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·Method·

Increasing the effectiveness of intracerebral injections in adult and neonatal mice: a neurosurgical point of view

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ABSTRACT

Intracerebral injections of tracers or viral constructs in rodents are now commonly used in the neurosciences and must be executed perfectly. The purpose of this article is to update existing protocols for intracerebral injections in adult and neonatal mice. Our procedure for stereotaxic injections in adult mice allows the investigator to improve the effectiveness and safety, and save time. Furthermore, for the first time, we describe a two-handed procedure for intracerebral injections in neonatal mice that can be performed by a single operator in a very short time. Our technique using the stereotaxic arm allows a higher precision than freehand techniques previously described. Stereotaxic injections in adult mice can be performed in 20 min and have >90% efficacy in targeting the injection site. Injections in neonatal mice can be performed in 5 min. Efficacy depends on the difficulty of precisely localizing the injection sites, due to the small size of the animal. We describe an innovative, effortless, and reproducible surgical protocol for intracerebral injections in adult and neonatal mice.

Keywords: rodents; surgery; neurosciences; stereotaxic injection; newborn mice; neonatal brain

INTRODUCTION

Intracerebral injections of tracers or viral constructs in rodents have contributed much to our understanding of the nervous system by targeting specific areas of the brain. These techniques are now commonly used in the neurosciences[1, 2]. Because experimental results depend on intracerebral injections, they have to be perfectly executed. Although intracerebral injection procedures in adult[3] and newborn[4-6] mice have already been described, the purpose of this article is to update these protocols. Our aim is to detail, step by step, an easy and reproducible procedure to allow the investigator to improve the effectiveness and safety of this surgery, and save time. Using our neurosurgical experience in humans, we apply the principles to rodents and propose tips to circumvent the difficulties of the procedures. Furthermore, for the first time, we describe a two-handed procedure for intracerebral injections in neonatal mice.

MATERIALS

Stereotaxic Intracerebral Injections in Adult Animals

REAGENTS

-Experimental mice (use of live mice must conform to institutional rules)
-Sterile saline (0.9% NaCl)
-Distilled water
-Ophthalmic gel
-Anesthetics: ketamine hydrochloride / xylazine hydrochloride solution (Sigma®) [or ketamine (Imalgene®) + xylazine (Rompun®)] or isoflurane (Iso-Vet®) (ketamine is a narcotic and should be used

according to the regulations of the host institution). Some investigators use tribromoethanol (Avertin®) as the anesthetic for rodents (it is not a narcotic).
-Analgesics: buprenorphine (Vetergesic®; this is a narcotics and should be used according to the regulations of the host institution), ketoprofene (Ketoprofene Arrow®)
-Injection fluid: virus, enzyme, tracer…
-Crushed ice
-Lidocaine (Xylovet®) (local anesthetic, optional)
-Iodine-based wash (Betadine®, optional)
-Hydrogen peroxide (optional)

EQUIPMENT (Fig. 1)
-Stereotaxic apparatus (e.g., Stoelting®, Digital Lab Standard for mouse, or David Kopf Instruments® for mouse or rat)
-Stereotaxic injection system (Harvard Apparatus®, Pump 11 Elite)
-Dissecting microscope (Leica® S6E)
-Temperature-controlled heating cage (VetTech Solutions® HE011),
-Surgical tools: surgical scissors, fine forceps, surgical hook or 26-gauge needle, black leader for human spinal anesthesia (Braun, Spinocan 4509900), needle holder
-Surgical braided absorbable suture (Vicryl 3-0, Ethicon®)
-High-speed dental drill with small drill-bits (Foredom® 38 000 rpm)
-10 µL injection syringe (Hamilton® 1700) with flexible (silica) 33-gauge needle (Phymep®), or rigid 33-gauge needle (Hamilton® 7762-03)
-10 µL calibrated micropipettes (Eppendorf® Research Plus)
-Container of crushed ice to preserve injection fluid
-Cotton swab
-Laboratory film (Parafilm®)
-Precision wipes (Kimtech Science®)
-10 mL syringes,
-Isoflurane vaporizer (optional)
-Electric shaver (optional)

Intracerebral Injections in Neonatal Animals

REAGENTS
-Experimental new-born mice (postnatal days 0–1 (P0–P1); use of live mice must conform to institutional regulations),
-Crushed ice
-Sterile saline (NaCl 0.9%)
-Distilled water
-Injection fluid: virus, enzyme, tracer…
-Iodine-based wash (Betadine®; optional)
-Mineral oil (optional)

EQUIPMENT (Fig. 1)
-Stereotaxic apparatus (Stoelting®, Digital Lab Standard)
-Stereotaxic injection system (Harvard Apparatus®, Pump 11 Elite)
-Dissecting microscope (Leica® S6E)
-Temperature-controlled heating cage (VetTech Solutions® HE011)
-Surgical tools: 30-gauge needle
-10 µL injection syringe (Hamilton® 1700) with rigid 33-gauge needle (Hamilton® 7762-03)
-10 µL calibrated micropipettes (Eppendorf® Research Plus)
-Polystyrene foam stage
-Container of crushed ice to preserve the injection fluid and induce anesthesia
-Petri dish
-Cotton swab
-Laboratory film (Parafilm®)
-Precision wipes (Kimtech Science®)
-Aluminum foil
-Syringes

