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Induction of leptomeningeal cells modification via intracisternal injection

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TITLE:

Induction of Leptomeningeal Cells Modification via Intracisternal Injection

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KEYWORDS:

Intracisternal injection; Leptomeninges; Transgenic mouse; Fate mapping; Connexin-30; Cerebrospinal fluid; Subarachnoid space.

SUMMARY:

We describe an intracisternal injection that employs a needle bent at the tip that can be stabilized to the skull, thus eliminating the risk of damage to the underlying parenchyma. The approach can be used for genetic fate mapping and manipulations of leptomeningeal cells and for tracking cerebrospinal fluid movement.

ABSTRACT:

The protocol outlined here describes how to safely and manually inject solutions through the cisterna magna while eliminating the risk of damage to the underlying parenchyma. Previously published protocols recommend using straight needles that should be lowered to a maximum of 1-2 mm from the dural surface. The sudden drop in resistance once the dural membrane has been punctured makes it difficult to maintain the needle in a steady position. Our method, instead, employs a needle bent at the tip that can be stabilized against the occipital bone of the skull, thus preventing the syringe from penetrating into the tissue after perforation of the dural membrane. The procedure is straightforward, reproducible, and does not cause long-lasting discomfort in the operated animals. We describe the intracisternal injection strategy in the context of genetic fate mapping of vascular leptomeningeal cells. The same technique can, furthermore, be utilized to address a wide range of research questions, such as probing the role of leptomeninges in neurodevelopment and the spreading of bacterial meningitis, through genetic ablation of genes putatively implicated in these phenomena. Additionally, the procedure can be combined with an automatized infusion system for a constant delivery and used for tracking cerebrospinal fluid movement via injection of fluorescently labelled molecules.

INTRODUCTION:

Leptomeningeal cells are a fibroblast-like population of cells organized in a thin layer overlaying

the brain and expressing genes implicated in collagen crosslinking (e.g., *Dcn* and *Lum*), and in the establishment of a brain-meningeal barrier (e.g., *Cldn11*)[1,2]. Leptomeningeal cells are implicated in a wide range of physiological functions, from strict control over the cerebrospinal fluid drainage[3] to guidance of neural progenitors in the developing brain[4,5]. A recent study has also proposed that leptomeninges in the newborn may harbor radial glia-like cells that migrate into the brain parenchyma and develop into functional cortical neurons[6].

Leptomeningeal cells are located in close proximity to surface astrocytes and share with them, as well as other parenchymal astroglia, expression of connexin-30 (Cx30)[7]. The surgical procedure outlined below allows widespread and specific labelling of these meningeal cells via a one-time delivery of endoxifen into the cisterna magna of transgenic mice conditionally expressing tdTomato in $Cx30^+$ cells (i.e., using a CreER-loxP system for fate mapping). Endoxifen is an active metabolite of Tamoxifen and induces recombination of CreER-expressing cells in the same way as Tamoxifen does. It is, however, the recommended solution for topical application because it dissolves in 5-10% DMSO, instead of high concentrations of ethanol. Additionally, endoxifen does not cross the brain-meningeal barrier, thereby enabling specific recombination of leptomeningeal cells, without labelling of the underlying $Cx30^+$ astroglial population (see Representative Results).

The technique presented here aims at manually and safely injecting the compound in the cerebrospinal fluid, via direct access to the cisterna magna. Unlike other, more invasive procedures requiring craniotomy, this approach allows to infuse compounds without causing damage to the skull or the brain parenchyma. Thus, it is not associated with the induction of inflammatory reactions triggered by activation of parenchymal glia cells. Similar to other injection strategies described before[8-10], the present approach relies on the surgical exposure of the atlanto-occipital dural membrane covering the cisterna magna, after blunt dissection of the overlaying neck muscles. However, unlike for other procedures, we recommend the use of a needle bent at the tip, which can be stabilized against the occipital bone during administration. This will prevent the risk of the needle penetrating too deep and damaging the underlying cerebellum and medulla.

This surgical procedure is compatible with lineage tracing investigations that aim at mapping changes in cell identities and migration routes through parenchymal layers. It can also be adapted to genetic ablation studies that intend to probe the role of leptomeningeal cells in health and disease, such as their contribution to cortical development[5] or the spreading of bacterial meningitis[3,11]. Finally, it can be utilized to track cerebrospinal fluid movement when combined with delivery of fluorescent tracers in wildtype animals.

PROTOCOL:

The surgical procedures hereby presented were approved by Stockholms Norra Djurförsöksetiska Nämnd and carried out in agreement with specifications provided by the research institute (Karolinska Institute, Sweden).

NOTE: Intracisternal injection can be flexibly adapted for multiple research purposes. We present below a procedure developed to efficiently label leptomeningeal cells for fate mapping based on injection of endoxifen in a transgenic mouse line carrying R26R-tdTomato[12] and CreER, the latter under the Cx30 promoter[13]. Labelling of this population of cells may be achieved through injection of viral constructs using the same procedure outlined below. Finally, this approach can be employed for tracking cerebrospinal fluid flow, by infusion of fluorescent tracers.

1. Preparation of the injection system

NOTE: Carry out the procedure in a suitable surgical room, and in aseptic conditions. Surgical tools may be sanitized via soaking in 70% ethanol or other disinfectant, or heat (e.g., using a glass bead sterilizer). Rinse the instruments before use when employing chemical disinfection or allow them to cool down when sanitized with heat.

