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TITLE:

Direct Cannula Implantation in the Cisterna Magna of Pigs

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glymphatic system, cerebrospinal fluid, cisterna magna, cannulation, pig

SUMMARY:

This article presents a step-by-step protocol for the direct cannula implantation in the cisterna magna of pigs.

ABSTRACT:

The glymphatic system is a waste clearance system in the brain that relies on the flow of cerebrospinal fluid (CSF) in astrocyte-bound perivascular spaces and has been implicated in the clearance of neurotoxic peptides such as amyloid-beta. Impaired glymphatic function exacerbates disease pathology in animal models of neurodegenerative diseases, such as Alzheimer's, which highlights the importance of understanding this clearance system. The glymphatic system is often studied by cisterna magna cannulations (CMc), where tracers are delivered directly into the cerebrospinal fluid (CSF). Most studies, however, have been carried out in rodents. Here, we demonstrate an adaptation of the CMc technique in pigs. Using CMc in pigs, the glymphatic system can be studied at a high optical resolution in gyrencephalic brains and in doing so bridges the knowledge gap between rodent and human glymphatics.

INTRODUCTION:

Cerebrospinal fluid (CSF) is an ultrafiltrate of blood which is found within and around the central nervous system (CNS)[1,2]. Apart from giving buoyancy to the brain or absorbing damaging mechanical forces, CSF also plays a pivotal role in clearing metabolic waste from the CNS[3]. Waste clearance is facilitated by the recently characterized glymphatic system which permits the convective flow of CSF through the brain parenchyma via perivascular spaces (PVS), which encircle penetrating arteries[3–5]. This process has been shown to be dependent on aquaporin-4 (AQP4), a water channel expressed primarily on the astrocytic endfeet, bound to the PVS[4,6]. The

study of the glymphatic system is achieved by both *in vivo* and *ex vivo* imaging, using either advanced light microscopy or magnetic resonance imaging (MRI), following the introduction of a fluorescent/radioactive tracer or contrast agent into the CSF[7–11].

An effective way to introduce a tracer into the CSF without incurring damage to the brain parenchyma is through cisterna magna cannulation (CMc)[12,13]. A large majority of all glymphatic studies, thus far have been carried out in rodents and avoided in higher mammals because of the invasiveness of CMc coupled to the practical simplicity of working with a small mammal. Additionally, the thin skulls of mice permit *in vivo* imaging without the need for a cranial window and subsequently allow for an uncomplicated brain extraction[11,14]. Experiments carried out in humans have yielded a valuable macroscopic *in vivo* data on the glymphatic function, but relied on intrathecal tracer injections in the distal lumbar spine and, furthermore, utilize MRI which does not yield sufficient resolution to capture the microanatomy the glymphatic system[7,15,16]. Understanding the architecture and extent of the glymphatic system in higher mammals is essential for its translation to humans. In order to facilitate glymphatic translation to humans, it is important to apply techniques that are carried out in rodents to higher mammals so as to allow for direct comparisons of the glymphatic system across species of increasing cognition and brain complexity[17]. Pig and human brains are gyrencephalic, possessing a folded neuroarchitecture, while rodent brains are lissencephalic, thereby having substantial difference among each other. In terms of the overall size, pig brains are, also, more comparable to humans, being 10-15 times smaller than the human brain, while mouse brains are 3,000 times smaller[18]. By better understanding the glymphatic system in large mammals, it may be possible to utilize the human glymphatic system for future therapeutic intervention in conditions such as stroke, traumatic brain injury and neurodegeneration. Direct CMc in pigs *in vivo* is a method that allows for the high-resolution light microscopy of the glymphatic system in a higher mammal. Furthermore, due to the size of the pigs used, it is possible to apply monitoring systems similar to those used in human surgeries making it feasible to tightly document and regulate vital functions in order to assess how these contribute to the glymphatic function.

PROTOCOL:

All procedures were carried out in accordance with the European directive 2010/63/EU and were approved by Malmö-Lund Ethical committee on Animal Research (Dnr 5.2.18-10992/18) and conducted according to the CODEX guidelines of the Swedish Research Council.

1. Preparation

1.1. Tracer

1.1.1. Prepare artificial CSF (126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 26 mM $NaHCO_3$; pH 7.4)

1.1.2. To 500 μL of artificial CSF, add 10 mg of albumin from bovine serum (BSA) conjugated with Alexa Fluor 647 (BSA-647).

1.1.3. Centrifuge at 5,000 x *g* for 5 min and use the supernatant.

1.2. Cannula

1.2.1. Attach a 1 mL syringe to the female Luer connection of the intravenous (IV) line, 3-ways tap with 10 cm extension.

1.2.2. Attach an 18 G needle to the male end.

1.2.3. Open the 3-way stop lock to allow for continuity from the needle to the syringe.

1.2.4. Carefully unsheathe the needle and aspirate approximately 300 μL of the saline into the IV line.

1.2.5. Remove the needle from the saline and proceed to introduce in some air to create a small air bubble (5-10 mm) in the IV line.

1.2.6. Place the needle into the tracer and aspirate all 500 μL of the tracer. The saline in the IV line should be visibly separated by an air bubble.