PROCEDURES

Stereotaxic Intracerebral Injections in Adult Animals

Step 1: Anesthesia

Weigh the animal and calculate the appropriate dose for anesthesia. We use a mixture of ketamine and xylazine given intraperitoneally. Doses are summarized in Table 1. To inject the ketamine-xylazine mixture, restrain the animal with one hand, abdomen up. Inject the mixture intraperitoneally through a 21-gauge needle inserted into the lower left or right abdominal quadrant (Fig. 2A). Immediately place the animal in the heating cage to maintain body temperature. The animal should reach deep

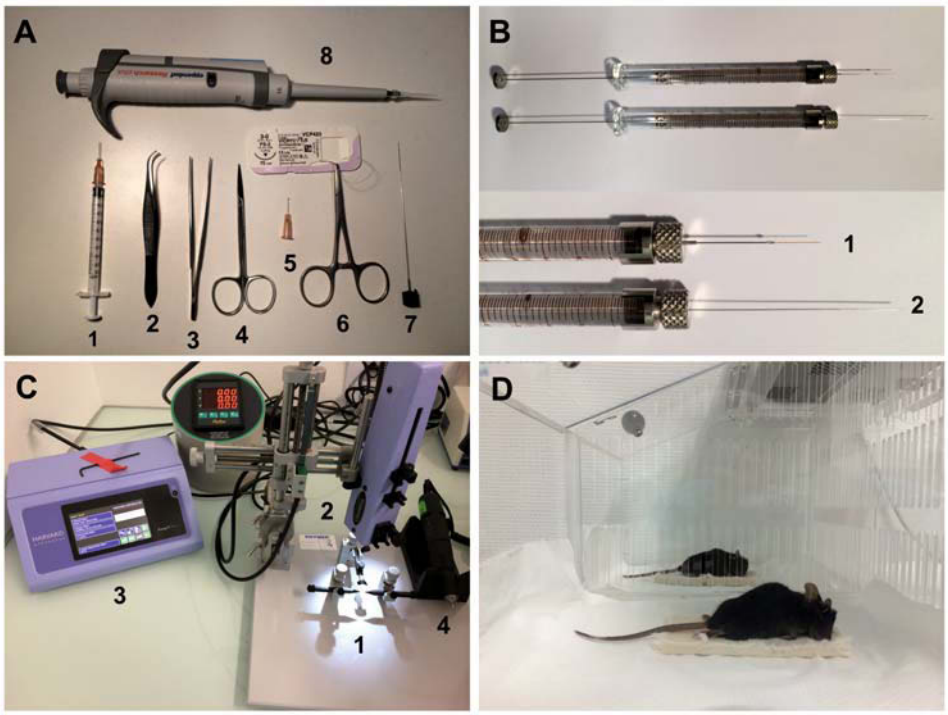

Fig. 1. Equipment required for intracerebral injections in mice. (A) Surgical tools. 1, 1 mL syringe for anesthesia; 2 and 3, fine forceps; 4, surgical scissors; 5, surgical hook; 6, needle holder with surgical braided absorbable suture; 7, black leader for human spinal anesthesia; 8, 10 µL calibrated micropipette. (B) 10 µL injection syringes with 1, flexible or 2, rigid 33-gauge needle. (C) Surgical area. 1 and 2, Stereotaxic apparatus with stereotaxic arm; 3, Stereotaxic injection system; 4, High-speed dental drill with small drill-bits. (D) Temperature-controlled heating cage.

anesthesia within ~10 min. Check the lack of response to nociceptive stimuli to confirm depth of anesthesia by pinching the tail. Instead of ketamine-xylazine mixture, vaporized isoflurane may be used to obtain surgical anesthesia (Table 1). *Pre-anesthesia with atropine is unnecessary for adult animals, but you may perform local anesthesia using lidocaine given subcutaneously (optional) (Table 1).*

Step 2: Preparation of the Animal

Shave the fur on the skull, clean the skin with iodine-based wash (optional because infection in rodents is almost nonexistent)[7]. Apply lubricant ophthalmic gel to both eyes to avoid keratitis during surgery. Pull out the tongue using forceps to facilitate breathing (Fig. 2B). Make a straight midline incision through the skin with surgical scissors from the back of the neck to the interpupillary line (the incision must run far enough along the anterior–posterior axis to expose stereotaxic markers). Softly push aside the connective tissue on top of the skull as needed for a clear view.

Step 3: Fixation of the Animal in the Stereotaxic Apparatus (for Right-handers)

To place the animal in the stereotaxic apparatus, fix the left ear bar to a predefined scale (e.g., 4 mm). Position the animal's head to lead its left ear canal onto the ear bar; the tip of the ear bar should pull the skin to expose the skull. An auxiliary ear bar (EB-5N, Narishige Co. Ltd., Tokyo, Japan) may assist the investigator in fixing the animal's head. Keep the animal's head in place using your left index finger to sustain the head and position the right ear bar to the same scale applying soft pressure to complete the fixation (Fig. 2C). In the right position, the animal's head is straight and symmetrical to the ear bars and cannot move laterally. If the animal's head position looks incorrect or is unstable, repeat the procedure.

