Video Article

Construction and Implantation of a Microinfusion System for Sustained Delivery of Neuroactive Agents.

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Abstract

Sustained delivery of neuroactive agents is widely used in neuroscience, but poses many technical challenges. It is necessary to deliver the agent with high precision while minimizing localized trauma and inflammation. Also, the ability to customize the system to accommodate animals of different species and sizes is desirable. This video presentation demonstrates the construction of an infusion system that can be fitted to any particular research animal. The delivery microcannula diameter is approximately 10-fold smaller than most infusion cannulas presently used. This translates into enhanced accuracy and reduced trauma to the brain region under study. The delivery cannula can also be sculpted to fit the contour of the surface of the animal's skull, thereby allowing closure of the scalp incision neatly over the infusion system, precluding the need for a skull-mounted pedestal, reducing risk of infection, and ensuring a greater level of comfort to the animal. The system is assembled in an air-free environment and requires the researcher to fashion glass micropipettes with a heat source. These construction methods require special skills that are best acquired, if not in person, using video instruction. (This article is based on work first reported in J Neurosci Methods. 2008 Jan 30;167(2):213-20. Epub 2007 Aug 28.).

Video Link

The video component of this article can be found at https://www.jove.com/video/716/

Protocol

Introduction

Direct infusion of neuroactive agents allows specific brain regions to be studied while bypassing the blood brain barrier. The applications of this approach in neuroscience are diverse and include altering the level of brain activity in discrete subregions (Berretta et al., 2004; Gliddon et al., 2005; Kim et al., 2000), investigating the actions of psychotropic agents (Clinton et al., 2006; Di Benedetto et al., 2004), providing controlled models of brain inflammation (Hauss-Wegrzyniak et al., 1998; Marchalant et al., 2007; Rosi et al., 2004), studying the mechanisms of addiction (Kim et al., 2005; Lockman et al., 2005; Zhang et al., 2006), and enabling long-term administration of trophic factors (Naert et al., 2006; Radecki et al., 2005; Takahashi et al., 2006).

Standard methods for chronic delivery of agents often utilize a mini-osmotic pump (e.g., ALZET Osmotic Pumps, Cupertino, CA) loaded with a neuroactive agent, which is delivered within the brain through a stainless steel cannula with a diameter that may range from 0.25 mm (Williams et al., 1987) to 0.5 mm, but most commonly is approximately 0.3 mm (Plastics One, Roanoke, VA ; see Fig 6A). While delivery cannulas of this size may be suitable for many applications in which high levels of precision are not required and trauma to the microenvironment is not a major concern, these cannulas are suboptimal for delivering agents to small, delicate sites in which the cannula is comparable in size to (or larger than) the targeted structure itself, or where the function must not be compromised by traumatic insult.

Presented here is a method for the preparation and implantation of a chronic infusion system for delivery of neuroactive agents within the brain utilizing a "microcannula" with a much reduced tip diameter. This technique allows small substructures to be targeted and reduces trauma to the of the area of interest. The present procedure is thus taught as an alternative to conventional methods for researchers who desire to minimize disruption to the brain region under investigation.

Materials and methods

Animals and design

Eighteen adult (400-450 gm) male Sprague-Dawley rats were used to demonstrate the procedure and test the function of the present method. Twelve animals were implanted with microinfusion systems and contributed to a time-course evaluation of sustained function over 4 days. Six animals were used to compare level of trauma and reactive gliosis 5 days after implantation of either a standard 28 ga delivery cannula (N=3) or

a microcannula (N=3) attached to a saline-filled mini-osmotic pump. Animals were housed in clear plastic cages, and maintained on a 12-hour light/dark schedule, with food and water provided ad libitum. All procedures were approved by the McLean Hospital Institutional Animal Care and Use Committee, in compliance with applicable federal and local guidelines for experimental use of animals.

Microinfusion system assembly

Figure 1 illustrates the components and assembly of the microinfusion system. We recommend constructing and implanting the system using sterile technique. In addition, in order to prevent introduction of air into the system, it is assembled while submerged within a saline bath. For the present tests, mini-osmotic pumps (MOPs) were employed (ALZET, model #1002, Cupertino, CA) with a 14 day duration, flow rate of 0.25 µL /hr, and a fill capacity of 98.6 µL. However, since excessive flow rates can deluge smaller areas of interest in the brain and cause structural damage, the flow rate for these studies was decreased to 0.125 µL /hr by coating one half of the MOP with paraffin as instructed by the manufacturer. For evaluation of local trauma, MOPs were filled with sterile saline. For evaluation of sustained function of microinfusion systems, Fast Green (1% in saline, Fisher Scientific, Park Lane Pittsburgh, PA) was chosen as a test solution because of its low toxicity, its ability to diffuse within viable brain tissue, and because it has a molecular weight (808.84) at the upper end of the range for "small molecules" (<1000) that are commonly of interest for chronic infusion (e.g. muscimol, picrotoxin, fluoxetine, etc.).

