into the lumen of PE-10 catheter (i). The cannula/needle assembly (vi) is made by inserting a 23 G needle into the lumen of the guide cannula (iii). (**B**) The delivery catheter assembly (v) is made by connecting tubing adapter to one end of the PE-50 catheter and connecting the cut 30 G needle into the other end using a piece of PE-10 catheter (ii) as an adaptor. During the surgery, the 30 G needle end of the delivery catheter assembly (v) is connected to the top of the implanted catheter (vi), after the other end of the implanted catheter is inserted into the intrathecal space of the animal.

Figure 2: Identification of injection site and incision line. (**A**) With the abdominal of the rat supported by a 50 mL conical tube, the two pits between muscles above the pelvis are easily seen (arrows). (**B**) With one hand holding the pits, use the other hand to gently press and feel the spine and find the intervertebral space between the L5 and L6 vertebrae, i.e., the injection site (* in panel A). The dotted line in panel A shows the incision line with the injection site at its center.

Figure 3: A single bolus IT injection of ASOs reduces rat Malat1 in vivo. We injected a single bolus of either PBS or 300 µg of Malat1 ASO; two weeks after the surgery we collected different

regions of the CNS and quantified the expression levels of Malat1 RNA. We obtained good KD of Malat1 RNA in all regions analyzed, with some variability among regions (cerebral cortex = 87% KD, striatum = 77% KD, cerebellum = 74% KD, spinal cord = 94% KD; error bars = ± SEM).

DISCUSSION:

The present article shows a powerful method to deliver therapeutic agents directly into the rat CNS. In theory, a similar technique can be also performed in mice, though due to the smaller size, the method can be more challenging. Therefore, our group performs intracerebroventricular (ICV) injections in mice for CNS drug delivery, which reach the same goals through a different route of administration. That method has been described in another study[16].

The advantage of the method described here is that it does not require expensive equipment, nor any special tools. We recommend preparing the catheter/wire assembly shown in **Figure 1A** ahead of time. One should prepare at least as many catheter/wire assemblies as there are rats in the study, although we suggest preparing some extra catheter/wire assemblies in case some are damaged or need to be replaced during the surgery.

Once the technique is mastered, the whole procedure described requires about 25 min per rat, thus allowing treatment of many rats within one day. If one person performs the surgery on the first rat, and a second person does surgical preparation on the next rat, two rats can be processed at the same time to reduce per animal time. Establishing techniques such as the one described here, is crucial for the development of a robust pre-clinical research pipeline that can advance CNS-targeting therapies. Indeed, IT delivery of ASOs as a therapeutic intervention is a method that is currently being explored for treatment of many disorders of the CNS[17-20]. Nusinersen, an ASO-based treatment for spinal muscular atrophy (SMA) patients, was recently approved in several markets worldwide, demonstrating the applicability of this method also to pediatric patients[21-23].

ACKNOWLEDGEMENTS:

We would like to thank Ionis Pharmaceuticals for supplying the ASOs described in the article.

DISCLOSURES:

The authors are all employees of Biogen, Inc. or Ionis Pharmaceuticals. The authors receive the antisense oligonucleotides described in the article from Ionis Pharmaceuticals.