With your right hand, insert the incisor adapter into the animal's mouth until the animal's incisors 'fit' in the opening of the adapter. The animal's head can be freely moved up by using your left index finger on the back of its neck to

facilitate the insertion of the incisor adapter (Fig. 2D). Then slightly pull back the incisor adapter to check its correct position and allow moderate extension of the animal's head. Fix the incisor adapter in place. Finally, place the snout clamp just below the eyes using low pressure. At this point in the procedure, the animal's head should be perfectly fixed in the stereotaxic apparatus. *For left-handers, reverse the sides of the procedure.*

Step 4: Alignment of Bregma and Lambda ("Flat-Skull" Step)

Make sure that the top of the animal's skull is in the horizontal plane. For this, the head position may be adjusted using the screw at the incisor adapter. Then place the injection syringe into the holder of the stereotaxic arm.

The following step is optional if your target injection is neocortical or superficial. Use a dissecting microscope at ×20 magnification. Make sure that bregma and lambda are easily distinguishable (Fig. 2E). Level the animal's head horizontally by measuring the dorsal-to-ventral (Z) coordinates of bregma and lambda and adjusting the head position so that they become equal using the adjustment screw at the incisor adapter (Fig. 2F). A 50-μm difference between bregma and lambda can be tolerated.

Step 5: Craniotomy and Opening the Dura

Use a dissecting microscope at ×20 to ×40 magnification. Consider the position of bregma as the reference (stereotaxic zero) of the *X* and *Y* coordinates. Using predefined stereotaxic coordinates, mark on the skull the intended site of injection. Adjust the drill to 20,000 rpm. Make a single burr-hole in the skull at the injection site. For this purpose, refine the bone by circular movements of a small drill-bit while applying very gentle pressure on the skull. Too much pressure may cause the drill to penetrate the skull and directly damage the brain parenchyma. Keep the drill-bit moist with sterile saline, applied drop-wise throughout the drilling to avoid heat generation and make the bone transparent. Stop when the drill-bit reaches the internal cortical layer of the skull. A thin layer of skull may remain. Using a 26-gauge surgical hook, remove the small pieces of any remaining skull to expose the dura. Flush the site with sterile saline to remove bone dust and possible blood. Open the dura by making a small incision with the leader for spinal anesthesia (*opening of the dura is optional if you use a rigid needle or micropipette, but necessary if you use a flexible needle*). Finally, position the syringe over the craniotomy and check that it is not diverted by the edge of the burr-hole. If the syringe is diverted, widen the craniotomy.

Step 6: Preparation for Injection

Adjust the features of fluid aspiration on the injection system (the aspirated volume should be 10% larger than the injected volume, or at least +100 nL, for small volumes). Pipet the desired volume of fluid onto a precut square of laboratory film; then bring the needle of the syringe into the drop under visual control with the dissecting microscope and suck up the fluid. If you are not comfortable with this, you can place the laboratory film on top of the skull or directly apply aspiration into the tube containing the fluid. Aspiration speed can be up to 38 μL/min (slower aspiration is preferred for viscous fluid). Check that the drop has been aspirated on the laboratory film. *Critical*: Make sure that the needle is not clogged by ejecting a small volume of fluid (e.g. 50 nL) under visual control. A droplet should be seen forming at the tip.

Step 7: Injection

Position the syringe over the burr-hole. Lower the syringe until the needle touches the cortical surface and use this point as "zero" (*Z zero*). Lower the syringe needle to the desired depth (*Z* coordinate of the injection site) in the brain parenchyma (Fig. 2G). Adjust the features of fluid injection on the injection system. The injection of the fluid should be very slow to avoid an acute increase of intracranial pressure and facilitate diffusion of the fluid (rate of injection should not be >100 nL/min). Depending on the total volume injected, this step may take up to 10 min. We limit the maximal injection volume to no more than 1500 nl in one session.

Step 8: Anesthesia of the Next Animal (Ketamine-Xylazine Anesthesia Only)

During the intracerebral injection, perform anesthesia of the next animal by injecting the ketamine-xylazine mixture intraperitoneally, and immediately place the animal in the temperature-controlled cage.

Step 9: Withdrawal of the Syringe and End of the Procedure

When the injection is completed, allow a minimum of 2 additional minutes rest time before starting to withdraw the syringe from the brain. Withdraw the syringe slowly, in two stages, allowing another 2 min rest time at the halfway point to avoid backflow of the fluid to the surface. The burr-

hole does not need to be covered. Remove the animal from the stereotaxic apparatus. *Clean the skin with iodine-based wash (optional).* Pull the edges of the skin together and suture the skin at 3 separate points.

Immediately (before suturing) rinse the syringe to avoid clot formation in the needle, by alternating aspiration and ejection of distilled water several times (in most stereotaxic apparatus this can be easily done using the "withdraw/inject" program). Check the patency of the syringe by ejecting a large volume of distilled water.

Step 10: Postoperative Management

Place the animal in the temperature-controlled cage until full recovery. Subcutaneous rehydration is unnecessary but provided free access to food by putting wet chow in the cage. Once the animal recovers, analgesic treatment may be administered, according to the protocol of your institution, for at least 2 days after surgery (Table 2). CAUTION: do not administer analgesic before the animal has fully recovered. Then return the animal to its home cage. Monitor the well-being of the animal for at least 3 days or according to local regulations.

Clean and disinfect tools and the surgical area with 70% ethanol. This surgical procedure (steps 2–9) can easily be performed in 20 minutes.

Intracerebral Injections in Neonatal Animals

No stereotaxic coordinates: the skull is too soft for fixation in a stereotactic apparatus.

