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Corresponding Author:	Martin Doughty, Ph.D. Uniformed Services University of the Health Sciences Bethesda, MD UNITED STATES
Corresponding Author's Institution:	Uniformed Services University of the Health Sciences
Corresponding Author E-Mail:	martin.doughty@usuhs.edu
Order of Authors:	Orion Furmanski
	Michael D. Nieves
	Martin Doughty, Ph.D.
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TITLE:

Controlled Cortical Impact Model of Mouse Brain Injury with Therapeutic Transplantation of Human Induced Pluripotent Stem Cell-Derived Neural Cells

AUTHORS AND AFFILIATIONS:

Orion Furmanski[1,2], Michael D. Nieves[1,2,3], Martin L. Doughty[1,2,3]

[1]Center for Neuroscience and Regenerative Medicine, Uniformed Services University, Bethesda, MD, USA

[2]Department of Anatomy, Physiology, and Genetics, Uniformed Services University, Bethesda, MD, USA

[3]Graduate Program in Neuroscience, Uniformed Services University, Bethesda, MD, USA

Corresponding Author:

Martin L. Doughty (martin.doughty@usuhs.edu)

Email Addresses of Co-Authors:

Orion Furmanski (orion.furmanski.ctr@usuhs.edu)

Michael D. Nieves (michael.nieves.ctr@usuhs.edu)

KEYWORDS:

Traumatic brain injury, cortex, craniectomy, sensorimotor, transplantation, stem cell

SUMMARY:

This protocol demonstrates methodologies for a mouse model of open-skull traumatic brain injury and transplantation of cultured human induced pluripotent stem cell-derived cells into the injury site. Behavioral and histologic tests of outcomes from these procedures are also described in brief.

ABSTRACT:

Traumatic brain injury (TBI) is a leading cause of morbidity and mortality worldwide. Disease pathology due to TBI progresses from the primary mechanical insult to secondary injury processes, including apoptosis and inflammation. Animal modeling has been valuable in the search to unravel injury mechanisms and evaluate potential neuroprotective therapies. This protocol describes the controlled cortical impact (CCI) model of focal, open-head TBI. Specifically, parameters for producing a mild unilateral cortical injury are described. Behavioral consequences of CCI are analyzed using the adhesive tape removal test of bilateral sensorimotor integration. Regarding experimental therapy for TBI pathology, this protocol also illustrates a process for transplanting cultured cells into the brain. Neural cell cultures derived from the human induced pluripotent stem cells (hiPSCs) were chosen for their potential to show superior functional restoration in human TBI patients. Chronic survival of hiPSCs in the host mouse brain tissue is detected using a modified DAB immunohistochemical process.

INTRODUCTION:

Traumatic brain injury (TBI) is a general term for the acquired injury to the brain due to either indirect mechanical forces (rotational acceleration/deceleration or contra-coup) from blows to the head or direct damage from objects or blast waves. TBI has been estimated to be the cause of roughly 9% of worldwide deaths and observed in an estimated 50 million cases per year[1,2]. A 2017 report from the Centers for Disease Control and Prevention estimated that in 2013, there were a total of 2.8 million hospital visits and deaths due to TBI in the United States[3]. Many milder TBIs go unreported every year. Serious TBI can lead to lifelong impairment of cognition, motor function, and overall quality of life. The consequences of mild TBI, especially repetitive sport-related TBI, have been only recently appreciated for their insidious health effects[4,5].

Preclinical modeling is a vital component of developing new mechanistic insights and potential restorative therapy for TBI. The controlled cortical impact (CCI) model of TBI is an open-head model of mechanical contusion injury to the cortex. The impact parameters can be modified to produce CCI injuries that range from mild to severe[6]. CCI injuries are focal rather than diffuse, as seen with other closed head models of TBI. CCI can be performed to induce a unilateral injury, such that the contralateral cortex can serve as an internal comparator. This protocol demonstrates the characteristics of a mild CCI to a portion of the cortex that encompasses primary somatosensory and motor regions. This cortical area was chosen for its involvement in sensorimotor behaviors for which numerous behavior tests can detect injury-induced deficits[7]. Behavioral improvements due to therapeutic interventions for TBI can be detected, as well.

A hallmark of TBI is widespread neural dysfunction in the injured region. Injured neurons undergo cell death, and neuronal network connectivity is disrupted[8,9]. TBI disrupts recruitment of endogenous stem cells, which leads to further downstream behavior deficits[10,11]. Transplantation of neural stem cells and stem cell-derived cells has been explored as a possibility to restore function in the injured brain. In addition to the potential to restore damaged neural circuitry, transplanted cells exert paracrine effects that promote neuronal survival and functional recovery from TBI[12]. A variety of cell types have been transplanted preclinically to evaluate their restorative potential in models of neurologic disorders[13-15]. The recent popularization of induced pluripotent stem cell technology[16] has facilitated the development of numerous human stem cell lines for experimental use. Preclinical testing with hiPSC-derived cells is an important first step to characterizing a given cell line's potential therapeutic efficacy against human diseases. This laboratory has developed protocols for differentiating hiPSCs to neural phenotypes[17] in pursuit of transplantable cells to aid recovery from traumatic brain injury.

Experiments in this protocol use a unilateral CCI to induce TBI to the left somatosensory and motor cortex of adult mice. A mild CCI injury results in a sustained functional deficit in the right forepaw that is used to track the effects of hiPSC-derived neural cell engraftment on functional recovery. Forepaw sensorimotor testing in this protocol was adapted from the methodology established by Bouet and colleagues[18] and demonstrated previously by Fleming and colleagues[19]. This protocol outlines a complete workflow for performing an experimental brain

injury, therapeutic transplantation of hiPS cells, and behavioral and histologic analysis of experimental outcome measures.

PROTOCOL:

All experiments described in this protocol were reviewed and approved by the Uniformed Services University Animal Care and Use Committee.

1. Craniectomy and controlled cortical impact

1.1. Preparation of the controlled cortical impact device and surgical supplies.

1.1.1. Load a 1 mL slip-tip syringe with 0.5 mL of sterile saline for wound irrigation. Attach a 25 G needle to the syringe to control irrigation.

1.1.2. Prepare a dilute solution of CsA in DMSO to final concentration of 1 mg/mL. Load a second 1 mL slip-tip syringe with 0.5 mL of cyclosporine A (CsA) solution for immunosuppression. Attach a 25 G needle or larger to the CsA syringe.

1.1.3. Attach the controlled cortical impact piston to an arm on a stereotaxic frame and set to an angle of 15°. Attach a 3 mm impactor probe to the piston.

1.1.4. Set the velocity of impact to 1.5 m/s and the impact dwell time to 0.1 s to produce a mild cortical injury.

1.2. Perform a unilateral craniectomy

1.2.1. Place the mouse in an anesthesia induction chamber connected to an isoflurane vaporizer with compressed oxygen source. Induce anesthesia with ~3% isoflurane at ~0.7 L/min oxygen. Check for the depth of anesthesia by the lack of response to the toe-pinch.

1.2.2. Shave the scalp using electric clippers and wipe away any loose fur.

1.2.3. Place the mouse in a stereotaxic frame with attached anesthetic delivery nose cone.

1.2.3.1. Place a warming pad set to 37 °C on the stereotaxic frame under the mouse to maintain body temperature under anesthesia. Fix the head in place with ear bars and a bite bar and orient the head such that the skull frontal bone is horizontal. Maintain anesthesia at ~1.5%–2% isoflurane for the duration of the surgery.

1.2.4. To perform preoperative care and aseptic surgical preparation, apply antibiotic ophthalmic ointment to the eyes using a sterile cotton swab. Apply an iodine-based solution to the shaved scalp area. Remove this with 70% ethanol. Cover the animal with a fenestrated surgical drape so that the top of the head is visible but the eyes are covered.

1.2.5. Make a midline incision (1.5–2 cm) on the scalp using a scalpel or scissors. Use sterile cotton swabs to clean the wound and to clear the fascia left of the midline at bregma.

1.2.6. Use the impactor probe to identify the craniectomy site.

1.2.6.1. Set the stereotaxic reference point (X = 0, Y = 0) to bregma. Adjust the probe laterally to 2 mm left of the midline. Outline a 5 mm diameter circle around the probe using a fine-tip surgically safe marker. Raise and rotate the impactor out of position.