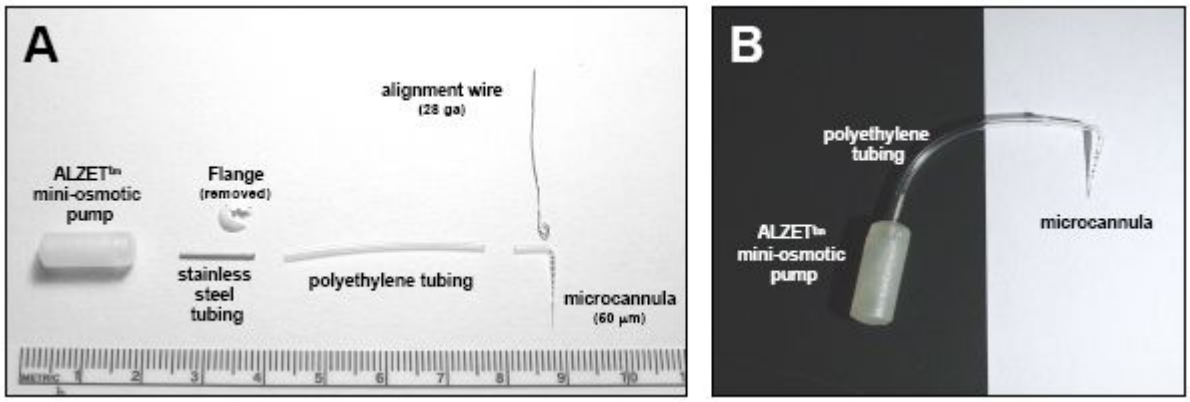

Figure 1: Assembly of the microinfusion system. (A) The components of the system placed in their relative positions for assembly. Note the flange from the flow modulater tubing has been broken away. We recommend the alignment wire be secured just before implantation (see text). (B) Assembled microinfusion system prior to attachment of alignment wire and implantation.

Microcannula preparation

The microcannula can be fashioned using a standard horizontal or vertical electrode puller (Stoelting Co., Wood Dale, IL) with settings for producing a glass electrode with a long, gently tapering shank (Fig 2A). Borosilicate tubing is suitable for this purpose (part 1B100F-6, 1.0 mm, 6 in; World Precision Instruments, Inc., Sarasota, FL), as it has a relatively low melting point, allowing subsequent bending with focused heat. The distal-most portion of a straight microcannula can be cut with dissection scissors or a controlled break can be made with microforceps (World Precision Instruments, Inc., Sarasota, FL) to give the desired final tip diameter (50 µm for the present demonstrations), and a microscope stage micrometer is useful to guide and confirm the desired diameter. The cut tip can be briefly heated, or "fire-polished", to remove rough or sharp edges. The heating coil of the electrode puller or a bunsen burner is then used to impose a right angle onto the microcannula at a predetermined distance from the tip of the microcannula (Fig 2B) by placing the borosilicate tubing within the heating coil (or a bunsen flame) and applying gentle force at the distal portion with forceps as the tubing is heated. The predetermined length of the microcannula distal to the imposed right angle is decided based on the depth of the brain region to be targeted (e.g., 5 mm) and taking into account the skull thickness (~1 mm) and working distance above the skull (e.g., 1-2 mm). Thus the predetermined distance from the tip of the microcannula to the imposed right angle for the present demonstrations was 7-8 mm. A diamond pencil is then used to cut the borosilicate tubing approximately 8 mm proximal to the right angle.