1.2.7. Discard the needle and close the 3-way stop lock.

1.3. Animal

1.3.1. Sedate a pig by intramuscular (i.m.) injection of tiletamine (3.75 mg/kg) and zolazepam (3.75 mg/kg) and dexmedetomidine (37.5 μg/kg). Wait for it to become unconscious.

1.3.2. Prepare an intravenous line by inserting a 20 G cannula into the ear vein.

NOTE: Make sure the cannula is in the vein by injecting 5-10 mL of saline through the cannula. If the vein has been missed, this will be noticeable by small edema in the ear tissue.

1.3.3. Intubate the pig to ensure that the breathing rate can be regulated throughout the surgery.

NOTE: Ensure successful intubation by applying pressure on the pig's thorax and confirm that forcibly expired air is coming out of the intubation tube.

1.3.4. Attach the intubation tube to a ventilator set to a breath rate of 14 breaths/min.

1.3.5. Connect a pulse oximeter and cuff to the tail to monitor the heart rate (HR), blood pressure (BP), and oxygen saturation (sats). Insert a rectal thermometer to monitor the core temperature.

1.3.6. Prepare an IV bag of ketaminol (5 mg/kg/min), midazolam (0.25 mg/kg/min), and fentanyl (2.5 μg/kg/min), in saline and begin to infuse through the ear vein at approximately 10 drops/s.

NOTE: Throughout the surgery, the infusion rate may need to be increased or decreased based on the animal's vitals.

1.3.7. With the pig in the prone position, palpate the back of the head and neck of the animal to locate and mark the occipital crest and spine of the first thoracic vertebrae and the base of each ear.

1.3.8. Draw a straight line between the crest and the vertebrae along the longitudinal axis. Draw two lines from the crest to the base of each ear by following the base of the skull (**Figure 1A**).

1.3.9. Check that the animal is in a deep sleep by carefully clamping the tail and watching for a reflex.

NOTE: If the animal is still reflexive, the anesthetic infusion rate should be incrementally increased until the animal no longer exhibits a reflex.

2. Surgery

NOTE: All through the surgery, it is necessary to have at least one assistant to suction the light bleeding and cauterize any severed vessels.

2.1. Using a scalpel with a # 21 blade, make a dermal incision along the longitudinal line down to the muscle.

2.2. Extend two perpendicular dermal incisions further along the shoulders, 10-15 cm in length.

2.3. From the occipital crests, make dermal incisions along the line down to the base of each ear.

2.4. Gripping the skin corners formed at the occipital crest with anatomical forceps, carefully separate the skin from the underlying muscle by lightly running the scalpel blade over the fascia, moving from the rostral to caudal. Once the skin has been resected following each of the five incisions, parts of the trapezius muscles should then be visible.

2.5. Make a longitudinal incision with the scalpel, approximately 1 cm deep, where the trapezius comes together at the midline.

NOTE: When cutting through the muscles, there is an increased propensity for bleeding, so the cauterizer should be ready. If a larger vessel is severed, one person should quickly compress it with the gauze, while the other person uses the cauterizer.

2.6. Using a combination of straight and curved surgical forceps, perform blunt dissection working along the longitudinal cut in the muscles. This will separate the bellies of the trapezius, as well as the underlying semispinalis capitus biventer muscle.

2.7. Sever any persisting muscle fibers with a scalpel and continue blunt dissection until semispinalis capitus complexus becomes visible.

2.8. Sever the origins of the trapezius and semispinalis capitus biventer muscles along the posterior aspect of the skull. Carefully separate them longitudinally with the scalpel performing blunt dissection until the semispinalis capitus complexus is fully visible.

2.9. Retract the trapezius and semispinalis capitus biventer muscles using self-retaining retractors.

2.10. Where the bellies of the semispinalis capitus complexus come together in the midline, make a longitudinal incision with the scalpel approximately 1 cm deep.

NOTE: Be aware for any additional bleeding here. Bleeding can be managed using a combination of cotton swabs and cauterization.

2.11. Using surgical forceps, perform a blunt dissection working along the longitudinal cut between the muscle bellies until both the atlas and axis are palpable.

2.12. Sever the origins of the semispinalis capitus complexus muscles along the posterior aspect of the skull and separate it longitudinally from the underlying vertebrae by scalpel and blunt dissection.

2.13. Retract the semispinalis capitus complexus muscles using another set of self-retaining retractors.

2.14. Using a scalpel, carefully remove any remaining tissue overlying the region where the atlas meets the skull base.

2.15. Placing one arm under the animal's neck and one finger at the juncture of the atlas and skull, simultaneously elevate the head and flex the neck while palpating with the finger to reveal the cisterna magna using the other hand.

NOTE: The cisterna magna is recognizable when palpating as a strong elastic structure with a small amount of rebound as pressure is released with the finger.

3. Cannulation and injection

NOTE: This step also requires at least two people and is carried out with the animal's head elevated and neck flexed.

3.1. Ensure that one person elevates and flexes the head and neck of the animal whilst the other palpates for the cisterna magna making a note of its anatomical location.