1.2.7. Use the high-speed rotary micromotor kit hand tool to make an open hole in the skull using a round-tip 0.6 mm or 0.8 mm burr drill bit at ~70%–80% maximum speed. Apply light pressure to the skull while drilling along the 5 mm circumferential outline to thin this border.

1.2.7.1.1. Do not apply excess pressure while drilling. This can cause cortical injury due to vibration, compression, or accidental penetration. Allow the speed of the drill bit to do the work.

1.2.7.1.2. Do not drill in any given spot for too long to avoid excess friction heating of the skull. Irrigate the craniectomy occasionally with sterile saline to remove debris and to reduce heating from the rotary tool.

1.2.7.1.3. Pay close attention while drilling over the coronal suture line as these points are vulnerable to hemorrhage.

1.2.7.2. Use a pair of fine tweezers to remove the skull flap when the craniectomy outline is sufficiently thinned. Grasp the flap medially, and gently lift and pull laterally with a radial motion.

1.2.7.2.1. Do not to damage the dura mater when lifting the flap; this can cause severe injury and hemorrhaging.

1.3. Perform a mild controlled cortical impact injury

1.3.1. Clean the impactor probe with a sterile alcohol prep pad. Move the impactor probe back into position over the exposed cortex. Lower the probe until it touches the dura mater surface. Mark this position as Z = 0.

1.3.2. Withdraw the piston and move to Z = -1.0 mm. Discharge the piston to impact the cortex.

1.3.3. Quickly raise the piston and move the arm out of position. Apply generous amounts of saline to irrigate the cortex after injury. Rinse the surgery site with saline as needed and suture the scalp incision using simple interrupted stiches with 5.0 silk suture.

1.4. Perform postoperative care on the mouse

1.4.1. Discontinue anesthesia. Deliver CsA by subcutaneous injection into scruff at 10 mg/kg dose. Place the mouse in a clean and pre-warmed postoperative cage.

1.4.2. Provide acetaminophen analgesic in the drinking water at 1.0 mg/mL.

NOTE: Provide analgesia according to the appropriate IACUC standard operating procedure and in consideration of experimental outcome variables (e.g., sedation, neuroinflammation).

1.4.3. Provide moistened chow food in a warmed recovery cage to aid in rehydration and recovery.

1.5. Proceed from section 2 to section 4 above when performing craniectomy-only (sham) controls.

2. Stereotaxic transplantation of cell suspension

2.1. Begin cell transplantation procedure roughly 24 h after craniectomy.

2.2. Prepare the cell transplantation equipment and surgical supplies

2.2.1. Fill a 1 mL slip-tip syringe with sterile saline for wound irrigation. Attach a 25 G needle to the syringe to control irrigation.

2.2.2. Fill a 1 mL slip-tip syringe with CsA solution for immunosuppression. Attach a 25 G needle or larger to the CsA syringe. Refill the CsA syringe as needed between surgeries.

2.2.3. Prepare glass needles from 1.0 mm OD borosilicate glass capillary pipettes using standard methods.

2.2.4. Use fine tweezers to break the needle tips to approximately a 200 µm diameter. Ensure that the cylindrical shaft of the needle is no longer than 2.5 cm.

2.3. Calibrate the syringe pump for use with a 10 µL syringe. Enter a flow rate of 0.2 µL/min to deliver a total volume of 2.0 µL.

2.4. Prepare human induced pluripotent stem cell (iPSC) suspension.

2.4.1. Perform all cell handling in a cell culture BSL-2 hood using standard sterile handling techniques.

2.4.2. Prepare cell cultures in advance according to standard conditions defined for the cell type. Refer to Lischka et al.[17] as an example.

NOTE: Experiments shown in this demonstration used various neural phenotype cells derived from hiPSCs.

2.4.3. Gently dissociate the cells into a single-cell suspension using a cell detachment solution, or other preferred enzymatic or chemical means.

2.4.4. Count cells in suspension, then dilute the suspension to 5 x 10^4 cells/µL in minimal cell culture medium (e.g., DMEM) in a 1.7 mL flip top test tube.

2.4.5. Observe the following notes for transplantation:

2.4.5.1. Maintain the cells in suspension at 37 °C for the duration of procedures.

2.4.5.2. Load the syringe only immediately prior to performing intraparenchymal injection (step 4.5 below).

NOTE: Gravity can cause the cell suspension to settle or to cling to the side of the syringe if laid on its side. This leads to irregularities in the number of cells injected.

2.4.5.3. Allot ~5 x 10^5 cells (10 µL suspension) per mouse if performing multiple cell transplantation procedures in one day.

2.5. Perform stereotaxic transplantation surgery

2.5.1. Place the mouse in an anesthesia induction chamber connected to an isoflurane vaporizer with compressed oxygen source. Induce anesthesia with ~3% isoflurane at ~0.7 L/min oxygen.

2.5.2. Place the mouse in a stereotaxic frame with attached anesthetic nose cone.

2.5.2.1. Fix the head in place with ear bars and a bite bar and orient the head such that the skull frontal bone is horizontal. Maintain anesthesia at ~1.5%–2% isoflurane for the duration of the surgery.

2.5.3. Perform preoperative care and aseptic preparation

2.5.3.1. Apply hydrating ophthalmic ointment containing antibiotic to eyes using a cotton swab.

2.5.3.2. Lavage the incision site with sterile saline to clean the site and to loosen sutures. Gently apply 70% ethanol with a cotton swab to sterilize the incision site.

2.5.4. Remove sutures using fine tweezers and ophthalmic scissors. Irrigate surgery site and craniectomy with abundant sterile saline.

2.5.4.1. Consider the animal for exclusion if the cortex displays disqualifying characteristics including excessive herniation, discoloration, disrupted vascularization, or hemorrhage.

2.5.5. Load the cell transplant syringe

2.5.5.1. Move cell suspension from the 37 °C incubator to a cell culture biosafety hood. Gently swirl or tap the tube to ensure a homogeneous cell suspension.

2.5.5.2. Use a micro pipettor to load ~7.5 µL cell suspension into the Hamilton syringe through the plunger end.

2.5.5.2.1. Hold the syringe at a ~120° angle with the plunger end facing down. Insert the plunger, taking care not to introduce an air bubble between the suspension and plunger tip.

2.5.5.3. Attach the gasket assembly to the pipette needle, then attach the needle to the syringe.

2.5.5.4. Push the plunger to move cell suspension into the pipette needle. If there is resistance against suspension outflow, use fine tweezers to break the needle tip to enlarge the diameter.

2.5.6. Attach the syringe to the stereotaxic syringe pump. Advance the plunger to make sure the syringe pump assembly is working properly.

2.5.7. Move the needle into the coordinates for injection.

2.5.7.1. Align the needle tip to bregma. Set the X and Y coordinates to 0. Then move the needle tip over the craniectomy to 2.0 mm lateral and -1.0 mm posterior to bregma. Touch the needle tip to the dura mater surface and set the stereotaxic coordinate to Z = 0.

2.5.7.2. Push the plunger to ensure the cell suspension is flowing adequately before introducing the needle into the brain.

2.5.7.3. Introduce the needle into the brain to a depth of Z = -1.4 mm. These stereotaxic coordinates place the graft at the gray matter-white matter border of the deep cortex[20].

2.5.8. Start the syringe pump to infuse cell suspension. Set the lab bench timer to 15 min and start the timer. Use a long working distance microscope to monitor cell suspension outflow.

2.5.8.1. Irrigate the surgery site with sterile saline during injection to maintain tissue hydration.

2.5.8.2. At 15 min, slowly withdraw the transplantation needle. Irrigate the surgery site with saline and close the incision with sutures.

2.5.9. Perform postoperative care

2.5.9.1. Discontinue anesthesia. Deliver CsA by subcutaneous injection into scruff at 10 mg/kg dose. Place the mouse in a clean and pre-warmed postoperative cage.

2.5.9.2. Provide acetaminophen analgesic in the drinking water at 1.0 mg/mL.

NOTE: Provide analgesia according to the appropriate IACUC standard operating protocol (SOP) and in consideration of experimental outcome variables.