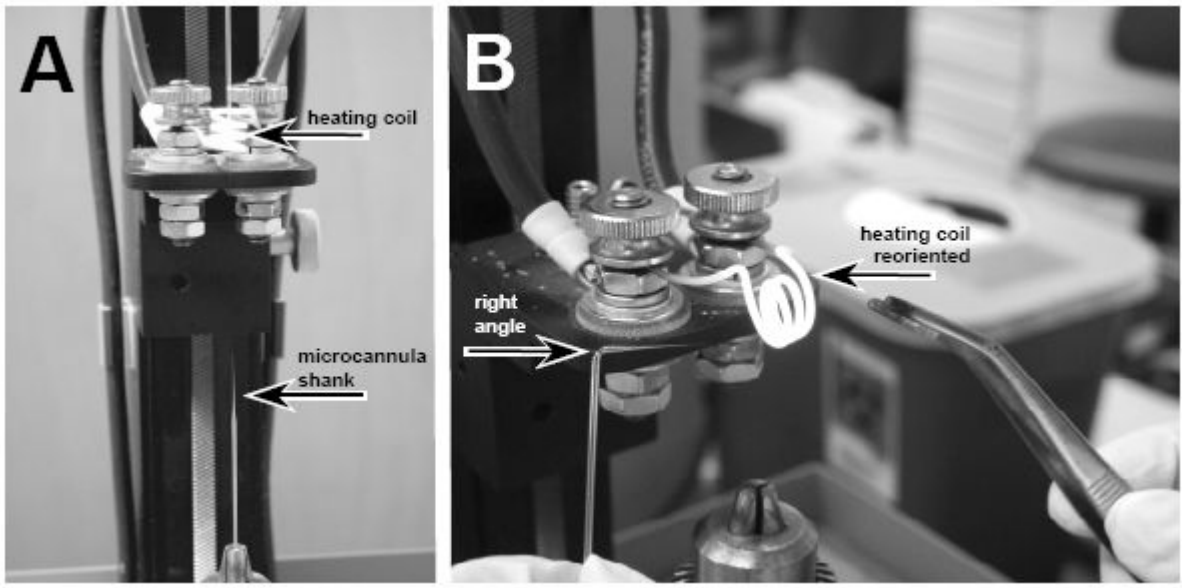

Figure 2: Preparation of microcannula. (A) An electrode puller (Stoelting Co. Wood Dale, IL) is used to produce a microcannula with a long, tapering shank. (B) The heating coil may be reoriented for ease of use, and the microcannula is placed within the coil, allowing a right-angle bend to be formed using forceps as the glass tubing is heated.

After the desired number of microcannulas has been made, we recommend sterilization with ethylene oxide or using an autoclave. The distal-most 1-2 mm of the microcannula can be colored with a sterile surgical marker (or permanent ink prior to sterilization) to allow the microcannula tip to be easily visualized during the surgical procedure.

MOP preparation

MOPs were prepared according to the manufacturer's instructions. Briefly, the plastic flange from the stainless steel MOP tubing ("flow modulator") is first removed using scissors or rongeurs. A length of polyethylene tubing (Plastics One, Roanoke, VA, #C312 VT; OD, 1.22 mm; ID: 0.72 mm) is attached to the area of stainless steel tubing previously occupied by the flange (~4 mm) by inserting the stainless steel tubing into the lumen of the polyethylene tubing and securing with a suitable adhesive around the outer circumference of the connection. LumaBond (myNeuroLab, Inc., St. Louis, MO) is particularly useful as it cures within seconds upon exposure to focused light (e.g., from a fiber optic lamp). The length of tubing should approximate the distance between the base of the animal's skull and the rostral-most aspect of the scapulae (e.g., approximately 1.5-3.0 cm for laboratory rats). The pump is filled as instructed, and the stainless steel ALZET tubing, with the length of polyethylene tubing attached to its end, is inserted into the filled pump. A small volume of solution from the pump will be forced around the outside of the pump-tubing interface (and should be dabbed away) and also into the proximal region of the polyethylene tubing. The filled pump with attached tubing is then incubated in sterile saline for 12 hours at 37°C. If the pump is functioning properly, after this incubation period, fluid will be seen to have traveled a few millimeters within the polyethylene tubing.

Construction of microinfusion system

The pump with its attached tubing is immersed in a 10 cm Petri dish containing sterile saline, and the remaining air within the polyethylene tubing is removed using a syringe filled with the same solution contained in the pump and which has been fitted with small-diameter tubing that can be inserted within the polyethylene tubing. Solution can thus be injected into the polyethylene tubing to replace the air. Similarly, the microcannula is immersed into the saline bath and air is removed by injecting with solution. This is easily achieved with a second solution-filled syringe fitted with flexible (e.g., silicone) tubing that fits over the large (proximal) end of the microcannula.

The proximal end of borosilicate tubing is then inserted into the polyethylene tubing. Note that this may require dilation of the polyethylene tubing by firmly pressing the closed tips of microforceps into the lumen thereby flaring the opening for easy insertion of the borosilicate tubing. The air-free infusion system is then removed from the saline bath and placed on a sterile surface and gently dried with gauze or a cotton swab. The microcannula-polyethylene tubing connection is secured with a circumferential coat of adhesive (e.g., LumaBond).

If the system has been assembled properly, and if the MOP continues to function as it should, during the final step of this procedure a droplet of solution will usually appear at the tip of the microcannula as the osmotic mechanism within the pump continues during preparation. MOP flow rate can be decreased proportionately by coating the appropriate surface area with paraffin as instructed by the manufacturer. For the present demonstrations, 50% of each MOP was coated by briefly dunking it into molten paraffin and allowing it to cool, thus reducing the flow rate by half to 0.125 µL/hr. The completed microinfusion system is then submerged in sterile saline and stored at 37°C until implantation.