1.1. Using forceps, bend the needle of the injection syringe to 30° at 3 mm from the tip.

NOTE: Use Hamilton syringes with a 30 G beveled needle.

1.2. Prepare 1 mg/mL of endoxifen solution, diluted in 10% DMSO and backfill the syringe with the bent needle.

NOTE: Administer 5 µL of the compound to ensure widespread exposure of meningeal cells in the adult C57Bl/6j mouse (ca. 25-30 g), although pilot experiments that test different concentrations and injection volumes may be necessary when treating animals of different ages and strains.

1.3. Adjust the mouse head holder so that the mouth piece is at approximately 30° from the surface of the surgical table.

NOTE: A stereotactic frame with three-point fixation (i.e., ears and mouth) can also be used for this procedure. In this case, however, the animal will only be fixed with the mouth piece, whereas ear bars can be extended under the animal's forelimbs and be used to support the animal's body during the procedure.

2. Induction of anesthesia

2.1. For injectable anesthetics, use concentrations and modes of administration recommended by the veterinary unit at the local institution.

2.2. For inhalational anesthetics, such as isoflurane, prepare the administration unit according to the manufacturer's specifications.

2.3. With isoflurane, set concentration of the compound at 4% for induction of anesthesia.

2.4. Deliver air at a rate of 400 mL/min.

2.5. Allow the anesthesia unit to fill the chamber with the anesthetic for a few minutes and place the mouse in the chamber afterwards.

NOTE: For the present experiments, we have used adult (>2 months old and approximately 30 g) male and female transgenic mice carrying Cx30-CreER and R26R-tdTomato, and bred on a C57Bl/6j background.

2.6. Monitor the animal during induction of anesthesia. The breathing pattern should slow down when the mouse is under full anesthesia.

2.7. Remove the animal from the chamber and check for suppression of the paw reflex by delicately pinching the hind paws.

NOTE: The reflex may still be present but delayed a couple of seconds. If that is the case, place the animal back in the chamber until complete suppression of the paw reflex has been achieved.

2.8. Administer analgesic (e.g., Carprofen, 5 mg/kg) to aid post-operative pain management.

3. Positioning of the animal for the procedure

3.1. Fix the animal's head onto the head holder. To improve accessibility to the cisterna magna, position the animal's body at approximately 30° from the surface of the table and the head titled downwards, to establish an angle of 120° with the rest of the body and extend the back of the neck to facilitate access to the cisterna magna (**Figure 1A**).

3.2. Add paper towels underneath the animal to support its body throughout the procedure.

3.3. Secure anesthesia delivery through the mouth piece and reduce isoflurane concentration to 2.5% and air delivery to 200 ml/min.

3.4. Apply ophthalmic ointment.

4. Exposure of the cisterna magna

4.1. Shave the back of the animal's neck and sanitize the area with 70% ethanol and Betadine.

4.2. Using surgical scissors, perform a midline incision (ca. 7 mm in length) starting at the level of the occipital bone and extending it posteriorly.

4.3. Gently separate superficial connective tissue and neck muscles pulling sidewise from the midline with fine tip tweezers. This will expose the dural membrane overlaying the cisterna magna, shaped as an inverted triangle.

4.4. Use sterile absorption spears or cotton swabs to control any resultant bleeding.

4.5. Position a small surgical separator to maintain the neck muscles pulled aside and enable visualization of the cisterna magna throughout the procedure.

5. Intracisternal injection

5.1. To gain access to the cisterna magna, identify the caudal end of the occipital bone and insert the needle that was previously bent immediately underneath.

NOTE: There will be a sudden drop in resistance as the dural membrane is punctured. However, the tip of the needle will only penetrate slightly underneath the meningeal surface, thanks to its hooked shape.

5.2. Once the dura has been perforated, allow the bent tip of the needle to penetrate underneath the dural surface by gently pulling the syringe upwards and parallel to the animal's body, in order to "hook" the needle to the skull (see **Figure 1B**). This will ensure better stability and will prevent the needle from penetrating deeper, thus avoiding the risk of damaging the underlying cerebellum or medulla.

5.3. Inject the compound slowly to avoid interference with the cerebrospinal fluid's natural flow.

NOTE: Depending on the purpose of the experiment, the infusion rate may vary. If slow and steady infusion rate is required (e.g., when using the procedure to trace cerebrospinal fluid movement), it may be advisable to use an automatized microinfusion system in combination with the syringe.

5.4. After the injection, let the needle rest in site for 1 min and carefully remove it. Use fine tip forceps to aid retraction of the needle from its hooked position.

NOTE: The use of a thin needle (e.g., 30 G) does not lead to substantial damage of the meningeal membrane, and consequent outflow of the cerebrospinal fluid. If larger needle sizes are required, we recommend sealing the punctured site with cyanoacrylate adhesive following injection, to prevent extensive loss of fluid.

6. Concluding the procedure and post-operative care

6.1. Remove the separator and allow the muscles to go back to their original position overlaying the dural membrane.

6.2. Close the skin incision using a few drops of cyanoacrylate adhesive.

NOTE: Alternatively, use absorbable sutures (e.g., 5-0 vicryl sutures) and close the skin incision with interrupted stitches.

6.3. Apply local anesthetic (e.g., 100 µL of 5% lidocaine) at the incision site.

6.4. Remove the animal from the holder and place in a clean cage placed on a heating pad. Monitor the animal until it regains consciousness.