3.2. Slowly and carefully introduce a 22 G cannula through the dura and into the cisterna magna at an oblique angle to the longitudinal axis.

NOTE: Do not insert the cannula too deep, as this can cause damage to the brain. Knowing how far to insert the cannula comes with experience in understanding how it feels for the cannula to pierce the dura. Essentially, just as the dura has been pierced, the cannula is then deep enough for a successful tracer injection. This depth is approximately 3-5 mm but will differ based on the size or age of the animal. Successful cannulation should be immediately evident through the visualization of clear, pulsatile CSF ascending the cannula. For the best outcome, it is recommended to practice several cannulations beforehand in euthanized animals to get one's understanding of dural piercing.

3.3. Retract the needle from the cannula and place a cap on the lock.

3.4. First, apply superglue and an accelerator where the cannula enters the tissue, followed by the application of the dental cement. Wait for 5 min for the cement to harden.

3.5. Carefully remove the cap from the cannula and attach the male end of the previously prepared IV line tap with 10 cm extension, with the tracer, to the cannula.

3.6. Slowly inject the tracer by hand or using a micro-infusion pump at a rate of 100 µL/min. Remove the 3-ways IV line tap with 10 cm extension and replace it with the cap. Tracer should now be visible pulsating at the base of the cannula (**Supplementary Video 1**).

NOTE: If injecting by hand, do this until the tracer is just still visible in the cannula shaft, approximately 1-2 mm above where the dental cement is covering the shaft.

3.7. Following injection, place sandbags under the neck to maintain some flexion. The head may then be released, and the animal is left in a resting prone position.

3.8. Release the self-retaining retractors and place muscles as they lay before. Bring the skin together over the muscles using surgical towel clamps.

3.9. Cover towel clamps and incision with gauze and then a blanket to limit heat loss.

3.10. Allow the tracer to circulate for the desired time before sacrificing the animal by i.v. Pentobarbital injection.

4. Brain extraction and processing

4.1. Using a scalpel with 20-blade, extend the longitudinal dermal incision from the occipital crest to approximately 7 cm above the nose.

4.2. Reflect the skin overlying the dorsal aspect of the skull using the scalpel.

NOTE: There are several ways to cut and remove the dorsal aspect of the pig skull on an animal-by-animal basis. What follows is the procedure that has worked most often for this experiment.

4.3. Using a handheld compact saw, make a coronal cut in the skull, approximately 3 cm above the two large veins seen exiting the skull. Extend to two further vertical cuts from the coronal cuts and two more further cuts to bring the vertical cuts together in the midline.

NOTE: Maintain a firm grip of the saw when making the skull bone cuts as it will tend to pull away upon the first contact with the bone or tissue, which can lead to a severe injury.

4.4. Ensure the skull cuts are through the entire thickness of the bone by following up with a hammer and narrow chisel (10 mm) to each of the cuts.

4.5. Using the hammer, finally knock a wide chisel (25-30 mm) into the coronal cut. With one person supporting the head, ensure that the other person applies leverage on the chisel to wince open the dorsal skull.

4.6. Once the dorsal skull fragment has been removed, dissect out the overlying dura mater using curved surgical scissors.

4.7. Use a spatula to severe the spinal cord from the cerebellum at the rostral aspect. Then proceed to guide the spatula under the brain from the front, severing the olfactory bulbs, pituitary gland, and cranial nerves.

4.8. Place the spatula behind the cerebellum and apply a fair amount of pressure to dislodge the brain from the cranial cavity, carefully lifting it out once loose.

4.9. Immediately fix the whole brain by tissue immersion in 4% paraformaldehyde overnight.

NOTE: After this step, it is possible to carry out the whole brain imaging using a stereoscope (**Figure 1E**).

4.10. The next day, make coronal slices of the brain using a salmon knife and fix the slices overnight by tissue immersion in 4% paraformaldehyde.

4.11. Finally, place the slices in 0.01% azide in PBS for long-term storage.

REPRESENTATIVE RESULTS:

Once the pig is unconscious, it is palpated, and its surface anatomy is marked, starting at the occipital crest (OC) and working towards the thoracic vertebrae (TV) and each ear base (EB). It is along these lines that the dermal incisions are made (**Figure 1A**). The three muscle layers including trapezius, semispinalis capitus biventer and semispinalis capitus complexus are

resected and held open by two sets of self-retaining retractors to expose the cisterna magna (CM) (**Figure 1B**). The head is then flexed to open up the space between the back of the skull and the atlas and ease the access to the CM (**Figure 1C**). An 18 G cannula is inserted carefully 3-5 mm into the CM and fixed in place by both super glue and dental cement (DC). Tracer can then be injected at a fixed rate. Once the tracer has been injected, the IV-line and syringe are replaced with a cannula cap (**Figure 1C-D**). The muscles are then put back into the place and the pig is covered and kept warm for the time the tracer is circulated. After circulation, the animal is euthanized, and the brain is quickly removed. It is possible to generate stitched macroscopic images of the dorsal surface of the brain, which facilitate in providing detailed insights into the distribution patterns of tracer on the dorsal brain surface across the sulci and fissures (**Figure 1E**). Similar images can be generated from the ventral and lateral surfaces of the brain where the tracer distribution may be investigated in the temporal lobe (TL) and lateral fissure (LF) (**Figure 1E**). A stereoscope may additionally be utilized to produce higher magnification images of the brain surface where it is possible to see the tracer in the PVS along arteries (**Figure 1F-G**). Macroscopic coronal brain sections, approximately 8 mm thick, are cut using a salmon knife and provide further insight into the depth of tracer penetration in the interhemispheric fissure (IHS), as well as subcortical tracer distribution in structures such as the hippocampus (HPC) and striatum (STR), (**Figure 1H**).