Implantation of microinfusion system

The microinfusion system is implanted using standard stereotaxic methods as previously described (Cooley, 1990). After preparation of the surgical field, a burr hole is drilled for entry of the microcannula. If desired, skull screws can also be positioned to stabilize the bonding compound used to secure the microcannula. The compound used in this laboratory (Geribond, Den-Mat Inc., Santa Maria, CA) does not require additional anchoring; however, the skull surface must be prepared by cleaning with an acetonedampened cotton swab. A 1-2 mm diameter "hook" is made at the distal end of the alignment wire (see Fig. 1), allowing it to encircle the bend in the borosilicate tubing. It is then secured (using LumaBond),

in an orientation along the same axis as the distal portion of the microcannula (that is to be advanced into the brain, see Fig. 3B). The infusion system is then mounted on the stereotaxic carrier (Fig. 3), clamping the alignment wire onto the holder. Sterile suture is used to tether the infusion pump to the top of the stereotaxic manipulator, thus suspending it and preventing loss of orientation during the procedure due to the weight (and torque) of the pump (Fig. 3B). The microcannula can then be positioned in the desired orientation (e.g. precisely vertical) by using forceps to gently bend the alignment wire distal to the carrier clamp. The microcannula tip is positioned over a point of reference (e.g., bregma or lambda) and then maneuvered to the point of entry into the brain. The coordinates used for the animals in these experiments were: Bregma +0.2 mm, lateral 3.2 mm, and ventral 5.0 mm beneath dura with the animal's head in a skull-flat position.

After the microcannula is advanced to the appropriate depth within the brain, the system is fixed into position with a pedestal compound (e.g., Geribond). The pedestal can be sculpted close to the surface of the skull to facilitate wound closure. After the pedestal compound has cured (~2 min), the alignment wire is cut flush with the pedestal using the drill's cutting wheel. A small amount of compound may be used to cover and smooth the remaining barb from the severed wire. The MOP is then inserted subcutaneously, positioned between the animal's scapulae. Finally, the wound is lavaged with sterile saline, and the incision closed using suture or wound clips (Fig. 3, panel D).

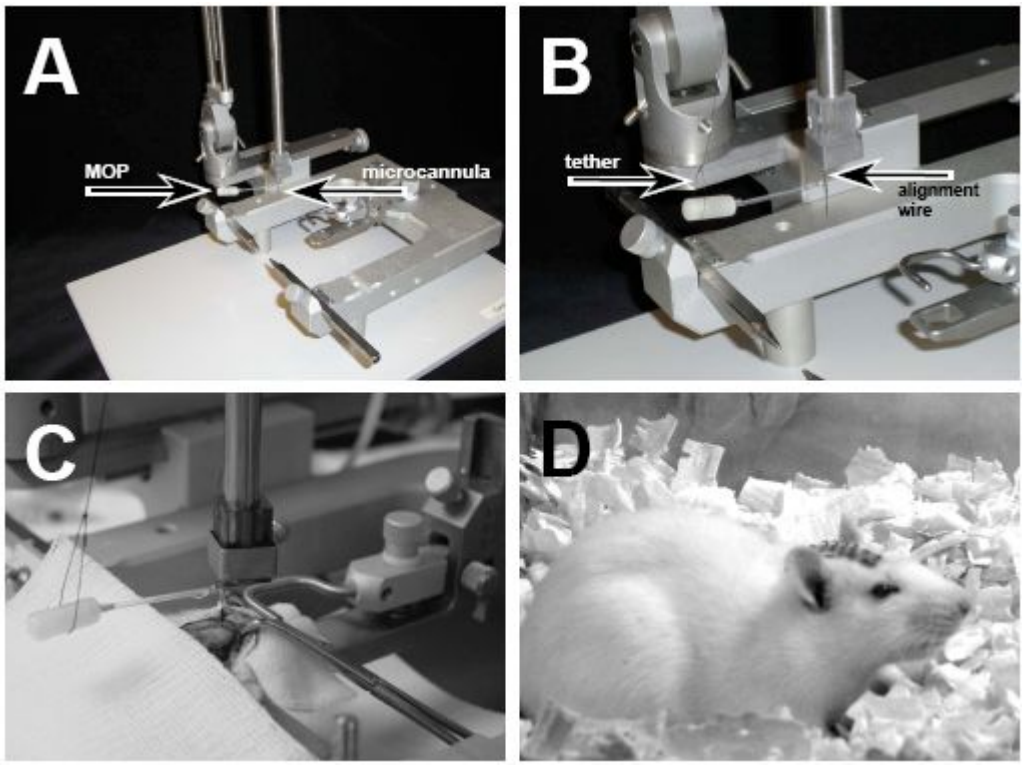

Figure 3: Implantation of microinfusion system. (A) Positioning of microinfusion system onto stereotaxic instrument. (B) The MOP is tethered using sterile suture to prevent its weight from disrupting the orientation of the microcannula. The microcannula is positioned for precise vertical entry into the brain using the alignment wire. (C) A microinfusion system is positioned for placement and fixation within an animal. (D) The design of the microinfusion system allows easy closure of the incision over a low-profile pedestal, reducing the risk infection and minimizing discomfort to the animal.

