Chapter 38

Postnatal Neural Stem Cells in Treating Traumatic Brain Injury

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Abstract

Traumatic brain injury (TBI) is one of the leading causes of death and disabilities worldwide. It affects approximately 1.5 million people each year and is associated with severe post-TBI symptoms such as sensory and motor deficits. Several neuro-therapeutic approaches ranging from cell therapy interventions such as the use of neural stem cells (NSCs) to drug-based therapies have been proposed for TBI management. Successful cell-based therapies are tightly dependent on reproducible preclinical animal models to ensure safety and optimal therapeutic benefits. In this chapter, we describe the isolation of NSCs from neonatal mouse brain using the neurosphere assay in culture. Subsequently, dissociated neurosphere-derived cells are used for transplantation into the ipsilateral cortex of a controlled cortical impact (CCI) TBI model in C57BL/6 mice. Following intra-cardiac perfusion and brain removal, the success of NSC transplantation is then evaluated using immunofluorescence in order to assess neurogenesis along with gliosis in the ipsilateral coronal brain sections. Behavioral tests including rotarod and pole climbing are conducted to evaluate the motor activity post-treatment intervention.

Key words Neural stem cells, Neonatal mouse brain, Neurosphere, Controlled cortical impact, Traumatic brain injury, Perfusion, Immunofluorescence, Rotarod, Pole climbing

1 Introduction

Traumatic brain injury (TBI) is the damage of the brain tissues and structures, which is caused by various outside mechanical forces that strike the head such as in car accidents, falls, or being struck by or against a moving or stationary object [1]. The injury is characterized by both morphological and physiological changes, where TBI can cause disturbances at the level of cellular integrity and/or

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affect the homeostasis of brain tissues and cells, thus causing reversible or irreversible cellular dysfunction or death [2]. Due to the fact that the brain is composed of different populations of cells, including neurons, and a closely interacting population of supporting cells, TBI is considered to be complex. Recently, scientists have demonstrated the existence of an interaction between cells of the central nervous system (CNS) and those of the immune system, thus raising the degree of TBI complexity [3]. For these reasons, TBI is considered one of the leading causes of death and morbidity [1]. Experimental models of TBI have been developed to mimic human TBI. Such models allow evaluation and understanding of the morphological, physiological, and behavioral changes associated with TBI. The most widely used model is the controlled cortical impact (CCI) experimental brain injury model, proposed by Smith et al. [4]. It has the advantages of being able to control the severity and the magnitude as well as level of injury. The CCI model mimics focal brain injury and cortical damage as well as axonal injury along with neuronal cell loss [4]. These characteristics will be discussed fully in Chapters 4, 5, and 11.

TBI is associated with a wide range of post-injury affliction, and scientists are currently trying to find fully effective neurotherapies aiming to ameliorate TBI symptoms. One promising strategy is the use of neural stem cells (NSCs) originating from embryonic, neonatal or adult brains [5] whereby NSCs are harvested from the sub-ventricular zone (SVZ) of neonatal mouse brain. These cells are a promising tool for the study of regenerative therapy in TBI, since they are able to become mature and functional cells to replace degenerated ones [6].

A significant amount of work has focused on NSCs. Transplanted cells into the site of injured mouse brain show significant cell differentiation, migration, and long-term survival post-transplantation. Also, the motor and spatial learning functions of the injured animal improve [7].

When neuronal replacement is the primary goal of a cell therapy regimen, the efficacy of NSC-based therapy could be increased via promoting neuronal differentiation. Application of poly-unsaturated fatty acids (PUFAs) [8–10] shows promising results in this regard. Docosahexaenoic acid (DHA) is one of the main PUFAs that promote hippocampal neuronal development and synaptic function in the developing hippocampus [8]. As NSC transplantation in a hostile environment can lead to severe gliosis [11], it may be beneficial to treat NSCs with PUFAs before transplantation and/or co-transplanting NSCs with the neurogenic promoting PUFAs.

In this chapter, we describe the methodology of culturing and harvesting NSCs, and, inducing TBI using the CCI mouse model. In addition, we describe the process of intra-cardial perfusion in mice prior to brain removal. We also explain the detailed methodology for the detection of neurogenic markers using immunofluorescence assays (IF) on brain sections, and, hematoxylin and eosin (H&E) staining to

evaluate the injury. Finally, we illustrate rotarod and pole climbing tests used for assessing motor activity post-NSC transplantation.

2 Materials

2.1 Harvesting Neural Stem Cells from Neonatal Mouse Brain

2.1.1 NSC Isolation

1. 15 ml Tubes.
2. 50 ml Tubes.
3. Eppendorf tubes.
4. Petri dishes.
5. Pads (towel-like mesh, placed on the bench used to absorb blood and liquid).
6. DMEM-F12 HAM, 7 ml (Sigma, D8437 Ca, USA)
7. Ethanol 75%.
8. Distilled water.
9. Blade (Paragon, 0086, USA).
10. Straight Iris scissors (RS-5650—Roboz Surgical Instrument Co.).
11. Curved narrow pattern forceps (RS-5671—Roboz Surgical Instrument Co.).
12. Narrow pattern forceps (Roboz, Switzerland).

2.1.2 NSC Culture and Neurosphere Formation

1. Tissue Dissociation Solution (ATV; Aqueous Trypsin and Versene EDTA chelating agent):
   (a) NaCl 8 g/l (Sigma, 71383, Switzerland)
   (b) KCL 0.4 g/l (Sigma, 746436, Switzerland)
   (c) Glucose 1 g/l (Merck, K22651037, Germany)
   (d) $NaHCO_3$ 0.85 g/l (Fisher Scientific, S233-500, USA)
   (e) Trypsin 2 g/l (Lonza, BE02-007E, Belgium)
2. Blocking solution:
   (a) DMEM/F12 HAM (Sigma, D8437 Ca, USA)
   (b) Fetal bovine serum 10% (FBS) (Sigma, F9665, USA)
   (c) DNase 20 μg/ml (Roche, 10104159001, Germany)
3. Complete medium:
   (a) DMEM/F12 (Sigma, D8437 Ca, USA)
   (b) Insulin 50 mg/ml (Humulin R (U100), USA)
   (c) B27 (Gibco, 17504-044, USA)
   (d) N2 (Gibco, 17502-048, USA)
   (e) Glucose 16.25 mM (Merck, K22651037, Germany)
   (f) HEPES 1 M (Amresco, PH = 7, J848)
   (g) Penicillin/streptomycin (Lonza, DE17-602, Belgium)

4. Trypan blue (Sigma, RNBC2340, Germany)
5. EGF 20 ng/ml (Sigma, E4127, USA)
6. FGF 20 ng/ml (Sigma, F5392, USA)

2.2 Controlled Cortical Impact (CCI) Injury Model

1. Ketamine (Panpharma, 30692)/Xylazine (Interchemie) (90 mg/kg and 10 mg/kg, respectively).
2. Betadine solution (Basle, Mundipharma Ag, 12H145K3, Switzerland).
3. (0.9%) Saline.
4. Blade.