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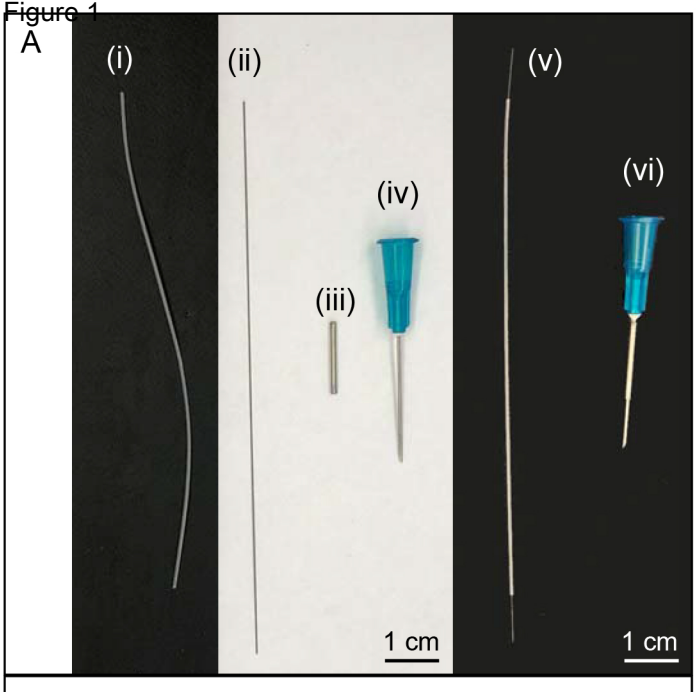
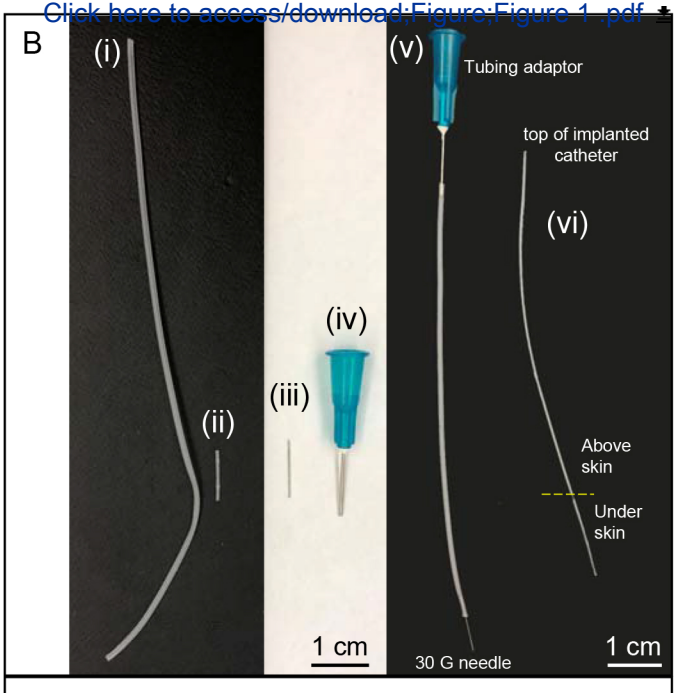
Figure 1



(i) PE10 catheter
(ii) stylet wire
(iii) guide cannula made from 19G needle
(iv) 23G needle
(v) catheter/wire assembly
(vi) cannula/needle assembly



(i) PE50 catheter
(ii) adapter made from PE10 catheter
(iii) 30G needle (cut to 1 cm in length)
(iv) Tubing adapter
(v) delivery catheter assembly
(vi) implanted catheter