Preparation and implantation of standard infusion system

The procedure for preparation of the standard infusion system is identical to that for the microinfusion system except the microcannula is replaced by an "osmotic pump connector cannula" with a diameter of 300 µm (28 ga; Plastics One, Roanoke, VA, #3300PM/SPC; see Fig 5a). Implantation of the standard system is also identical to that of the microinfusion system, including the attachment of an alignment wire to allow precise vertical positioning of the cannula of entry into the brain.

Histology and Analysis

To examine the microinfusion system's ability to provide sustained delivery of Fast Green, groups of three animals were sacrificed at 12 hr, 1 day, 2 days, and 4 days after surgery. Subjects were deeply anesthetized with sodium pentobarbital (120 mg/kg, ip) and transcardially perfused with 200 mL of 0.1M phosphate buffered saline (PBS) followed by 400 mL of 4% paraformaldehyde in 0.1M phospate buffer. Perfusion solutions were kept at 4°C, with a pH of 7.4 and perfused at a rate of 50 mL per minute using a peristaltic pump. In order to remove the cannulas without damaging the postmortem tissue, the perfused animal was fixed in a stereotaxic frame, the pedestal was secured with adhesive to a rigid wire attached to the manipulator arm clamp, and the skull was carefully removed from around the pedestal using a dental burr. The pedestal and the underlying cannula were thereby removed along the same trajectory in which the cannula was advanced for placement. Brains were then removed from the calvaria and immersed for 12 hours in the same fixative.

For evaluation of sustained infusion of Fast Green, sections with a thickness of 200 µm were cut using a Vibratome (myNeuroLab, St. Louis, MO) and wet mounted on glass slides. Representative sections were imaged using a Canon CanoScan LiDE 600F scanner. For evaluation of local trauma and reactive gliosis, 70µm Vibratome sections were first wet-mounted and photographed unstained to prevent tissue distortion

that may occur with histological processes, such as drying, staining, and dehydration. These same sections were then allowed to dry onto their gelatin-coated glass slides, and they were stained with hematoxylin and eosin (H&E), dehydrated with graded alcohols, and cover-slipped with Permount.

Adjacent 70 µm sections underwent a standard immunofluorescence procedure to detect glial fibrillary acidic protein (GFAP), which is expressed by astrocytes during the process of reactive gliosis in response to trauma (Ding et al., 2000; Ma et al., 1991). Briefly, free-floating sections were rinsed with PBS and blocked with 10% normal donkey serum (NDS) and 3% bovine serum albumin in 0.1 M PBS with 0.3% Triton X-100 (PBS/Triton) for one hour and then incubated in rabbit anti-GFAP antibody (1:500; Sigma Chemical Co., St. Louis, MO) in PBS/Triton with 1% NDS for 12 hours at 4°C. Sections were then rinsed with PBS and incubated with Alexa Fluor® 594 donkey-antirabbit secondary antibody (1:500; Molecular Probes, Inc., Eugene, OR) in PBS with 5% NDS for one hour at room temperature. The sections were thoroughly rinsed again with PBS and counterstained with NeuroTrace® green fluorescent Nissl stain (1:500 in PBS for 5 minutes; Molecular Probes, Inc., Eugene, OR). After a final rinse with PBS, sections were mounted on gelatin-coated slides and coverslipped with Slow-Fade (Molecular Probes). All photomicrographs were acquired using a Zeiss Axioskop microscope with AxioVision software (Carl Zeiss, Inc., Thornwood, NY).

Results

In vitro testing

Prior to evaluation in animals, three microinfusion systems were prepared by placing their Fast Green-filled MOPs into in one compartment (15 ml conical tube) and placing their microcannulas in a second compartment (1 ml Eppendorf tube), both containing sterile saline at 37°C. Each system demonstrated continuous flow of dye over 12 hours of observation. Figure 4 illustrates function of a typical system over 3 hours.