2.5.9.3 Provide moistened chow food recovery cage to aid in rehydration and recovery.

2.6. Continue daily CsA injections at 10 mg/kg throughout the survival duration of the mouse.

3. Adhesive tape removal test of sensorimotor integration

3.1. Cut the electrical tape into 3 mm x 5 mm strips using a small razor knife prior to performing the behavior test. Use a smooth glass surface for cutting the adhesive strips.

N OTE: Use yellow and red tape, as mice have difficulty distinguishing between these colors[21].

3.1.2. Select a small mirror that fits well inside the clear plastic box.

3.1.2.1. Fix the mirror in place at a roughly 45° angle with modeling clay or adhesive tape in order to view animal behavior from below.

3.1.3. Place the box and mirror assembly on a bench in a quiet dedicated behavior testing room. Arrange the cylinder on the plastic box above the mirror.

3.1.4. Arrange the handling cloth, tweezers, and adhesive strips on the bench next to the behavior testing box.

3.1.5. Clean the box and cylinder with 70% ethanol and paper towels. Allow surfaces to dry thoroughly.

3.1.6. Bring the mice into the behavior testing room. Allow the mice to acclimate to the testing room for at least 30 min prior to behavior testing.

3.1.7. Remove water supplies from the cages to minimize urination events during testing, which can interfere with efficient test performance.

3.2. Perform the adhesive removal test

NOTE: This behavior test is best performed by two to three investigators: one to operate the stopwatches, and one or two to handle the mice and observe the behavior.

3.2.1. Use each tweezer to peel one adhesive strip of each color.

3.2.1.1. Choose which strip color corresponds to which forepaw and remain consistent throughout the trial.

3.2.2. Use the handling cloth to restrain a mouse by the scruff of the neck and back such that the mouse holds the forepaws away from its body and head.

3.2.3. Use tweezers to place an adhesive strip on the plantar surface of each forepaw. Use delicate and consistent finger pressure to secure the strips to the paws.

3.2.4. Quickly place the mouse into the plastic cylinder. Start the two stopwatches when the mouse has all four paws on the plastic box.

3.2.5. Use the two stopwatches to record the latencies for the following four events: left paw notice, left paw removal, right paw notice, right paw removal.

3.2.5.1. Record a notice event when the animal makes unambiguous recognition of the adhesive strip by shaking or flinching the paw or biting the strip.

3.2.5.2. Record a remove event when the animal effectively removes the strip from the forepaw plantar surface.

NOTE: The remove event is not disqualified by strip readherence or if the strip clings to the lateral surface of the forepaw.

3.2.5.3. Stop the timer at 120 s if the corresponding strip has not been removed.

3.2.5.4. Record the elapsed times for the four events on the data sheet.

3.2.6. Perform a second trial on each mouse

3.2.6.1. Allow a minimum of 5 min to elapse between trials for an individual mouse to reduce stress, which can interfere with efficient performance on the test.

3.2.6.2. Clean the apparatus with paper towels between testing mice to remove waste when testing multiple mice from a single cage.

3.2.6.2.1. Clean the apparatus thoroughly with 70% ethanol and paper towels when testing mice from multiple cages.

3.2.6.3. Reverse the color-paw placement order for the second trial of the session to randomize any effect of color.

3.2.7. Calculate the average latency of each event between two trials per daily session for each animal.

3.3. Clean the apparatus and handling cloth thoroughly with water, replace the cage water supplies, and return the mice to their holding facility.

3.4. Repeat steps 3.1–3.2 on successive experimental timepoints for a repeated measures trial design.

NOTE: Repeat steps 3.2.1–3.2.5.3 daily for 5 days prior to any data collection to acclimate the animals to the task. Baseline data acquisition prior to surgery is recommended.

4. Diaminobenzidine (DAB) immunohistochemical analysis of graft survival and injury pathology

4.1. Euthanize the animals and perform a transcardial perfusion.

4.1.1. Prepare solutions of 0.1 M phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA) in PBS. Balance the pH of both solutions to 7.4.

4.1.2. Place the two solutions on ice in a fume hood. Place a peristaltic pump in the fume hood, and run the pump to fill the tubing with PBS. Set the pump flow rate to ~7 ml/min. Connect a 25 G needle to the outflow tube of the pump.

4.1.3. Place a drainage tray with a soft substrate in the hood. Raise one end of the tray at a slight angle so that perfusion fluids drain to the lower end.

4.1.4. Place a mouse in an anesthesia induction chamber. Fill the chamber with 4% isoflurane using compressed 100% oxygen vehicle gas.

4.1.5. Move the anesthetized mouse from the chamber to an anesthesia nose cone. Decrease the isoflurane to 2%.

4.1.6. Perform a thoracotomy to expose the heart. Discontinue anesthesia, as the thoracotomy causes euthanasia.

4.1.7. Carefully place the perfusion pump outflow needle in the left ventricle along the long axis of the heart. Do not pierce the heart septum, as this will cause poor perfusion.

4.1.8. Use small scissors to cut the right atrium, then immediately turn on the peristaltic pump.

4.1.9. Continue the PBS flow until the fluid leaving the right atrium runs clear of blood.

4.1.9.1. Turn off the pump. Move the pump intake tube to the container of PFA. Restart the pump. Continue perfusing PFA until either the animal is sufficiently rigid or until a 20 mL volume has been pumped through.

4.1.9.2. Turn off the pump. Remove the outflow needle from the heart. Move the intake tube from the PFA to the PBS, and thoroughly flush out PFA from the tubing.

4.2. Remove the brain from the skull by careful dissection. Place the brain in a small container filled with 4% paraformaldehyde, and allow the brain to post-fix overnight at 4 °C.

4.3. On the day after post-fixation, replace the P with PBS and 0.1% sodium azide. Store the brains in this solution until preparation for histologic sectioning.

4.4. Collect 40–50 µm sections of formaldehyde-fixed brain tissue via a freezing microtome or room temperature vibratome.

4.5. Pretreat tissues in 0.3% hydrogen peroxide in water to inactivate endogenous peroxidases, which can produce nonspecific staining.

4.6. Perform antigen retrieval using citric acid buffer (10 mM sodium citrate, 0.05% polysorbate-20, pH 6.0) at 60 °C for 30 min.

4.7. Perform antibody labeling by standard methods. Refer to Lundell et al.'s report[22] from this laboratory for more details.

4.7.1. Use a mouse IgG neutralization kit (see **Table of Materials**) to reduce cross-reactivity with endogenous antibodies. Incubate the tissues in IgG neutralization buffer for at least 1 h at room temperature (RT), following the manufacturer's directions.

4.7.2. Use a mouse anti-human nuclear antigen primary antibody (hNA; 1:500 dilution) when transplanting human iPSC-derived cells to locate the transplanted cells. Incubate tissues with the primary antibody at 4 °C for 48–72 h using gentle agitation.

4.7.3. After rinsing away primary antibody solution, incubate tissues with HRP-conjugated anti-mouse secondary antibody at 1:250 dilution for 2 h at RT.

4.7.4. Perform DAB chromogen reaction by standard methods to reveal immunolabeling. Allow DAB reaction to develop for 5 to 10 min at RT.

4.8. Attach stained tissues to slides and apply cover slips using standard methods.

4.9. Quantify numbers of labeled cells using unbiased stereology as described previously[23,24]. Perform analysis using 20 µm optical sections and a between section interval of three.

REPRESENTATIVE RESULTS:

Craniectomy surgery facilitates experimental brain injury and therapeutic cell transplantation: the controlled cortical impact model of brain injury and subsequent cell transplantation therapy require careful removal of the overlying skull. The craniectomy may be performed on any dorsal surface of the skull to permit manipulations to the brain region of interest. The diagram in **Figure 1** depicts a 5 mm diameter craniectomy schematic to uncover primary somatosensory and motor cortices (**Figure 1A**). At 24 h after craniectomy, a second surgery was performed to inject human iPSC-derived neural cell suspension into deep layers of the cortex (**Figure 1B**). Some cerebral edema is normal on the first day following craniectomy, and particularly after CCI. However, cerebral vasculature sparing during all phases of this procedure is crucial for survival of the cortex. **Figure 2** illustrates the cell transplantation procedure in a mouse with minimal cerebral herniation, minimal bleeding, and extensive cortical vascularization. These features are good prognostic indicators of a successful surgery.