Step 1: Preparation of Surgical Area

Put crushed ice into the Petri dish and cover it with aluminum foil (this is necessary to maintain hypothermia-induced anesthesia during surgery). As previously described[4], a homemade stage is used to position the body and stabilize the head. However, the dimensions of the stage should be adjusted according to the strain and size (or weight) of the mouse. For example, we used P0 C57BL6 mice (mean weight: 1.3 g) and precut the stage according to the features shown in Fig. 3A. Place and fix

Table 1. Features of systemic and local anesthetics used in adult mice

Anesthetics		Dose			Anesthesia duration	Sleep duration
		Concentration	Volume	Dose		
Ketamine (Imalgene®)	Ketamine	100 mg/mL	1.5 mL	150 mg/kg		
+ Xylazine (Rompun®)	Xylazine	20 mg/mL	0.75 mL	15 mg/kg	10 min	60–120 min
	Saline	sterile	7.75 mL	-		
	Mixture	-	10 mL	0.1 mL/10 g (IP)		
Tribromoethanol (Avertin®)				250 mg/kg	2 min	40–90 min
				0.1 mL/5 g (IP)		
Isoflurane (Iso-Vet®)				3% - 200 mL/h induction	Unlimited	Unlimited
				1.5% - 200 mL/h maintenance		
Lidocaine (Xylovet®)				17.5 mg/kg (SC)	3 min	Local anesthesia

IP, intraperitoneal; SC, subcutaneous.

Table 2. Management of postoperative analgesia

Analgesics	Dose	Analgesia duration	Protocol
Buprenorphine (Vetergesic®)	0.1 mg/kg SC	12 h	2/day for at least 2 days
Ketoprofene (Ketoprofene Arrow®)	5 mg/kg SC	12 h	2/day for at least 2 days (optional)

SC, subcutaneous.