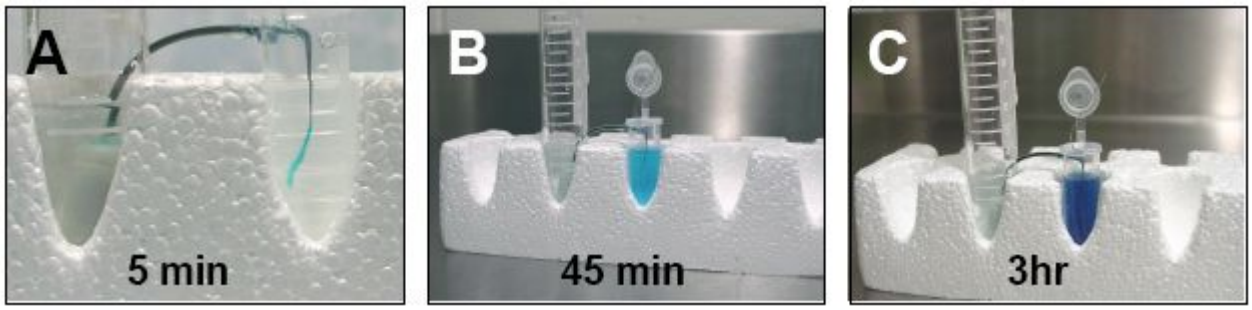

Figure 4: In vitro demonstration of microinfusion system function. (A) Flow of Fast Green dye is easily seen unimpeded from the microcannula at the beginning of an in vitro test period. (B) As flow continues, the reservoir solution becomes saturated (B), and ultimately opaque with coloration (C).

Evaluation of in vivo microinfusion system

After perfusion, the removed microcannulas and their connections were carefully inspected, and all were found to be entirely intact. Thus, there occurred no breakage of the borosilicate glass within tissue, nor were there any defects in the connections of system components. A 10 .L saline-filled Hamilton syringe with a short length of silicon tubing attached to its needle was used to apply gentle flow to the cut end of the polyethylene tubing attached to the microcannula. All twelve of the microcannulas allowed continued flow and did not appear to be obstructed. Furthermore, after removing each MOP from the animals, the volume of the remaining solution was measured and found to be appropriate for the MOP's flow rate (0.125 µL/hr) and the amount of time it was allowed to function.

In vivo delivery

Evaluation of coronal sections of tissue infused with Fast Green demonstrated little variability in distribution between animals within groups at any given time point (12 hrs, 1 day, 2 days, and 4 days). Figure 5 illustrates representative areas of Fast Green diffusion at these time points showing a progressive increase in dye infiltration over four days. Note that prior experiments using higher concentrations of Fast Green (e.g., 5- 15%) resulted in rapid opacification of the parenchyma, thus obscuring accurate evaluation of the microinfusion system's function over time. While the full extent of 1% dye diffusion can be difficult to accurately determine on gross inspection, areas of infiltration were easily discernable at 2.5X magnification, and are indicated with dashed lines in Figure 5.