NOTE: The procedure is not expected to cause long-lasting pain or distress in the animal. Nevertheless, the mouse should be monitored closely for the first postoperative days and pain relief measures may be undertaken whenever deemed necessary, and in accordance with recommendations provided by the veterinary unit at your institution.

REPRESENTATIVE RESULTS:

Intracisternal injection of endoxifen in transgenic mice expressing CreER under the Cx30 promoter[13] and an inducible fluorescent reporter allows for specific recombination of leptomeningeal cells without labelling the neighboring Cx30-expressing surface and parenchymal astrocytes in the cortex (**Figure 1**). In order to gain access to the cisterna magna, the anesthetized animal is positioned with its body and its head at an angle of approximately 120°, thus, allowing the back of its neck to be stretched (**Figure 1A**). The atlanto-occipital portion of dural membrane is then exposed through blunt dissection of the neck muscles, thus gaining access to the underlying cisterna magna. A safe manual injection is performed with a needle bent to approximately 30° at 3 mm from the tip. This allows the syringe to be held against the occipital bone of the cranium, thus improving stability during administration (**Figure 1B**). Taking advantage of the physiological movement of the cerebrospinal fluid, the endoxifen solution is distributed throughout the subarachnoid space to efficiently recombine leptomeningeal cells overlaying olfactory bulbs, cortex, and cerebellum (**Figure 1C**). As demonstrated in **Figure 1**, the solution does not cross the brain-meningeal barrier and does not come in contact with astroglial cells of the parenchyma, as opposed to systemic administration through oral gavage (2 mg/mL per day, on five consecutive days; **Figure 1C-E**).

FIGURE AND TABLE LEGENDS:

Figure 1. Specific labelling of leptomeningeal cells via intracisternal injection of endoxifen.

Panel **A** and **B** illustrate the procedure developed for intracisternal administration. In order to gain access to the cisterna magna, the back of the neck should be stretched. The anesthetized animal is, therefore, positioned at an approximate angle of 120° between the body and the head, which is tilted downwards (**A**). The hooked needle allows to secure the syringe to the skull and to safely proceed with a manual administration of the solution (**B**). Panel **C** displays a sagittal section of the brain after intracisternal administration of endoxifen in a transgenic mouse model carrying CreER under the Cx30 promoter and inducible expression of tdTomato fluorescent reporter. The asterisk (*) marks the injection site and demonstrates the absence of operative damage following the procedure. Endoxifen selectively induces genetic recombination and reporter gene expression in cells in the meningeal layer overlaying the olfactory bulb (Ob), cortex (Ctx), and cerebellum (Cb). Only a few astrocytes in the midbrain (arrowhead) become

recombined after intracisternal delivery of endoxifen. Panels **D**-**F** are magnifications of the boxed area in **C** in animals that were treated with vehicle solution (**D**), subjected to intracisternal injection of endoxifen (**E**), or treated systemically through oral gavage (**F**). Whereas intracisternal administration specifically labels cells of the leptomeninges, systemic delivery also leads to recombination of Cx30-expressing astrocytes throughout the cortical layers (L1 to L6). Panel **G** illustrates recombination of leptomeningeal cells (asterisk), identified through Pdgfra reactivity, after intracisternal injection. By contrast, surface (arrowhead) and parenchymal (arrow) astrocytes expressing Gfap remain unlabeled. Scale bars: (**C**) 1000 μm; (**D**-**F**) 150 μm (**G**) 40 μm.

DISCUSSION:

The protocol outlined here presents a straightforward and reproducible procedure to label leptomeningeal cells for fate mapping. We use intracisternal injection of endoxifen, an active metabolite of Tamoxifen, to induce expression of tdTomato fluorescent reporter in Cx30-CreER; R26R-tdTomato mice[12,13].

Compared to other protocols used for gaining access to the cerebrospinal fluid through the cisterna magna[9], our approach ensures a safe manual administration thanks to the use of a bent needle that can be stabilized to the occipital bone of the skull. Once the dural membrane of the cisterna magna is perforated, there is a sudden drop in resistance. At this point, other protocols recommend lowering the needle to a maximum of 1-2 mm from the dural surface and manually keeping it in a steady position throughout the procedure[9]. As opposed to a straight needle, the hooked needle is secured to the skull and cannot penetrate deeper in the tissue, thus eliminating the risk of damaging the underlying cerebellum or medulla. Our hooked system allows for a safer administration of solution, particularly when using a slow rate of infusion.

The procedure outlined here is not expected to cause long-lasting discomfort to the operated animal. Care must be taken, however, when administering large volumes of solution. A fast delivery rate may lead to alterations in the intracranial pressure and the development of neurological symptoms in the mouse. We suggest injecting volumes up to 5-10 μL to avoid this risk or assemble the syringe onto a micromanipulator that has control over the delivery rate. This is particularly important when adapting this procedure to the study of the cerebrospinal fluid movement. It is recommended to avoid manual injection and use a slow rate of infusion (e.g., 1 μL/min) to prevent excessive perturbance of the physiological flow. Furthermore, the present protocol is designed to perform a single intracisternal injection, which efficiently labels leptomeningeal cells. We recommend considering ethical specifications, as well as the animal's ability to withstand multiple surgical procedures, should the study require repeated administration of compounds.

