METHOD

Rat Cerebrospinal Fluid Treatment Method through Cisterna Cerebellomedullaris Injection

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Abstract Drugs that lack the ability to cross the blood-brain barrier (BBB) need to be placed directly into the central nervous system. Our laboratory studies the involvement of the glutamatergic system in the aggressiveness of glioma, and some ligands of glutamate receptors cannot permeate the BBB. Here, glioma-implanted rats were treated by a technique that delivers ligands directly into the cerebrospinal fluid by puncture into the cisterna cerebellomedullaris. Rats were anesthetized and fixed in a rodent stereotactic device. The head was gently tilted downwards at an angle that allowed exposure of the cisterna. Injection into the cisterna was done freehand using a gingival needle coupled to a microsyringe. The efficiency of intracisternal injection was demonstrated using a methylene blue solution. This type of injection is adaptable for any rodent model using small volumes of a variety of other drugs, and is an interesting method for neuroscience studies.

Keywords Neurobiology · Neuroscience · Drug administration · Intracisternal injection · Cisterna magna · Cerebrospinal fluid treatment · Central nervous system · Surgical technique · Rodent

Introduction

The brain protects itself from pathogens and toxins derived from blood by severely restricting the transport of cells, particles, and most hydrophilic molecules across the vascular endothelium of its blood vessels. This protective mechanism is known as the blood-brain barrier (BBB) [1]. Some drugs are unable to cross the BBB, requiring direct central nervous system (CNS) delivery. To circumvent the BBB, these drugs can be delivered directly into the cerebrospinal fluid (CSF). CSF is initially derived from blood serum that has been filtered across BBB and improved by the choroid plexuses, which are found in each ventricle. Therefore, CSF is generated in the ventricles and flows unidirectionally from the lateral ventricles through the 3rd ventricle into the 4th ventricle, then empties into the cisterna cerebellomedullaris (also known as cisterna magna in humans) where it is distributed over the outer surface of the brain (subarachnoid space). From there, the CSF is reintegrated into venous blood through arachnoid villi and arachnoid granulations [2].

Metabotropic glutamate receptors (mGluRs) are G-protein-coupled receptors that participate in the modulation of synaptic transmission and neuronal excitability. mGluRs are classified into three groups based on sequence homology, G-protein coupling, and ligand selectivity: group I (mGluRs 1 and 5), group II (mGluRs 2 and 3), and group III (mGluRs 4, 6, 7, and 8) [3]. Their ligands have demonstrated neuroprotection *in vitro*, but limited applicability in animal models due to the low BBB penetration. Although in recent decades many potent and selective mGluR ligands, which are known to be effective in *in vivo* studies [4], have been synthesized, some group-III-selective agonists, *e.g.*, L-(+)-2-amino-4-phosphonobutyric acid, L-Serine-O-phosphate, and (RS)-4-

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phosphonophenylglycine, do not present oral bioavailability or BBB penetration [5]. Several studies using mGluR ligands have demonstrated their involvement in the progression, aggressiveness, and recurrence of a highly malignant type of CNS tumor, glioblastoma (GBM) [4]. To evaluate the role of mGluRs as biomarkers and therapeutic targets in this neoplasia, our research group has performed *in vivo* experiments in rats implanted with C6 lineage cells and treated with the same mGluR ligands generally used *in vitro*.

The most-cited routes for direct CNS delivery in rodents are intracerebroventricular and intracisternal, although other studies have also described direct injection into cerebral tissue. The intracerebroventricular injection method is often carried out stereotactically and histological verification of the accuracy of the injection is required to generate reliable data [6]. Most studies perform these treatments in the lateral ventricles or in the 4th ventricle [7, 8]. Intracerebral injection (e.g., intrahippocampal or intrastratial) is also accomplished using stereotactic vectors to locate the cerebral structure within the rodent brain [9–11]. The most common intracisternal route is the cisterna cerebellomedullaris, at the base of the skull [12]. Frequently, these procedures involve stereotactic placing of an injection cannula in a specific brain region. For chronic treatment, a cannula connected to an injection device makes it possible to infuse microliter quantities of drugs.