Figure 2

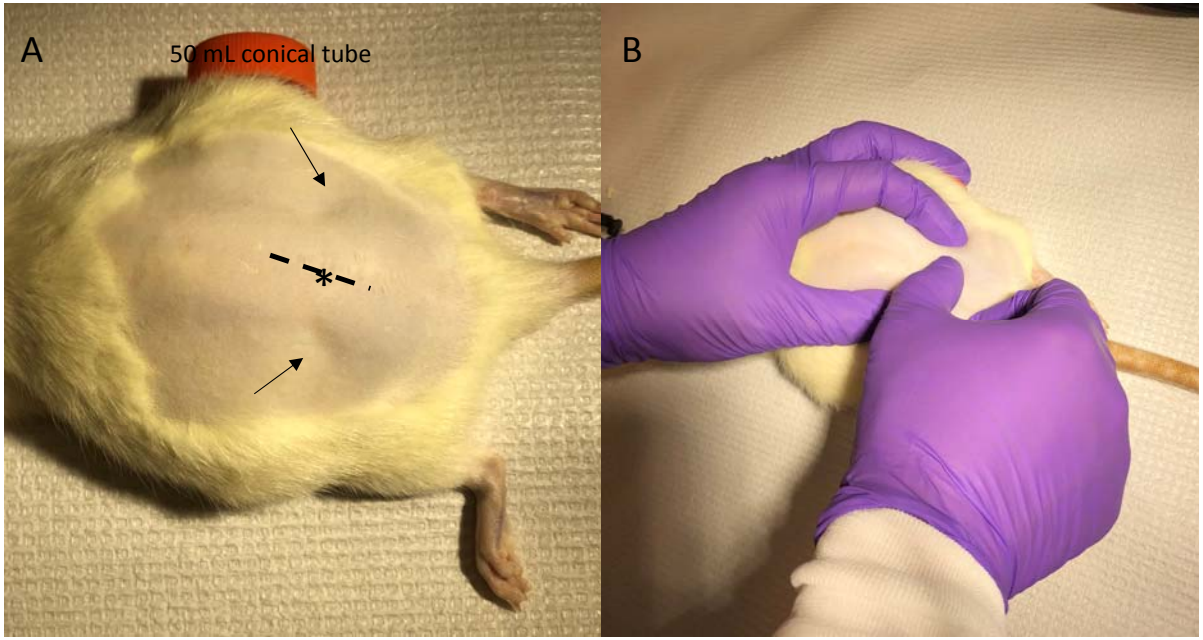


Figure 3

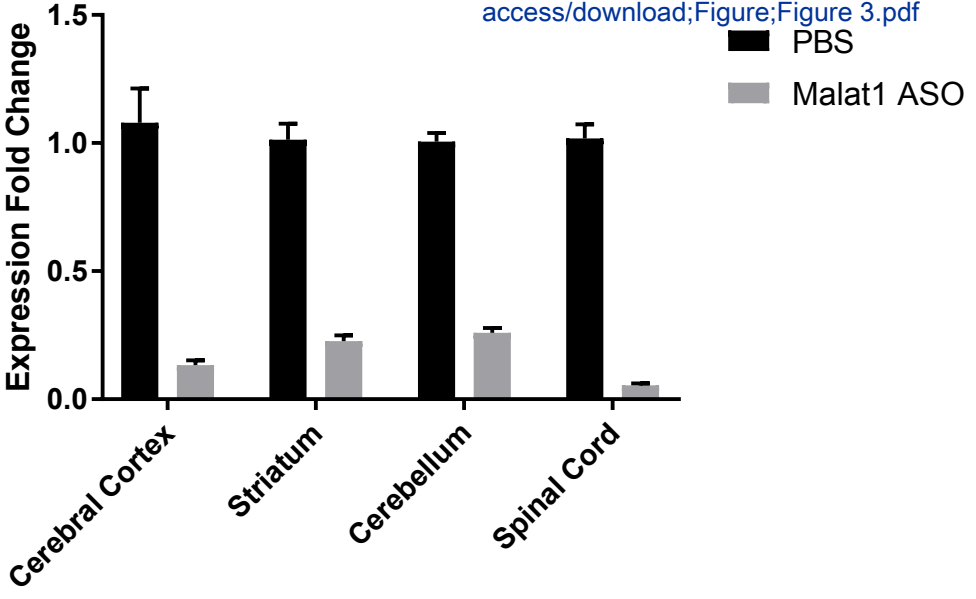


Table of Materials

Rat IT material

Name	Vendor	Description	Order information and/or Numbers	Notes
3M Steri-Drape Small Drape with Adhesive Apertu	3M		1020	
70% ethanol	Decon Laboratories, Inc		8416-160Z	
Alcohol swab sticks	Dynarex		NO 1204	
BD General Use Syringes 1 mL Luer-Lok tip	BD	1ml TB Luer-Lok tip	BD 302830	
BD Intramedic PE Tubing	BD	Polyethylene tubing PE50 Diameter 0.023 in	BD 427400 (10ft, Fischer Scientific 22-204008) or 427401 (100ft, Fischer Scientific 14-170-12P)	
BD Intramedic PE Tubing	BD	Polyethylene tubing PE10 Diameter 0.011 in	BD 427410 (10ft, Fischer Scientific 14-170-11B) or 4274011 (100ft, Fischer Scientific 14-170-12B)	
BD Intramedic PE Tubing Adapters	BD	23 gauge intramedic luer stub adaper	BD 427565 or Fisher Scientific 14-826-19E	120V 1.2A
BD PrecisionGlide Single-use Needles 30G	BD		BD 305128	
Buprenorphine Sustained Release-lab	ZooPharm	Prescription required		
Ethylene oxide sterilizer	Andersen Sterilizer INC.	AN 74i, gas sterilizer	AN 74i	
Guide cannula	BD	19G x 1 WT (1.1 mm x 25mm) needle	BD 305186	
Hamilton syringe 100ul	Hamilton company	Hamilton syringe 100ul		
Hot bead Sterilizer	Fine Science Tools		STERILIZER MODELNO FST 250	
Ophthalmic ointment	Dechra veterranery product		17033-211-38	
Pocket Pro Pet Trimmer	Braintree Scientific		CLP-9931 B	
Povidone scrub	PDI		S48050	
Saline	Baxter	Sodium Chloride 0.9% Intravenous Infusion BP 50ml	FE1306G	
Scalpel	Feather	disposable scalpel	No. 10	
Small animal heating pad	K&H Manufacturing		Model # 1060	
Stylet Wire	McMaster-Carr	1749T14	LH-36233780	
Surgery Towel drape	Dynarex		4410	
Surgical scissors and forceps	FST and Fisher Scientific			
Sutures	Ethicon		4-0 or 5-0	
Tool to make the Guide cannular	Grainger	Rotary tool (Dremel)	14H446 (Mfr: EZ456)	1.5” diameter, Pk5
		EZ lock cut off Wheel	1PKX5 (Mfr: 3000-1/24)	1.5”, Pk2
		Grinding Wheel, Aluminum Oxide	38EY44 (Mfr: EZ541GR)	
		EZ lock Mandrel	1PKX8 (Mfr: EZ402-01)	1.5” diameter
		Diamond wheel floor Tile	3DRN4 (Mfr: EZ545)	