2.3 Neural Stem Cell Transplantation

1. Ketamine/xylazine (90 mg/kg and 10 mg/kg, respectively).
2. Betadine solution.
3. 0.9% Saline
4. Hamilton syringe (10 μl) (Hamilton Company, Reno, Nevada, USA).
5. DMEM/F12 medium (Gibco, 31331-028, USA).

2.4 Perfusion, Brain Removal, and Brain Slicing

2.4.1 Intra-cardial Perfusion

1. 10 ml Syringe and 27 G needle for anesthesia.
2. Anaesthetics: Ketamin and xylazine.
3. Ethanol 75%.
4. PBS 1× (Lonza, BE17-517Q, Belgium).
5. Paraformaldehyde (PFA, 4%) (Sigma, 16005, Germany).
6. Butterfly catheter (23 G) with blunted needle.
7. Straight Iris scissors.
8. Curved Iris scissors (Germany, RS-5671).
9. Chemical fume hood.

2.4.2 Brain Removal

1. Freshly prepared PFA (4%).
2. Sucrose (30%) (Sigma, 16104, Germany).
3. Curved narrow pattern forceps (RS-4980).

2.4.3 Brain Slicing

1. Ethanol 75%.
2. PBS 1×.
3. Cryopath.
4. 6-Well plate.
5. Filter paper.

2.5 Immuno-fluorescence (IF) Assay

1. PBST (PBS-1%; Triton), Triton (100×) (Sigma, T8787-250ML, USA).
2. PBS.
3. Blocking solution: 1 ml FBS (Sigma, F9665, USA) + 9 ml PBST.

4. Antibody dilution: 1 ml Blocking solution + 9 ml PBST.
5. Primary antibodies:
   (a) GFAP: rabbit polyclonal antibody, 1/1000 (Abcam, 7260, USA)
   (b) NeuN: rabbit Polyclonal antibody, 1/500 (Abcam, 104225, USA)
   (c) DCX: goat Polyclonal antibody, 1/500 (Santa Cruz, sc-8066, USA)
   (d) Iba1: rabbit Polyclonal antibody, 1/200 (Wako, 091-19741, USA)
6. Secondary antibodies: Anti-rabbit, anti-mouse and anti-goat (Molecular probes).
7. Mounting solution (Sigma, F4680, USA).
8. Anti-fade (Life Technologies, P36930, USA).
9. Coated slides (OMEGALAB, 217105, USA).

2.6 Hematoxylin and Eosin Stain (H&E)

1. Coated slides.
2. Distilled water.
3. Hematoxylin (Merck, Hx247326, Germany).
4. Eosin (0.5%) (Kaltek, 1123, USA).
5. Ethanol (95%; 100%).
6. Xylol (# 3905, Belgium).
7. Mounting solution.

3 Methods

3.1 Animals

All animal experimentation was performed in compliance with Institutional Animal Care and Use Committee (IACUC) guidelines at the American University of Beirut. C57BL6 mice were obtained from Charles Rivers Laboratories maintained in the animal care facility at the American University of Beirut (AUB). All animals were handled under pathogen-free conditions and fed chow diet. Female mice were coupled with males for one night before removal of the male. Females were then tested for vaginal plug and positive mice were considered pregnant (0.5 day of gestation). Females gave birth to ~5–10 pups at days 20–21 of pregnancy. A total of ten pups were used for harvesting NSCs for each experimental group.

3.2 Harvesting Neural Stem Cells from Neonatal Mouse Brain

A thin layer of tissue is isolated from the sub-ventricular zone (SVZ) and the septal region surrounding the lateral ventricles of mouse pup brain (Fig. 1). Tissues from 7 to 10 pups are pooled to generate NSCs for each experimental group. For further details on how to harvest SVZ tissue, please refer to the video protocol by Azari et al. [12].

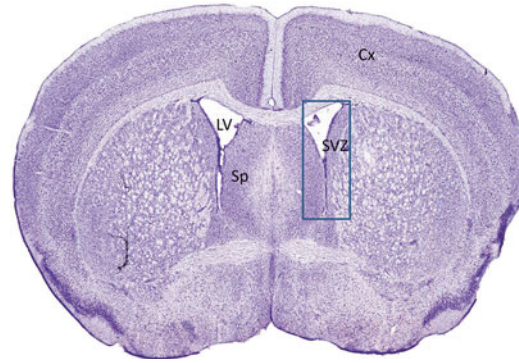

Fig. 1 Coronal section of a mouse brain showing the SVZ for harvesting NSCs. The SVZ and the septal region are marked by a *blue rectangle*. The brain is sliced into coronal sections using a blade. Image is modified from "The Mouse Brain in Stereotaxic Coordinates," George Paxinos and Keith B. J. Franklin, Second Edition, 2001. Cx; cortex, LV; lateral ventricle, Sp; septum, SVZ; subventricular zone

3.2.1 NSC Isolation

1. Sterilize the work area in order to prevent any contamination (*see* **Note 1**).
2. Prepare a couple of Petri dishes containing DMEM/F12 (Sigma-Aldrich) in which the removed brains and sections would be placed (*see* **Note 2**).
3. Anesthetize pups on ice before cutting their heads using scissors. Each brain is removed from the skull and then a coronal section is performed at the level of optic chiasm using a razor blade. The front part of the brain that contains the SVZ can be either micro-dissected as a whole to harvest the SVZ from the lateral walls of the lateral ventricles or it can be further cut into coronal sections of about 350 μm in thickness. The resulting sections are transferred under the microscope to micro-dissect the SVZ tissue containing NSCs.
4. Dissect under the microscope a rectangle that frames the SVZ including the septal region surrounding the lateral ventricles, then drop the tissue in a 15 ml tube containing DMEM/F12 (*see* **Note 3**).
5. Repeat the same procedure until all the brain tissues are harvested.

3.2.2 NSC Culture and Neurosphere Formation

1. Remove excess DMEM using a pipette cone.
2. Put the harvested tissues in a sterile petri dish and gently mince using a sterile scalpel blade. Then, add 2.5 ml ATV solution, collect all tissues in a 15 ml conical tube, and incubate for 10–15 min at 37 °C with gentle shaking (*see* **Note 4**).
3. Inhibit the activity of ATV solution by adding 5 ml of DMEM, 10% FBS (500 μL/5 ml DMEM), and DNase (200 μL/5 ml DMEM) for a total of 5 min (*see* **Note 5**).