Adhesive tape removal testing reveals sensorimotor deficits after unilateral brain injury: the parameters of the brain injury model described above were predicted to affect forelimb sensory and motor function. The adhesive tape removal test was chosen to evaluate the severity of forelimb functional deficits, and the potential therapeutic benefits of cell transplantation. Mice were trained on the testing procedure for 5 days, then allowed to rest for two days prior to baseline behavior testing. Surgeries were performed on the day following baseline testing. Behavior tests in this study were performed on postoperative days 1, 3, 5, 7, 10, 14, 21, 28, 35, and 42. **Figure 3** shows results from a pilot experiment in which forelimb function in mice with craniectomy alone (sham) and with CCI injury were compared to forelimb function in naïve mice (n = 11 naïve, 12 sham, 11 CCI). Mice that underwent surgery exhibited transient increased latencies to notice adhesive stimuli for 1–3 days immediately after surgery (**Figure 3A,B**). Mice showed transient postoperative deficits in adhesive removal from the ipsilateral forepaw as well (**Figure 3C**). However, mice that underwent CCI exhibited significant deficits in motor performance in the forepaw contralateral to injury compared to naïve mice out to postoperative day 28 (**Figure 3D**). These data also describe the unexpected severity of sensorimotor loss in craniotomized mice without CCI, indicating that surgical craniectomy to this area also induces TBI-related neurofunctional deficits.

Immunodetection of human induced pluripotent stem cell (iPSC)-derived cell grafts in mouse brain sections: experiments were performed to determine whether human iPSC-derived neural cells would survive long-term transplantation in the mouse brain. Human neural stem cells (NSCs) derived from iPSCs were differentiated into either immature neurons or astrocytes *in vitro* using established methods[17]. Transplants of each of the three neural cell phenotypes were tested in our CCI model of traumatic brain injury using the procedure described above and

depicted in **Figure 2.** The mice were euthanized for histologic analysis at 7 days after transplantation. Mouse brain sections were immunostained for the human nuclear antigen (hNA). Human cell grafts could be clearly distinguished from host tissue in sham surgery and CCI brains (**Figure 4**). Astrocyte grafts (n = 3 sham, 2 CCI) showed poor survival compared to NSCs (n = 12 sham, 15 CCI) and neurons (n = 11 sham, 10 CCI), and were not considered for future experiments.

FIGURE LEGENDS:

Figure 1: Coordinate parameters of surgical manipulations. Cartoon depictions of mouse brain regions of interest. Red circles indicate a ~5 mm diameter craniectomy. A red cross indicates the craniectomy central point 2 mm lateral to bregma. (**A)** The shaded region of cerebral cortex in the upper diagram is affected by mild CCI when a craniectomy is performed as shown in lower diagram. (**B)** The blue arrow in upper diagram indicates the approximate location of cell injections at 1.4 mm depth from cortical surface. The blue cross in the lower diagram indicates the placement of cell injection 2 mm lateral and 1 mm posterior to bregma.

Figure 2: Intraoperative monitoring of cell suspension injection. Photograph taken through a long working distance microscope during intraparenchymal cell injection. Anatomic features are annotated for clarity. The scalp partially obscures the surgery site to minimize dehydration during the procedure. Minor bleeding may occur during needle penetration as shown, which is not cause for concern if large cortical vessels remain intact.

Figure 3: Behavioral evaluation of sensorimotor integration after brain injury. Mice that underwent craniectomy and CCI were compared to naïve controls and to mice that underwent only sham surgery (n = 11 naïve, 12 sham, 11 CCI). Data are presented as group mean latencies, with error bars indicating SEM. (**A)** Mice that underwent CCI exhibited increased latency to recognize adhesive stimuli applied to the ipsilateral forepaw on the first postoperative day. (**B)** Mice that underwent craniectomy or CCI exhibited substantially increased latency to notice adhesive stimuli applied to the contralateral forepaw on postoperative days 1 and 3. (**C)** Mice that underwent craniectomy or CCI exhibited substantially increased latency to remove adhesive stimuli from the ipsilateral forepaw on postoperative days 1 and 3. (**D)** Mice that underwent craniectomy or CCI exhibited substantially increased latency to remove adhesive stimuli from the contralateral forepaw on postoperative days 1–5. Motor deficits in mice with CCI persisted strongly for 28 days after injury.

Figure 4: DAB immunohistochemistry for human cell grafts in mouse brains. Human iPSCs were differentiated into neural stem cells (NSCs), neurons, or astrocytes *in vitro.* Cell cultures were transplanted into mouse brains with or without CCI. Mice were euthanized for histologic analysis seven days after cell transplantation. Micrographs depict representative results of human nuclear antigen staining. Black insets depict markers for stereologic quantification of cell numbers (cyan) and graft volume (red).

DISCUSSION:

Mild CCI as a model system for testing experimental regenerative therapy

The CCI model is a valuable tool for investigating mechanisms of tissue dysfunction after mechanical injury to the cortex. The tunability of the injury parameters is an attractive feature of this model. Altering the Z depth of impact, the velocity, or dwell time can increase or decrease severity of the injury as desired by the investigator[10,25]. The mild CCI model of contusive brain injury, when performed correctly, should cause modest cortical cell death and minimal cavitation. Craniectomy and skull flap removal must be performed with great care. Excessive downward force applied while drilling the craniectomy trench can cause cortical injury due to heating and vibration. Mechanical disruption of the dura mater during skull flap removal almost uniformly predicts severe cortical injury. Disruption of major cortical blood vessels is likely to result in excessive lesioning of the cortex and is grounds for excluding the animal from the experiment. Unfortunately, the signs of an exacerbated injury can be subtle on the day after surgery. Neither edema nor small cortical vessel rupture are necessarily negative indicators. Hematomas and abnormal coloration due to ischemia are clearer indicators of surgical complication. Documenting intraoperative events and correlating complications with histopathologic outcomes are crucial to refining good craniectomy technique.

It must be noted that mTBI modeling in animals comes with certain caveats. There are numerous preclinical models of mTBI other than the model presented here. Experimental TBI can be induced through mechanical forces, blast waves through air, or a combination of these forces[26-28]. Mild to severe injuries are judged by a combination of histopathological and behavioral outcomes (reviewed in Petraglia[29] and Siebold[30]). Behavior deficits in rodents can resolve within days to weeks[31] whereas human mTBI patients' deficits can persist for months in the form of post-concussive syndrome[32]. Although no single model is a complete analog for clinical mTBI, preclinical testing reveals physiologic mechanisms that cannot be assessed in the human condition.

The most important steps in the cell transplantation procedure are the handling and injection of the cell suspension. Rough handling causes cell lysis, leading to the release of sticky genomic DNA and aggregation of the surviving suspended cells. The needle tip diameter must be wide enough to permit smooth outflow of the suspension; restricted flow causes discontinuous delivery as the suspension sediments inside the needle. When following this protocol and avoiding the pitfalls discussed here, robust grafts were visible after 7-day survival times (**Figure 4**). Long term graft survival in a preclinical model is key to determining potential therapeutic efficacy of this approach.

Application of the adhesive tape removal test to contusive brain injury modeling

The adhesive removal test reveals positive neurologic deficits in terms of increased latency to remove the adhesive stimuli. The main potential drawback to this test is the likelihood of inhibited performance due to factors not related to injury. Handling stress can reduce mouse exploratory behavior[33], so it is crucial that the animals undergo extensive restraint acclimation prior to baseline data collection. It is important to remove water supplies at least 30 min prior to testing in order to mitigate urination-related freezing events. Finally, the testing apparatus

must be cleaned often as animals can become distracted into investigatory sniffing of odor cues from unfamiliar animals.