Immunohistochemical staining for AQP4 expressed at astrocytic endfeet, glial-fibrillary acidic protein (GFAP) expressed throughout astrocytes and smooth muscle actin (SMA) located around arterioles showed that the tracer localized both within the PVS and moves into the brain parenchyma (**Figure 1I-N**). AQP4 and GFAP staining are used to identify astrocytes and more specifically, the astrocyte foot processes that form the outer surface of the PVS while lectin and glut-1 stain the endothelial cells that form the inner surface of the PVS (**Figure 1I-M**). By carrying out these stains to define the PVS boundaries, it is possible to then identify CSF-injected tracer localized to the PVS space. This supports the notion that CSF gains access to the gyrencephalic brain via extensive PVS transport which then facilitates glymphatic influx into the brain parenchyma. SMA staining identifies arteries and arterioles by binding to smooth muscle cells found in arterial walls and can be used to show that PVS influx occurs along arteries as opposed to veins, which constitutes the basic physiology of normal glymphatic function (**Figure 1N**).

FIGURE AND TABLE LEGENDS:

Figure 1. Cisterna magna cannulation in pigs. (**A**) Pig prepped prior to the start of the surgery and marked where dermal incisions will be performed starting from the occipital crest (OC) then posterior to thoracic vertebrae (TV) and lateral to each ear base (EB). (**B**) Head in the relaxed position with the trapezius, semispinalis capitus biventer and semispinalis capitus complexus muscles retracted, thus exposing cisterna magna (CM). (**C**) Head flexed manually to increase access to CM for cannulation and injection. (**D**) Close up image of a cannula inserted into CM after injection and fixed in place with the dental cement (DC). (**E**) Dorsal, ventral, and lateral brain surfaces respectively after fluorescent imaging with accompanying structural white light images. Areas of interest that are visible at these surfaces include the interhemispheric fissure (IHS), temporal lobe (TL), and lateral fissure (LF). (**F**) Structural white light image of the artery and veins on the brain surface. (**G**) Fluorescent image of (**F**) showing the tracer distribution along the

surface artery. (**H**) Macroscopic slices from the anterior and posterior cerebral regions show two-dimensional tracer dispersion and distribution in fissures (LF, IHS) and subcortical structures like the striatum (STR) and hippocampus (HPC). (**I-J**). Confocal images showing the tracer in the PVS, bounded by lectin-stained endothelial cells internally and AQP4 on astrocyte foot processes externally. (**L-M**). Confocal images showing the tracer in the PVS, bounded by cells internally with astrocyte foot processes visible forming an outer boundary. (**N**) Confocal image showing the tracer in the PVS around an arteriole stained for smooth muscle actin (SMA) with tracer also visible in and around, surrounding the brain parenchyma. CM, cisterna magna; DC, dental cement; EB, ear base; HPC, hippocampus; IHS, interhemispheric fissure; LF, lateral fissure; OLB, olfactory bulb; OC, occipital crest; STR, striatum; TL, temporal lobe; TV, thoracic vertebrae.

Supplementary Video 1: CSF pulsation after the tracer injection. Close up video of the cisterna magna after the tracer injection. Blue tracer is visible in the cannula neck pulsating at the rhythm of the CSF and is indicative of a successful cannulation and injection.

DISCUSSION:

Herein, is described, a detailed protocol to perform the direct cannulation of the cisterna magna in pigs, including the necessary preparation, surgical procedure, tracer infusion and extraction of the brain. This requires someone with experience and certification for working with large animals. If carried out correctly, this allows for the delivery of desired molecules with surety directly into the CSF, after which a series of different advanced light imaging modalities can be used to explore CSF distribution and glymphatic function at high resolution in a large mammal.

It is important to note that although this is the same procedure as cisterna magna cannulation in rodents, it is slightly more challenging and requires several hours of training. Such training includes the handling of large mammals under laboratory conditions, an understanding of the anatomy and the musculoskeletal system, specifically in pigs, and some degree of proficiency in using surgical instruments. Once these criteria have been met, it is possible to carry out this technique, which thus far has a 100% success rate compared to an 80-90% success rate in mice. The most critical point for performing the procedure correctly, is elevating the head and flexing the neck while inserting the cannula and infusing the tracer. Although tracer was injected here by hand, it was done so in a controlled manner of 100 μL per minute. In mice 10 μL of tracer is typically injected and when directly comparing brain sizes this would translate to approximately 2 mL in a 50 kg pig[6,11,18]. Therefore, the injection of 500 μL of tracer was in fact a conservative volume and should not have produced extended perturbances in intracranial pressure (ICP). Additionally, it has recently been shown that perivascular glymphatic function is not simply an artifact of transient increases in ICP but persists when ICP is maintained at the baseline using a dual syringe method further strengthening the notion that these findings do not reflect artifacts of altered ICP[19].

