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TITLE:

Intra-Arterial Delivery of Neural Stem Cells to the Rat and Mouse Brain: Application to Cerebral Ischemia

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SUMMARY:

A method for delivering neural stem cells, adaptable for injecting solutions or suspensions, through the common carotid artery (mouse) or external carotid artery (rat) after ischemic stroke is reported. Injected cells are distributed broadly throughout the brain parenchyma and can be detected up to 30 d after delivery.

ABSTRACT:

Neural stem cell (NSC) therapy is an emerging innovative treatment for stroke, traumatic brain injury and neurodegenerative disorders. As compared to intracranial delivery, intra-arterial administration of NSCs is less invasive and produces a more diffuse distribution of NSCs within the brain parenchyma. Further, intra-arterial delivery allows the first-pass effect in the brain circulation, lessening the potential for trapping of cells in peripheral organs, such as liver and spleen, a complication associated with peripheral injections. Here, we detail the methodology, in both mice and rats, for delivery of NSCs through the common carotid artery (CCA, mouse) or external carotid artery (rat) to the ipsilateral hemisphere after an ischemic stroke. Using GFP-labeled NSCs, we illustrate the widespread distribution achieved throughout the rodent ipsilateral hemisphere at 1 d, 1 week and 4 weeks after postischemic delivery, with a higher density in or near the ischemic injury site. In addition to long-term survival, we show evidence of differentiation of GFP-labeled cells at 4 weeks. The intra-arterial delivery approach described here for NSCs can also be used for administration of therapeutic compounds, and thus has broad applicability to varied CNS injury and disease models across multiple species.

INTRODUCTION:

Stem cell (SC) therapy holds tremendous potential as a treatment for neurological diseases, including stroke, head trauma and dementia[1-6]. However, an efficient method to deliver exogenous SCs to the diseased brain remains problematic[2,6-13]. SCs delivered through peripheral delivery routes, including intravenous (IV) or intraperitoneal (IP) injection, are subject to first-pass filtering in the microcirculation, especially in the lung, liver, spleen and muscle[8,9,13,14], increasing chances of accumulation of cells in non-target areas. The invasive intracerebral injection method results in localized brain tissue damage and a very restricted distribution of SCs near the injection site[2,6,8,14-16]. We have recently established a catheter-based intra-arterial injection method to deliver exogenous neural SCs (NSCs), which is described here applied in a rodent model of focal ischemic stroke. We induce transient (1 h) ischemia-reperfusion injury in one hemisphere using a silicone rubber coated filament to occlude the left middle cerebral artery (MCA) in the mouse or rat[17-19]. In this model we have reproducibly observed approximately 75-85% depression of cerebral blood flow (CBF) in ipsilateral hemisphere with Laser Doppler or Laser speckle imaging[17,19], yielding consistent neurological deficits[17-19].

For time-saving purposes, the video is set to play at twice the normal speed and routine surgical procedures such as skin preparation and wound closure with suture and the use and setup of the motorized syringe pump are not presented. The method of intra-arterial delivery of NSCs is demonstrated in the context of the middle cerebral artery occlusion (MCAO) model of experimental stroke in rodents. Therefore, we include the transient ischemic stroke procedure in order to later demonstrate how the second surgery, the intra-arterial injection, is performed using the previous surgical site on the same animal. The feasibility of intra-arterial NSC delivery in rodent stroke models is demonstrated by assessing the distribution and survival of exogenous NSCs. The efficacy of NSC therapy to attenuate brain pathology and neurological dysfunction will be reported separately.

PROTOCOL:

All the procedures on animal subjects were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Kentucky, and appropriate care was taken to minimize stress or pain associated with surgery.

1. Preparation of injection catheter and surgical hooks

1.1. Construct the injection catheter (**Figure 1**). Gather necessary materials including: MRE010, MRE025, and MRE050 tubing, 20 G, 26 G and 27 G injection needles (**Figure 2A**), 600 grit sandpaper, superglue and two-component 5-minute epoxy.

1.1.1. Cut 20 G and 26 G needles at 1 cm from the needle hub and polish the end on sandpaper (**Figure 2B**). Flush the needles with 10 mL of double distilled water to clean the needle bore.

NOTE: Two different designs (**Figure 1**) are used. Design 1 has a single connector and is used for injection of solutions or suspensions. Design 2 has 20 G and 26 G Luer lock connectors for

injection of cells (20 G needle) and flush of the dead volume (26 G needle) to ensure delivery of the full volume of NSC-containing solution.

1.2. Design 1: Insert a 3-4 cm length MRE010 catheter into a 15 cm length MRE025 catheter and secure with superglue.

1.2.1. Connect the other end of the MRE025 tube to a segment of MRE050 catheter, and secure with superglue. Insert a dulled 20 G needle into the remaining end of the MRE050 catheter and secure with superglue (**Figure 1**).

1.2.2. Further reinforce the connection sites with epoxy glue. This catheter design is optimal for injection of reagents (like chemical or drug solutions or other biologics such as cytokines).

1.3. Design 2: Insert a 3-4 cm length MRE010 catheter into a 15 cm length MRE025 catheter and secure with superglue.

1.3.1. Connect the other end of the MRE025 tube to a segment of MRE050 catheter, and secure with superglue. Insert a dulled 20G needle into the remaining end of the MRE050 catheter and secure with superglue.

1.3.2. Insert a dulled 26 G needle into the MRE050 tube near the tip of the first needle, following the direction of injection flow, and secure with superglue (**Figure 1** and **Figure 2C**). Reinforce both needles and the segment of MRE050 tube with clear epoxy (**Figure 2C**). This design allows injection of vehicle solution through needle 2 (26G) after NSC injection through needle 1 (20 G) to flush the dead volume in the catheter into the brain circulation, achieving more precise control of injection volumes.

1.3.3. Use a 20G needle for NSC injection in order to minimize damage to the NSCs, which could adversely affect viability.

1.4. After construction, flush the catheters with 10 mL of double distilled water, followed by 70% ethanol, and then soak them in 70% ethanol overnight.

1.5. Before the surgery, remove the catheters from 70% ethanol and flush with 10 mL of sterile PBS, and place them in an autoclaved surgical tool box for storage and transportation.

1.6. Preparation of the surgical hooks

1.6.1. Cut a 1.5- 2 cm long needle shaft from a 27 G needle, and polish both ends on sandpaper until dull. Then use a small hemostatic clamp to bend the shaft into a hook at one end and a ring-shape at the other end.

1.6.2. Insert a 10-15 cm long MRE025 catheter through the ring and secure with clear surgical tape (**Figure 2D**). Make 2 more hooks using the same method.

1.6.3. Soak all hooks and catheter systems in 70% ethanol until use.

2. Animal preparation: Delivery, housing, environment adaptation

2.1. Use male and female C57BL/6 mice (10-12 weeks, n=10/time point) and Wistar rats (10-12 weeks, n=10) in this study.

2.2. House them in an environmentally controlled animal vivarium with food and water ad libitum.

2.3. Allow them to adapt to the environment at least 1 week before the stroke surgery.

NOTE: One mouse and one rat died at 1 d after stroke surgery and one mouse was euthanized at 3 d post-stroke prior to NSC injection for humane reasons because of severe paralysis.

3. Culture of mouse and rat neural stem cells (NSCs)

NOTE: NSCs were isolated and cultured following an established protocol[20].

3.1. Mouse

3.1.1. Isolate wildtype (WT) and GFP-labeled NSCs from the E18 embryonic cortex from timed-pregnancy female C57BL/6 mice mated with GFP-positive male mice (B6 ACTb-EGFP). To identify GFP (+) embryos, observe the harvested embryos on a fluorescence microscope using the FITC channel. GFP (+) embryos yield green fluorescence signal while WT embryos show only weak auto-fluorescence (**Figure 3A**).