4. Centrifuge the mixture for 5 min at 110 × *g* and remove the supernatant.
5. Add 1 ml complete media to the pellet; gently triturate cells by pipetting up and down to make sure that the cells are resuspended in the media.
6. Count the cells using a hematocytometer by taking 50 μL of cell suspension and diluting it in 30 μL DMEM and 20 μL trypan blue in an Eppendorf tube. Mix well and place 20 μL on both sides of the counting chamber (*see* **Note 6**).
7. Seed cells in T25 flasks to a total of 100,000 cells per flask (5 ml complete media/flask). For example, if 2×10^6 (2 million cells/1 ml of complete media) are obtained, prepare three flasks needed, each containing 1×10^5 cells as described in the next step.
8. Prepare a total of 3×10^5 cells in 15 ml complete media (5 ml/flask × 3 flasks); take 150 μL of the cell suspension into 15 ml complete media, and then distribute to the three flasks (10^5/5 ml/flask).
9. Incubate the flasks at 37 °C and 5 % CO_2.

   At this stage the cells will give rise to primary neurospheres (**P0/D0**) (Fig. 2a); it takes 5–6 days to observe neurospheres in the flask.

10. Transfer the supernatant into new labeled flasks the next day (**D1**).
11. Repeat the above transfer for two consecutive days (**D3**) and then add 2.5 ml of complete media containing 0.5 μL EGF and 0.25 μL FGF (2.5 ml/flask) (*see* **Note 7**).
12. Repeat the addition of 2.5 ml complete media after 3 days (**D6**), and supplement with growth factors in the same way. Starting on **D7** up till **D10**, 1–4 days after the last addition of complete media and growth factors, neurospheres should be ready for the first passage. Ideally, neurospheres should have an average size of 110 μm, thus ranging in size between 70 and 140 μm before the passage. Neurospheres should not be grown for longer periods as this may lead to increased cell density at the center of the spheres (dark region) and, eventually, cell death and/or differentiation.
13. Collect the cell suspensions from all flasks in one 15 ml or 50 ml tube and centrifuge the neurospheres at 110 × *g* for 5 min (*see* **Note 8**).
14. Transfer the supernatant into a new 15 ml tube after filtering it using a syringe and a blue strainer (0.20 μM). The collected suspension is called conditioned media.
15. Add 1 ml ATV to the neurosphere pellet for about 5–10 min at 37 °C with gentle shaking (*see* **Note 9**).

16. Prepare 5 ml DMEM containing 500 µL of 10% FBS and 200 µL DNase. Make sure to filter as indicated above. Stop ATV activity by adding the mix directly onto the neurosphere pellet containing dissociated cells and ATV.
17. Wait for 5 min to ensure complete inactivation of ATV activity, and then centrifuge the suspension at $110 \times g$ for 5 min.
18. Remove supernatant and tap the base of the tube.
19. Add 1 ml of conditioned media, then take 50 µL of the cell suspension, and add it to 30 µl DMEM and 20 µl trypan blue.
20. Take 20 µL to perform cell counts using a hematocytometer.
21. Depending on the number of cells obtained after dissociation, distribute 1×10^5 cells in each T25 flask (*see* **Note 10**).
22. Incubate the flasks at 37 °C and 5% CO_2.

At this stage, cells grow into secondary neurospheres (**P1/D10**). It takes 4–5 days to observe neurospheres in the flask.

23. Three days after passage 1 (**D13**), add 1.5 ml complete media to each flask supplemented with growth factors (0.1 µL EGF, 0.15 µL FGF).
24. Five days after passage 1 (**D15**), add 2.5 ml complete media for each flask and supplement with factors (0.5 µL EGF, 0.25 µL FGF).
25. Seven days after passage 1 (**D17**), neurospheres are ready for the second passage (repeat **step 13** to **24**). **TBI** is performed during that same day as described below.
26. At this stage, tertiary neurospheres are obtained (**P2/D20**) (Fig. 2b) and are ready to be dissociated and injected 2 days (**D22**) after the last addition of media and growth factors [13].

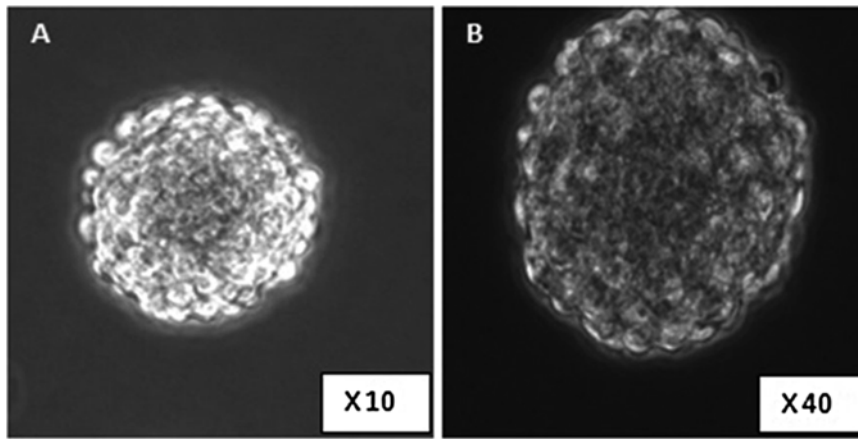

Fig. 2 Different stages of neurosphere culture with little morphological differences. Primary (**a**) and tertiary neurosphere (**b**) at different magnifications

3.3 Controlled Cortical Impact Injury Model (CCI)

An open head injury (penetrating), known as CCI, is performed to induce TBI (Fig. 3), using a Leica Angle Two system (Leica Microsystem, UK). It is a computer-assisted model that allows delivery of an easy and accurate impact to the mouse cortex.

1. Using the mouse coronal brain atlas software of the CCI machine, set the target region parasagitally between Bregma and Lambda (somatosensory area of the parietal cortex) (Fig. 4).
2. Adjust the velocity of the probe to 2 m/s and the dwell time to 150 ms.
3. Anesthetize the mouse by intramuscular injection of ketamin/xylazine (3 μL/g and 0.5 μL/g, respectively) (*see* **Note 11**) and position it in a stereotaxic frame, using the nose clamp and the ear bars.
4. Trim the scalp using scissors and apply Betadine to the underlying skin. A midline incision is made to expose the skull.
5. Locate Bregma and Lambda points manually, using the 1 mm impactor tip (*see* **Note 12**).
6. Move the impactor to "zero" in the instrument medio-lateral (ML) and antero-posterior (AP) coordinates. At this point, the impactor is right above the target site. Using the dorso-ventral (DV) drive, the impactor is lowered until it reaches the skull, confirmed by the contact sensor.
7. The skull is then marked at this particular point and the impactor is retracted in order to perform manual craniotomy, using a dental drill.
8. Lower the probe again until it reaches the brain, then retract it, and lower it again for 1 mm depth (DV axis) in order to per-