This is not the only technique that can be used to perform CMc in pigs, and although it is substantially more invasive, it appears to give a more accurate tracer infusion. Another way to perform CMc in pigs is by lying them on their side in the lateral recumbency position and going in blind with a 150 mm spinal needle[20]. Although this was an attractive method due to its minimal

invasiveness, the potential success rate was perceived to be lower. Since the back of the pig head is flat and CM sits very deep (10-12cm) from the surface, the spinal needle has a long distance to travel before penetrating the CM, thus limiting the certainty of successful cannulation. Apart from the large distance to the CM the diameter of the CM itself is only about 10 mm, further reducing the chance of successful cannulation. In contrast, by utilizing the direct CMc method, it is possible to directly visualize the cannulation and thus know with certainty that it has been successful and that the agents have been delivered to the CSF and not leaked out into surrounding soft tissue. Ensuring successful cannulation is important for such experiments owing to the high cost of pigs, surgery facility, and fluorescent tracers, as well as to minimize the number of pigs used.

The limitations of this method, apart from the invasiveness, is that the cost and time discourages many repetitions as compared to rodents. The first surgery carried out took around 3 hours, but it is currently being performed in approximately 45 minutes. This represents a significant time improvement, however, to perform a cannulation in a mouse, it takes less than 5 minutes for a skilled researcher, meaning the actual surgery time upon reaching proficiency is still 9 times longer than in mice. Additionally, the large brain means the tracer circulation times in the pig are more extensive, for example, 2-6 hours, while in mice a standard circulation time is 30 minutes. Apart from the high cost of the tracer, needed in large volumes for the pig, the actual cost of the pig itself as well as its housing, anesthetics, and cost of using a full operating theater make the end cost of this procedure for one pig 15 times more expensive than in a single mouse. An additional time-related challenge is the time taken for the brain extraction after the tracer circulation. Previous reports have shown that some movement of the tracer through PVS persists after euthanasia[21]. This makes it important to extract brains, as quickly as possible, to minimize any confounding effects from this phenomenon. While the mouse brain extraction only amounts to a few minutes, pig brain extractions take approximately 15-20 minutes of time. The brain should be removed as quickly as possible to limit this effect but with the thickness and architecture of the pig skull it is difficult to reduce the current extraction times.

Although direct cannulation makes the procedure fairly invasive, overall blood loss only averaged 100 mL per surgery, which constitutes a loss of less than 3% of the total blood volume. Furthermore, the animal receives a continuous saline infusion with the anesthetics and an additional IV line of Ringers' lactate, mitigating the risk of hypovolemia.

Future studies are needed to explore the translation of the glymphatic physiological drivers identified in mice, as well as glymphatic function in awake or naturally sleeping swine removes the impact of anaesthetics[22,23]. In order to investigate the natural sleep or awake state, it will be necessary to adapt the current protocol such that tracer can be delivered via less invasive means whilst still maintaining a high success rate. This could potentially be achieved by carrying out CM injections under computed tomography fluoroscopy, which has been previously utilized for lumbar puncture in pigs[24]. Going forward, it would be of great interest to combine this technique with genetic manipulations of the AQP4 water channel to understand its role in glymphatic function in a large mammal. In exploring the full extent of the glymphatic system in a large mammal, the field moves closer to understanding glymphatic function in humans and how it