3.2. Rat

3.2.1. Isolate NSCs from the subventricular zone (SVZ) of young adult WT rats. Label them with DiI just prior to injection following manufacturer's instructions[21].

3.3. Culture mouse or rat NSCs until they develop into neurospheres, and passage them when the diameter of sphere reaches around 100 µm. Use the NSCs for injection between passages 3 and 5.

3.4. Verify their stem cell properties using an embryonic stem cell marker panel (**Figure 3C**).

3.5. On the day of injection, collect NSC spheres and dissociate with the cell detachment solution, suspend in calcium- and magnesium-free PBS to a concentration of 10^7 cells/mL, and place on wet ice until injection.

4. Surgical preparation

4.1. Before surgery, mark a dot on the commercial MCAO suture with a silver marker pen at 9 mm (for mouse) or 15 mm (for rat) from the tip for in-surgery reference of insertion length. Autoclave the surgical tools (scissors, forceps) and instruments before each surgery, and heat sterilize them in a glass bead sterilizer between operations.

4.2. Induce anesthesia in animals with 5% isoflurane via inhalation and maintain anesthesia with 1-2% isoflurane. Evaluate the depth of anesthesia through observation of general conditions (breathing pattern, whisker movement, and spontaneous body correction posture), corneal reflex and response to toe pinch.

4.3. Lay animal supine on a heating pad, prepare the surgical site on the animal by clipping and scrubbing with betadine solution followed by 70% ethanol. Protect the animal's eyes from drying by applying ophthalmological ointment (e.g., artificial tear ointment) during surgery.

4.4. Have surgeons thoroughly scrub their hands with a bacteriocidal scrub and wear a mask, sterile gloves, and a clean lab coat.

5. Middle cerebral artery occlusion (MCAO) stroke surgery

NOTE: The surgeries to induce ischemic stroke in one hemisphere of mouse or rat are similar in that a suture is introduced into the internal carotid artery (ICA) to occlude blood flow (**Figure 4**)[17-19,22]. However, the artery selected for suture insertion differs based on the available operation space required for the subsequent stem cell injection. The rat has ample space in the external carotid artery (ECA) segment to permit two separate, sequential surgeries (stroke and NSC injection), but the mouse does not, requiring an alternate approach. Stroke-induced cerebral blood flow changes, brain infarct size and neurological deficits have been reported as in the authors' previous reports[17-19].

5.1. To induce ischemic stroke, begin both mouse and rat surgeries with a midline incision on the cervical area, and isolation of the left common carotid artery (CCA), ECA and ICA (**Figure 4**). Exercise caution to not stretch, displace or squeeze the CCA or vagus nerve. Since the selection of artery and surgical steps are different thereafter, MCAO surgery on the mouse and rat will be described separately.

5.2. MCAO surgery on mouse (**Figure 4A**)

5.2.1. Place three braided 6-0 nylon sutures under the CCA (**Figure 4A**, step 1), and make one tight surgical knot to occlude the vessel as far from the bifurcation as possible using the proximal string (**Figure 4A**, step 2). Trim down the suture ends.

5.2.2. Make a slipknot at the distal side of CCA (caution: do not over-tighten as it will be released in step 6) and one loose slipknot in between the two tightened knots (**Figure 4A**, step 2).

5.2.3. Cut a small incision (~ ¼ - ⅓ of the circumference) close to the proximal knot on the CCA with microscissors (**Figure 4A**, step 3), and carefully insert the commercial silicone rubber coated 7-0 solid nylon suture (**Figure 4A**, step 4). Secure this suture with the middle string, tightening sufficiently (**Figure 4A**, step 5) to ensure no blood leakage from the incision and no movement of the silicone rubber coated nylon filament by the backflow from ICA, while still allowing advancement of the suture toward the ECA with a gentle push by the tweezers.

5.2.4. Release the upper (distal) slipknot (**Figure 4A**, step 6) and advance the nylon suture into the ICA until its tip passes the bifurcation for 9 mm (using the silver marker on the suture as a reference). Tighten the upper two slipknots to secure the suture and prevent blood backflow.

5.2.5. Withdraw the filament 1 h later (**Figure 4A**, step 7) and ligate the CCA using the middle knot to prevent bleeding (**Figure 4A**, steps 5-7 in reverse order, final results as seen in step 8). Release the upper knot. Close the wound with 4-0 surgical suture.

5.3. MCAO surgery on rat (**Figure 4B**)

5.3.1. Place two braided 6-0 nylon sutures under the ECA (**Figure 4B**, step 1), and make one tight knot at the distal end as far as possible (**Figure 4B**, step 2).

5.3.2. Place vessel clips on the ICA and CCA to occlude the arterial blood flow (**Figure 4B**, step 3). A slipknot can be used as an alternate for a vessel clip.

5.3.3. Make a small incision on the ECA with microscissors (**Figure 4B**, steps 3-4), insert a commercial silicone rubber coated 6-0 nylon filament (**Figure 4B**, step 5), and secure properly with a slipknot on the ECA.

5.3.4. Release the vessel clip on the ICA, advance the filament into the ICA until the silver marker (15 mm) reaches the bifurcation (**Figure 4B**, step 6), and then secure the suture with the 2nd knot on the ECA (**Figure 4B**, step 6).

5.3.5. After 1 h of ischemia, withdraw this filament and ligate the incision to prevent bleeding (**Figure 4B**, step 7), remove the vessel clip from CCA (final result as in step 8), and close the wound with 4-0 surgical suture.

6. Recovery

6.1. After stroke surgery, place animals on a heating pad until they fully regain consciousness.

6.2. Provide analgesia via subcutaneous injection. Return animals to their home cages with access to water and food ad libitum.

7. Intra-arterial injection

7.1. Wash the whole catheter with 70% ethanol and soak overnight until use. Right before the injection, connect the Luer lock of the needle with a sterile syringe, and wash the entire lumen side of the catheter system with 10 mL of sterile PBS.

7.2. Time window and preparation for NSC injection

NOTE: Based on experience and reports from other research teams, the timing for NSC injection is crucial for survival of both subjects and exogenous NSCs. In our pilot study, injection of NSCs at early time points (within the first 6 h after reperfusion) led to higher mortality. Thus, we tested later injection time points and determined the time window between 2 d (48 h) to 3 d (72 h) after stroke is safe and tolerable for animals, and is efficient in achieving intraparenchymal distribution of NSCs. Results presented herein are from animals received NSC injection at 3 d after injury.

7.2.1. Set the syringe pump injection rate at 20 µL/min for mice and 50 µL/min for rats. Excessive speed or duration of the injection can result in systemic volume overload, to which mice are more vulnerable than rats.

7.2.2. In brief, at 3 d after stroke surgery, anesthetize the animals with isoflurane and lay them supine on a heating pad.

7.2.3. Reopen the cervical wound and expose the ECA, ICA and CCA again (**Figure 5**, step 1). As in the stroke surgeries, determine the injection route based on the species. Utilize the CCA for NSC injection in the mouse, and the ECA for the rat[23].

7.3. Intra-arterial Injection through the CCA in mouse

7.3.1. Place two 6-0 braided nylon sutures under the CCA. Create a loose slipknot with each of them between the bifurcation and lower knots from the previous stroke surgery (**Figure 5**, step 2).

7.3.2. Tighten the upper slipknot and then make a small incision above the lower knot (**Figure 5**, step 3). Insert a MRE010 catheter through the incision (**Figure 5**, step 4) and secure with the middle knot without blocking the injection flow (**Figure 5**, step 5). Backflow of blood should be visible in the catheter when releasing the upper knot and adjust the catheter position.