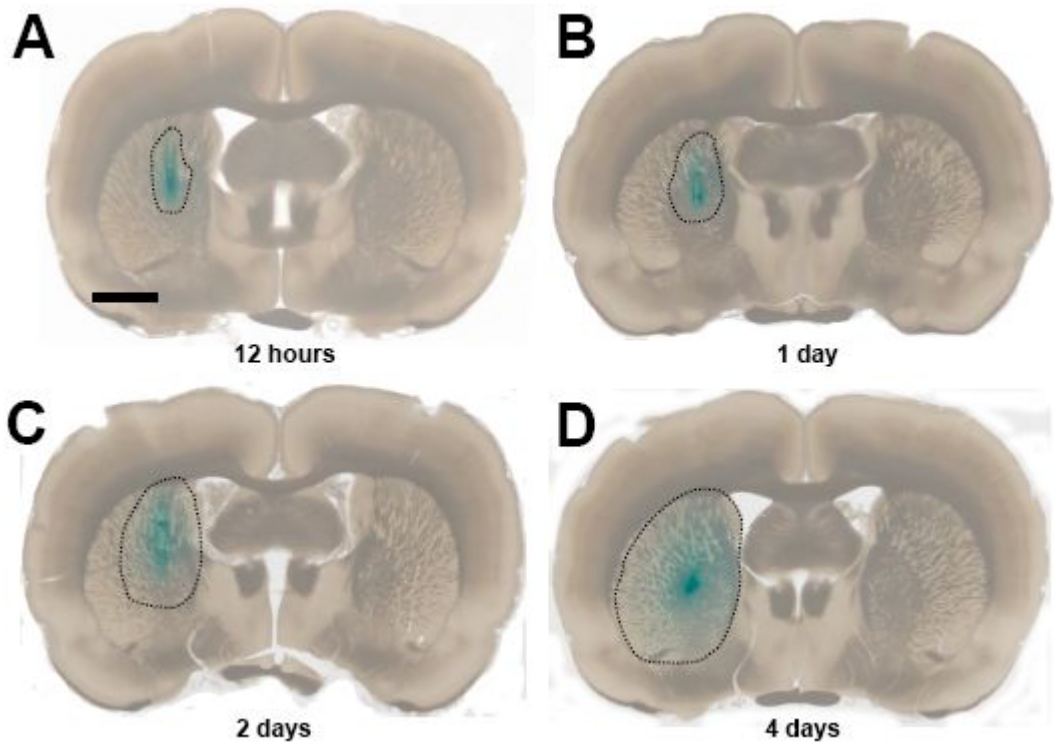

Figure 5: In vivo demonstation of sustained microinfusion of Fast Green. (A) Twelve hours after intrastriatal placement of a Fast Green-loaded microinfusion system, dye is seen diffusing into the parenchyma surrounding the microcannula site. Observations at 1 day (B), 2 days (C), and 4 days (D) indicate continued delivery of Fast Green solution with increasing areas of dye infiltration. The outer limit of diffusion was observed under low-power microscopy and is indicated here with dashed lines. Scale bar, 2 mm.

Evaluation of trauma and reactive gliosis

Gross observation of freshly cut, unstained tissue sections showed that the microinfusion system resulted in a reduction in localized trauma at the site of delivery. The standard cannula was invariably seen to disrupt and displace a greater area of tissue (Fig. 6B & C), which is better appreciated at higher power with H & E (Fig. 6D & E). Moreover, blood was frequently seen accumulated at the standard infusion site (Fig. 6D), suggesting a greater degree of compromise to the vasculature and implying decreased integrity of the blood brain barrier.

The microinfusion system also resulted in greatly reduced reactive gliosis as demonstrated by decreased immunoreactivity for GFAP in the vicinity of infusion via a microcannula (Fig. 6G) when compared to a standard cannula (Fig. 6F). While microcannula-induced GFAP staining was elevated above normal levels (Fig. 6H), the striking escalation in reactive gliosis with standard cannula placement and infusion (6F) was not seen when the microinfusion system was employed.