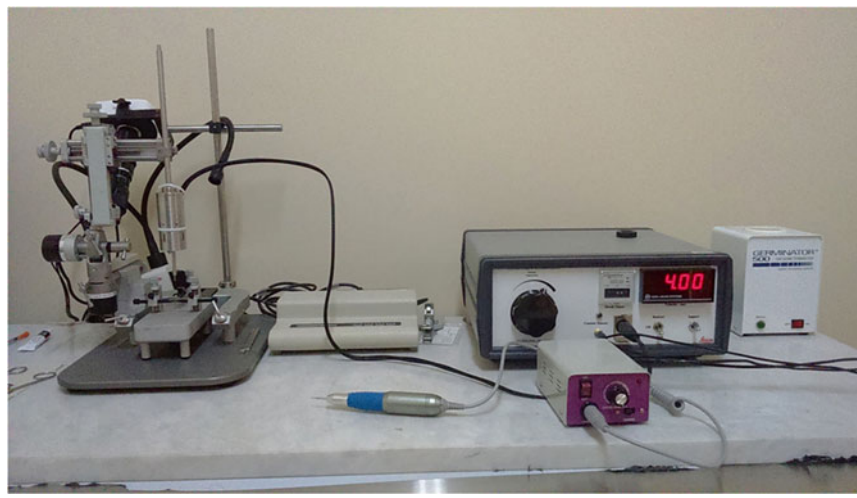

Fig. 3 Controlled cortical impact (CCI) apparatus. A computer-assisted model that induces TBI in the cortex

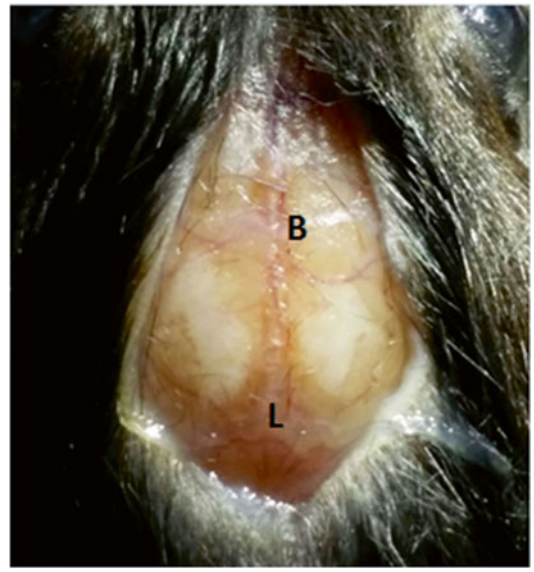

Fig. 4 Bregma and Lambda location. The mouse is positioned in a stereotaxic frame to induce injury

form the impact injury. An injury to the depth of 1 mm is induced to simulate a mild traumatic brain injury.

9. Remove the mouse rapidly from the stereotaxic frame, suture the skin, and keep in a holding cage until recovery from anesthesia (*see* **Note 13**).

TBI is performed on day 17 of cell culture so that the cells would be ready for injection 1 week after inducing TBI.

3.4 Neural Stem Cell Transplantation

The derived neurospheres, supplemented with 1.5 ml and 2.5 ml complete media and associated growth factors on D19 and D21, respectively, are labeled with Hoechst [14] and ready to be injected into the mouse brain at the injury site on D22.

1. Add 2.4 μL Hoechst (stock) to the flask containing 9 ml of complete media and growth factors at this stage.
2. Incubate the flask for 15–30 min.
3. Wash by transferring the suspension into 15 ml tube, add 5 ml PBS (1×) to the neurospheres pellet, and centrifuge for 5 min at 110 × *g*
4. Repeat **step 3** three times.
5. Remove supernatant (PBS), then add 1 ml ATV solution, and incubate for 10 min at 37 °C until neurospheres are dissociated.
6. Add 5 ml of DMEM containing 500 μL FBS and 200 μL DNase using a syringe and a blue filter directly above the pellet to stop ATV activity.

7. Keep the tube in the hood for 5 min until the ATV activity is completely inhibited. Then centrifuge for 5 min at 110 × *g*.
8. Remove supernatant, tap well the base of the tube, and add 1 ml of DMEM.
9. For cell counting, take 50 μL of the cell suspension and add 30 μL trypan blue and 20 μL DMEM (refer to Subheading 3.2.2) (*see* **Note 14**).
10. After cell counting, prepare a total of 3 μL of DMEM containing 1×10^5 cells to be injected in each mouse (refer to **Note 14**).
11. In order to avoid cell death, cells in Eppendorf tubes should be placed on ice and injected immediately.
12. Anesthetize the mouse, wait until the reflex (???elaborate which one or describe??? dissappears, and place the mouse in the stereotaxic frame using the same technique used in CCI.
13. Replace the impactor on the CCI machine with the Hamilton syringe, and fill it with the 3 μL suspension containing 1×10^5 cells.
14. A midline incision is made to expose the skull, and then Bregma and Lambda points are located manually using the 10 μL Hamilton syringe. The syringe is then moved to "zero" in the instruments medio-lateral (ML) and antero-posterior (AP) coordinates; at this point, the syringe is right above the target site. Using the dorso-ventral (DV) drive, the syringe is lowered until it reaches the brain (site of TBI). This is followed by lowering by 1 mm (DV axis) in order to perform the injection (Fig. 5) (*see* **Note 15**).

Fig. 5 Transplantation of NSCs using the CCI apparatus. The mouse is placed in a stereotaxic position. The Hamilton needle is placed on the surface of the brain, directly at the TBI site. The DV is lowered 1 mm and the cells are transplanted in the vicinity of the site of injury

3.5 Perfusion, Brain Removal, and Brain Slicing

One week after injecting NSCs at the site of TBI, brains are perfused, removed and sectioned into brain slices.

3.5.1 Intra-cardial Perfusion of Mice

1. Inject the mouse with ketamine/xylazine mixture. The suitable dose to avoid lethality is 90 mg/kg and 10 mg/kg, respectively [15].
2. Place the mouse in the cage until it is completely anesthetized and no reflex is observed at the pelvic limbs.
3. Place the mouse on its back and rinse the abdomen with 70% ethanol (Fig. 6a).