7.3.3. Place a vessel clip on the ECA, inject 1 x 10^6 GFP-NSCs through this catheter at 20 µL/min for 5 min with a syringe pump, followed by a flush with 50-100 µL of PBS at the same speed.

7.3.4. After injection, ligate the CCA above the incision with the upper slip knot and withdraw the MRE010 catheter (**Figure 5**, step 6). Tighten and trim the middle knot and the upper knot. Remove the vessel clip from the ECA. Refer to the final image in **Figure 5**, step 7.

7.3.5. Close the wound with 4-0 surgical suture.

7.3.6. After providing adequate recovery on a heating pad and subcutaneous analgesic injection, return animals to their home cage.

7.4. Intra-arterial injection through the ECA in rat

7.4.1. Temporarily occlude the ECA and CCA with vessel clips (**Figure 5**, step 2).

7.4.2. Make a small incision at the proximal side of ECA (**Figure 5**, step 3), insert the MRE010 catheter, and secure with a knot (**Figure 5**, step 4).

7.4.3. Remove both vessel clips, inject 5 x 10^6 NSCs in 100 µL of PBS at 50 µL/min for 2 min, followed by a flush with 50-100 µL of PBS (**Figure 5**, step 5) at the same speed, using a motorized syringe pump.

7.4.4. After injection, occlude the CCA and ECA with vessel clips again and ligate the ECA at the proximal side of the second incision after withdrawal of the injection catheter (**Figure 5**, step 6).

7.4.5. Remove the two vessel clips (**Figure 5**, step 7) and close the wound with 4-0 surgical suture.

7.4.6. After providing adequate recovery on a heating pad and subcutaneous analgesic injection, return animals to their home cage.

7.5. Histological assay

7.5.1. Collect brains from mice that received ischemic stroke followed by injection of NSCs or vehicle solution after euthanasia and intracardiac perfusion with 4% paraformaldehyde at 1 d (mouse and rat), 7 d (mouse) and 30 d (mouse) after injection. Each of these four groups consisted of 5 NSC and 5 vehicle injected animals.

7.5.2. Fix brains overnight and cryopreserve in 30% sucrose for 3 d.

7.5.3. Embed the brains into OCT, sliced at 40 µm thickness, and examine the distribution of NSCs after immunostaining with cell specific markers, including glial fibrillary acidic protein (GFAP, astrocytes), Tuj1 (mature neurons), and doublecortin (DCX, immature neurons).

NOTE: Because of the lack of a rat strain that expresses GFP, we utilized DiI, a transient fluorescent label, for rat NSCs, which allows only relatively short-term observation. Hence, NSC distribution was only examined at 1 d after stroke in rats.

REPRESENTATIVE RESULTS:

GFP-labeled NSCs were readily detected in the ischemic brain, mostly in the ipsilateral hemisphere, especially in the penumbra and along the injury rim (**Figure 6**). The examiner was single-blinded during imaging and analysis.

For example, at 1 d after injection, NSCs were detected within the mouse hippocampus. A subset of NSCs showed co-expression of the immature neuron marker DCX in the dentate gyrus even at this early time point (**Figure 6A**).

At 10 d after stroke (7 d after NSC injection), exogenous GFP-NSCs were observed at the highest density at the rim of injury (watershed area) in the striatum and cortex (**Figure 6B**). It is notable that by 7 d after injection many of the GFP-NSCs also expressed DCX (shown by blue circles), indicating their neuronal fate. Compared to animals that received vehicle solution injection, NSC injection also increased DCX staining (red) in ipsilateral hemisphere.

At 30 d after injection, NSCs were still detected in the injured cortex, and a portion of them showed expression of glial marker GFAP (**Figure 6C**) or mature neuronal marker Tuj1 (**Figure 6D**), indicating the potential of exogenous NSCs to differentiate into either a neuronal or glial fate, and survive up to 1 month in the injured brain.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic designs of injection catheters. We introduce two designs, Design 1 for compound solution injection and Design 2 for cell injection.

Figure 2: Preparation of catheter for NSC injection and of surgical hooks. (**A**) Materials for catheter construction: MRE010, MRE025 and MRE050 catheters at 3 cm, ~10-15 cm, and 3 cm lengths, respectively. (**B**) Cut off needle tips and polish until dull. (**C**) Connect each segment and secure with superglue, and then embed both needle Luer locks and MRE050 segment in epoxy for enhancement. (**D**) Make surgical hook using 27 G needle shaft and MRE025 catheter. Scale bar: 5 mm.

Figure 3: Culture of GFP (+) neural stem cells. (**A**) Identify GFP (+) embryos with fluorescence microscope using the FITC channel. (**B**) Isolate and culture cortical NSCs until they form neurospheres. Scale bar: 100 µm. (**C**) Examine neurosphere properties using a stem cell marker panel. Scale bar: 50 µm.

Figure 4: Schematic images of step-by-step middle cerebral artery occlusion (MCAO) stroke surgery on mouse or rat. Refer to the video for detailed surgical operation. ICA, internal carotid artery; ECA, external carotid artery; CCA, common carotid artery.

Figure 5: Schematic images of intra-arterial neural stem cell (NSC) injection in mouse or rat. Refer to the video for detailed surgical operation. ICA, internal carotid artery; ECA, external carotid artery; CCA, common carotid artery. The green arrow indicates direction of flow during injection.

Figure 6: Distribution, survival and differentiation of neural stem cells (NSCs) in the ischemic brain. (**A**) Detection of GFP (+) NSCs within the hippocampal dentate gyrus at 1 d after injection. Stem cells fluoresce green; doublecortin (DCX) immunostaining shown in red. The white arrow

indicates a GFP (+) NSC with DCX expression (red). (**B**) Schematic map of GFP (+) cells and DCX labelled cells at 10 d after Ischemia-Reperfusion (I-R) in sham controls (no injection) and vehicle (I-R) or NSC (I-R + NSC) injected mice. The topography of the ischemic insult is depicted in the last schematic, where lighter and darker orange represent the area subject to ischemic challenge and the necrotic core, respectively. The blue ribbon indicates the "watershed" area. The gray rectangles depict the locations where images for (**C**) and (**D**) were taken. (**C,D**) Exogenous NSCs can differentiate into a glial fate (GFAP, C) or a neuronal fate (Tuj1, D) by 30 d after delivery. No significant signals were observed in the FITC (GFP) channel in the stroke animals that received vehicle injection (vehicle in **C** and **D**), while in NSC injected mice, surviving GFP-NSCs were visualized and colocalized with GFAP (**C**) or Tuj1 (**D**) staining. Arrows indicate overlay of 2 channels. Scale bar: 50 μm.

DISCUSSION:

Stem cell therapy for neurological diseases is still at an early exploratory stage. One major issue is there is no established method for sufficient delivery of SCs or NSCs into the brain.

Although exogenous SCs/NSCs can be detected in the brain following intravenous (IV), intraperitoneal (IP) or intraparenchymal/intracerebral injection, each delivery approach has drawbacks. The detectable population within the brain is estimated to be very low with peripheral injection (IV or IP), representing only a small fraction of the cells injected or infused. Intracerebral injection yields a very focal distribution, and may directly induce brain injury[2,6-13]. Therefore, we tested the feasibility of intra-arterial injection as an alternative method for NSC delivery following ischemic stroke. This method delivers NSCs through the ipsilateral cerebral perfusion after a stroke insult. If injected early after stroke, exogenous NSCs can cross the disrupted blood-brain barrier (BBB), achieving a broad distribution throughout the brain. One advantage to intra-arterial injection is that it utilizes a first-pass effect within the CNS, maximizing the potential for exogenous NSCs to settle in the brain, in contrast to peripheral delivery routes in which the cells first pass through the rich microcirculation of filtering organs such as the lung and liver.