Alternative source for pre-made and sterilized materials for this procedure				
Dosing catheter system	SAI Infusion Systems		RIDC-01	
Guide cannula	SAI Infusion Systems		RIDC-GCA	
Internal Catheters	SAI Infusion Systems		RIDC-INC	
Stylet Wire	SAI Infusion Systems		RIDC-STY	

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July 5, 2019

Dear editors,

We would like to thank the reviewers for their thorough revision of our manuscript "Intrathecal delivery of antisense oligonucleotides in the rat CNS". We feel that their comments and concerns have indeed given us the opportunity to make our paper more accessible and useful to the reader.

Please, find below all our answers to both editorial and reviewer's comments. We have tracked all the changes within the attached text. Also, we have added one more figure (now figure 2).

Best regards,

Giulio Srubek Tomassy, PhD

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.
2. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

We have added the ethic statement

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We have edited accordingly

4. Section 3: Please specify all surgical tools used throughout.

We have edited accordingly

5. In the protocol, please briefly describe how to obtain presented in Figure 2.

We have edited accordingly

6. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-

[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

We have edited accordingly

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

We have revised the manuscript to address all Editorial comments.

Reviewer #1:

Major Concerns:
Some of the specific issues with regard to the description of the protocol are as follow:
1. It should be specified if this approach is limited to using animals of a specific age, weight, etc.

Information is added in section 2.1

2. In section 2.1, please give alternatives to anesthesia, as using vaporizers is very often not common.

We have only performed the procedure using vaporizers per Institution IACUC protocol. A sentence is added in section 2.2 to acknowledge the possibility of using an alternative means of anesthesia under Institution IACUC approval.