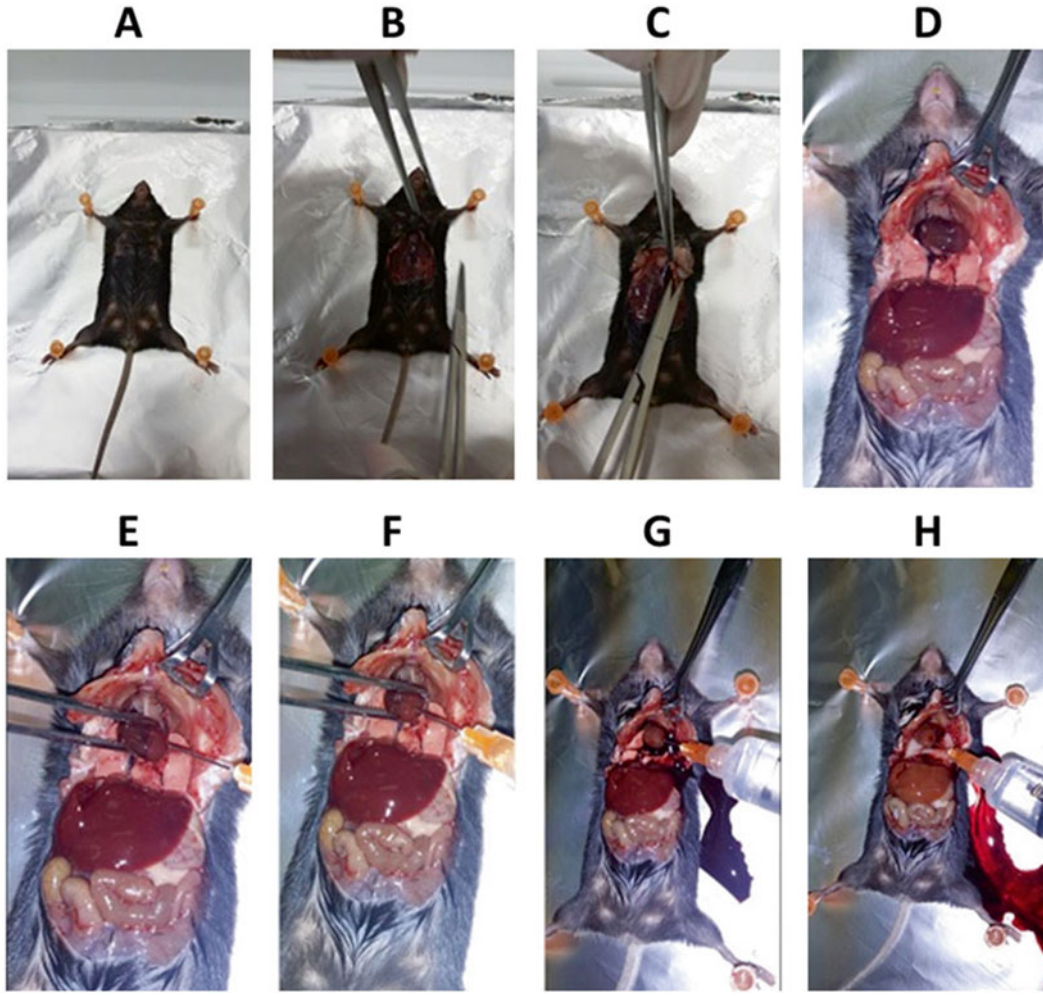

Fig. 6 Perfusion procedure. (**a**) Place the mouse on its back and rinse the abdomen with 70% ethanol. (**b**) A midline incision is made from the thoracic inlet to the pelvis, where the abdomen is opened using scissors. (**c**) Use forceps to grasp the xiphoid (white tip of the sternum), where an incision is made through the diaphragm, and then down the costal cartilage. (**d**) Flip the sternum to visualize the heart well. (**e**, **f**) Introduce a butterfly needle into the apex of the left ventricle. (**g**) Press on the syringe allowing PBS buffer to enter circulation, and then cut the right auricle immediately allowing perfusate to exit from the circulation. (**h**) When the mouse is clear of blood, perfuse using 4% PFA

4. Perform a midline incision from the thoracic inlet to the pelvis.
5. Open the abdomen using scissors (Fig. 6b).
6. Grasp the xiphoid (white tip of the sternum) using forceps, where an incision is made through the diaphragm, and then down the costal cartilage (Fig. 6c).
7. Flip the sternum to visualize the heart well (Fig. 6d).
8. Introduce a butterfly needle into the apex of the left ventricle, which is thicker and of lighter pink than the right ventricle (Fig. 6e, f).
9. Press on the syringe allowing PBS (1×) buffer to enter circulation, and then cut immediately the right auricle allowing the perfusate to exit the circulation (Fig. 6g) (*see* **Note 16**).
10. Once the mouse is clear of blood, perfuse using 4% paraformaldehyde (PFA) (Fig. 6h) (*see* **Note 17**).

3.5.2 Brain Removal

1. Make a midline incision using the scissors and flip the skin of the head to expose the skull (Fig. 7a).
2. Cut the optic nerves and remove the eyes using the scissors (Fig. 7b).
3. Cut along the sagittal suture after inserting Iris scissors caudally to the interparietal bone (Fig. 7c, d) (*see* **Note 18**).
4. Tilt one side of the parietal bone; again tilt the other side of the bone to reveal the brain using curved narrow pattern forceps (Fig. 7e–g).
5. Cut through the most anterior part of the skull between the eyes in order to expose the frontal lobe. This will ease the removal of the brain.
6. Cut the meninges beneath the skull, insert the curved forceps under the anterior part of the brain (olfactory bulbs), and then tilt the brain gently upward (Fig. 7h).
7. Separate the brain from the underlying tissue, by cutting the optic and cranial nerves (Fig. 7i).
8. Gently, lift the brain out of the skull (Fig. 7j).
9. Place the removed brain in 50 mL conical tube containing 10 mL PFA and place at 4 °C.

3.5.3 Brain Slicing

The next day, remove the brain from PFA and place it in 10 mL sucrose (30%) for 2–3 days. Change sucrose solution every day until the brain is ready for slicing (*see* **Note 19**). The aim of brain slicing is to perform histological staining such as IF and H&E.

1. Cut the cerebellum and the most anterior part of the brain using a blade.
2. Adjust the microtome temperature to −40 °C, speed to obtain 40 μm brain slices.