The intra-arterial approach described here is versatile, and can be adapted to accommodate different types of delivery paradigms and injury or disease models. Although in the current study only one intra-arterial injection is performed, the MRE025 catheter can be connected to a microport that is embedded subcutaneously, through which animals can receive repetitive intra-arterial injections[12]. Moreover, with the simpler, single lumen design, this injection method can be used for delivery of reagents in solution[12,23]. If delivery of multiple therapeutics is required, the dual lumen design could be utilized to deliver an initial solution simultaneously or sequentially with a second drug or compound. For applications to rodent models of neurodegeneration or traumatic brain injury, where there is no need for the first stroke surgery, surgery for installation of the injection catheter in the mouse can be performed on the ECA (same protocol as that for rat, appropriately adjusting the injection volume and rate in Step 7.4 for mice), to avoid potential disturbance of cerebral blood flow through the CCA.

Several disadvantages and potential adverse consequences of this intra-arterial injection should

be considered. Animals receive a second surgery, which carries the potential for complications related to anesthesia or surgery. Cerebral blood flow through the ipsilateral CCA is disturbed, albeit transiently (less than a few minutes), which may induce another transient episode of mild depression of CBF. In addition, BBB disturbance or opening is critical for intra-arterial NSC delivery, which limits the therapeutic window. In the pilot study, almost no GFP (+) NSCs were detected in naïve brain after intra-arterial injection. However, if the subject can tolerate medications that can transiently open BBB, such as high osmolality mannitol or saline, this could be used to create a transient window of BBB opening for NSC injection at later time points. In preliminary studies, we found that intra-arterial injection within the first 6 h after stroke resulted in higher mortality than observed with stroke alone. This may be related to a second invasive surgery after a relatively short period of recovery after the first surgery. Alternatively, after ischemic insult, the injured cerebrovasculature may have a higher tendency to constrict in response to any additional stimuli, such as the introduction of the catheter, additional fluid loading, or attachment of exogenous NSCs to the luminal wall after injection. Another reasonable concern regarding NSC delivery after stroke is that NSCs could form emboli that further occlude or disturb microvessels. In agreement with previous reports[8,16,24], we did not find significant evidence of GFP (+) emboli in the microvasculature, although we did find GFP (+) NSCs in the perivascular space (Virchow-Robin space) in the early days after injection. After we optimized the time window for injection, there was no difference in complication or mortality rate between stroke groups that received vehicle or NSC injection in the current study. Therefore, properly designed intra-arterial NSC injection is a safe and efficient method of NSC treatment targeting neurological diseases.

To achieve successful NSC injection and improve animal outcomes, several aspects should be handled with caution during the stroke surgery or NSC injection. General surgical support and care, such as protection of cornea and maintenance of core temperature, should be practiced. Here we introduce some potential complications of this specific surgery and guidance to minimize their occurrence.

There can be stress on the vagus nerve. During surgery, the vagus nerve should not be stretched, crushed, ligated or stimulated. Incidental stimulation of the vagus nerve can induce arrhythmia such as bradycardia, cardiac arrest, or even death.

Improper placement or tightening of suture, or misplacement or slipping of a vessel clip may result in arterial bleeding from the proximal end of CCA (from cardiac output) or distal end through the Circle of Willis. At each step, ensure the vessel clip or knots are placed properly to occlude the blood flow. If bleeding occurs, try to restore the correct placement of knot or vessel clips. If the visual field is blurred with blood, put the tip of a sterile cotton swab on the CCA and hold with pressure to stop the blood flow. Hemoglobin from bleeding will facilitate closing of the incision on the artery. After the bleeding stops, tighten the knot or place the vessel clip at the correct location, clean the blood in the visual field and continue the surgery.

There can be injury or complications from catheter insertion. Trim the MRE010 tip at a 45° angle, so it can enter the small incision on the artery easily, without inducing any vessel injury. In rare

cases, an over-sharpened tip may penetrate the artery or enter the space between the basement membrane and tunica externa. To avoid these injuries, make a proper size incision on the artery. We recommend a size of ¼-⅓ the circumference of the artery, which is big enough to allow the entry of catheter tip, but retains enough strength in the vessel wall to wrap outside the catheter. Too large an incision may lead to tearing of the artery at the incision site. Gently guide the MRE010 catheter tip to enter the incision. Do not force the entry of the catheter tip or advancement of the catheter. If necessary, sharp forceps can be used to lift the edge of the incision. Advance the catheter with a low angle relative to the artery so that the catheter and artery are almost parallel.

There are also potential injection-related complications. One common complication from intra-arterial injection is excessive volume loading, which can lead to acute cardiac overload and pulmonary edema. Rapid injection rates can amplify these risks and cause damage to the vessel wall[8]. Thus, both rate and total volume should be carefully controlled. We recommend 20 µL/min as generally safe for mice when used over a short period such as 5 minutes. If symptoms of volume overload are noted, such as quick, shallow breath, pink bubbles from nares, or dysphoria-like aberrant movement, the injection should be stopped or aborted, and the animals allowed to recover. Another possible complication is the formation of NSC emboli in the cerebrovascular system. The suspension solution should not contain calcium or magnesium, which are known to promote cell aggregation. To reduce the chances of inducing emboli, single cell suspensions of NSCs should be examined under microscope just before injection to confirm an absence of cell clusters. If cell clusters are present, titrate with a sterile 1 mL pipet until single cell suspension is achieved.

This study establishes the feasibility of the intra-arterial delivery approach for mice and rats, and reveals several important features of this intra-arterial injection of NSCs in the context of ischemic stroke. In comparison to the relatively focal distribution of NSCs surviving in the brain parenchyma typically reported with intra-cerebral injection[1,7,9,11,15,16], we observed a diffuse distribution throughout the ipsilateral hemisphere, including the cortex, hippocampus and striatum. Thus, intra-arterial delivery is well suited not only to stroke, but also to multiple injury types or diseases that involve diffuse brain damage. In the setting of MCAO, the highest concentration of NSCs were found along the rim of the injury site. The increased density of exogenous NSCs in the penumbra zone may be due to increased delivery to this region via collateral flow from re-established blood perfusion and opening of the BBB as well as to migration of NSCs toward the damaged area. Although IV delivery of SCs can result in a diffuse distribution, the number of cells that reach the brain is estimated to be a small fraction of the total delivered, in part due to filtration by peripheral organs[8,13]. Based on a previous study on brain metastasis[12], intra-arterial injected luciferase-labeled D122 tumor cells took advantage of the first-pass effect to settle down in the cerebral vasculature and develop metastatic sites in the brain rather than the peripheral organs. Cerebral metastatic sites due to exogenous tumor cells were detected in the brain ipsilateral to the injection as early as 1 week post-injection using an IVIS imaging system to detect the bioluminescent signal through the intact skull and scalp. In contrast, luminescent signal (indicating tumor burden associated with the exogenous tumor cells) from the peripheral organs, such as liver, lung, and muscle, were not detected until 3-4 weeks after intra-arterial

injection. Therefore, we expect, in a similar scenario, intra-arterial NSC delivery will also benefit from the first-pass effect in the cerebral circulation to greatly increase localization to the brain as compared to peripheral organs.

Although direct intracerebral injection can be used to deliver large numbers of cells to the injured brain, the approach results in cellular damage or hemorrhage due to needle penetration of the parenchyma which triggers localized neuroinflammation, potentially compromising survival and integration of the newly delivered cells[14-16,25,26]. The intra-arterial approach for NSC delivery is advantageous in that it avoids this localized brain damage and neuroinflammation, and supports long-term survival of NSCs[3,8,9,14,24]. We observed survival and differentiation of injected GPF-NSCs in the injured brain at time points up to 30 d after injection. Although we found NSCs that had differentiated into mature neurons and astrocytes, detailed studies are required to determine the relative distribution of various cell types generated from GFP-NSCs and the proportions that survive into the chronic postinjury period. More importantly, whether surviving, exogenous NSCs can interact with constitutive brain cells to rebuild the cerebral network and alter neurological function is still unclear and should be explored.