3. Images of an animal showing its positioning before surgery (lines 122-123) and some of the procedure steps are very desirable and would be actually helpful, e.g. to indicate where an incision is to be made, how the 6th lumbar vertebra looks like (lines 130-132).

We include Figure 2 showing the positioning before the surgery, the landmarks to locate the incision line.

4. Similarly, how to know exactly the 6th lumbar spine vertebra (section 3.1) - do you count the spines from the pelvic region, other ways? Please give to an experimenter some hints to recognize the site.

Section 3.1 and Figure 2 are added to describe the location of the incision.

5. The appearance of blood usually indicates a broken blood vessel. Have the authors common advice to distinguish between 'it does not indicate that harm has been done' and in case if the needle has been posited in a wrong way (lines 135-137)?

We sometimes see a tiny hint of blood in the CSF and never observed any complications afterwards. We revised section 3.2 and discussed what to do if large amount of blood or uncontrolled blooding occur.

6. What is the volume range that could be injected i.th? Does the volume of injected drug depend on the area intended for targeting (e.g. spinal cord, the brain tissue regions? (sections 3.8 and 3.10)

We routinely injected 30 uL of ASO and have not tested other injection volumes. In section 3.9, we pointed out that if other volume were to be used, pilot experiment should be performed to determine the safety and efficacy. We also cite published literature reporting the possible impact of injection volume on drug distribution.

7. Should animals be kept postoperatively individually per cage (lines 170-171)?

Two rats are housed per cage. We included this information in section 3.12.

8. Was the period of two weeks after delivery of genetic material established empirically (lines 193-195)? Can the authors show the time-dependence of the effects produced by oligonucleotides (other drugs/chemicals)?

We have not accessed in vivo knockdown efficiency earlier than 2 weeks post-surgery. In our experiences with several ASOs, we detected significant knockdown of the target genes up to 6-8 weeks post-surgery. We have not performed time-course experiment with other drugs/chemicals. We have revised the manuscript to include the above information in the "representative results" session.

The goal of this manuscript is to provide a visual description of how to perform the procedure. Evaluation of kinetics and distribution of any specific compound is case-dependent and is beyond the scope of this manuscript.

9. The authors recommend comparing the drug distribution and efficacy between various regions (lines 197-198). How do they propose to assess the distribution? Especially if oligonucleotides are not fluorescently tagged?

Since we only evaluated efficacy (i.e. knockdown efficiency) in various regions in the experiment described in this manuscript, we have deleted the statements about distribution. The distribution of ASO has been evaluated by our collaborator IONIS pharmaceuticals using various bioanalytical methods previously (Rigo et al . J Pharmacol Exp Ther 350:46–55, July 2014).

10. It is not clear if the approach could be useful in case repeated injection(s) are needed? (sections 3.11 and 3.12)

We have only used this approach for single injection, though it is compatible with repeated injections. We included the statement about this point in section 3.12.

Reviewer #2:

Manuscript Summary:

The manuscript by Yi et al describes a procedure for delivering ASOs into the CSF by intrathecal injection

in rats. Overall, the manuscript is well written and illustrated and has sufficient details to allow other researchers to replicate the procedure. I recommend publication with the following minor edits.

Minor Concerns:
1. Page 2, line 77 - delete "to" the development….
2. Page 2, lines 95, 97, 100; Page 3, line 106; - accept changes so red text not visible.
3. Page 5, last line change to "…..works via an RNaseH1-mediated mechanism that degrades the RNA…knock-down (KD) of gene expression".
4. Page 7 - first paragraph of discussion is one sentence. Suggest breaking it up.
5. Page 7, line 238 - delete "are" in the study; also suggest deleting "some" from "some extra ones" as it appears twice and replacing "ones" with catheters.