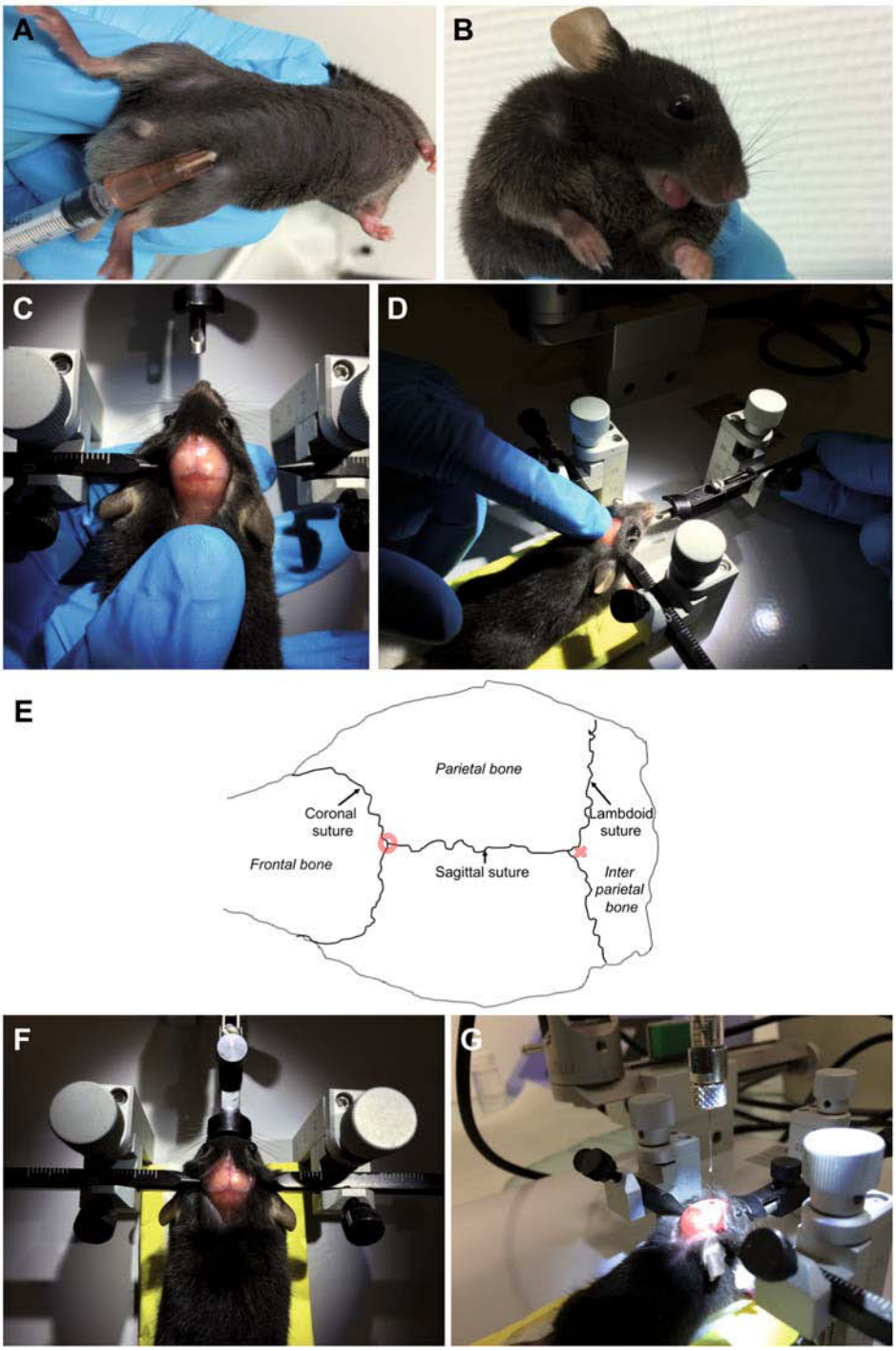

Fig. 2. Procedure of stereotaxic intracerebral injection in adult mice. (A) The ketamine-xylazine mixture is injected intraperitoneally through a 21-gauge needle inserted into the lower right abdominal quadrant. (B) The tongue of the mouse is pulled out to facilitate breathing. Lubricant ophthalmic gel is applied on both eyes to avoid keratitis during surgery. (C) The head of the mouse is held in place to allow positioning of the right ear bar. (D) The head of the mouse is moved up to insert the incisor adapter. (E) Stereotaxic landmarks on the skull of the mouse. Bregma is represented by the red circle and lambda by the red cross. (F) The snout clamp is placed just below the eyes. Before beginning injection, the skull is perfectly flat. (G) The syringe needle is lowered through the craniotomy to the desired depth; then the injection is started.

the stage to the center of the Petri dish. Finally, place the Petri box on the stereotaxic apparatus and keep it movable. Position the injection syringe on the stereotaxic arm and fix the stereotaxic arm above the Petri dish (Fig. 3B).

Step 2: Anesthesia

Place the newborn mouse in a container of crushed ice for ~2 min until the onset of hypothermia-induced anesthesia. When anesthesia is sufficient to start surgery, the skin becomes slightly blue and the animal does not move (Fig. 3C). This type of anesthesia can only be used for very young pups, until P2–P4 [4].

Step 3: Preparation for Injection

While waiting for anesthesia to set in, adjust the features of fluid aspiration on the injection system (aspirated volume should be slightly larger than injected volume). Pipet the desired volume of fluid onto a precut square of laboratory film; then bring the needle of the syringe into the drop under visual control with the dissecting microscope and suck up the fluid. If you are not comfortable with this, you can place the laboratory film on the stage or directly apply aspiration into the tube containing the fluid. Check that the drop has been aspirated. Make sure that the 33-gauge needle is patent by ejecting a very small volume of fluid (e.g. 50 nL) outside of the brain, under visual control, before proceeding. Adjust the features of fluid injection on the injection system.

Step 4: Preparation and Positioning of the Animal

Once anesthetized, place the newborn on the stage in the appropriate orientation for injection to obtain a "flat skull".

Step 5: Injection (for Right-Handers)

Hold the animal's head with your left index finger by applying moderate pressure to the back of its neck, and slightly stretch the head skin to facilitate penetration of the needle through the skin and the skull. Importantly, the animal's head should remain held in this position by your left index finger throughout the injection. The edge of the Petri dish should be blocked between your left third and fourth fingers (Fig. 3D). With your right hand, draw a dot on the skin surface at the desired needle insertion point under a dissecting microscope. First use a 30-gauge needle to make a small puncture at the needle insertion point (Fig. 3E). Slide the Petri dish so that the syringe is above the puncture site. With your right hand, lower the syringe until the needle touches the cortical surface and use this point as the "zero" (*Z zero*) on the stereotaxic apparatus. Lower the syringe needle to the desired depth (*Z* coordinate of the injection site) in the brain parenchyma (Fig. 3F and G). Inject the desired volume of injection fluid in one minute. We use a maximum volume of 1000 nL per session in our experiments. *For left-handers, reverse the sides of the procedure.*

Step 6: Withdrawal of the Syringe and End of the Procedure

When the injection is completed, allow a minimum of one additional minute rest time before beginning to withdraw the syringe from the brain. Withdraw the syringe slowly. Remove the animal from the surgical area.

Step 7: Postoperative Management

Immediately place the newborn in the heating cage until full recovery. After ~2 min, the skin appears pink and reaction to touch is normal. Once the newborn recovers, return it to its home cage with its mother and litter. Newborns do not need to be treated with analgesics (buprenorphine and ketoprofene can be toxic in newborn animals) or cleaned with iodine-based wash. Monitor the health and well-being of the animal for at least 7 days.

While performing hypothermia-induced anesthesia of another newborn animal, rinse the syringe to avoid clot formation in the needle by alternating aspiration and ejection of distilled water (in most stereotaxic apparatus this can easily be done using the "withdraw/inject" program). Check the patency of the syringe by ejecting of a large volume of distilled water (>5 µL).

Clean and disinfect the tools and surgical area with 70% ethanol. This surgical procedure (steps 2–6) can be performed in 5 min.

RESULTS

We performed 300 intracerebral injections (270 in adult mice and 30 in neonates) and retrospectively analyzed the targeting of the injection site. Stereotaxic intracerebral injections in adult mice gave 92% (249/270) efficacy in targeting the injection site (Fig. 4A). Concerning injections in neonatal mice, we demonstrate that surgery can be performed by a single operator in a very short time (a litter of 8 neonates was injected in <1 h). Efficacy depends on the precision with which injection sites can be defined, due to the small size of the animal (Fig. 4B).