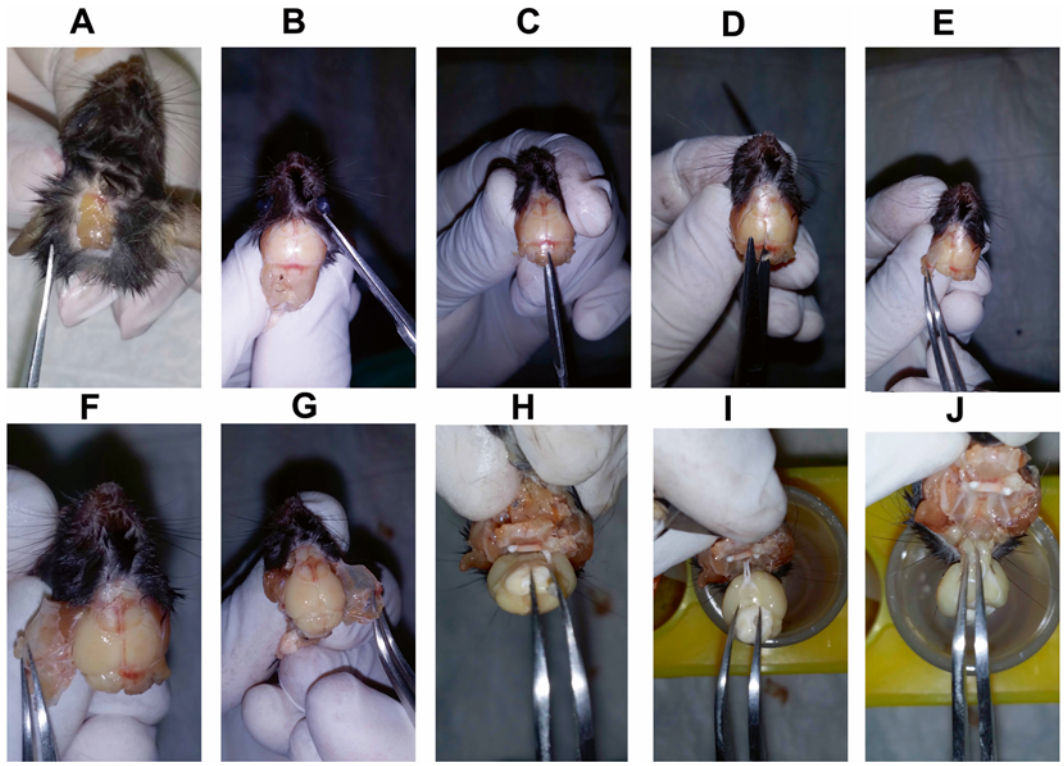

Fig. 7 Brain removal. (**a**) Use the scissors to make a midline incision and flip the skin to expose the skull. (**b**) Cut and remove the eyes. (**c**, **d**) Cut along the sagittal suture after inserting Iris scissors caudally to the interparietal bone. (**e**, **f**) Tilt one side of the parietal bone using curved narrow pattern forceps. (**g**) Tilt the other side of the bone to reveal the brain. Again, cut through the most anterior part of the skull between the eye lobes to expose the frontal lobe. This will ease the removal of brain. (**h**) Lift the brain out of the skull gently, and cut the cranial nerves. (**i**) Separate the brain from underlying tissue by cutting the optic nerve. (**j**) Cut the meninges and the most anterior part of the brain (olfactory bulbs). The removed brain is placed in 50 mL conical tube containing 10 mL PFA and stored at 4 °C overnight

3. Soak filter paper in PBS (1×), fold it into half, and place it on the metal of the microtome to avoid contact of brain tissue with the metal.
4. Place the brain on its posterior side (cerebellum side) on the filter paper in a straight position and spray the tissue with cryopath for instant freezing.
5. Start slicing the brain and distribute the slices into 6-well plates containing 1× PBS (*see* **Note 20**).

3.6 Immunofluorescence (IF) Assay

This technique uses specific antibodies raised against protein antigens in order to target fluorescent dyes to specific biomolecular targets within a cell, and therefore allows visualization of protein expression and distribution throughout the sample.

1. Wash the 40 μm brain slices previously placed in 6-well plates two times with PBST for 5 min at room temperature (RT) with gentle shaking (*see* **Note 21**).
2. Remove PBST after the second wash and add blocking solution (1 mL/well) for 1 h with continuous shaking.
3. Remove the blocking solution and add primary antibody (500 μL/well is enough to cover brain slices) overnight at 4 °C (*see* **Note 22**).
4. Remove primary antibody and perform three washes with PBST (15 min each, RT, on shaker).
5. Add 500 μL of the secondary antibody (specific for primary antibodies) for 1 h at RT with gentle shaking in the dark (*see* **Note 23**).
6. Remove secondary antibody and perform three washes with PBST (15 min each, RT, on shaker), followed by two washes with PBS (1×) (5 min each, RT, on shaker, in the dark) (*see* **Note 24**).
7. Mount the slices on microscope slides (star frost) using a mounting solution (2–3 drops).
8. Add a cover slip and make sure not to introduce any air bubbles (*see* **Note 25**).
9. Visualize using an upright fluorescent microscope.
10. Make sure to include a negative control (lacking primary antibody) for each assay in the immunofluorescence procedure (*see* **Note 26**).

3.7 Hematoxylin and Eosin (H&E) Stain

Most cells are colorless and transparent, and therefore histological sections are stained in order to make them visible. The techniques used can either be nonspecific, thus staining most of the cells in much the same way e.g. using H&E, or specific, selectively staining particular chemical groupings or molecules within cells or tissues e.g. by IF. Staining usually works by using a dye that stains a bright color some of the cellular components together with a counter stain that stains the rest of the cell by a different color. There are six wells per plate: five wells are used for IF assays and one well for H&E.

1. Translocate brain slices from Petri dishes into coated slides (star frost); tissues should be organized from anterior to posterior. This step is performed as slowly as possible to avoid tissue rupture (*see* **Note 27**).
2. Soak filter paper in PBS (1×) to stick slices on the slide and the slide is left on bench for 1 day until it dries.
3. Place the prepared slides in distilled water (dH_2O) for 3 min for hydration.

4. Transfer to hematoxylin for up to 1 min. Hematoxylin is a dark blue or violet stain that is basic (positively charged) and binds to basophilic substances (DNA/RNA) that are acidic and negatively charged.
5. Place the slides under running tap water. The use of tap water (as opposed to dH_2O) is to provide alkalinity necessary for "bluing" process.
6. Immerse the slides in eosin for up to 1 min. Eosin is a pink stain that is acidic (negatively charged) and binds to acidophilic substances (proteins) that are basic and positively charged.
7. Place slides in 95% ethanol two times, 3 min each.
8. Immerse in 100% ethanol for 5 min to prevent cell lysis.
9. Finally place slides in xylol for 1 min. Xylol acts as a clearing solvent to remove alcohol from tissues.
10. Add 2–3 drops of mounting solution followed by cover slip; avoid formation of bubbles.
11. Visualize the slides under a bright field microscope.

3.8 Behavioral Tests

In order to compare changes in behavior with or without stem cell transplantation, two behavioral tests are used for the study of mouse motor activity and coordination: the rotarod performance test and the pole climbing test, respectively.

3.8.1 Rotarod Performance Test

This test is based on a rotating rod with forced motor activity being applied, usually by a rodent (Fig. 8). The test measures parameters such as riding time (in seconds). Some of the other functions associated with the test include evaluating balance, grip strength and motor coordination of the subjects, especially after TBI.