In addition to endoxifen administration, the technique outlined here can be combined with delivery of viruses carrying reporter genes under a leptomeningeal cell-specific promoter. Furthermore, the present delivery system can be utilized for acute tracing of the cerebrospinal fluid flow[10]. For this purpose, fluorescent tracers such as Cell Tracker (ca. 700 Da) or Dextran Fluorescin (ca. 3000 Da) can be delivered through the cisterna magna. Furthermore, the syringe may be mounted onto a micromanipulator, which enables better control over the rate of infusion

of the compound. This may be important in order to avoiding excessive disturbance of the natural cerebrospinal fluid movement in tracing experiments.

Leptomeningeal cells express claudin-11 and other proteins associated with tight junctions, which contribute to the establishment of a blood-cerebrospinal fluid barrier in the subarachnoid space and to the homeostatic control of fluid and nutrients circulation[3]. The approach outlined here may be combined with conditional ablation of genes implicated in the junctional control of the barrier to probe their putative role in maintaining strictly regulated cerebrospinal fluid composition. Additionally, cells from the leptomeninges play a role in development, where they provide extrinsic signals that contribute to the generation of cortical neurons[5] and the formation of callosal connections[4]. Our method can also be adapted to gain further insight into the role of the leptomeninges in correct cortical development and axonal pathfinding. Finally, bacteria such as *Neisseria meningitidis* have been shown to attach to human leptomeningeal cells[14], and animal models for the disease have been developed to study bacterial invasion and resulting neurological damage[15,16], although surface ligands responsible for the infection are yet to be fully determined. Selective recombination of leptomeningeal cells achieved with our technique could aid identification of the adhesion sites used by bacteria to infect the subarachnoid space. Of note, the protocol hereby presented may require modifications to account for additional ethical and safety requirements necessary to carry out procedures that entail bacterial infections.

In conclusion, the intracisternal injection herein described represents a simple and well-tolerated surgical approach that offers the opportunity to investigate a wide range of leptomeningeal and cerebrospinal fluid functions, when combined with gene-editing approaches or infusion of labelled molecules.

ACKNOWLEDGMENTS:

This study was supported by grants from the Swedish Research Council, the Swedish Cancer Society, the Swedish Foundation for Strategic Research, Knut och Alice Wallenbergs Stiftelse and the Strategic Research Programme in Stem Cells and Regenerative Medicine at Karolinska Institutet (StratRegen).

DISCLOSURES:

The authors declare no competing interests.

REFERENCES:

1 Vanlandewijck, M. et al. A molecular atlas of cell types and zonation in the brain vasculature. *Nature.* **554** (7693), 475-480 (2018).

2 Whish, S. et al. The inner CSF-brain barrier: developmentally controlled access to the brain via intercellular junctions. *Frontiers in Neuroscience.* **9**, 16 (2015).

3 Weller, R. O., Sharp, M. M., Christodoulides, M., Carare, R. O., Mollgard, K. The meninges as barriers and facilitators for the movement of fluid, cells and pathogens related to the rodent and human CNS. *Acta Neuropathologica.* **135** (3), 363-385 (2018).

4 Choe, Y., Siegenthaler, J. A., Pleasure, S. J. A cascade of morphogenic signaling initiated by the meninges controls corpus callosum formation. *Neuron.* **73** (4), 698-712 (2012).

5 Siegenthaler, J. A. et al. Retinoic acid from the meninges regulates cortical neuron generation. *Cell.* **139** (3), 597-609 (2009).
6 Bifari, F. et al. Neurogenic Radial Glia-like Cells in Meninges Migrate and Differentiate into Functionally Integrated Neurons in the Neonatal Cortex. *Cell Stem Cell.* **20** (3), 360-373 e367 (2017).
7 De Bock, M. et al. A new angle on blood-CNS interfaces: a role for connexins? *FEBS Letters.* **588** (8), 1259-1270 (2014).
8 Ramos, M. et al. Cisterna Magna Injection in Rats to Study Glymphatic Function. *Methods in Molecular Biology.* **1938**, 97-104 (2019).
9 Xavier, A. L. R. et al. Cannula Implantation into the Cisterna Magna of Rodents. *Journal of Visualized Experiments.* (135) (2018).
10 Iliff, J. J. et al. A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta. *Science Translational Medicine.* **4** (147), 147ra111 (2012).
11 Coureuil, M., Lecuyer, H., Bourdoulous, S., Nassif, X. A journey into the brain: insight into how bacterial pathogens cross blood-brain barriers. *Nature Reviews Microbiology.* **15** (3), 149-159 (2017).
12 Madisen, L. et al. Transgenic mice for intersectional targeting of neural sensors and effectors with high specificity and performance. *Neuron.* **85** (5), 942-958 (2015).
13 Slezak, M. et al. Transgenic mice for conditional gene manipulation in astroglial cells. *Glia.* **55** (15), 1565-1576 (2007).
14 Hardy, S. J., Christodoulides, M., Weller, R. O., Heckels, J. E. Interactions of Neisseria meningitidis with cells of the human meninges. *Molecular Microbiology.* **36** (4), 817-829 (2000).
15 Colicchio, R. et al. The meningococcal ABC-Type L-glutamate transporter GltT is necessary for the development of experimental meningitis in mice. *Infection and Immununity.* **77** (9), 3578-3587 (2009).
16 Ricci, S. et al. Inhibition of matrix metalloproteinases attenuates brain damage in experimental meningococcal meningitis. *BMC Infectious Diseases.* **14**, 726 (2014).