might be utilized therapeutically.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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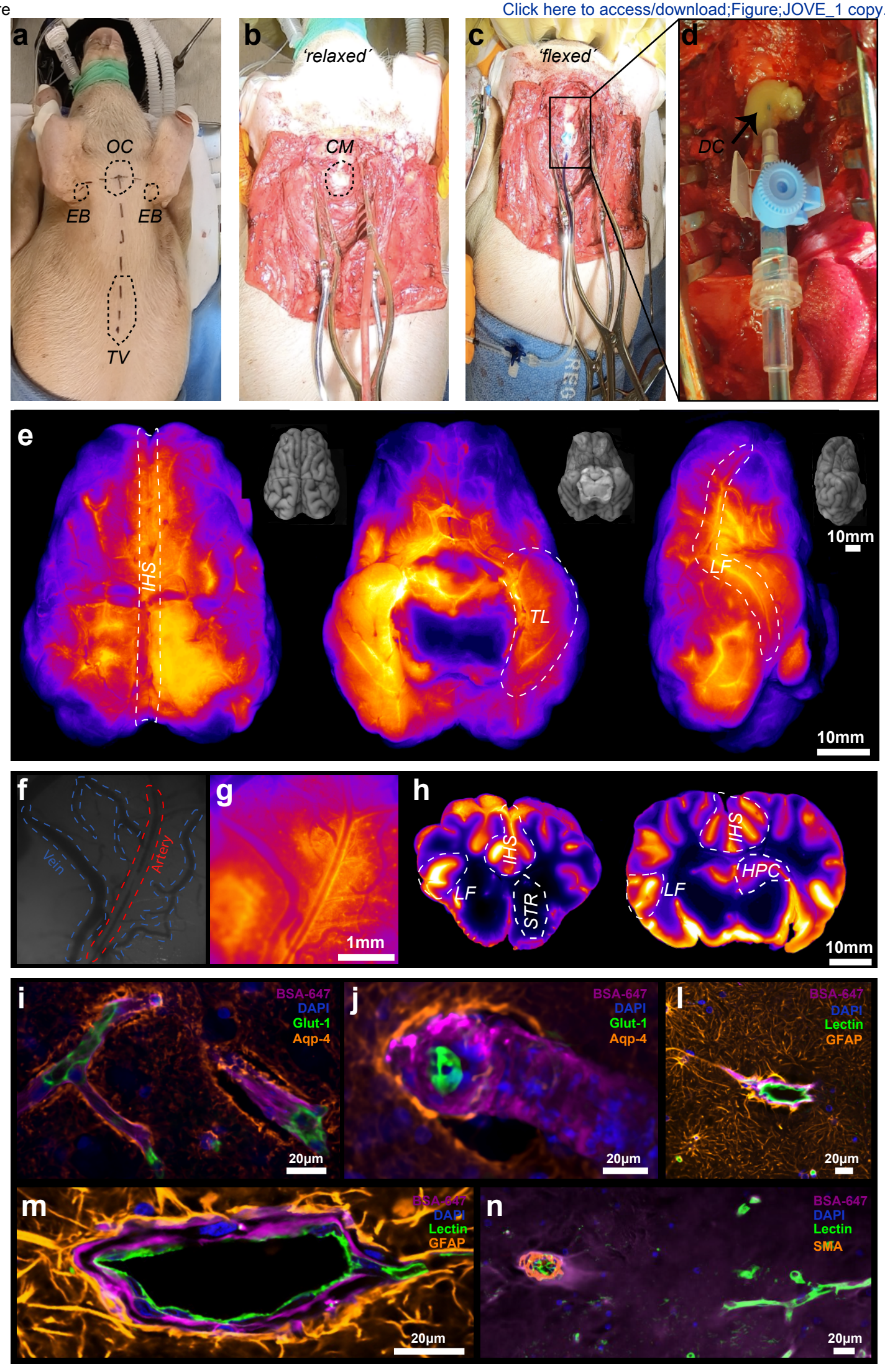

Video or Animated Figure

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Video or Animated Figure

Pulsation.mp4

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_20210407.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
0.01% azide in PBS	Sigmaaldrich	S2002	
18G needle	Mediq		
1ml Syringe	FischerSci	15849152	
20G cannula	Mediq	NA	
22G cannula	Mediq	NA	
4% paraformaldehyde	Sigmaaldrich	P6148	
Anatomical forceps	NA	NA	
Bovine serum albumin Alexa-Fluor 647 Conjugate	ThermoFischer	A34785	2 vials (10mg)
$CaCl_2$	Sigmaaldrich	C1016	
Chisel	ClasOhlson	40-8870	
Dental cement	Agnthos	7508	
compact saw	ClasOhlson	40-9517	
Glucose	Sigmaaldrich	G8270	
Hammer	ClasOhlson	40-7694	
Insta-Set CA Accelerator	BSI-Inc	BSI-151	
IV line TAP, 3-WAYS with 10cm extension	Bbraun	NA	
KCl	Sigmaaldrich	P9333	
Marker pen	NA	NA	
$MgCl_2$	Sigmaaldrich	M8266	
MilliQ water	NA	NA	
NaCL	Sigmaaldrich	S7653	
NaH_2PO_4	Sigmaaldrich	S8282	
$NaHCO_3$	Sigmaaldrich	S5761	
No. 20 scalpel blade	Agnthos	BB520	
No. 21 Scalpel blade	Agnthos	BB521	
No. 4 Scalpel handle	Agnthos	10004-13	
Saline	Mediq	NA	
Salmon knife	Fiskers	NA	
Self-retaining retractors	NA	NA	

Superglue	NA	NA
Surgical curved scissors	NA	NA
Surgical forceps	NA	NA
Surgical towel clamps	NA	NA

Reviewer #1:

Manuscript Summary:

In this manuscript the authors present a scaled up version of the typical cisterna magna injection of tracer to the CSF system in order to study the glymphatic system. The level of detail provided in the protocol is sufficient for someone well-versed in large animal surgical techniques to follow and complete the procedure. The authors present only the surgical techniques with the post-mortem analysis methods left up to the reader.

Minor Concerns:

In the methods describing creation of the cannula the authors introduce an air-bubble to separate saline and tracer but they do not state what the purpose of this air bubble is. Could not the whole syringe cannula system be filled with tracer and only 500 uL injected? If the air bubble is used to denote when to stop injecting I would question how one can see that the air bubble has reached the tip of the implanted cannula as it is obscured under dental cement. Also, would it be a problem if a small volume of air is injected into the cisterna magna? Perhaps these are addressed in the video but the purpose/use of the air bubble is not mentioned anywhere in the manuscript.