GBMs expand and eventually move brain structures, changing their stereotactic coordinates [13]. Thus, implantation of an injection cannula in rats implanted with C6 cells is impractical, since it is not possible to be certain that a treatment reaches the expected location. In addition, if animals have already undergone GBM-implantation surgery, another surgical procedure could cause more suffering. Hence, intracisternal injection is a methodological option for *in vivo* treatment with mGluR ligands, through adaptation of a CSF puncture protocol realized in rodents. This type of injection resembles the intrathecal administration of drugs in humans. Using this method, the rats receive treatment directly into the CSF by puncture of the cisterna cerebellomedullaris once a week for a total of 3 treatments. The advantage of this strategy is that stereotactic surgery is not needed, so it is less invasive than other methods and allows faster recovery. The animal is only positioned in a stereotactic apparatus for head immobilization, and injection is done manually.

Materials and Method

This protocol was approved by Ethics Committee on the Use of Animals of the Universidade Federal do Rio Grande do Sul (CEUA-UFRGS Protocol 31573) and was in

compliance with the National Institutes of Health Guidelines for the Use of Experimental Animals. Wistar rats (60 days old; $n = 10$ for the mGluR ligand study; $n = 2$ for demonstration of the injection area with methylene blue solution) were used. Two researchers participated for the maximally efficient implementation of this procedure, one to puncture the cisterna with the needle and the other to inject the treatment solutions.

Preparation of Treatment Solutions

1. Prepare the treatment solutions in a sterile environment.
2. Store the treatment solutions in well-identified 1.5 mL microcentrifuge tubes.

Materials

1. Inhalation anesthetic delivery system and inhalation anesthetic.
2. Medical oxygen.
3. Confinement chamber adapted to receive the inhalation anesthetic.
4. Inhalation mask adapted for rodents retained in the stereotactic frame with the head lowered (an adaptation with a plastic Pasteur pipette is possible; Fig. 1C).
5. Stereotactic frame.
6. Insulation between the stereotactic frame and the animal to prevent loss of body heat.
7. Precision microsyringe (10 μ L).
8. Support base for the microsyringe (approximately 3 cm high).
9. Adhesive tape.
10. Insulin syringe (0.45 mm $\times$ 13 mm; 26 G).
11. Gingival needle (0.33 mm $\times$ 22 mm; 30 G).
12. Ultrafine and flexible tube used in high-performance liquid chromatography (HPLC) devices (30 G).
13. Dental floss, timer, light fixture, heating pad.
14. Sterile gauze and sterile saline as injection fluid and for eye lubrication.
15. 70% ethanol.
16. Treatment solutions and shelf for centrifuge microtubes.
17. Personal protective equipment.

Pre-injection Procedure

1. Thoroughly clean the work area with 70% ethanol and organize the materials.
2. Place isoflurane solution inside the anesthesia system and turn on the heating pad and oxygen/isoflurane system.

Fig. 1 Materials used to assemble the injection setup, as well as the assembled structure. **A, B** Apparatus for intracisternal injection: gingival needle (0.33 mm × 22 mm; 30 G), ultrafine and flexible tube used in HPLC devices (30 G), and precision microsyringe (10 µL). The free-hand injection rate was controlled by moving the plunger of the microsyringe to each marking, always observing the timer. **C** Mask adapted for inhalation anesthesia (plastic Pasteur pipette).

Preparation for Treatment

The following procedures can be carried out by one of the researchers while the other managing the animal (next section).

1. Fill a sterile insulin syringe with saline solution.
2. Gently attach the syringe needle to the ultra-thin tube. Be CAREFUL not to pierce the tube or cut your finger.
3. Open the back protector of one of the sterile gingival needles and attach the needle to the free end of the tube. Be CAREFUL to not pierce the tube or cut your finger.
4. If the gauge of the tube is larger than that of the needle, use dental floss to firmly tie the tube to the base of the needle barrel to prevent overflow.
5. Remove the gingival needle protector and inject the saline contained in the insulin syringe to fill the entire tube and gingival needle. Remove the drop of solution that forms on the bevel by touching the needle on sterile gauze and reattach the needle protector.
6. Remove the insulin syringe from the tube, reattach the needle protector, and leave this syringe in an accessible place for use before the next treatment.
7. Connect the free end of the tube to the microsyringe. Secure the syringe to the support base with adhesive tape, leaving the volume markings visible.