Results presented here show significant forepaw motor deficits contralateral to mild CCI up to 28 days after injury. By contrast, concurrent testing using the cylinder test[34] and accelerating rotarod[3] showed injury-induced functional deficits that resolved within 5–10 days (data not shown). Adhesive tape removal has been used in a variety of experiments evaluating unilateral deficits in sensorimotor integrative behavior[7,35]. The test was also recently used to assess motor function recovery following regenerative intervention for cervical spinal cord injury[36]. The dynamic range of performance on this test can be tuned for latency or species variation by selecting adhesives of different strength. Here, it is suggested that 3M electrical tape is an optimal stimulus for mice based on availability, durability, and substantially increased latency to remove stimuli following mild CCI. Although the preliminary experiments shown here do not combine cell transplantation and behavior testing, ongoing experiments in our laboratory will assess transplant survival and concurrent effects on sensorimotor behavioral recovery from brain injury at 56 days after transplantation.

Refinement to DAB immunodetection of human cell grafts

DAB immunohistochemistry was chosen in order to produce strong, persistent labeling of human cells in mouse tissue for subsequent stereologic quantification. Pretreatment with hydrogen peroxide is crucial for reducing nonspecific staining in brain tissue due to endogenous peroxidases in erythrocytes. Nonspecific staining in these experiments was further reduced using a mouse IgG neutralization kit. This kit uses proprietary chemicals to reduce antigenicity of endogenous mouse IgGs, which can extravasate into brain tissue after TBI[37]. Early attempts employed a combination of mouse anti-hNA primary antibody, biotin-conjugated secondary antibodies, and streptavidin-HRP conjugate tertiary labeling using the Vector Laboratories ABC kit. The ABC conjugate exhibited extensive nonspecific DAB staining in the cortex ipsilateral to the craniectomy (data not shown). Subsequent staining trials employed secondary antibodies directly conjugated with HRP. This modified protocol produces high-resolution nuclear staining with greatly reduced background for all hiPS-derived cell types and surgery conditions (**Figure 4**). Unpublished experiments from this laboratory using this modified DAB staining technique detect hNA-positive cells in mouse brains 56 days after mild CCI. Overall, a binary antibody labeling procedure saved time and produced clearer immunostaining compared to the traditional tertiary labeling ABC kit procedure. This protocol could be useful for other preclinical studies of human cells transplanted into the mouse central nervous system.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

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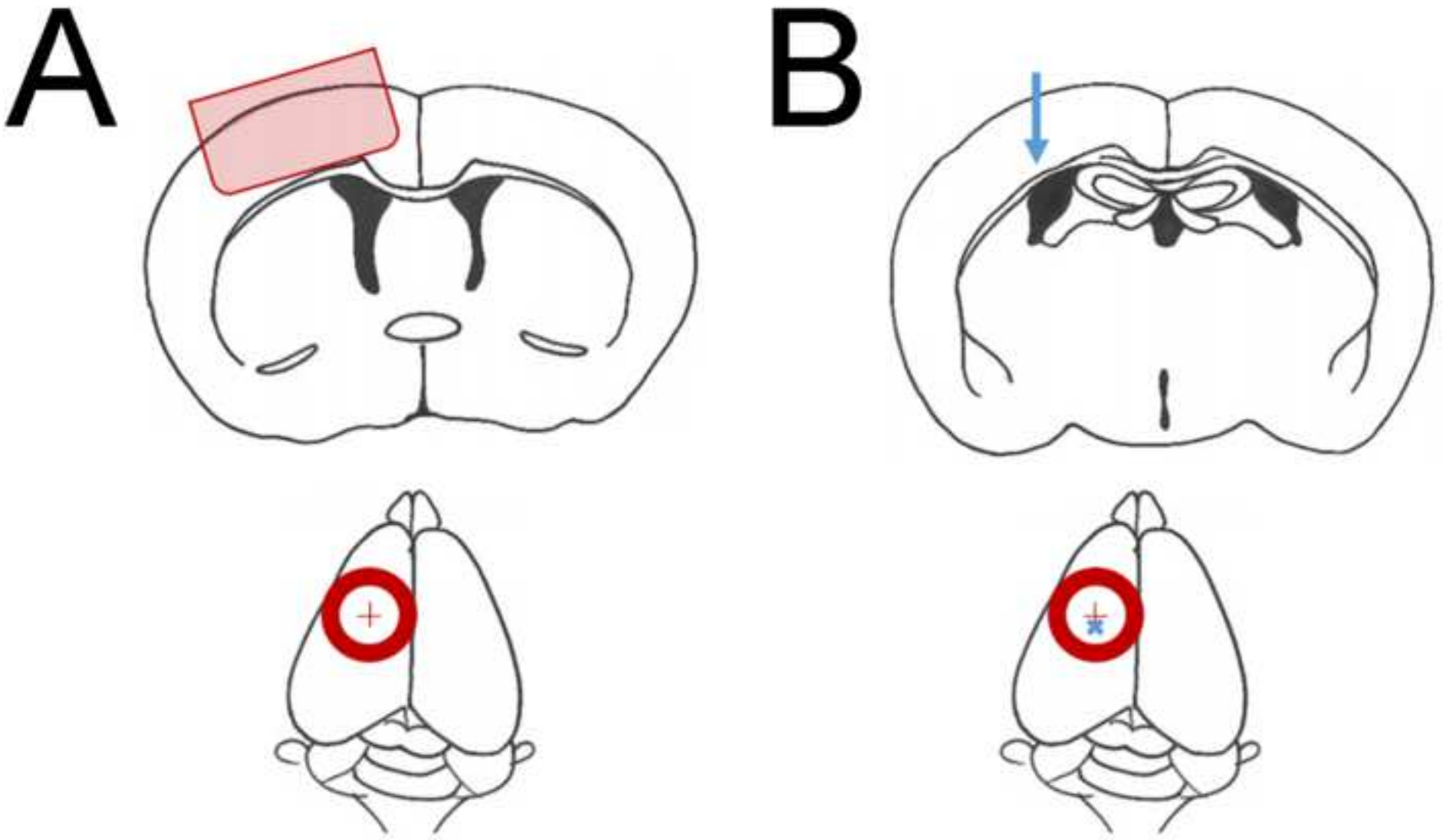

Figure 2

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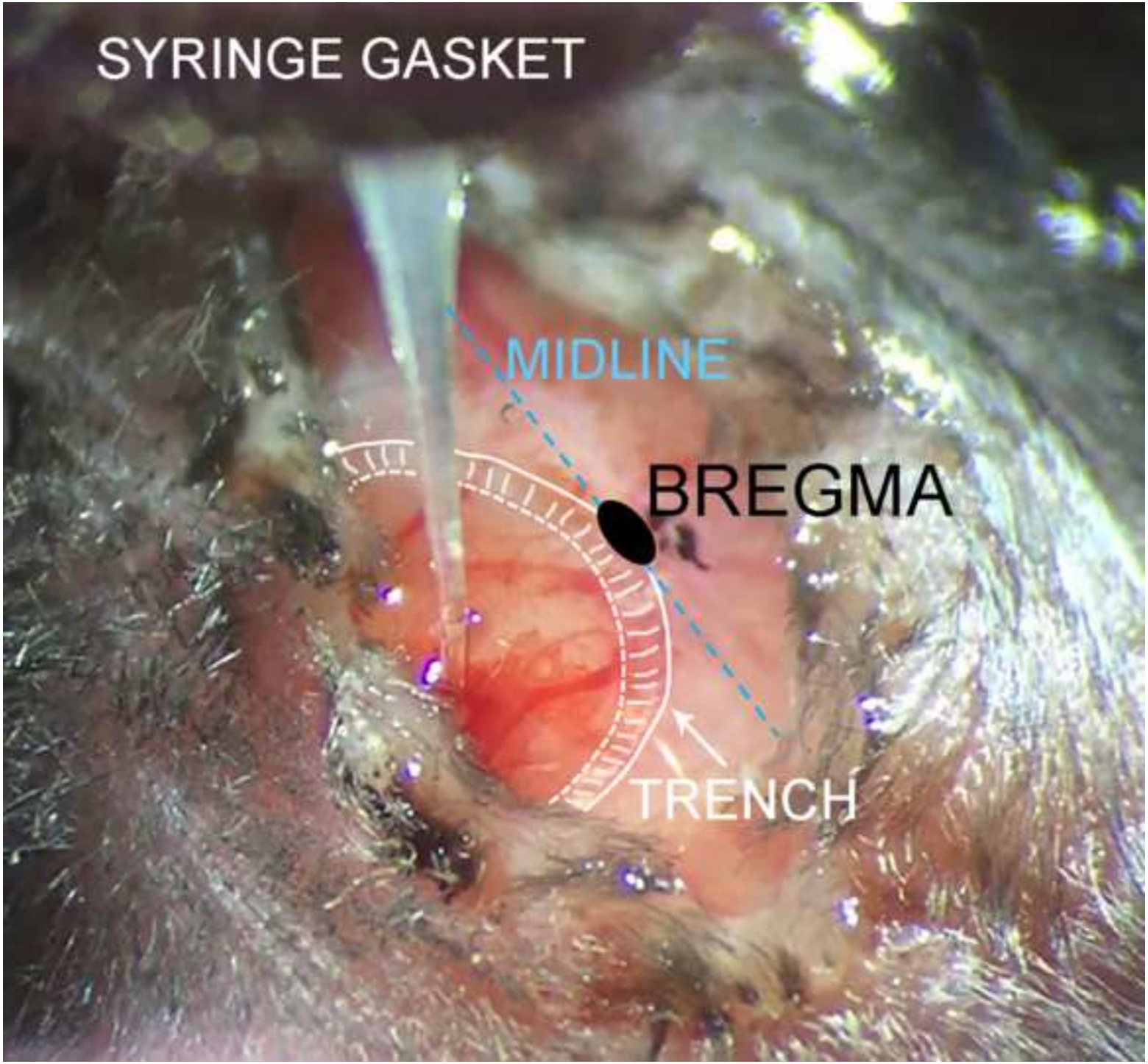