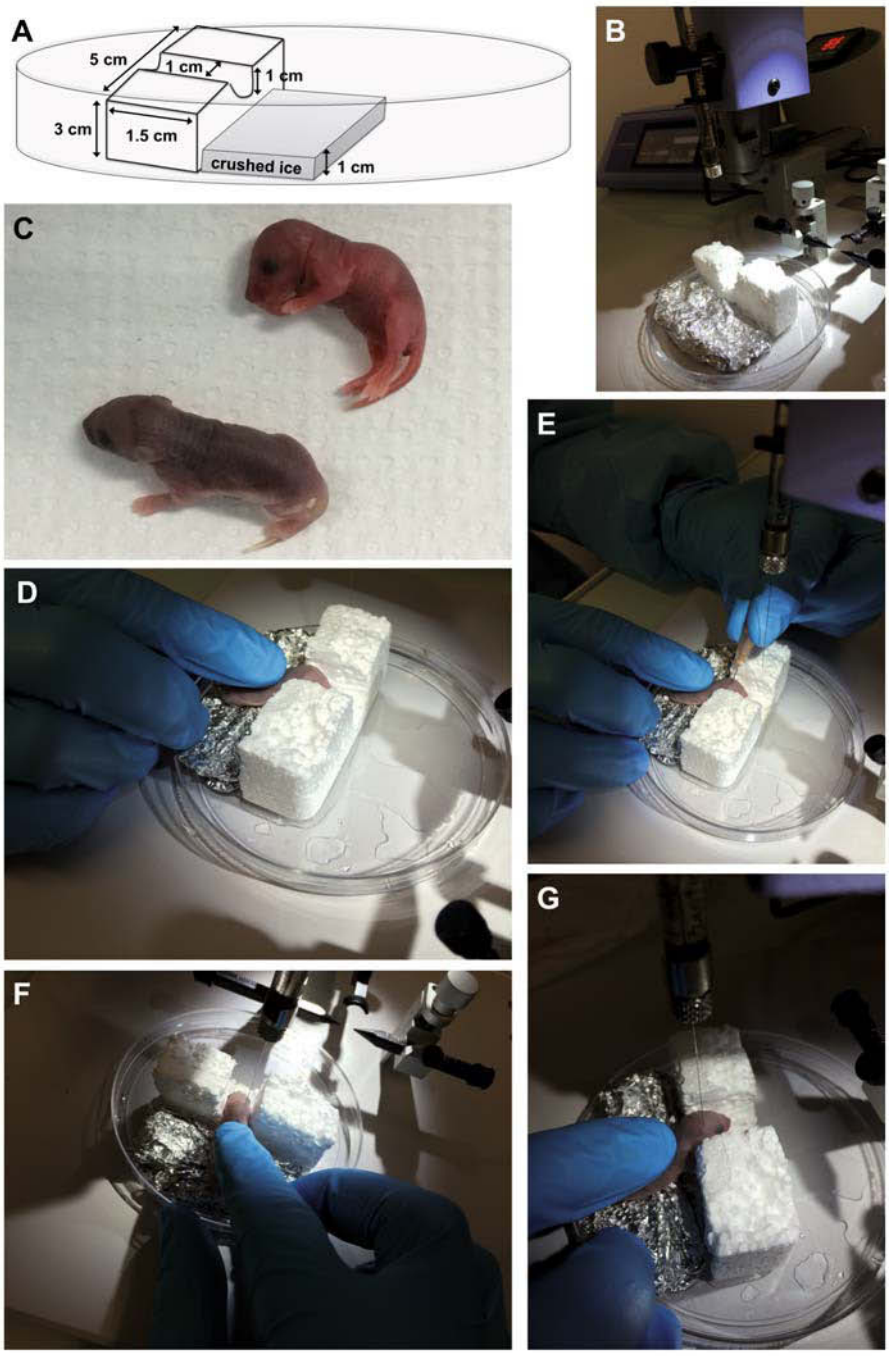

Fig. 3. Procedure of intracerebral injection in neonatal mouse. (A and B) Surgical area. The tailored stage is placed and fixed into the Petri dish. A carpet made with crushed ice surrounded by aluminum foil is placed in front of the stage. The Petri dish remains movable on the stereotaxic apparatus while the syringe is positioned above. (C) At the top of the picture, the neonatal mouse is awake (skin is pink). At the bottom of the picture, the neonatal mouse is under hypothermia-induced anesthesia (skin appears slightly blue). (D) The mouse's head is held with the index finger by applying moderate pressure on the back of its neck while the edge of the Petri dish is blocked between the third and fourth fingers. (E) A small puncture is made in the skull at the syringe needle insertion point with a 30-gauge needle. (F and G) The syringe needle is lowered through the puncture to the desired depth; then the injection is started. The mouse's head remains held by the operator's index finger throughout the injection. *The procedure shown on pictures D-G is performed by a left-handed operator.*