Taken together, we introduce an intra-arterial delivery method to deliver NSCs into the ischemic brain, demonstrating long-term survival in the ischemic hemisphere and differentiation into neuronal and glial cell types. The intra-arterial delivery approach is adaptable for numerous species and multiple models of CNS injury and disease and can be used for delivery of other cell types or single or multiple therapeutic compounds or biologics, providing broad utility for the neuroscience community.

ACKNOWLEDGMENTS:

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DISCLOSURES:

None.

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623
624

Figure 1

Click here to access/download;Figure;Fig 1 catheter design 1+2.jpg

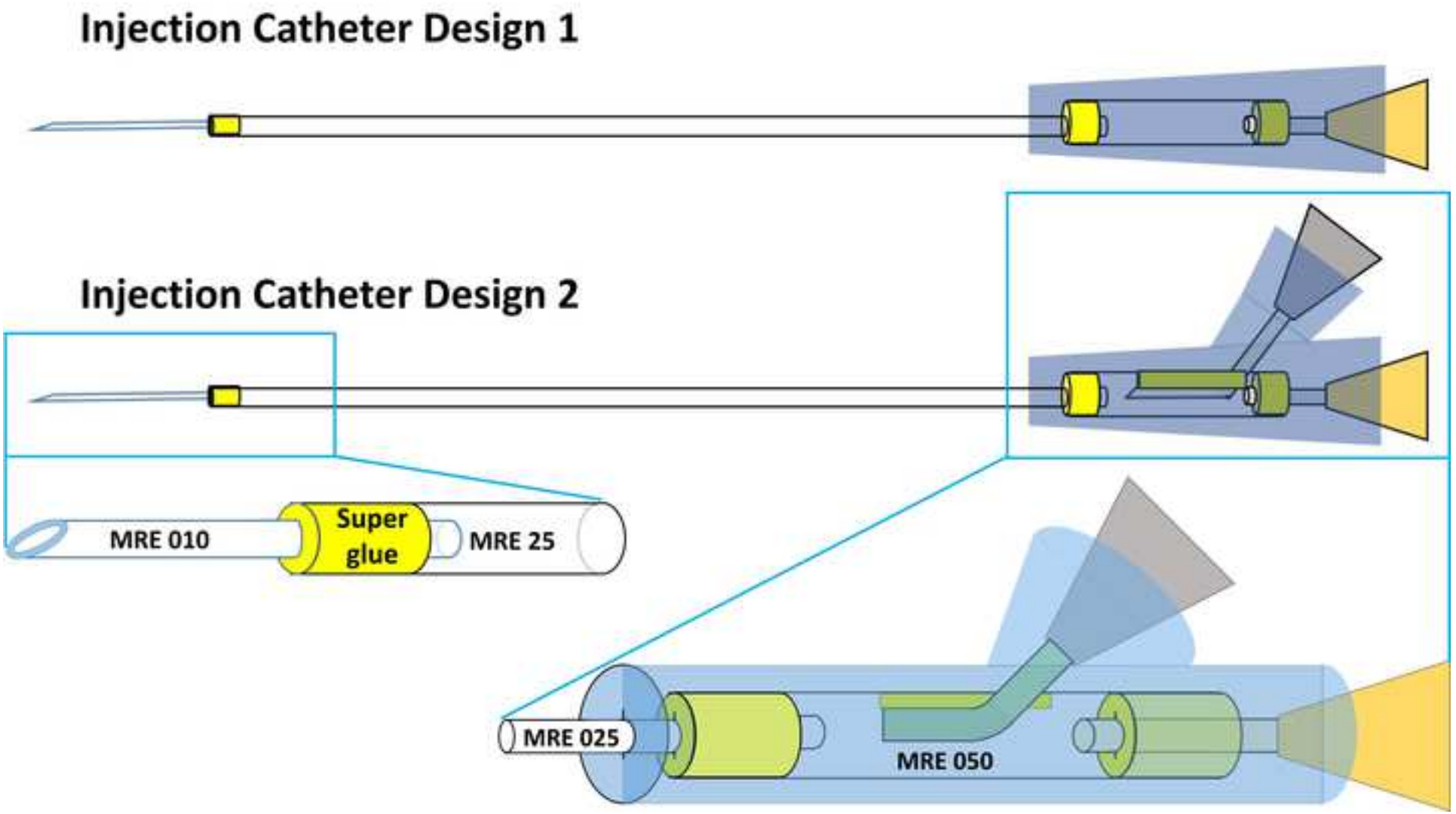