We thank the reviewer for the opportunity to clarify these points. The air bubble is indeed used to denote when to stop injecting the tracer. The reviewers observation is correct in that the dental cement conceals the final millimeters of the cannula. Thus when injecting we do so until tracer is visible 1-2mm above where the cannula becomes concealed by the dental cement. To clarify that no air is injected, we have added this as a NOTE in the manuscript:

"NOTE: If injecting by hand this should be done until tracer is just still visible in cannula shaft, approximately 1-2mm above where dental cement is covering the shaft."

In the discussion, the authors make note of several limitations of this scaled up version as compared to the typical rodent procedure including the cost of the procedure as well as the extended operating and tracer circulation time. Another factor that may be of interest to someone attempting to use this technique in their study is the approximate time required to extract the brain from the skull after euthanasia and the implications of that time delay. In Ma et al. (https://doi.org/10.1007/s00401-018-1916-x) it was reported that in mice there was a rapid movement of tracer from the paravascular space into brain parenchyma after euthanasia. In rodents the brain can be extracted from the skull within minutes, possibly even under a minute, and placed into fixative to mitigate this movement or alternatively the rodent could be transcardially perfused with fixative prior to removing the brain. Presumably it takes a substantially longer time to extract the pig brain, during which time the phenomenon described by Ma et al. would be taking place and potentially confounding results. So it would be good to know whether the authors feel that this is a limitation which should be recognized and, if so, how it can be mitigated or addressed.

We thank the reviewer for bringing this to our attention. The time needed for brain extraction in pigs is indeed another challenge for this technique. It goes without saying that the brain should be extracted and fixed as quickly as possible to avoid the artifacts described by Ma et al., however this minimum time of extraction is significantly longer than in small rodents which is difficult to reduce in thick skulled pigs. However if all brains are extracted over similar time periods this could minimize data variability based on Ma et al. findings. We would not encourage transcardial perfusion fixation both because it is not so feasible in the pigs and more so because

of the consequence of PVS collapse as described in Mestre et al, 2018 (PMID: 30451853). We have added a section in the discussion to address some of these points:

”An additional time-related challenge is the time taken for brain extraction after tracer circulation. Previous reports have shown that some movement of tracer through PVS persists after euthanasia[21]. This makes it important to extract brains as quickly as possible to minimize confounding effects from this phenomenon. While mouse brain extraction only amounts to a few minutes pig brain extractions take approximately 15-20 minutes. Needless to say that the brain should be removed as quickly as possible to limit this effect but with the thickness and architecture of the pig skull it is difficult to reduce current extraction times.”

Secondly, several studies in the glymphatic literature indicate that anesthesia affects the glymphatic system with significant differences in effects based on the anesthetic used. In this method, the animal is fully anesthetized throughout the whole protocol including during tracer circulation. Future researchers attempting to utilize this approach would probably like to perform some sort of experimental treatment in order to answer their question that would require either an awake behaving animal or at least to not have the confounds associated with the glymphatic system and anesthesia. This is probably out of the scope of this paper as its primarily a demonstration of methods (which it did well!) but I'd be curious to know how the authors foresee addressing this issue or adapting their protocol to accommodate future experimental needs.
We thank the reviewer for raising these points. We have added a some points concerning future expansion of this work and how it could be possible to achieve experiments in an awake animal.

“Future studies are needed to explore the translation of the glymphatic physiological drivers identified in mice as well as glymphatic function in awake or naturally sleeping swine absent the impact of anaesthetics[22, 23]. In order to investigate the natural sleep or awake state, it will be necessary to adapt the current protocol such that tracer can be delivered via less invasive means whilst still maintaining a high success rate. This could potentially be achieved by carrying out CM injections under computed tomography fluoroscopy which has been previously utilized for lumbar puncture in pigs[24]”

Reviewer #2:
Manuscript Summary:
Bechet et al., illustrated direct cannula implantation method in the cisterna magna of pigs to study glymphatic clearance.
Although cisterna magna cannulation has been often carried out in rodents, understanding the glymphatic system in higher mammals is essential for its translation to humans. The authors should consider adding following information.

Major Concerns:
1) This reviewer would suggest the authors to provide justification to use pigs as higher mammals to study glymphatic clearance. How closely do the pigs resemble the human compared to rodents in terms of neuroanatomical structures?
We thank the reviewer for raising this question. Overall the main takeaway is that the pig brain is more similar to humans than that of a rat or mouse as there are indeed several differences between pig brains versus humans and other primates. However, apart from

overall brain size, a fundamental neuroanatomical similarity of importance for us and the glymphatic field when comparing pig and human brains is the presence of sulci and gyri as opposed to the smooth surfaced lissencephalic rodent brain. We hypothesize that the folded cortical architecture differentially impacts overall CSF distribution as compared to lissencephalic brains and this is why it is important to study the glymphatic system at high resolution in a gyrencephalic brain. In terms of other similarities pre and post-natal CNS development between humans and pigs is highly similar (Dickerson & Dobbing, 1967, PMID: 24796035; Lind et al, 2007, PMID: 17445892) and the topology of the hippocampus points to a level of encephalization between rodents and primates (Holm & West, 1994, PMID: 8061750). Additionally although far less complex than the human brain pigs possess approximately 430 million (Jesling at al, 2006, PMID: 16574805) neocortical neurons versus 21 million in rats (Korbo et al, 1993, PMID: 8508308).
To address this we have added more information to the introduction:

"Pig and human brains differ substantially from rodents in that they are gyrencephalic, possessing a folded neuroarchitecture, while rodent brains are lissencephalic. In terms of overall size pig brains are also more comparable to humans being 10-15 times smaller than the human brain while mouse brains are 3000 times smaller."

2) Direct cannula implantation would potentially cause damage in brain. Can the authors describe how deep they insert the cannula below the dura in 3. Cannulation & Injection? How do the authors ensure that brains are intact during this procedure?
We thank the reviewer for raising these points. Concerning absolute depth we introduce the cannula 3-5mm into the CM, however this depth may vary based on individual variation and size of the animal used. The best metric to gauge successful cannulation is by stopping cannula insertion immediately after feeling the piercing of the dura and waiting for visual confirmation of pulsatile, clear CSF in the cannula. If this is observed the cannula has been correctly introduced to a suitable depth for injection of tracers. To address this we have added an additional NOTE in the methods section:

"NOTE: It is important not to insert the cannula too deep as this can cause damage to the brain. Knowing how far to insert the cannula comes with experience in understanding how it feels when the cannula pierces the dura. Essentially just as the dura has been pierced the cannula is then deep enough for successful tracer injection. This depth is approximately 3-5mm but will differ based on the size or age of the animal. Successful cannulation should be immediately evident through the visualization of clear, pulsatile CSF ascending the cannula. For the best outcome it is recommended to practice several cannulations beforehand in euthanized animals to get one's own understanding of dural piercing."

LUND UNIVERSITY

Biomedical Centre
Department of Experimental Medical Science
Glia-Immune Interactions

The Editorial Office
JOVE

3rd March 2021

Dear Dr. Goldman & Dr. Al Dalahmah,

We would like to submit the attached manuscript, entitled ***"Direct cannula implantation in the cisterna magna of pigs"*** by Bèchet et al as an article in the methods collection, *Understanding Astrocyte Function: Current Methods and Techniques,* in the *Journal of Visualized Experiments.*

The glia-lymphatic (glymphatic) system is a brain-wide system that acts to clear the neuropil of metabolic waste, including toxic proteins such as amyloid beta and tau. The basis of this clearance rests upon the movement of cerebrospinal fluid (CSF) from perivascular spaces (PVS) into the brain neuropil, which clears the waste as it moves, and can be mapped by introducing fluorescent tracers into the CSF via cisterna magna (CM) cannulation. The PVS-to-neuropil CSF motion is highly dependent on astrocytes, whose foot processes form the outer bound of the PVS and which exhibit a polarised expression of the water channel, aquaporin-4 (AQP4). Subsequently knockout of AQP4 in mice results in significant declines in global glymphatic function.

Yet, the implication of astrocytes and AQP4 in glymphatic function has been limited only to rodents. Some glymphatic work has been carried out in humans and non-human primates using magnetic resonance (MR) imaging, however, the low spatial resolution only lends insight to macroscopic phenomena of glymphatic function. Thus, there are fundamental gaps in our knowledge concerning the glymphatic system in large mammalian brains, specifically concerning the microscopic machinery that has been so well described in rodent experiments.

Thus, in order to investigate the glymphatic system and its microscopic machinery in a large mammal with a gyrencephalic brain similar to that of humans it was necessary to translate the CM cannulation procedure used in rodents to pigs. Herein, we describe the technique of direct cannula implantation in the CM of pigs in order to introduce fluorescent tracers and study the glymphatic system at microscopic resolution in a large mammal. This permitted visualisation of the microscopic features of the glymphatic system in the gyrencephalic brain. The demonstration of tracer penetration into the brain parenchyma from perivascular spaces surrounded by astrocyte foot processes expressing aquaporin-4 water channels confirms the phenomenon observed in rodents.

CM cannulation in pigs allowed for the first detailed study showing that the glymphatic system described and characterised in rodents persists in the gyrencephalic mammalian brain. We believe that the use of CM cannulation in pigs pushes us closer to understanding the nature of the glymphatic system in humans and will help strengthen the notion that it is possible to derive therapeutic benefit to neurodegenerative disorders through glymphatic manipulation. It is our hope that this technique serves as an inception point for a new stage of glymphatic experiments that will include animal models that more accurately depict human neurophysiology.

We would like to suggest the following reviewers: Per Christian Eide (human glymphatic research), Lauren Hablitz (glymphatic system), Joanna Wardlaw (glymphatic system) and Aravind Asokan (glymphatic system). We would like to exclude Roxana Carare, Roy Weller and Alan Verkman as reviewers due to competing interests.

Sincerely,

Iben Lundgaard