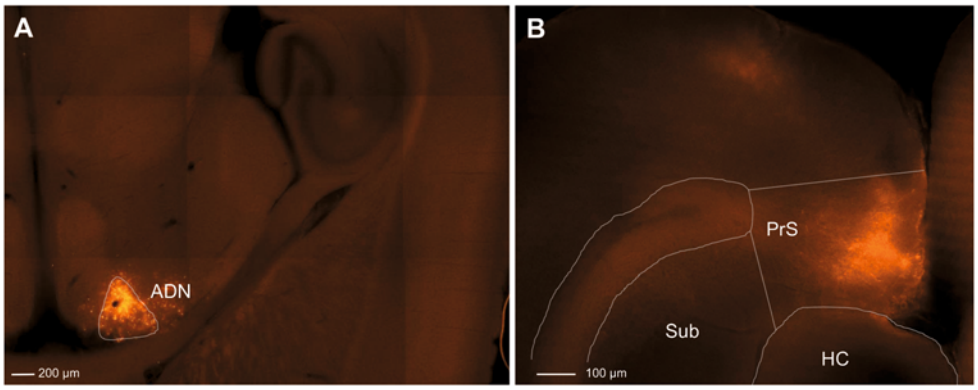

Fig. 4. Accuracy of injection sites. (A) Horizontal slice (DV = -2.85 mm). Stereotaxic injection of m-cherry expressing anterograde glycoprotein-deleted rabies virus (RABV ΔG (VSV G^{RtmC}); 300 nL)[13] in the anterodorsal nucleus of the thalamus (ADN) in an adult mouse (P28). The animal was sacrificed at P36. (B) Horizontal slice (DV = -2.00 mm). Injection of adeno-associated virus AAV1.CAGGS.Flex.ChR2-tdTomato (Addgene® 18917; 500 nL)[14] in the presubiculum (PrS) in a neonatal mouse (P0) which was sacrificed at P21. The virus expresses tdTomato in a cre-dependent fashion, here in an Sst-IRES-Cre mouse (Jax #013044). AAV, adeno associated virus; ChR2, channelrhodopsin-2; DV, dorsoventral; HC, hippocampus; IRES, internal ribosome entry site; PrS, presubiculum; RABV, rabies virus; Sst, somatostatin; Sub, subiculum.

DISCUSSION

Surgical Tips

Stereotaxic Intracerebral Injections in Adult Animals

Planning the injection (coordinates and trajectory)

-Define the injection site that allows the easiest trajectory. The injection site should be as superficial as possible; in the best case, the trajectory should avoid ventricles; when the needle enters the cortex, it should be orthogonal to the cortical surface (it is possible to work with oblique trajectories depending on the stereotaxic apparatus).

-Calculate tailored coordinates. After choosing coordinates using *The Mouse Brain in Stereotaxic Coordinates* (George Paxinos and Keith B.J. Franklin, Academic Press, 2005), verify the injection site by injecting an inert tracer (e.g. 300 nL of Fluororuby or Chicago blue) in several animals and assess the reproducibility of your injections under a microscope. If your injection site is incorrect, correct it by calculating deviation between the expected site and the current site.

-Privilege one-phase injections. Should you need to inject two different fluids (e.g. virus and tracer) in the same animal, inject them during the same procedure (if possible) because postsurgical fibrosis can mask skull sutures rendering a second injection more difficult.

Preparation of surgical area and tools

-Careful handling of injection syringe and needle. Make sure that the needle is correctly inserted and fits tightly into the syringe. If you observe air bubbles inside the syringe, remove the needle, reinsert it into the syringe, replace the hub, and tighten. While the syringe is unused, we strongly suggest keeping it fully filled with distillated water (do not use sterile saline which can crystallize in the syringe). To avoid clogging of the syringe, use the procedure described above (aspiration and ejection of distilled water) and avoid direct contact between the needle and blood during the surgical procedure (stop bleeding and wash the craniotomy with sterile saline if necessary). Finally, make sure that the needle is not bent, and if so, replace it.

-Isolate stereotaxic apparatus. Vibrations of the environment can be transmitted to the syringe during intracerebral injection and lead to damage of the brain parenchyma. To avoid transmission of vibrations, the base of the stereotaxic apparatus may be isolated by setting it up on an air table.

Anesthesia

-Choose anesthetics: The advantages of isoflurane are: speed of induction and recovery, greater control of depth of anesthesia, less metabolism of the drug in

the liver and significantly less sensitization of the heart to catecholamines. The disadvantages of isoflurane include the cost and logistics of using precision vaporizers, the risk of fatal overdose if an open system is used instead of a precision vaporizer, as well as depressed respiratory rate and decreased blood pressure. In addition, once animals awaken from gas anesthesia, there is no residual analgesic activity[8, 9]. The advantages of ketamine-xylazine combinations are that they may be combined in one syringe, and that they produce short-term surgical anesthesia with good analgesia[10]. The disadvantages of ketamine-xylazine combinations are that they do not reliably reach the surgical level of anesthesia in all cases, and that they can cause profound cardiac depression[8]. Tribromoethanol induces anesthesia rapidly and provides good surgical analgesia for about one hour. However, tribromoethanol is an irritant, especially at high doses or with repeated use, and can cause intestinal ileus several weeks after injection. Tribromoethanol degrades in the presence of heat or light to produce toxic byproducts. Degraded solutions can be both nephrotoxic and hepatotoxic and can lead to death. The effects of tribromoethanol are also somewhat unpredictable in mice younger than 16 days[11, 12].

-**Manage anesthesia.** If the animal is not anesthetized after 10 min using the ketamine-xylazine combination, inject an additional 20% of the dose intraperitoneally and wait 5 min. If a ketamine-xylazine combination is used for surgery longer than 20 min, animals will likely require additional anesthetic. Inject an additional 20% dose intraperitoneally and closely monitor if the anesthesia deepens. It is possible to redose with a lower dose of ketamine rather than the combination. However, redosing repeatedly with ketamine alone does not produce a surgical level of anesthesia. Preferably, isoflurane should be used for procedures longer than 30 minutes.

Surgical procedure

-**Choose the needle.** The choice of the appropriate needle should be made according to the advantages of each type: a rigid steel needle is not diverted by the ependyma for a transventricular trajectory and perforates the dura without prior opening; a flexible silica needle allows for the best healing of brain parenchyma after withdrawal of the syringe.