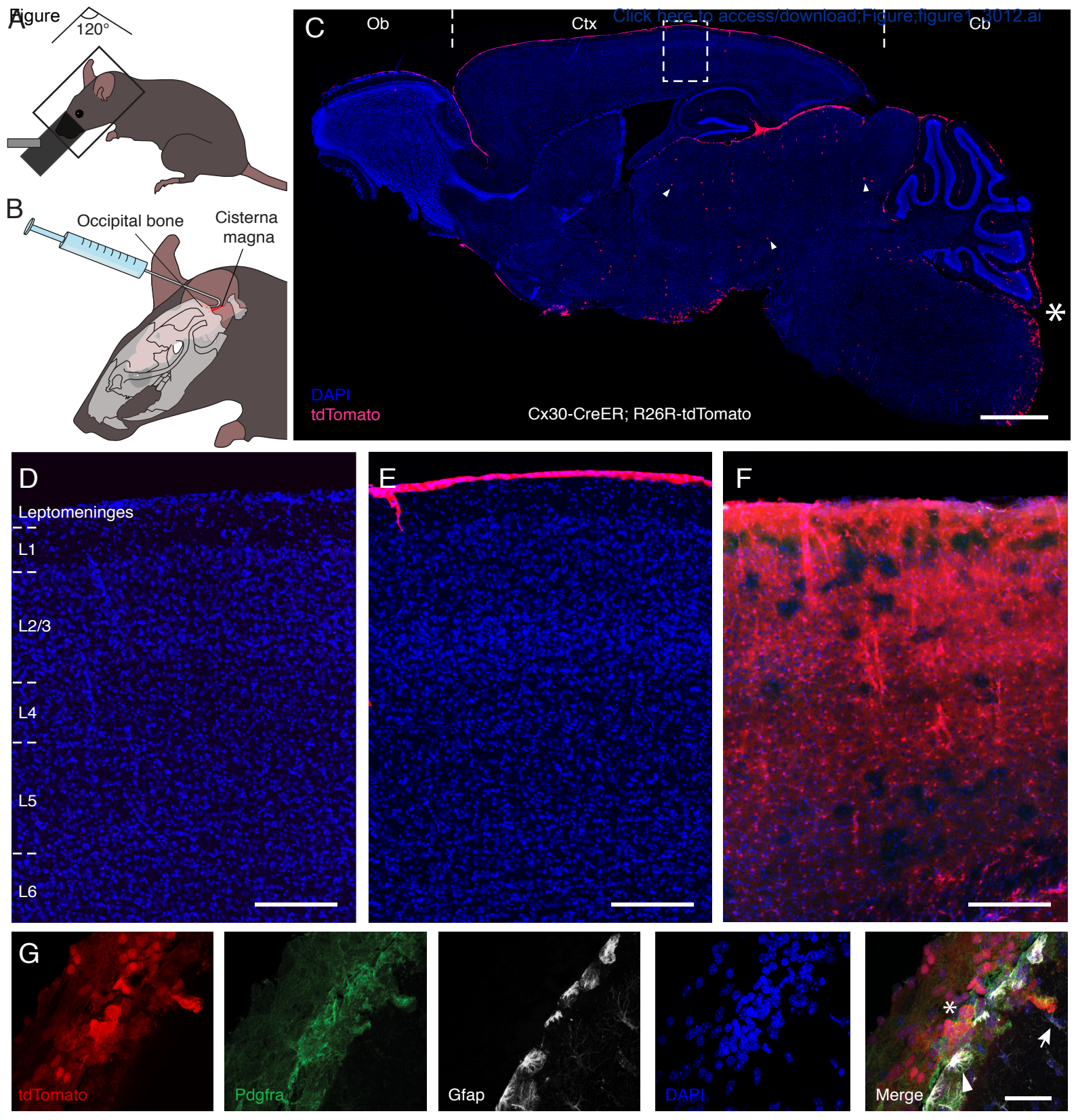


Table of Materials

Click here to access/download;Table of Materials;jove_materials.xls

Name of Material/ Equipment	Company	Provider	Catalog Number	Comments/Description
Anesthesia unit	Univentor 410	AgnTho's	8323102	Complete of vaporizer, chai
Anesthesia (Isoflurane)	Baxter Medical AB	Apoteket	000890	
Betadine	Sigma-Aldrich		PVP1	
Carprofen	Orion Pharma AB	Apoteket	014920	Commercial name Rymadil
Cyanoacrylate glue	Carl Roth		0258.1	Use silk 5-0 sutures, in alter
Medbond Tissue Glue	Stoelting		50479	
DMSO	Sigma-Aldrich		D2650	
Endoxifen	Sigma-Aldrich		E8284	
Ethanol 70%	Histolab		01370	
Hamilton syringe (30G beveled needle)	Hamilton		80300	
Lidocaine	Aspen Nordic	Apoteket	520455	
Mouse head holder	Narishige International		SGM-4	With mouth piece for inhal
Scissors	Fine Science Tools	AgnTho's	15009-08	
Shaver	Aesculap	AgnTho's	GT420	
Sterile absorption spears	Fine Science Tools		18105-01	Sterile cotton swabs are als
Surgical separator	World Precision Instrument		501897	
Tweezers	Dumont	AgnTho's	11251-35	
Viscotears	Bausch&Lomb Nordic AB	Apoteket	541760	

Notes

mber, and tubing that connects to chamber and mouse head holder

rnative

ational anhestetics. Alternatively, use a stereotactic frame

o a good option

Ophtalimc ointment

We would like to thank the editor and the reviewers for the constructive comments on the manuscript and for providing timely reviews. Below is our point-by-point response to the concerns and suggestions raised. Changes are highlighted in the manuscript and reported here.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

We made sure there are no spelling and grammatical errors in the manuscript.