Figure 2

Click here to access/download;Figure;catheter preparation 2020-3-4.tif

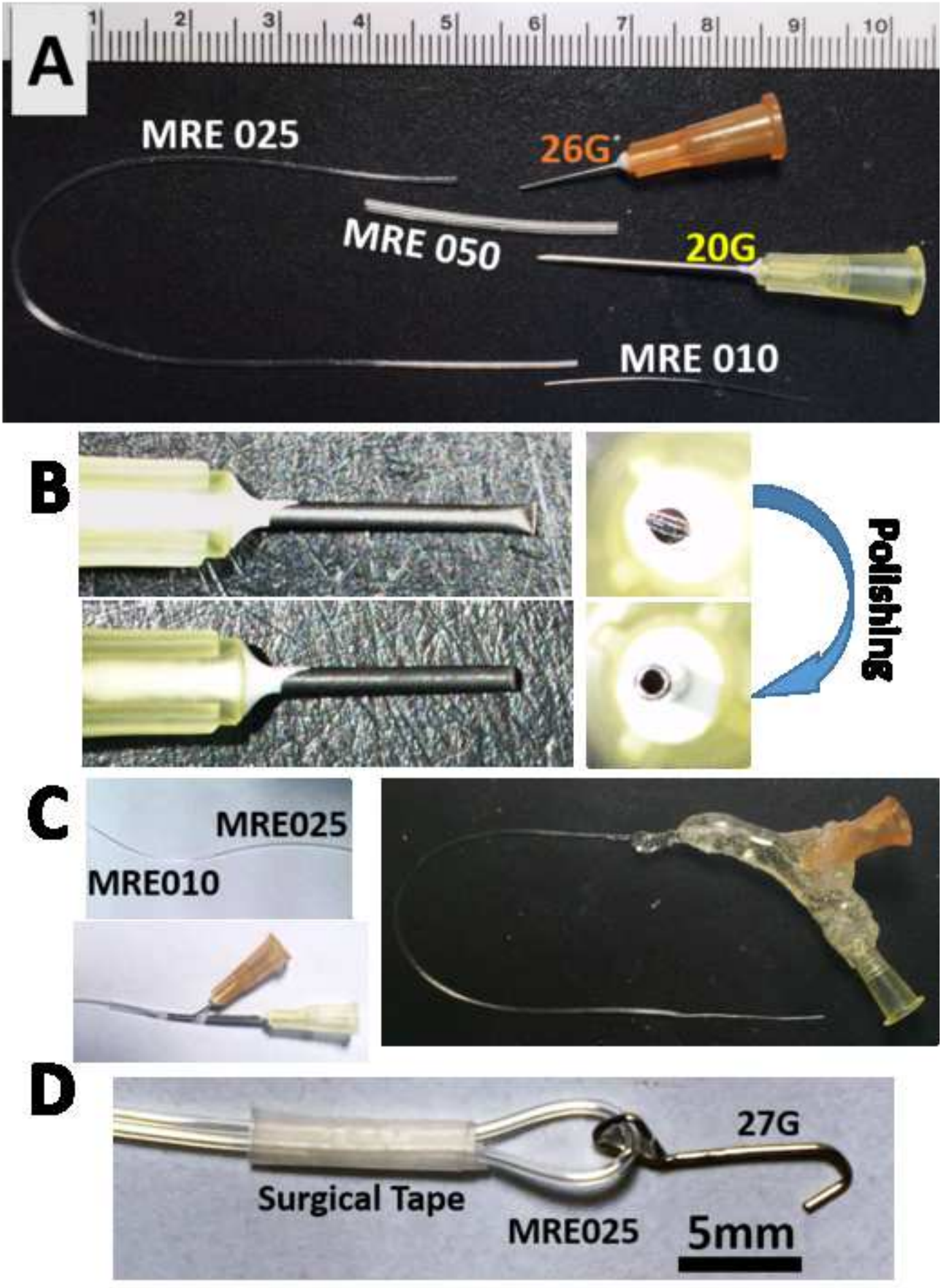

Figure 3

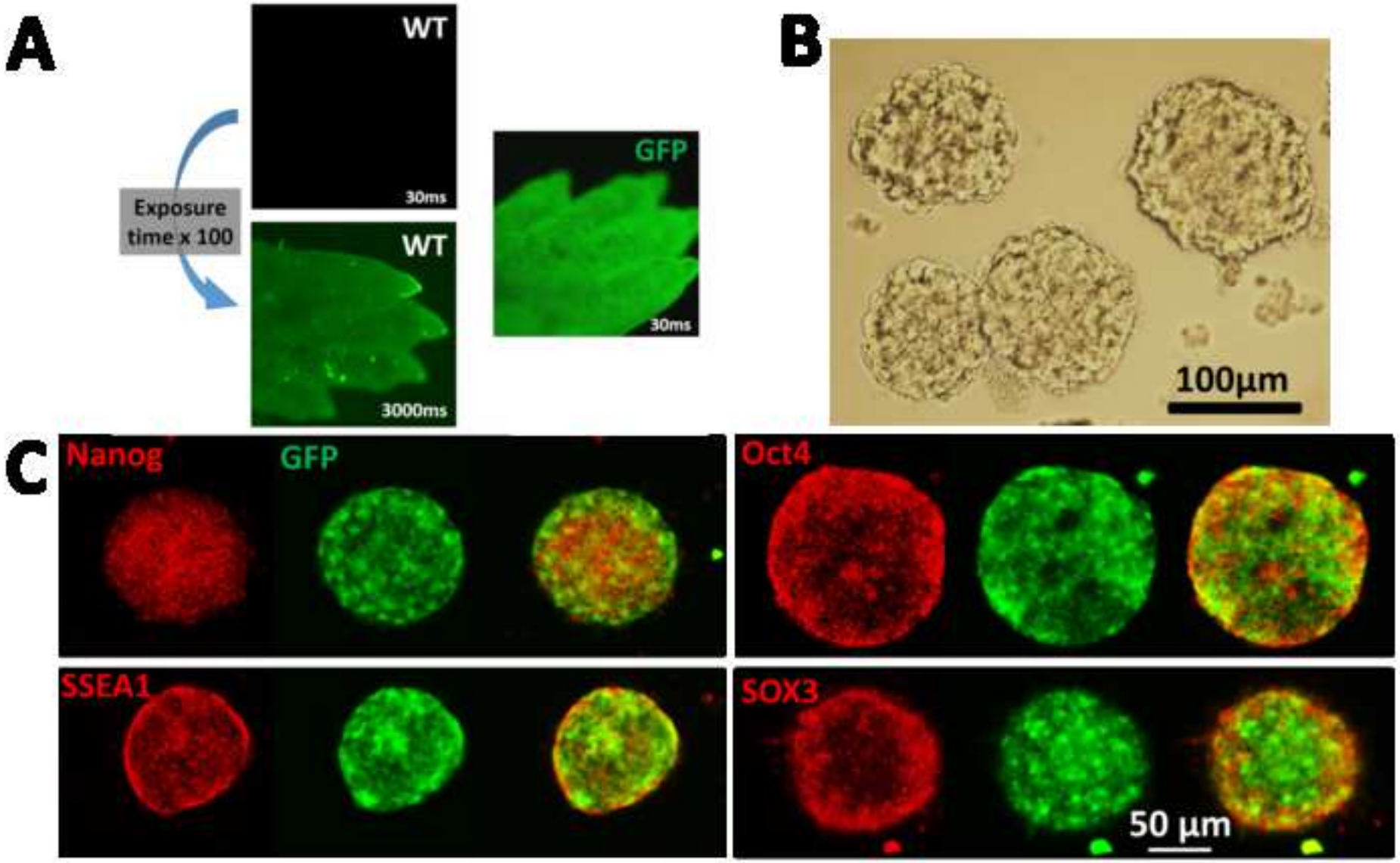

Figure 4

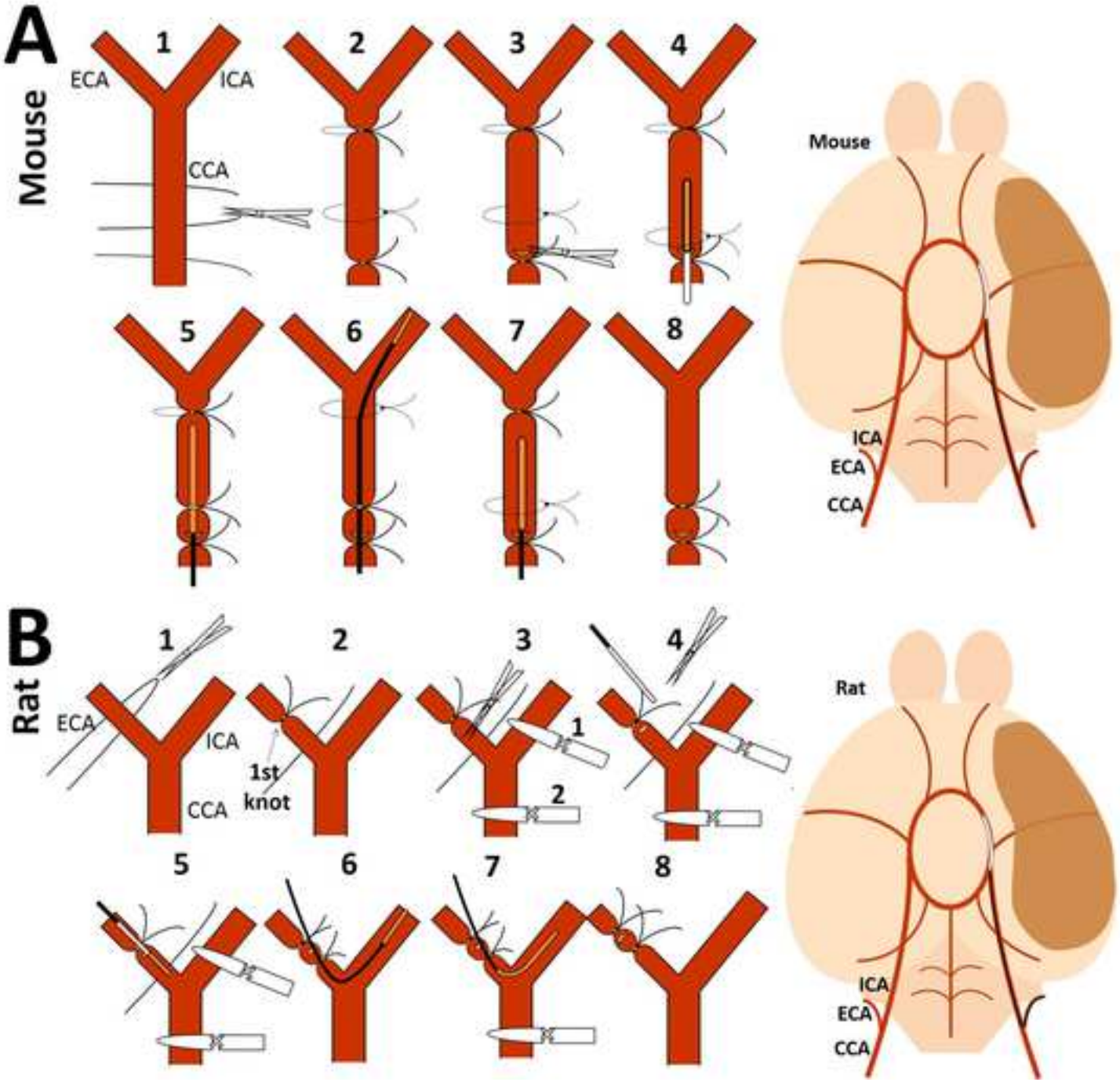

Figure 5

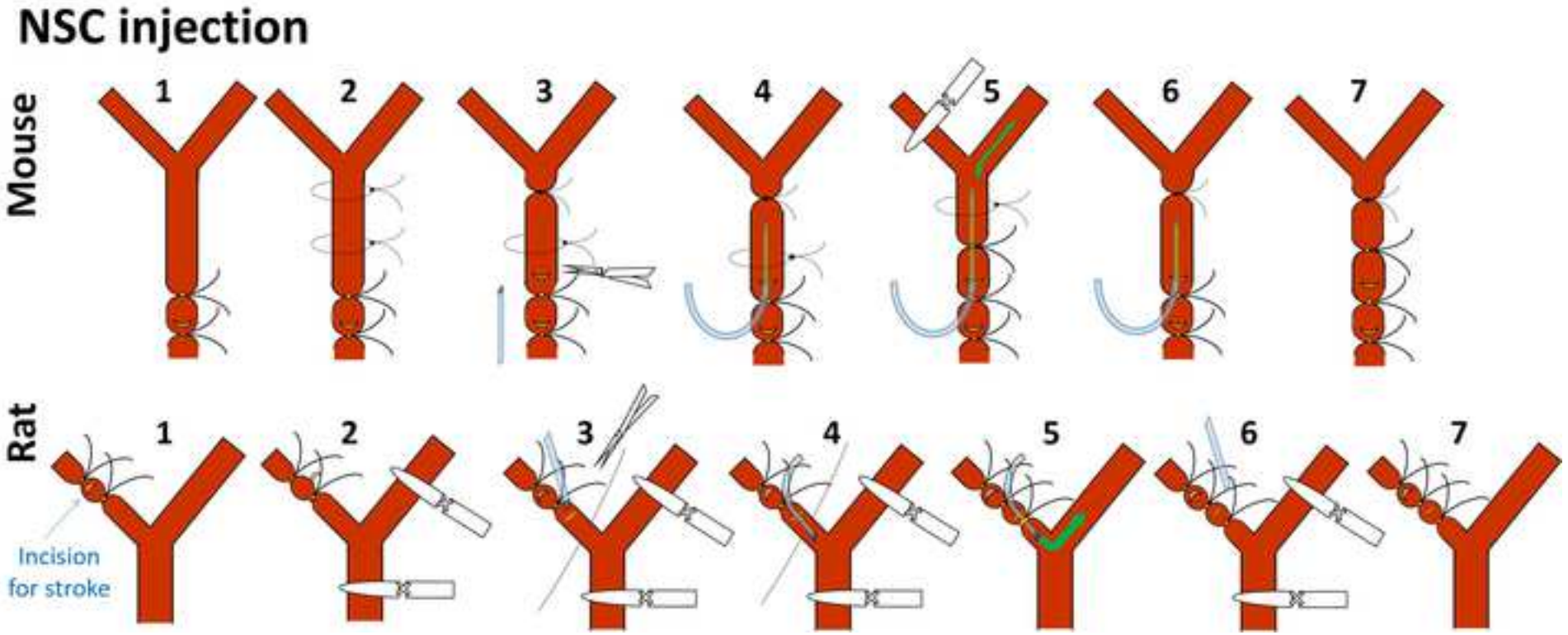

Figure 6

Click here to access/download;Figure;NSC distribution 1-7-30d 2020-4-8 final brown.jpg