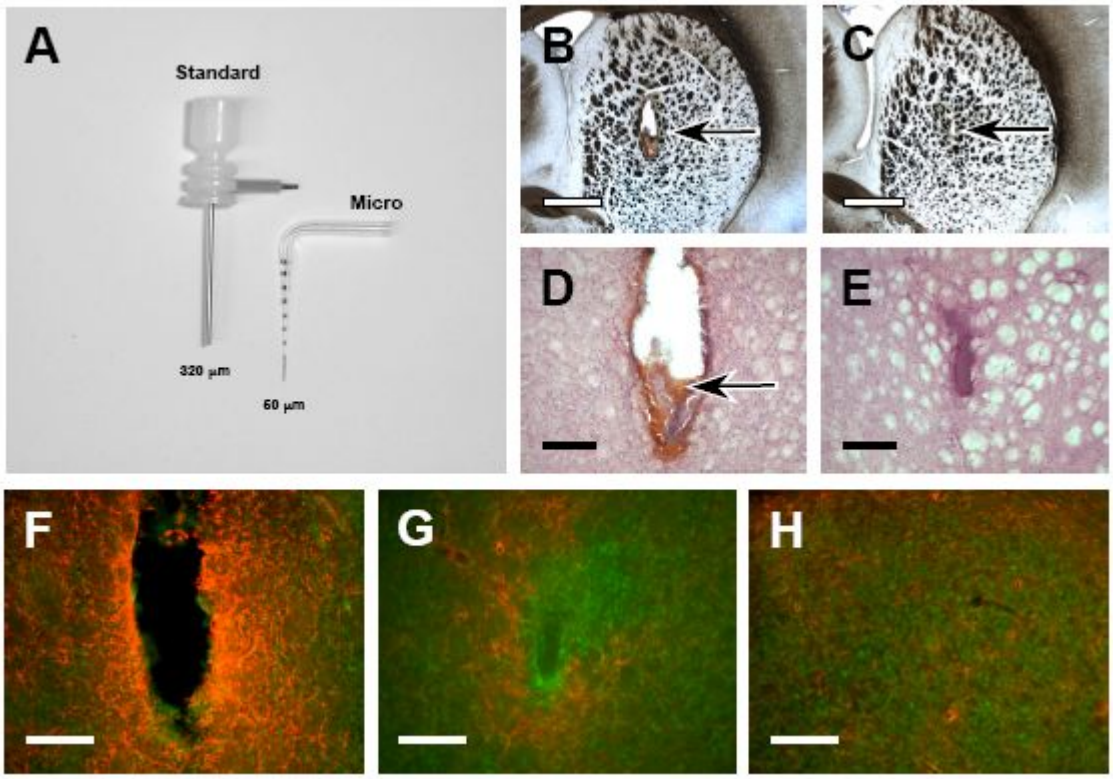

Figure 6: Localized trauma caused by a microcannula compared to a standard delivery cannula after 5 days of saline infusion. (A) A microcannula pulled from borosilicate tubing with a tip diameter of 50 µm allows free, unrestricted flow of most neuroactive agents, yet is 6X smaller than a standard delivery cannula (28 ga, 0.30 mm). (B, C) Unstained, wet mounts of coronal sections (thickness, 70 µm) illustrating greater local tissue damage (arrows) caused by a standard cannula (B) when compared to a microcannula (C). (D, E) Higher power photomicrographs of H&E stained sections shown in B and C, respectively. The standard cannula caused more extensive traumatic injury as illustrated in D, having displaced a larger area of tissue and causing greater insult to the vasculature as demonstrated by the collection of blood within the lesion cavity (arrow). The lesion caused by the microcannula, however, is considerably smaller and less disruptive to tissue at the site of infusion. (F, G, H) Immunofluorescence against GFAP demonstrates dramatically increased numbers of GFAP+ astrocytes around the lesion of the standard cannula (F) when compared to that of the microcannula (G) and normal, unlesioned striatum (H). Scale bars: A & B, 1 mm; D - H, 250 µm.

Discussion

Various studies have demonstrated that by decreasing the size of the delivery cannula for intracerebral infusions, tissue trauma and insult to the blood brain barrier are reduced (Perry et al., 1993), inflammation is decreased and the immune-response attenuated (Finsen et al., 1991), and reactive gliosis is diminished (Nikkhah et al., 1994). We present here a method for the chronic delivery of neuroactive agents within the brain via a microcannula with a diameter that is reduced 6-fold compared to that of conventionally-used delivery cannulas. We have demonstrated that this system reliably delivers a representative solution over time and the level of localized trauma and reactive gliosis is dramatically reduced. These studies employed a delivery cannula with a final tip diameter of 50 m; however, smaller tip diameters may be utilized. The primary virtue of such microinfusion systems is their ability to deliver agents to very small, discrete targets, while incurring minimal trauma in the vicinity of the infusion.

The microcannulas used in the construction of the infusion system we describe can be easily custom-built with predetermined lengths and tip diameters. The microinfusion system is designed to conform to the surface of the animal's skull allowing secure, low-profile fixation and precluding the need for a large cranial pedestal. The surgical wound can therefore be easily sutured over the streamlined microinfusion pedestal, thus reducing both the discomfort to the animal and the risk of infection. In addition, securing the system to the skull requires less skull surface area; therefore, additional or subsequent procedures may be performed without interference from a larger pedestal.

One should, however, take into account certain technical considerations when employing the methodology described here. A higher level of skill is required, and the production and implantation procedure is less time-efficient than it may be for standard infusion systems. On the other hand, this may be outweighed, or at least counterbalanced, by the time (and animals) spared by practicing high-precision technique. Another difficulty is that while the microcannula can be customized with virtually any diameter, a microcannula with a very small lumen can become obstructed, either by components of the infusate or by tissue encountered during implantation (e.g., blood, brain tissue). This risk is reduced by keeping the microcannula tip moistened (e.g., using a water- or saline-saturated cotton swab to prevent drying of infusate solutes) during the implantation procedure and by performing clean, "blood-free" surgery.

We recommend that the researcher establish a microcannula tip diameter that is appropriate for the particular composition and/or viscosity of the solution that will be loaded into the MOP. This is easily achieved by using an in vitro test system similar to the one described in the protocol (Fig. 4), or by simply immersing the assembled system in a 37°C saline bath and monitoring the bath composition and/or measuring the volume of

the MOP contents over time. A disadvantage is that the microcannula is relatively fragile, particularly if its tip diameter is very small (e.g., ~10-50 microns). Accidentally breaking the microcannula during surgery usually requires that the entire system be replaced, as repairing or rebuilding that unit would extend surgery time considerably. Scientists skilled in such techniques and who practice exacting method rarely experience breakage, however.

At the conclusion of the experiment, the microinfusion system can be inspected to ensure that the microcannula has not been damaged and that it has remained patent. The volume of the mini-osmotic pump can also be measured to ensure that the appropriate amount of neuroactive agent has been delivered; we do not recommend recycling or reusing the pumps, however. While the present methods were demonstrated in adult rats, use of a system that reduces trauma and requires less skull surface area for fixation may be considered preferable over conventional methods for experiments with smaller animals, such as mice or young rats.

Although the present method requires a somewhat more advanced level of proficiency and skill by the experimenter, it offers to increase precision perhaps by an order of magnitude, and to minimize experimental confounds associated with trauma to the region of interest. In our laboratory, we have found that this translates into more reliable and robust effects of the experimental intervention.

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