• **Protocol Language:** Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly, and actions should be described in the imperative tense wherever possible.
1) Examples NOT in imperative voice: 1.3, 1.5, 2.4

The text is now adjusted so that the imperative tense is used throughout the protocol. As suggested by the editor, we made changes for points 1.3, 1.5, and 2.4. Additionally, we modified the text for points 2.7, 3.1, 5.1, and 5.4. See changes underlined below:

Changes for 1.3:

1.2.Prepare 1 mg/mL of Endoxifen solution, diluted in 10% DMSO and backfill the syringe with the bent needle.

NOTE: We recommend administering 5 µl of the compound to ensure widespread exposure of meningeal cells in the adult C57Bl/6j mouse (ca. 25-30 g), although pilot experiments that test different concentrations and injection volumes may be necessary when treating animals of different ages and strains.

Changes for 1.5:
1.3.Adjust the mouse head holder so that the mouth piece is at approximately 30° from the surface of the surgical table.

NOTE: A stereotactic frame with three-point fixation (i.e., ears and mouth) can also be used for this procedure. In this case, however, the animal will only be fixed with the mouth piece, whereas ear bars can be extended under the animal's forelimbs and be used to support the animal's body during the procedure.

Changes for 2.4:
2.4. Deliver air at a rate of 400 ml/min.

Changes for 2.7:
2.7.Remove the animal from the chamber and check for suppression of the paw reflex by

delicately pinching the hind paws.

NOTE: The reflex may still be present but delayed a couple of seconds. If that is the case, we recommend placing the animal back in the chamber until complete suppression of the paw reflex has been achieved.

Changes for 3.1:

3.1.Fix the animal's head onto the head holder. To improve accessibility to the cisterna magna, position the animal's body at approximately 30° from the surface of the table and the head titled downwards, to establish an angle of 120° with the rest of the body and extend the back of the neck to facilitate access to the cisterna magna (Figure 1A).

Changes for 5.1:

5.1.To gain access to the cisterna magna, identify the caudal end of the occipital bone and insert the needle that was previously bent immediately underneath.

NOTE: There will be a sudden drop in resistance as the dural membrane is punctured. However, the tip of the needle will only penetrate slightly underneath the meningeal surface, thanks to its hooked shape.

Changes for 5.4:

5.4.After the injection, let the needle rest in site for 1 min and carefully remove it. Use fine tip forceps to aid retraction of the needle from its hooked position.

NOTE: The use of a thin needle (e.g., 30G) does not lead to substantial damage of the meningeal membrane, and consequent outflow of the cerebrospinal fluid. If larger needle sizes are required, we recommend sealing the punctured site with cyanoacrylate adhesive following injection, to prevent extensive loss of fluid.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Find below the changes to the text, which are here underlined.

1) 2.6: Mention animal age, sex, weight, strain.

We are now reporting details for the animal used as a note in point 2.5:

NOTE: For the present experiments, we have used adult (>2 months old and approximately 30 g) male and female transgenic mice carrying Cx30-CreER and R26R-tdTomato, and bred on a C57Bl/6j background.

2) 3.2: Use of the term "tissues" can be confusing in this context, "paper towel" may be a suitable alternative.

We have now removed the term "tissues" and replaced it with "paper towels" in point 3.2.

3) 4.2: Mention surgical tools used.

We have specified to use surgical scissors to perform the incision:

4.2.Using surgical scissors, perform a midline incision (ca. 7 mm in length) starting at the level of the occipital bone and extending it posteriorly.

4) 5.1: Is this the needle from 1.1? How deep should you insert?

The needle mentioned in 5.1 is indeed the one that has been prepared in 1.1. We have specified this and added that the needle will only penetrate slightly under the dural surface due to its hooked shape. In 5.2 we then added that the whole tip of the bent needle shall be inserted through the meningeal layer, in order to hook it to the occipital bone.

5.1.To gain access to the cisterna magna, identify the caudal end of the occipital bone and insert the needle that was previously bent immediately underneath.

NOTE: There will be a sudden drop in resistance as the dural membrane is punctured. However, the tip of the needle will only penetrate slightly underneath the meningeal surface, thanks to its hooked shape.

5.2.Once the dura has been perforated, allow the bent tip of the needle to penetrate underneath the dural surface by gently pulling the syringe upwards and parallel to the animal's body, in order to "hook" the needle to the skull (see Figure 1B). This will ensure better stability and will prevent the needle from penetrating deeper, thus avoiding the risk of damaging the underlying cerebellum or medulla.

5) 6.2: Mention suture material and pattern.

We now suggest suturing with absorbable sutures and interrupted stitches as an alternative to using skin glue.

6.2.Close the skin incision using a few drops of cyanoacrylate adhesive.