-**Select the right skull landmarks.** If bregma and lambda are not distinguishable, apply oxygenated water to the skull, and the sutures appear white and easily visible. If the sagittal suture is shifted from the superior sagittal sinus (only visible in young rodents), consider that the zero of the medial-to-lateral (*X*) coordinate is located above the superior sagittal sinus (Fig. 5A and B).

-**Open the dura.** Be careful not to push the dura too strongly downward while trying to penetrate it; this will cause intracerebral hemorrhage or an epidural hematoma. The leader for human spinal anesthesia is the safest tool for opening the dura and avoid injuring the brain surface.

-**Stop bleeding.** To stop epidural or cortical bleeding, dab the injured vessel for at least 10 s with a cotton swab, then wash out the blood clot with sterile saline.

-**Manage the transventricular trajectory.** Several injection sites are located below the ventricle (e.g. thalamus). For these specific sites, we strongly suggest using a rigid needle in order to avoid deflection of the needle by the ventricular ependyma (Fig. 5C-E). After injection, withdraw the syringe slowly, in two stages, allowing 2 min rest time at a point located below the ventricle to avoid intraventricular diffusion of the fluid (Fig. 5E).

-**Manage multisite injection.** To avoid clot formation in the needle during multisite injection, replace the syringe after performing the first injection. Then, after positioning the new syringe on the stereotaxic arm, return to bregma to define the reference (stereotaxic zero) of the *X* and *Y* coordinates.

Intracerebral injections in Neonatal Animals

All surgical tips described above also apply to injections in newborn animals where relevant.

-**Perform surgery at P0.** The superior sagittal and lateral sinuses are used as landmarks to identify the desired needle insertion point. In mice, the sinuses become difficult to discern and the skull becomes less flexible after P3. Thus, to facilitate the procedure, we recommend, as much as possible, to perform surgery at P0. Likewise, the local diffusion of most tracers

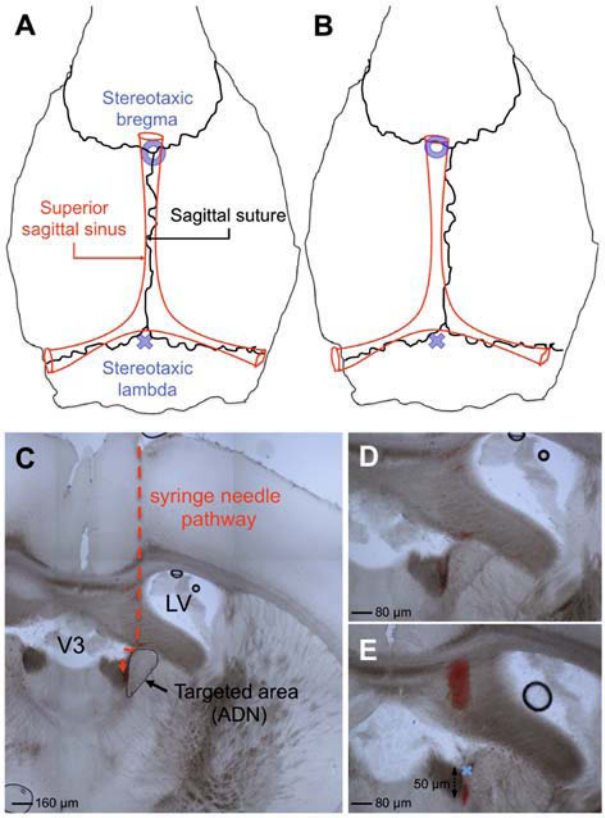

Fig. 5. Surgical tips. (A) Typical anatomical localization of the sagittal suture above the superior sagittal sinus. Correct stereotaxic landmarks are represented by the blue circle (bregma) and cross (lambda). (B) Frequent anatomical variation. The sagittal suture is shifted from the superior sagittal sinus. Correct zero of the medial-to-lateral (*X*) coordinate (stereotaxic bregma) is located above the superior sagittal sinus. (C-E) LV, lateral ventricle; V3, third ventricle; ADN, anterodorsal nucleus of thalamus. Coronal slice of mouse brain after injection of Fluororuby (red inert tracer) targeting the ADN. (C and D) Injection using a flexible 33-gauge needle. The flexible needle is deflected by the ventricular ependyma leading to an incorrect injection site. (E) Injection using a rigid 33-gauge needle. The rigid needle is not deflected resulting in a correct injection site. The blue cross represents the point where the operator should wait 2 min before continuing the withdrawal of the syringe to avoid intraventricular diffusion of the tracer.

throughout the neonatal brain is greater at P0 than that seen later in postnatal development.

-**Define landmarks.** After determining the coordinates of the injection site and the best entry point with the assistance of the *Atlas of the Developing Mouse Brain* (Paxinos *et al*., Elsevier, 2007), in addition to natural landmarks (sinuses), use the stereotaxic arm to increase the precision of the location of the entry point into the skull.

-**Manage the postoperative period.** To avoid parent mice killing their litters, minimize the total time away from the mother, ideally to <15 min [5].

CONCLUSIONS

We describe an innovative, effortless, and reproducible surgical protocol for stereotaxic intracerebral injections in adult mice that should give >90% efficacy in targeting an injection site. This procedure should allow any researcher to master these experiments quickly. Concerning injections in neonatal mice, we demonstrate that surgery can be performed by a single operator in a short time. The efficacy depends on the precision with which injection sites can be defined, due to the small size of the animal. However, our technique using the stereotaxic arm allows a higher precision than the freehand techniques previously described.

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