1. Adjust the time and speed of rotarod apparatus to 5 min and 4 m/s, respectively. The speed can be increased to 40 m/s as time progresses.
2. Perform rotarod test four times for each group (R0, R1, R2, and R3) where R0 is performed before the injury, while the rest is performed after TBI.
3. Perform each test for 4 consecutive days. The first 3 days consist of three trials each with an inter-trial time of 15 min. The fourth day is called the challenging session which consists of one trial. The differences between the first 3 days and the fourth day are the speed of rotating rod and the time set.
4. During the first 3 days, adjust the speed to 4 m/s and gradually increase it with time to reach 40 m/s for 5 min.
5. In the challenging session, adjust the speed at 4 m/s and then accelerate to 60 m/s for 8 min.

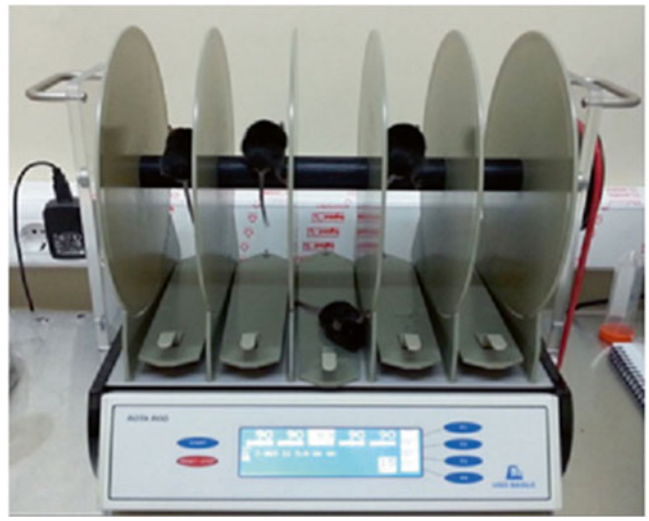

Fig. 8 Rotarod test. The mice are placed on a rotating rod; the speed and time at which the mice fall off the rod are recorded by the apparatus

6. R0 is performed 4 days before inducing TBI, R1 after TBI by 3 days, R2 after R1 by 4 days and R3 is performed after R2 by 4 days. When the R3 test is finished, the pole climbing test is performed immediately (*see* **Note 28**).

3.8.2 Pole Climbing Test

After R3 (rotarod test), the pole climbing is performed to study motor coordination in rodents.

1. Each mouse is trained for three consecutive trials during this test before starting with the experimental trials.
2. Place the mouse on the tip of a vertical rod where the mouse has to walk down the rod to reach the surface (Fig. 9).
3. Record four time points in this test, the t-turn (time at which the mouse turns on the rod), the t-half (time at which the mouse reaches half of the rod), the t-stop (time at which the mouse stops on the rod), and the total-t (time at which the mouse reaches the surface).
4. Repeat the test for five consecutive trials without break (*see* **Note 29**).

4 Conclusion

In conclusion, we have described in this chapter the detailed techniques and methods used in order to treat TBI in a CCI mouse model. We started with harvesting and isolating NSCs from the neonatal SVZ, then growing primary, secondary and tertiary

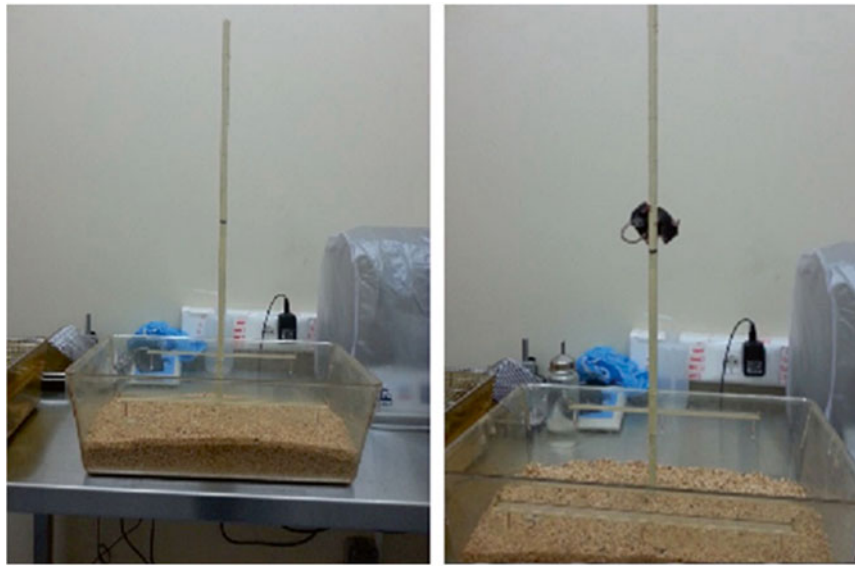

Fig. 9 Pole climbing test. The mouse is placed on the tip of a vertical rod. The t-turn thet-half, the t-stop and the t-total time are recorded as the mouse descends on the rod (see text for detail)

neurospheres. These tertiary neurospheres are then immunostained in order to confirm their undifferentiated state before injection. Mice underwent TBI using the CCI model, which is a highly reproducible brain injury model. One week following injury, NSCs are transplanted at the vicinity of the site of injury rather than in deep brain areas. Following transplantation, motor coordination and balance are evaluated using behavioral tests. The rotarod test is used to assess balance, grip strength and motor coordination of mice after TBI. It consists of placing the mouse on a rotating rod under continuous acceleration and recording the time it took to fall off. The pole climbing test was performed to study motor coordination of the mouse after training for three consecutive trials, followed by five consecutive experimental trials. The mouse was placed on the tip of a vertical rod (height = 60 cm, diameter = 1 cm) and the time at which the mouse reached the bottom (t-total) was recorded. This chapter also described how to sacrifice, perfuse and remove mice brains. Cardiac Perfusion is the method used to introduce a fixative in order to fix and preserve brain tissues. This is followed by removing the brains and splicing them into coronal sections using a microtome. This method is necessary for optimal results through the use of immunofluorescence assays in order to detect proteins in brain sections, as it demonstrates the presence of antigens (Ag) within tissue sections by means of specific antibodies (Abs). Immune reaction is then visualized by fluorescence after Ab-Ag coupling.