8. Pull the plunger of the microsyringe out to the 1 μL mark to form a bubble inside the gingival needle.
9. Remove the protector from the gingival needle and place it into the centrifuge microtube containing the treatment solution to be injected.
10. Gently pull the syringe plunger to pick up 3 μL –5 μL of the desired treatment solution. Observe while the tube is filled. Be very CAREFUL to not to form bubbles.
11. Check the 1 μL bubble between the fluid filling the tube and the treatment solution. Replace the protector of the gingival needle and leave the treatment components close to stereotactic frame.

Anesthesia of Animals and Positioning in the Stereotactic Frame

1. Turn the isoflurane to 5% and O_2 to 0.4 L/min and direct the gas flow to the anesthetic chamber. Place the rat in the chamber, wait until breathing is significantly slowed, and remove when it is completely unconscious.
2. Position the gas flow towards the stereotactic frame to keep the animal anesthetized. Place it on the inhalation mask positioned in the stereotactic frame for a little more time and perform a toe pinch to ensure that the rat is completely unconscious.
3. Place the anesthetized rat on the stereotactic frame. Secure the head with the ear bars, leaving the animal's head level and centered.
4. Gently tilt the animal's head downwards, leaving the snout at an angle of $\sim 90^\circ$ in the vertical axis and place the nasal clamp over the region just above the eyes. Be CAREFUL not to injure the animal. This position allows better exposure of the cavity in which the cisterna is located.
5. Check the cavity with your forefinger and shave the hair in this region.
6. Lubricate the animal's eyes with saline. Turn the isoflurane level down to 2%–3% for maintenance. It is important to occasionally check to make sure that rat is completely unconscious with a toe pinch throughout the injection procedure.
3. Position the needle at an angle of $\sim 60^\circ$ to the horizontal axis with the needle bevel facing the researcher.
4. Push the needle exactly into the middle of the cavity of the cisterna.
5. Puncture the cisterna delicately by lowering the needle at the same inclination (60°). First you feel the needle piercing the animal's skin. By gently lowering the needle you feel a small puncture resistance; at this point, you are piercing the dura mater and the arachnoid mater. The depth of needle insertion is ~ 8 mm. When you feel the needle pierce, advise your fellow researcher to gently pull on the microsyringe plunger.
6. If the needle bevel has exactly hit the cisterna cerebellomedullaris, CSF will rise after the plunger is pulled. This process is observed as a backflow of the treatment solution inside the syringe along with the 1 μL bubble between the saline and the treatment solution.
7. If the needle bevel has not reached the cisterna, CSF will not rise. In this case, the treatment solution does not rise, but saline backflows inside the syringe and the bubble formed between treatment and saline increases in size (vacuum formation).
8. If you have not reached the right spot, lower the needle a little more and ask your colleague to gently move the plunger back and forth until you find the point where the cisterna is located.
9. When you find the spot, ask your colleague to start the timer and inject the treatment solution at a very slow rate (~ 0.5 $\mu\text{L}/\text{min}$), always observing the timer.
10. After injection of the entire treatment solution (be CAREFUL to not inject the 1 μL bubble of air), remain holding the gingival needle for ~ 2 min to prevent backflow of the treatment solution. This period varies according to volume applied.
11. Remove the needle very gently to prevent backflow of the treatment solution.
12. Remove the animal from the stereotactic frame and place it on the heating pad until complete recovery of consciousness.
13. Monitor the animal daily for at least 3 days after injection.

Injection of Treatment Solutions Directly into CSF

1. Check again with your forefinger the depression in which cisterna cerebellomedullaris is located.
2. Remove the needle protector from the injection setup. Hold the needle guard with the thumb and forefinger of one hand and, using the forefinger of the other hand, gently lift the tube over the animal's head and hold the part of the tube to which the needle is connected with this hand.