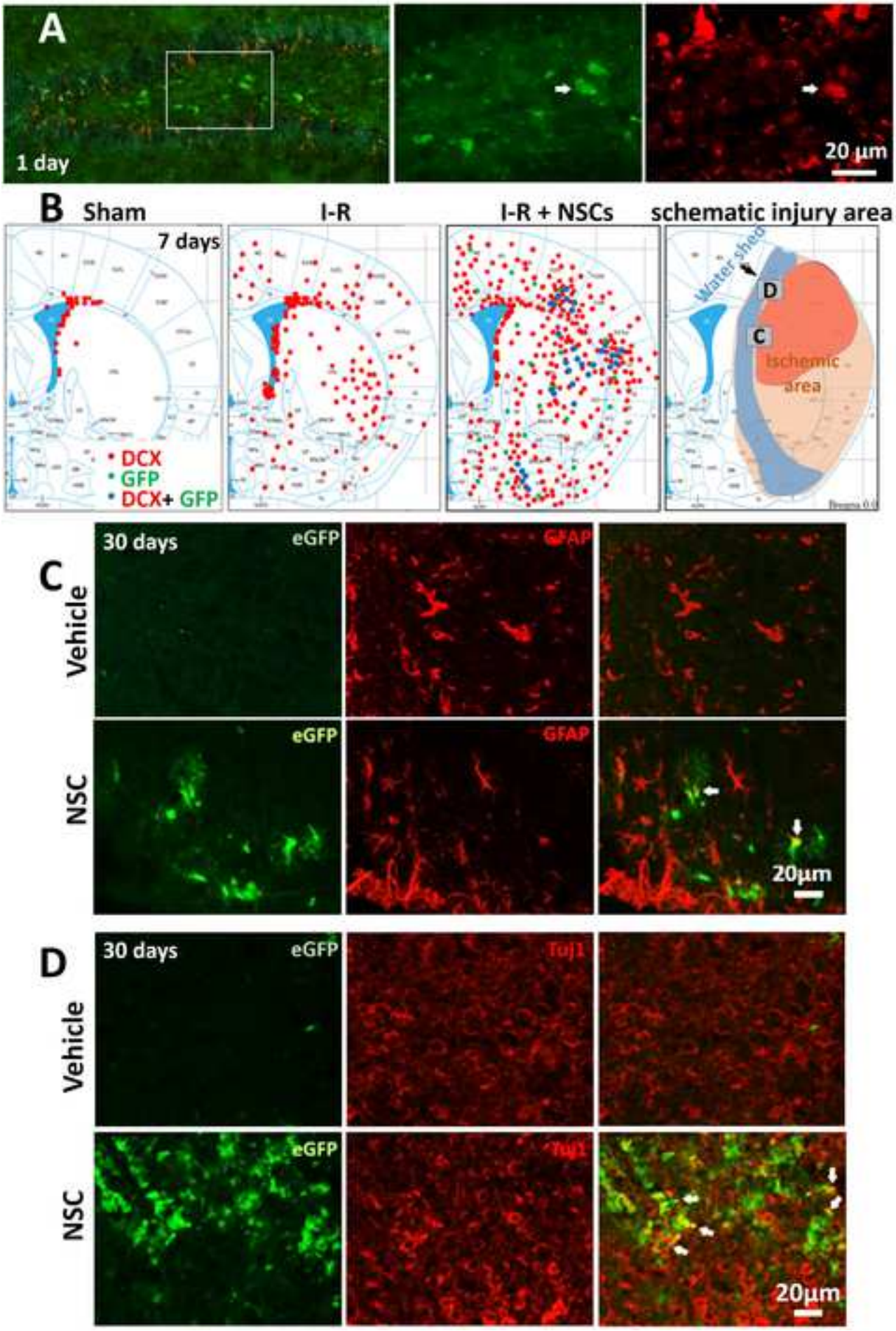

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials 2020-4-10.xlsx

Name of Material/Equipment	Company	Catalog Number
20 G needle	Becton & Dickinson	BD *PrecisionGlide 305175*
26 G needle	Becton & Dickinson	BD *PrecisionGlide 305111*
27 G needle	Becton & Dickinson	BD *PrecisionGlide 305136*
4-0 NFS-2 suture with needle	Henry Schein Animal Health	56905
6-0 nylon suture	Teleflex/Braintree Scientific	104-s
Accutase	STEMCELL Technologies	7922
blade	Bard-Parker	10
Buprenorphine-SR Lab	ZooPharm	Buprenorphine-SR Lab®
Calcium/magnisum free PBS	VWR	02-0119-0500
DCX antibody	Millipore	AB2253
GFAP antibody	Invitrogen	180063
Isoflurane	Henry Schein Animal Health	50562-1
MCAO filament for mouse	Doccol	702223PK5Re
MCAO filament for rat	Doccol	503334PK5Re
MRE010 catheter	Braintree Scientific	MRE010
MRE025 catheter	Braintree Scientific	MRE025
MRE050 catheter	Braintree Scientific	MRE050
Nu-Tears Ointment	NuLife Pharmaceuticals	Nu-Tears Ointment
S&T Forceps - SuperGrip Tips JF-5TC Angled	Fine Science Tools	00649-11
S&T Forceps - SuperGrip Tips JF-5TC Straight	Fine Science Tools	00632-11
Superglue	Pacer Technology	15187
syringe pump	Kent Scientific	GenieTouch
Tuj1 antibody	Millipore	MAb1637
two-component 5 minute epoxy	Devcon	20445
Vannas spring scissors	Fine Science Tools	15000-08
vascular clamps	Fine Science Tools	00400-03
Zeiss microscope	Zeiss	Axio Imager 2

Comments/Description
preparation of injection catheter
preparation of injection catheter
preparation of injection catheter
surgery
surgery
cell detachment solution
surgery
analgesia (0.6-1 mg/kg over 3 d)
NSC dissociation
immunostaining
immunostaining
surgery
surgery
surgery
preparation of injection catheter
preparation of injection catheter
preparation of injection catheter
eye care during surgery
surgery
surgery
preparation of injection catheter
surgery
immunostaining
preparation of injection catheter
surgery
surgery
microscopy

Response:

1. Manuscript: we have added more details in the surgery section at Line 208, 215,216, 230, 234, and 304.

2. Video:
   1). JOVE video format:
   (1).The title card is repeated at the end of the video.
   (2). A 3 second white background is added at the end of the video.

   2). Editing and pacing:
   (1). Short dissolve clips are added at edit points at 4:27, 4:37, 4:47, 6:55, 8:03, and 8:05 in the updated video.
   (2). The word “surgery” is fully extended to its end.
   (3). "Trim down the suture ends." - "Further expose the CCA beyond the bifurcation" - "Push the upper loose slipknot to the bifurcation and tighten it to prevent backflow of blood" have been rearranged and synchronized with video in the updated version at 6:30 – 6:43.

We have made following changes according to comment from the Veterinary Reviewer:

1. Images would be more easily visualized if on a solid background. It looks like they are placed on skin or a textured background. Improvement requires taking images on solid color non-textured background

Response: *We have taken new images and replaced the old photo in Figure 2 C and video from 1:31-1:55.*

2. Difficulty to tell where in the mouse this image is- need to zoom out. There is no mention of anesthesia / heat support / aseptic technique.

Response: *We have inserted additional video frames to show the starting of the surgery and the initial surgical area with bigger visual field (4:28-49), and outlined the zoomed-in surgical area with a black square 4:46- 49. We also introduced the anesthesia, surgical preparation and support in sections 4 and 7.2.3 in the text, and 8:13, 9:24, 9:54, 13:04 in the video.*

3. Labels are not on objects- difficult to tell location. It would also be helpful to know how skin is retracted. Improvement requires removal of labels, or place them directly on objects, not just next to them.

Response: *Since the objects in the surgical area are subject to surgical activity-induced motion, we included still photo shots from the surgical video to better visualize the anatomical landmarks; see 5:06-21. We now also show the process of retracting the skin (see 4:38-4:48).*

4. State whether this suture should be tightened to occlude the vessel.

Response: *Yes, this knot is tightened to occlude the CCA permanently. See section 5.2.1 in text and 6:11-20 in the video.*

5. Bifurcation of vessel mentioned in video is not visible. Label video to indicate bifurcation

Response: *The bifurcation is at the upper edge of the visible surgical area. During operation, follow the CCA to the distal end, and the bifurcation will be visualized as in 6:40-45.*

6. Insert MCO suture into CCA? (step 5)- this is unclear. Mention how far to insert into CCA, and show how to determine this.

Response: *In the mouse MCAO model, the filament suture is inserted into the CCA through a small incision cut with microscissors, as shown in Figure 4A, steps 4-5 and in the video from 6:53-7:06. The filament is inserted 9 mm past the bifurcation in the mouse (described in section 5.2.4) and 15 mm in the rat (section 5.3.4). This is determined using a silver mark on the MCO suture, as described in the surgical preparation steps, section 4.1.*

7. Skin closed and animal allowed to recover- no mention of support for animal

Response: *To keep the total video length within the JOVE limit, routine surgical procedures and postoperative care are described in manuscript, but not shown in the video. Postoperative support for the animal is described in sections 6, 7.3.4 and 7.4.5 of the manuscript.*

8. Animal is reanesthetized- no mention of how.

Response: *The animal was re-anesthetized with Isoflurane inhalation as in the first surgery, as described in the text, section 7.2.3.*

9. Video does not match audio- not sure what is being shown

Response: *We re-edited the video thoroughly, and the video/audio are synchronized now.*

10. How to close skin wound / recover

Response: *The wound was closed with suturing and the animal recovered on a heating pad until they fully regain consciousness. We added detail regarding wound closure in the text (sections 5.2.5, 5.3.5, 7.3.4 and 7.4.5) and recovery is described in sections 6 and 7.4.5. We specify in Note 1 of the text that routine surgical procedures and postoperative care steps are not shown in the video, in order to keep the total video length within JOVE limits.*

11. Tighten 2nd slip knot? This step is unclear.

Response: *Before insertion of catheter for NSC injection, the middle slipknot is loose, as in text 7.3.1*.

12. Syringe pump- there is no mention of syringe pump before this.

Response: *We included the syringe pump information in the material table and discuss its use in the text in sections 7.2.2, 7.3.3 and 7.4.3.*

13. "Ligate the upper knot"- unsure of what this means as ligate usually means to tie off. Does this mean tighten?

Response: *Yes, we ligated the upper knot to block blood leakage. In the new video, this narration is at 12:33-35. To reduce the potential for confusion, the term 'ligate' was replaced by 'tighten' in the text in Section 7.3.4 and in the Discussion within point 1b.*

14. No mention of how to close the wound or number of cells injected. Closure of wound should be mentioned or shown in video.

Response: *Wound closure is mentioned in the video at 9:22-25 and 13:06-09 and in the text, with additional detail, in sections 5.2.5, 5.3.5, 7.3.4 and 7.4.5. The number of cells used for injection is described in sections 7.3.3 and 7.4.3 of the text. We specify in Note 1 of the text that routine surgical procedures and postoperative care steps are not shown in the video, in order to keep the total video length within JOVE limits.*