Figure 3

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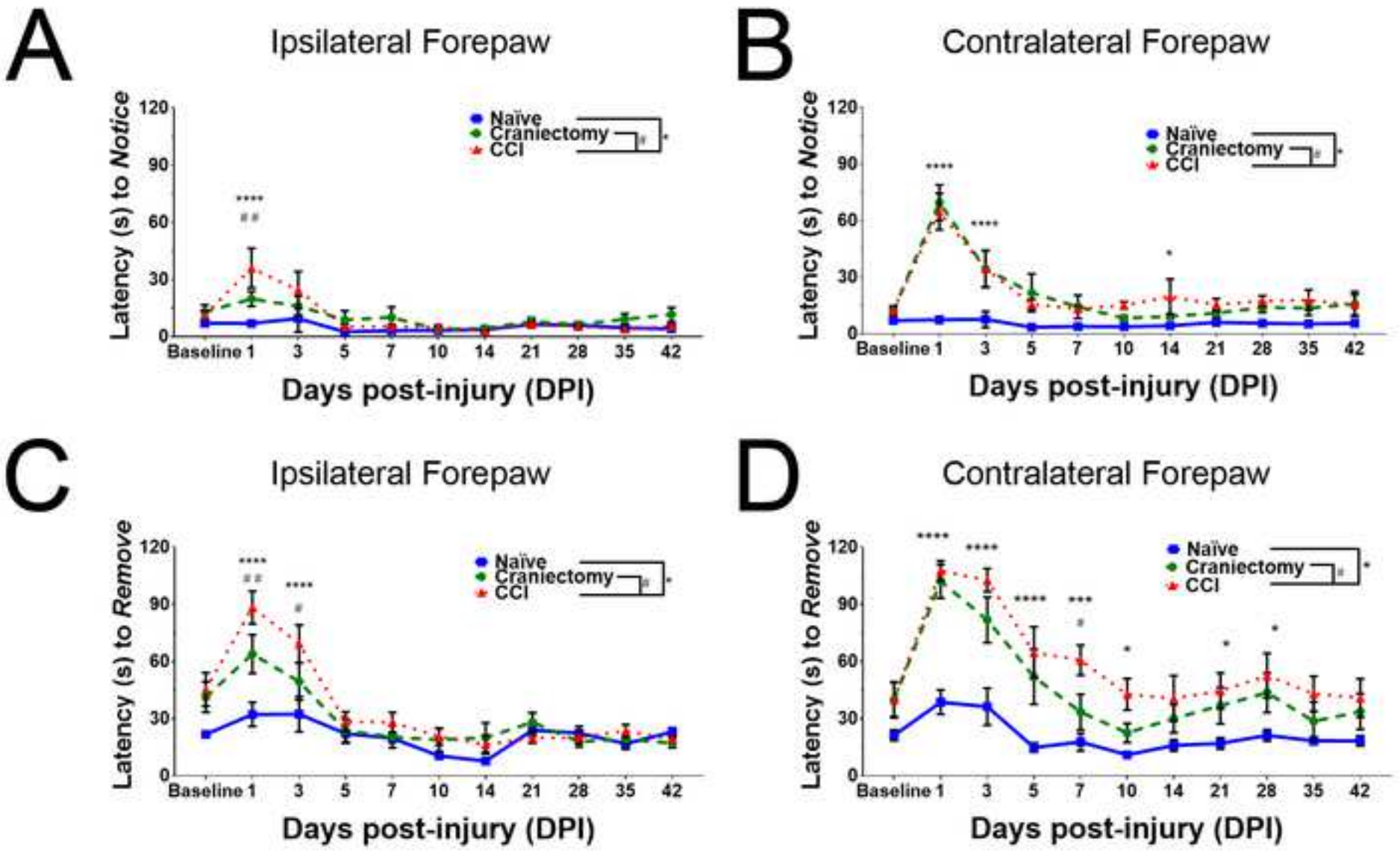

Figure 4

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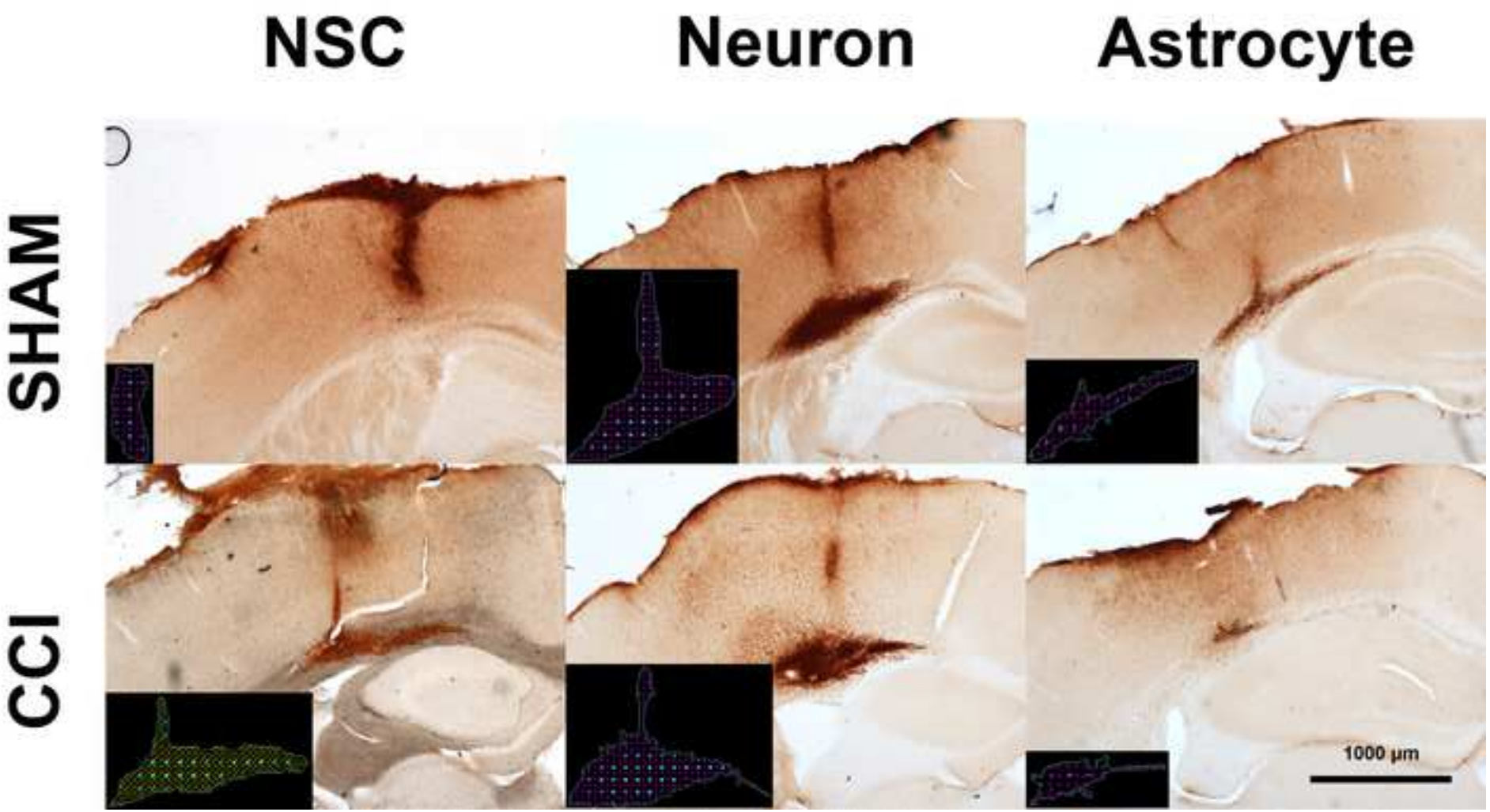