5 Notes

1. Isolation of NSCs should be done inside a laminar flow hood, where stringent sterile conditions need to be maintained to keep the harvested cells free of any contamination. Disinfection is performed by rinsing the area and the hood with sterilizing agent (Clorox), followed by distilled water and finally 70% ethanol. Moreover, all required equipment (microscissors, scissors, blade, curved forceps, and forceps) should be sterilized prior to use.
2. The first Petri dish is used to place removed brains and the second one is used for brain sections containing SVZ. Removal of brains should occur one by one.
3. Once the tissue is dropped into the tube, it should be placed on ice until all tissues are harvested from all pups. This is important to preserve tissues; otherwise, stem cells will be lost.
4. The base of the tube should be tapped gently every 2 min for 10–15 min, thus allowing dissociation of the tissue. Alternatively, the tube can be placed inside a shaker at 37 °C with gentle shaking.
5. To stop the activity of ATV, prepare 5 ml DMEM containing FBS and DNase in a 15 ml tube. Add the mixture to 5 ml syringe having a blue filter on its tip, and then filter the mixture directly above the tissue and ATV. Adding serum-containing medium might interfere with neurosphere formation or cause attachment of NSCs to the plate. It is highly recommended to add soybean trypsin inhibitor to the trypsin solution to inactivate it. Stopping the activity of ATV can be done by gently mixing the two mixtures via pipetting to ensure trypsin inactivation and also dissociation of tissue into single-cell suspension.
6. Count eight squares on both sides of the hematocytometer. In order to obtain the number of cells in the suspension, divide the obtained number of cells by eight and multiply by the dilution factor (two) and again multiply by ten to the power four ($X/8 \times 2 \times 10^4$). Another method to count the cells is to take 10 μl of the resuspended cells, mix with 90 μl of trypan blue, then transfer 10 μl from this mixture to a hematocytometer, and perform cell counting.
7. Since we have three flasks, and by referring to the example in **step 8**, a total of 7.5 ml of complete media should be prepared (2.5 ml/flask) in a 15 ml tube to which 1.5 μL EGF and 0.75 μL FGF are added.
8. Before collecting neurospheres in suspension, use 5–10 ml pipette (depending on suspension volume) with pipette aid in order to wash the base of the flask and hence collect the highest number of neurospheres.

9. After adding ATV solution, place the tube in the incubator at 37 °C for 7–10 min for complete dissociation. Tap the base of the tube regularly every 2 min to ensure dissociation and then observe dissociation under the microscope.
10. For example, suppose that 2×10^6 cells were obtained in 1 ml conditioned media and only three flasks of cells are needed with a total of 15 ml (5 ml/flask). A mix of conditioned media and complete media are prepared in one tube (10.5 ml complete media + 4.5 conditioned media + 3 μL EGF, 1.5 μL FGF) (3.5 ml complete media/5 ml; 1.5 ml conditioned media/5 ml; 1 μL EGF/5 ml and 0.5 μL FGF/5 ml). The volume taken from the cell suspension is dependent on the number of cells; so, if two million cells are collected and only 10^5 are needed for 5 ml, a total of 0.15 ml (0.05 ml × 3 the number of flasks = 0.15 ml) of suspension is added to 15 ml complete media + conditioned media. The 15 ml are then distributed equally to the three flasks (5 ml/flask).
11. Different ratios of ketamine and xylazine were tried on mice and caused immediate death after anesthesia. Therefore, changing the ratio will be a limiting step.
12. Bregma and Lambda points are used by the software to calculate the distance that the manipulator must move along each axis to reach the target site where TBI should be performed.
13. When the injury is sutured, antibiotics should be rubbed over the skin in order to prevent any inflammatory response. Moreover, the temperature of the cage should be kept around ~25–30 °C to prevent death of the mouse.
14. Example: Suppose we get 268 cells, so $(268/8) \times 2 \times 10^4 = 6.7 \times 10^5$ in 1 ml DMEM:

    (a) x $10^5 \rightarrow$ 1 ml $\Longrightarrow$

    (b) $105 \rightarrow$ x X = 0.15 μL of suspension placed in 3 μL DMEM/mouse
15. The volume and the rate of injection are adjusted (3 μL; 0.30 μL/min, 0.45 μL/min, or 0.50 μL/min, respectively). Depending on the adjusted rate, injection takes between 6 and 12 min. Therefore, after injecting 3 μL, keep the Hamilton syringe in the brain for 5 min to avoid loss of suspension. Then, the Hamilton syringe is withdrawn slowly from the brain and the lesion is sutured.
16. The flow of PBS into the circulation should be slow to avoid high pressure on the blood vessels; otherwise, the brain will not be well perfused. The volume of PBS used is usually 30–40 mL until no blood is observed through the auricle.

17. Since PFA is a hazardous chemical, perfusion must be done in a chemical fume hood for the best personal protection. The volume of PFA is 30–40 mL, where muscle contraction and white liver are indicators of good perfusion. PFA and other fixatives must be collected after the perfusion and disposed of appropriately as hazardous chemical waste.
18. Incline the scissors 45° to avoid cutting through the brain.
19. Sucrose is used to dehydrate the tissue and cryoprotect it, thus and preventing freeze artifact and loss of tissue architecture.
20. Coronal sections are made allowing matching levels along the rostr-caudal axis of the brain to be examined so that comparisons can be made between littermate controls and experimental animals. Coronal sections of the entire brain should be examined in order to detect small abnormalities. The distribution of brain slices should be in a well-organized manner (serial collection) so that one well can represent the whole brain. For instance, the first slice should be placed in the first well, the second slice in the second well, and so on until the brain is completely sliced. For long-term storage, brain slices are placed in PBS supplemented with sodium azide.
21. If the brain slices are kept in PBS with sodium azide, wash slices twice with PBS (1×) for 10 min each to remove the chemical and then transfer to PBST.
22. The primary antibody can be kept for 1–2 h on shaker at 4 °C. The primary antibodies used are GFAP (rabbit), NeuN (rabbit), Map2 (mouse), DCX (goat) and Iba1 (rabbit). The volume of primary antibody may vary depending on the dilution factor. The antibody stock should be diluted in antibody dilution buffer or block solution.
23. The secondary antibody should be added in the dark since it is light sensitive and the plates should be covered with aluminum paper during the whole experiment. The secondary antibodies are anti-rabbit, anti-mouse and anti-goat. The volume of secondary antibody can vary depending on the dilution factor (1:400 usually). Finally the antibody stock should be diluted using antibody dilution buffer or block solution.
24. To label the cells with Hoechst before injection, we usually add 1 mL Hoechst diluted in PBS (1/10,000) for 5 min, followed by washing with PBS (1×) two times (5 min, RT, on shaker, in the dark).
25. The slides should be placed in a slide-specific book at 4 °C.
26. The brain slices should be distributed into wells in a way that each well represents the brain from anterior to posterior (as described above). In order to avoid losing tissue, it is recom-

mended to use a 100–1000 pipette while washing brain tissues. A negative control is performed for each assay in addition to the experimental plates. In this control, no fluorescence should be observed; tissues are incubated with antibody dilution buffer and secondary antibody. On the other hand, experimental tissues are labeled with both antibodies.

27. The tissues should be placed in a certain order; for example all TBI samples should be on the right.
28. The behavioral tests (rotarod and pole climbing) should be done by the same manipulator and in a silent room to avoid stressful stimuli that may affect animal behavior.
29. After performing the pole climbing test, mice should be perfused and their brains removed. Data for both behavioral tests should be transferred to a computer program (Excel, GraphPad, etc.) in order to draw graphs and compare the results.

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