NOTE: Alternatively, use absorbable sutures (e.g., 5-0 vicryl sutures) and close the skin incision with interrupted stitches.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For

example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

Please find in the text the parts highlighted in yellow. Altogether they make up 2.5 pages of text (incl. spaces and headings).

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have addressed the important points in the discussion: for instance, we highlight the importance of slow delivery of the compound to avoid increasing intracranial pressure, and suggest modifications in case the experiment requires administration of large volumes (e.g., assembling the injection syringe onto a micromanipulator to regulate delivery rate), we mention how our method builds on, and improves, current strategies for intracisternal injection with the use of a hooked needle that is safely secured to the skull. Furthermore, we list a few applications for which our method can be applied (e.g., for the study of leptomeningeal functioning at the brain-meninges barrier, or the tracking of cerebrospinal fluid).

• **References:** Please spell out journal names.

We are now using full names for the journal. Please see the reference section in the manuscript for the changes.

Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
The manuscript entitled "Genetic modification of leptomeningeal cells via intracisternal injection" describes an intracisternal injection method which successfully injects solutions through the cisterna magna eliminating the risk of causing damage. The protocol is well-written with details that will ensure other researchers to perform the related experiment. In my opinion, there are some small additions to be made in order to improve the reading of the protocol.

We thank the reviewer for their positive feedback and address the minor concerns and suggestion raised below.

Major Concerns:
None

Minor Concerns:

Title
I suggest, to remove the word "Genetic" from the Title and modify the Title for example: "Induction of leptomeningeal cells modification via intracisternal injection".

We welcome the suggestion to revise our title and have changed it accordingly.

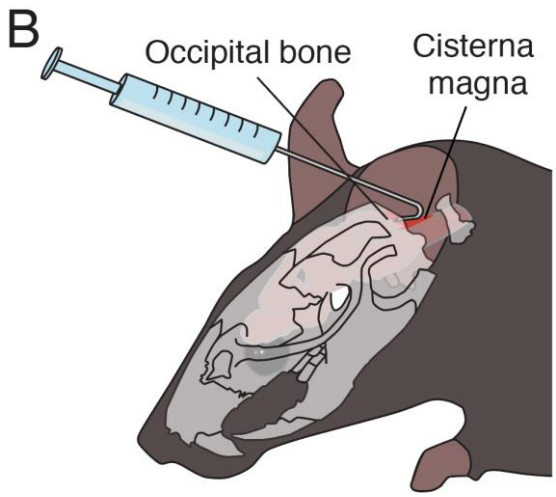
Introduction
In the introduction when the authors suggest that this method could be used to induce bacterial meningitis, references should be expanded (other references should be included such as: Colicchio et al., Infect Immun. 2019 Mar 25;87(4); Ricci et al., BMC Infect Dis. 2014 Dec 31;14:726; Zhang et al., J Vis Exp. 2018 Jul 1, 137).

We have now added references for the bacterial infection (see line 367). The following references were added:

15 Colicchio, R. *et al.* The meningococcal ABC-Type L-glutamate transporter GltT is necessary for the development of experimental meningitis in mice. *Infection and Immunity*. **77** (9), 3578-3587, (2009).
16 Ricci, S. *et al.* Inhibition of matrix metalloproteinases attenuates brain damage in experimental meningococcal meningitis. *BMC Infectious Diseases*. **14** 726, (2014).

Protocol
In order to improve the impact of this research and help the reader to better understand the technique and how it differs from other methods, it would be useful to clarify for those who are not familiar with the terms to which they refer anatomically intracisternal and cisterna magna. Most readers may not be familiar with the details of the cranial anatomy, therefore a brief but more detailed explanation is recommended.

To aid identification of the anatomical landmarks, Figure 1B is now specifying where occipital bone and cisterna magna are located. See updated panel below.



Discussion
The authors suggest that this protocol could be useful to study meningococcal meningitis and to understand the interactions between N. meningitidis and leptomeninges.
However, it should be clarified that in case of meningococcal manipulation some modifications should be made to the protocol, first of all on the safety for biological risk.

We welcome the suggestion and have now specified in the discussion (line 369-371) that researchers will need to consider additional ethical and safety measures when working with bacterial infections. See below:

Of note, the protocol hereby presented may require modifications to account for additional ethical and safety requirements necessary to carry out procedures that entail bacterial infections.

Reviewer #2:

Manuscript Summary:

This is a straightforward method paper that will benefit many. Overall, the manuscript is clear. I have just a few points;

We thank the reviewer for the positive feedback and address their comments below.

Major Concerns:

1. Page 3. Reference for expression of Cx30

Thank you for noticing we missed the reference. It is now added:

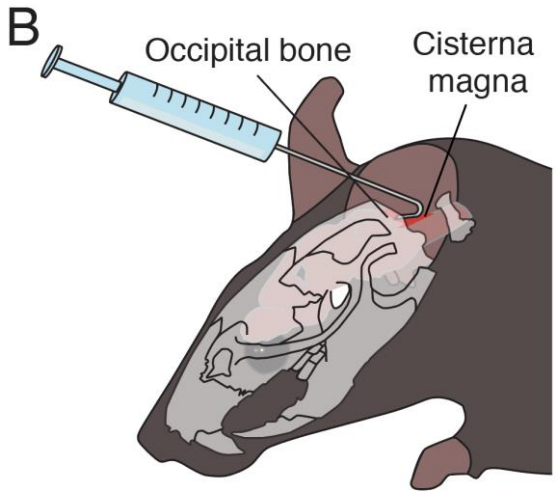
*De Bock, M. et al. A new angle on blood-CNS interfaces: a role for connexins? FEBS Letters **588** (8), 1259-1270, (2014).*

2. 3.2 indicate the exact tilting of the head that is optimal

We have now specified that the head should form an angle of 120° with the rest of the body, as shown in figure 1A, to facilitate access to the cisterna magna.