Representative Results

The aim of this study was to determine the efficiency of intracisternal injection through the application of methylene blue. For this reason, we do not present our data on the antitumoral effects of mGluR ligands. However, it is

important to emphasize that all 10 animals received delivery of mGluR ligands or saline solution at the cisterna cerebellomedullaris (100% of success rate). The photographs in Fig. 1 show the materials used to assemble the injection setup, as well as the assembled structure. Three microliters of methylene blue (10 mg/mL) were injected into the cisterna cerebellomedullaris (Fig. 2A, B). Five minutes after injection, the rat was euthanized. Fig. 2C shows a cross-section of the spinal cord stained by methylene blue injected into the cisterna. Fig. 2D shows in detail the cerebellum and part of the medulla oblongata. Between these two regions is the methylene blue staining exactly where the cisterna cerebellomedullaris was located.

Since decapitation was performed within a few minutes (5 min) after injection of methylene blue, it stained only the exact place of the injection. Thus, if we had left the methylene blue longer, it would have stained more brain structures around the cisterna cerebellomedullaris. It should be noted that at the time of decapitation, methylene blue was seen flowing along with the CSF.

Discussion

The wide diversity and heterogeneous distribution of mGluR subtypes in the CNS make these receptors particularly attractive drug targets [3]. Many studies have validated the therapeutic utility of mGluR ligands in neurological and psychiatric disorders including depression, anxiety disorders, schizophrenia, pain syndromes, epilepsy, Alzheimer's disease, and Parkinson's disease [3]. Several studies have demonstrated the involvement of mGluRs in the progression, aggressiveness, and recurrence of GBMs [4, 14]. Many *in vitro* studies have evaluated the mGluR-mediated signaling in GBMs, but only few have been able to assess their role in GBM malignancy using *in vivo* models [4]. This deficiency may have occurred because not all mGluR ligands have oral bioavailability or BBB penetration. Thus, one of the ways to use these BBB-impenetrable ligands in *in vivo* models is through direct CNS treatment. As we intended to evaluate the effect of *in vivo* administration of mGluR ligands on GBM-implanted rats and one of these does not cross the BBB,

Fig. 2 Intracisternal injection procedure. **A** Schematic of intracisternal injection showing how the rat's head is attached to the stereotactic frame. The animal is placed in the head-first prone position with the head remaining lowered due to approximation of the nasal clamp of the stereotactic frame at the head, just above the eyes. The hair around the cavity of the cisterna is shaved and a gingival needle connected to the microsyringe by a very fine and flexible tube is operated by one of the researchers to puncture exactly in the middle of this cavity. The

other researcher assists by manipulating the microsyringe plunger. **B** The procedure of intracisternal injection. The arrow indicates the bubble formed between the treatment solution and the fluid filling the tube. **C** Cross-section of the spinal cord. The arrow shows staining of the cord with methylene blue injected into the cisterna cerebellomedullaris. **D** The area between cerebellum and medulla oblongata stained by methylene blue. The marked location is the exact point where the CSF remained in the cisterna.

we decided to develop a less aggressive intracisternal injection protocol.

This intracisternal injection procedure is an adaptation of the rat cisterna cerebellomedullaris puncture protocol for obtaining CSF [15, 16]. A similar protocol has been used by Liu *et al.* [17]. This type of treatment was chosen because it is less invasive than other types of direct injection into the CNS reported in the literature, which require stereotactic surgical procedures. Like all types of direct injection into the CNS, small volumes of drugs are administered at a very slow rate to avoid a sudden increase in intracranial pressure. The gingival needle used for injection was left at the injection site for a few minutes post-delivery and then retracted very slowly in order to prevent fluid backflow through the injection channel.

Animals treated with mGluR ligands were closely observed for at least 3 days after injection in order to identify and/or differentiate any reactions caused by possible brain disruption or damage from the injection. Analyzing our preliminary data, we found that weekly ligand treatment of rats (one administration per week for 3 weeks) with this type of injection did not cause external damage. There was no inflammatory process at the external site of the injections and the rats did not appear to be suffering. In addition, none of the animals died due to this injection procedure. Through the experiments with methylene blue, it was possible to conclude that this type of intracisternal injection is effective in reaching exactly the site of the spinal cord bathed by CSF concentrated in cisterna cerebellomedullaris. In conclusion, although this treatment procedure requires two people, the noninvasive character of this type of injection of treatment solutions directly into the CSF stands out in relation to the others, being adaptable for any type of drug or rodent animal model.

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Compliance with Ethical Standards

Conflict of interest All authors claim that there are no conflicts of interest.

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