1-5. We have made the changes in the manuscript accordingly.

Reviewer 3:

Major Concerns:
1. Abstract: 1st paragraph contains mostly general statements and could be condensed to one sentence. At the same time, it would be useful if the main distinctive features of the procedure are specified here.

We have condensed the abstracted and added the sentence to emphasize the distinctive features of the procedure.

2. Similar comment about the Introduction:
p. 1 Lines 64-77 - can be condensed, and a brief review of other published IT catheter implantation techniques could be added.

We have condensed the introduction.

3. Additionally, the paper could be more informative if it contained the answers to the following questions:
a) What was the reason for a pre- and post-injection flushes?

The pre and post-injection flush is thought to reduce local sequestration of the compounds and improve their distribution to the rostral structures (Wolf et al JCI 2016). This point is added in section 3.10.

b) Were there any infections/complications, among the 20 injected animals and/or all animals injected in the lab using this method?

We did not observe injections or major complications in our experience. We occasionally observe minor incidents, for example, suture came off or minor swelling around the suture and they were

quickly mitigated. We revised the manuscript to comment on this in the last paragraph of the introduction.

c) Have you actually performed this procedure in mice?

We did not perform this procedure in mice. We clarified this point in the first paragraph of the Discussion.

d) Does ASO regional distribution in mice/rats after ICV differ from distribution after IT?

We do not have adequate data to address this question. Based on the ease of the procedure, we routinely perform ICV injection to deliver ASO in mouse and IT injection to deliver ASO in rats. We have not compared the two procedures in the same species. While the question is an important one, we believe it is beyond the scope of this manuscript.

4. It is good form to cite pioneering efforts in any field. The first rodent oligonucleotide IT delivery study was by Standifer et al. (Neuron, 1994) while corresponding ICV studies were by Wahlestedt et al. (Science, 1993; Nature 1993).

We included the recommended references.

Minor Concerns:
1. Line 82 'The protocol is very effective and causes very minimal discomfort to the animals.' - reference or explanation needed (effective-KD, mini-post-operation ops)

Explanation added

2. Surgery item 3.4. - not very clear from the description if the catheter would go rostrally or caudally

Description added

3. Lines 237-8 – typos

Corrected

4. Line 241 - 'Also, multiple animals can be done at one time reducing the per animal times.' - not clear

With one person does the surgical prep and a second person performs the surgery, two animals can undergo the procedure at the same time. We clarified this point in the manuscript.

Reviewer #4:

Manuscript Summary:

This manuscript describes a surgical method for surgical implantation of a cannula into the IT space of a rat for CNS delivery of drugs, in this case ASOs. It is a well written protocol for a useful technique. It includes instructions for low cost in house generation of surgical supplies, which is very nice. This is a very complex procedure that is explained well. Considering the ever increasing frequency of intra-CNS drug delivery, this has broad applications.

Major Concerns:

It would be helpful for the manuscript to include images or even diagrams of the various stages of the surgical procedure. As is, the images are only of the in house supplies and the representative data. Even a line drawing diagram of the various anatomical structures described would add greatly to the clarity and accessibility of the protocol.

We include Figure 2 showing the positioning before the surgery, the landmarks to locate the incision line. We feel that the accompanying video will adequately show the various stages of the surgical procedure and therefore and it is not necessary to include still images.

It would also be useful to include some discussion of the long-term potential for this placement and any methods for reducing the probability of infection, activity-related events, or other adverse events. –cite long term study While the procedure is described as being useful for long-term infusion, the representative data is from an acute bolus injection experiment. The flexibility to use the method for longer-term or intermittent dosing is a strength, but long-term care or limits of placement are not addressed.

We have only used this approach for single bolus injection, though the technique is compatible with repeated injections. We clarified this point in section 3.12. In our experience, we did not observe long-term adverse events up to 8 weeks post-surgery. We revised the manuscript to comment on this in the last paragraph of the introduction.

Minor Concerns:

none'