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 ml syringes	Becton Dickinson (BD)	309659	
1.7 ml flip top test tubes	Denville	C2170	
10 microliter syringe	Hamilton	7635-01	
25G Precision Glide syringe needles	Becton Dickinson (BD)	305122	
70% ethanol			Product of choice; varies by region
acetaminophen oral suspension	Tylenol (Children's)		Dilute to 1 mg/ml in water
anesthetic vaporizer	Vetland	521-11-22	
animal handling cloth			Purchase from department store
Betadine	Purdue Products	NDC-67618-151-32	
compressed oxygen			Product of choice; varies by region
cyclosporine A	Sigma-Aldrich	30024-100mg	
DAB staining kit	Vector Laboratories	SK-4100	
dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D8418-500ml	
DMEM	Invitrogen (ThermoFisher)	11965-092	
donkey anti-mouse IgG antibody, HRP conjugated	Jackson ImmunoResearch	715-035-151	
electrical tape	3M Corporation		Purchase from department store
fine tweezers	Fine Science Tools	11254-20	
forceps	Fine Science Tools	91106-12	
glass capillary pipettes, 1 mm OD, 0.58 mm ID	World Precision Instruments	1B100F-3	
High Speed Rotary Micromotor Kit	Foredom Electric Co.	K.1070 - K.107018	
Ideal Micro Drill Burr Set Of 5	Cell Point Scientific	60-1000	
Impact One Stereotaxic Impactor for CCI	Leica Biosystems	39463920	
isoflurane	Baxter	NDC-10019-360-60	
lab bench timers	Fisher Scientific	14-649-17	
Micropipette puller	MicroData Instruments, Inc.	PMP-102	Any puller will suffice
Microscope cover slips	Fisherbrand	12-545-E	
Microscope slide mounting medium			Product of choice
mirror			Purchase from department store
mouse anti-human nuclear antigen antibody	Millipore	MAB1281	
Mouse on Mouse blocking kit	Vector Laboratories	BMK-2202	
needle holder hemostat	Fine Science Tools	12002-12	
ophthalmic ointment	Falcon Pharmaceuticals	NDC-61314-631-36	
ophthalmic spring scissors	Fine Science Tools	15018-10	
plastic box			Purchase from department store
plastic cylinder			Purchase from department store
QSI motorized syringe pump	Stoelting	53311	
Removable needle compression fitting	Hamilton	55750-01	
small rodent stereotaxic frame	Stoelting	51925	
small scissors	Fine Science Tools	14060-09	
StemPro Accutase	Invitrogen (ThermoFisher)	A1110501	
Sterile alcohol prep pads	Fisherbrand	06-669-62	
sterile cotton swabs/Kendall Q-tips	Tyco Healthcare	540500	
Sterile saline	Hospira	NDC-0409-1966-07	
Stopwatches (2)	Fisher Scientific	06-662-56	
Superfrost Plus Gold microscope slides	Fisherbrand	15-188-48	
sutures - 5.0 silk with curved needle	Oasis	MV-682	

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CORRESPONDING AUTHOR

Name:	Martin L. Doughty, Ph.D.
Department:	Department of Anatomy, Physiology, and Genetics
Institution:	Uniformed Services University Bethesda, MD, USA
Title:	Associate Professor
Signature:	Martin L. Doughty
Date:	December 12, 2018

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Furmanski, Nieves, and Doughty – JoVE submission JoVE59561

Response to editorial and peer reviewer comments

We greatly appreciate the efforts of the editors and peer reviewers on behalf of our manuscript. The original critiques are shown below in bold font. Our responses are shown directly below each reviewer's critique in italics.

Responses to editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have extensively proofread the manuscript for inaccuracies in spelling and grammar.

2. Please define all abbreviations before use.

We clarified some abbreviations that were not initially defined (e.g. CsA).

3. Please provide an email address for each author.

Emails were added for authors Furmanski and Nieves.

4. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

A statement regarding adherence to Institutional Animal Care and Use Committee had been included in the Acknowledgement section at the bottom of the manuscript. This statement was revised and moved to the beginning of the step-by-step protocol as requested.

5. For the protocol section please have all the steps and headings numbered. For e.g. Please number Line 80., line 180, Line 315, etc. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

Protocol section headings were numbered as requested.

6**. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However two notes cannot follow each other.**

Several steps, clarifying statements, and suggestions were not constructed in the imperative tense. We worked extensively to either change these statements to the imperative, or moved them to the Discussion section as requested.

7. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

As above, several methodologic suggestions were moved to the Discussion section.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.

Steps in the protocol were streamlined wherever possible to limit each step to an individual action.

9. Please provide volume and concentrations of all the solutions/reagents used in your study. Please be as specific as you can with respect to your experiment.

Some steps were initially unclear regarding volumes of saline or cyclosporine A (CsA) to be loaded in syringes. Reagent volumes have been clarified as requested.

10. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

We have added detail to protocol steps where appropriate to improve clarity.

11. 1.1. What kind of syringe? How much saline? 12. 1.1. and 1.2 are different syringes? How much?

We clarified the types of syringes to use for saline irrigation and CsA injections. The syringes are separate syringes of the same model.

13. 4.1: What kind of cells?

We have clarified the protocol to specify neural stem cells derived from human induced pluripotent stem cells. However, this protocol can be used for transplanting many different kinds of mammalian cells in suspension.

14. Once all changes are done, please ensure that the highlights are 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. This will be used for generating script for the video.

The highlighted sections were revised. Headings that were not methodologic were removed from highlights. Some additional steps in the adhesive tape removal protocol were added to highlights. The highlighted protocol steps total roughly 2.75 pages.

15. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The Discussion section has been extensively revised. Some advisory statements from the protocol were moved to the Discussion as requested in Comment 6. Each section of the amended Discussion was revised to conform to the content suggested by the Editor.

16. Please expand the journal names in the reference section.

The bibliography was reformatted to include full journal titles. In-line citations are presented as superscript numbers.

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The figures used in this manuscript have not been previously published in journal article format and are not subject to copyright restriction.

18. Please alphabetically sort the table of materials. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.

The table of materials has been updated. We added additional details on the rotary tool used for craniectomy. The revised list of materials was alphabetized as requested.

Responses to Peer Reviewer #1:

1. Cyclosporin needs to be mentioned in parenthesis during the first instance of usage of CsA.

The first instance of Cyclosporine A in the text was spelled out as requested.

2. It is known that a higher speed setting on rotary tool can cause heat damage to the tissue during craniectomy. What speed setting are you using or recommend?

We regret not including specific settings on the rotary tool equipment to use for performing craniectomy. The adjustment dial on the base unit which dictates rotation speed does not indicate revolutions per minute (RPMs). Instead, we have indicated a range of RPMs as a percentage of maximum speed.

3. Craniectomy over the cranial sutures could lead to hemorrhage. Are there any precautions that the authors took?

Our experiences agree with the reviewer's observation that drilling over cranial suture joints can cause hemorrhaging. We have clarified the protocol to indicate that the sutures must be avoided during craniectomy.