3. Illustration of the mice at the stereotaxic frame with the bended needled in place during the injection is a key: it cannot be visualized by the scheme how the needle is safely kept in place and the anterior to posterior orientation of the syringe seems a bit awkward to access the CM.

We have modified figure 1B so that the head of the mouse is tilted downwards, similar to the depiction in 1A. We, furthermore, edited orientation of the syringe and looks of the needle to resemble reality more closely. We believe these adjustments will make it easier for the readers to understand the injection strategy. See below the updated panel 1B.



4. The manuscript does not mention how the perforated area of the dura membrane is sealed after removing the needle. CSF leakage will lead to loss of the injected compound and pathological changes in CSF flow.

We do not seal the dural membrane after injection. In our experience, the use of a thin needle (e.g., 30G) only causes a small aperture in the meninges that does not allow substantial amounts of CSF to flow out. The small puncture will, furthermore, be tapped by the overlaying connective tissue and neck muscles, thus, further preventing fluid loss. We have added a note to highlight this point in the protocol (point 5.4).

5.4. After the injection, let the needle rest in site for 1 min and carefully remove it. Use fine tip forceps to aid retraction of the needle from its hooked position.

> *NOTE: The use of a thin needle (e.g., 30G) does not lead to substantial damage of the meningeal membrane, and consequent outflow of the cerebrospinal fluid. If larger needle sizes are required, we recommend sealing the punctured site with cyanoacrylate adhesive following injection, to prevent extensive loss of fluid.*

Reviewer #3:
Manuscript Summary:
The manuscript describes a simple procedure for intra-cisterns saga injections of molecules into the CSF. This procedure appears to be more convenient with less risk of CNS tissue injury and erroneous injections.

We thank the reviewer for the comments and address the concerns below.

Minor Concerns:
The procedure could be commented on further with respect to frequency and numbers of injections allowed, and volume limits.

Per ethical permit, we are required to keep injection volumes < 10 ul. We have added a note at point 2.5 to recommend conducting pilot experiments to adjust for the optimal injection volume and concentration of the compound based on the details of the study.

NOTE: For the present experiments, we have used adult (>2 months old and approximately 30 g) male and female transgenic mice carrying Cx30-CreER and R26R-tdTomato, and bred on a C57Bl/6j background.

Furthermore, our ethical permit allows for a single intracranial injection, which prevents us from observing how well can the animal tolerate repeated treatment. A single administration of Endoxifen is however able to efficiently label cells from the leptomeninges, thus, not requiring the animal to undergo repeated surgeries. We have specified in the manuscript (at line 55) that the present protocol describes a single administration. We have, furthermore, added that one should consider revising ethical specification and the ability of the animal to withstand multiple surgeries in case the study requires repeated treatments (lines 340-344).

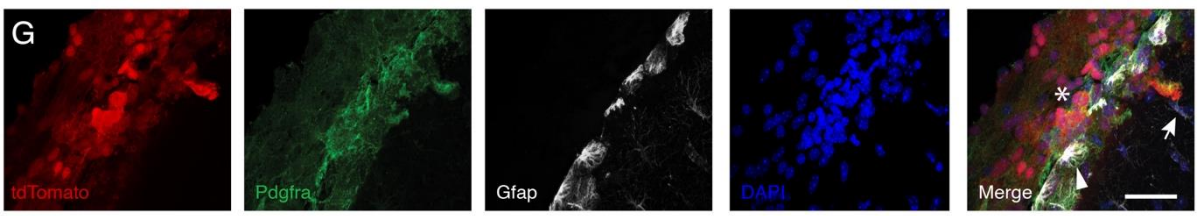
Line 55: The surgical procedure outlined below allows widespread and specific labelling of these meningeal cells via one-time delivery of Endoxifen into the cisterna magna of transgenic mice conditionally expressing tdTomato in Cx30^{+} cells.

Lines 340-344: Furthermore, the present protocol is designed to perform a single intracisternal injection, which efficiently labels leptomeningeal cells. We recommend considering ethical specifications, as well as the animal's ability to withstand multiple surgical procedures, should the study require repeated administration of compounds.

Also, for some of the conclusions about the type of cell targeted by the Endoxifen molecule, the authors could show immunohistochemistry to back up their claims that it is the type of cell that they say. Many cells express the connexion gene that they are demonstrating activation of with their reporter.

We have now added panels G in Figure 1 to highlight Pdgfra reactivity of the tdTomato-labelled cells, suggesting they are indeed leptomeningeal cells, and demonstrated that the underlying astroglial cells (also expressing Cx30) are not recombined with the current procedure.

*Panel **G** illustrates recombination of leptomeningeal cells (asterisk), identified through Pdgfra reactivity, after intracisternal injection. By contrast, surface (arrowhead) and parenchymal (arrow) astrocytes expressing Gfap remain unlabeled.*



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Author(s): Margherita Zamboni, Giuseppe Santopolo, Jonas Frisén

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