4. Acetaminophen is known as a neuroprotector (Expert Opin Emerg Drugs. 2009 Mar; 14(1): 67-84., J Neuroinflammation. 2009; 6: 10.). Would using acetaminophen affect the behavior results?

Acetaminophen was chosen on the basis of its minimal neurobehavioral side effect profile. NSAIDs generally are more acceptable than stronger opiate analgesics which may cause sedation. Please see our response to Reviewer #2 critique 3 for a more detailed explanation.

5. The authors need to mention the timeline for the adhesive tape test in the protocol.

We have elaborated on the timeline for performing adhesive tape behavior testing as requested by the reviewer. This timeline includes when training and baseline testing take place before craniectomy (with or without CCI). We go on to list the days on which postoperative behavior testing were performed in the experiment described. The postoperative testing schedule is mutable according to a given investigator's experimental paradigm.

6. Why are the craniectomy only mice performing worse than naïve mice?

The reviewer correctly observes that mice which underwent craniectomy performed worse in the adhesive removal test than naïve mice. Performance deficits were attributed to injury and inflammation from surgery. Transient cognitive dysfunction due to isoflurane anesthesia may have played a role as well. Naïve controls were truly naïve, and did not undergo anesthesia.

7. The CCI injury can cause IgG extravasation and hemorrhage in the cortical tissue. The needle tract also can contribute. The blood and IgG can react with DAB to give false positives. What modifications did the authors do for the IHC protocol to reduce this?

Our experience during optimization of human nuclear antigen immunohistochemistry agree with the reviewer's observation that endogenous erythrocyte peroxidases and endogenous IgGs can produce non-specific DAB staining in injured brain tissue. We added clarification to the protocol regarding the use of hydrogen peroxide to inactivate endogenous peroxidase activity. Already present in the protocol was the use of the Vector Laboratories Mouse On Mouse kit. This kit contains proprietary chemicals that block antigenicity of endogenous mouse IgGs, which may have extravasated into brain tissue after injury. We regret the lack of clarity on these two important aspects of DAB immunohistochemistry.

Responses to Peer Reviewer #2:

1) In Sec. 2.7, the authors state to use a high-speed rotary tool for the craniectomy. I recommend the authors state what specific type of tool is used, and burr/rotary blade, to best ensure replication by others. No mention of the rotary tool is made in the Equipment/Supply list, and this should be listed there, as well.

We appreciate the reviewer's attention to detail regarding the rotary tool equipment. Specific details have been added to the equipment list and protocol regarding the manufacturer and model of the rotary tool. We also added details on the size of the burr bits used during craniectomy.

2) The need for clarification of the type of cutting device is highlighted by the use of different terms such as "cutting" in 2.7.1.1 and "drilling" in 2.7.1.2. Are these different procedures, or just different terms for the same procedure and tool?

We regret the inconsistency in terminology for performing craniectomy. The term "drilling" is now used consistently throughout the protocol.

3) For post-surgical analgesia (2.10.2), is acetaminophen enough? Often buprenorphine or carprofen (or a combination of the two) is utilized. Is there a particular reason stronger analgesias are not used?

Both peer reviewers make specific mention of our choice of postoperative analgesic. Acetaminophen suspension is the minimum mandatory postoperative analgesic required by the Uniformed Services University (USU) Animal Care and Use Committee and Center for Laboratory Animal Medicine veterinarians. The use of acetaminophen suspension is also standard operating procedure in the Preclinical Studies core facility of the USU Center for Neuroscience and Regenerative Medicine. The caveat with the use of acetaminophen, or NSAIDs in general, in central nervous system injury modeling is the attenuation of cyclooxygenase-dependent inflammatory signaling. Reactive gliosis, cell death, and cytokine expression could all be negatively regulated. Opiate analgesics, such as buprenorphine, may provide greater relief of postoperative pain at the cost of side effects such as sedation or euphoria. In the end, acetaminophen was chosen on the basis of providing pain relief while minimizing side effects that could interfere with graft survival or cause performance deficits in the adhesive removal behavior test.

4) During the post-operative period, are there any physical manifestations of injury that should or could be monitored that may affect the status of the animal, e.g. hemorrhage following suture closure, worsening conditions and lack of expected recovery post-surgery? The "Representative Results" section mentions

vascular damage and related problems can affect lesion size. Though some description is provided in Section 5.4.1, it is unclear how disrupted vascularization is identified, as the CCI damages the cortex, correct? Shouldn't all animals have some vascular disruption post-impact? Figure 2 shows apparent vascular disruption, (though the authors state this is normal vascularization) but it is not clear if this is due to the injection. Though the parameters for this impact are for producing mild TBI, I am sure producing consistent results with no vascular disruption is difficult, even if the meninges are left intact. Based on the apparent vascular disruption shown in Figure 2, if the damage is due to the injection, this should be mentioned in the Figure Legend.

We observe animals daily for overall well-being and specifically for weight gain throughout the experiment. Inactivity, weight loss, ungroomed fur, and hunched posture are standard measures of postoperative distress under our IACUC guidelines. Ictal-like repetitive myoclonic behaviors have been documented infrequently, and are cause for exclusion from studies.

We regret that the lack of detail regarding intact vascularization versus disruption and hemorrhage were troubling to the reviewer. In our experience, widespread cortical vessel disruption and bleeding requiring extensive hemostasis are the clearest intraoperative indicators of negative surgical outcomes. Accidental tearing of the meninges during skull flap removal is one such cause of severe hemorrhage. Neurovascular complications can be qualitatively assessed when the animals undergo intraparenchymal cell transplantation on the day after craniectomy. We inspect the cortical surface for preservation of numerous cortical vessels, coloration suggesting adequate blood supply, and extent of edema. Extensive blood clotting within the craniectomy is a strong indication of excessive brain injury.

It is true that CCI injury will nearly always result in some ruptured cortical vessels. The most serious events are related to rupturing major vessels or the sagittal sinus. Occasionally, injury-induced cortical herniation can interrupt blood supply. These serious events were strongly correlated with severe cortical injury and/or cavitation. Isolated small-diameter vessel rupture after impact or after transplant needle penetration (as seen in Figure 2) did not correspond with negative outcomes. Nevertheless, as the reviewer requests, we have added a note to the Figure 2 legend that minor bleeding as shown is unlikely to indicate excessive injury.

5) For the Discussion section, it would be helpful to talk about the limitations of performing consistent mild TBI and how it is defined in the literature. The authors' model is just one model, and other models and definitions exist, which adds to the difficulty of studying the effects of mild TBI and any treatment effects.

We have added a discussion section comparing mild TBI animal models as the Reviewer suggests.

6) Are there any potential risks associated with injection of a volume of cells in mildly damaged tissue (as shown in Figure 4)? Injection of the cells could cause tissue damage as a result of pressure and tissue displacement if a cavity is not present. Again, if this is a possible issue (or why it isn't) could be provided in the Discussion.

It is true that rapid intraparenchymal injection can cause mechanical injury. The rate of injection described in the protocol, 0.2 microliters/minute, appears to be sufficiently slow to minimize injury due to volume displacement. We do not observe overt cell death (cavitation, pyknosis) or excessive reactive gliosis (GFAP, Iba1) as a result of injection in brain tissues collected within 7 days of surgery.

Minor Concerns:

7) In section 2) the idea of draping or isolating the surgical field should be highlighted. The eyes are exposed (even with ophthalmic ointment) and performing a craniectomy puts the animal at risk for debris in and on the eyes. Even if the video does not show it, mention of a drape and isolation of the field should be made, and that it has been excluded from the video for demonstration purposes.

We have added an instruction to the craniectomy section regarding the use of a fenestrated surgical drape, as requested by the reviewer.

8) In section 5.8.3, is there a specific reason why -1.4mm is chosen for the depth? A description here might be helpful (and a reference).

The graft site parameters were chosen based on mouse brain atlas coordinates[1] that centered the graft at the gray matter-white matter junction. We sought to place cell transplants deep to the injured cortex to test two central ideas. (1) Superficial injury could exert less negative impact on graft cell survival. (2) Graft cells could support injured overlying cortex through paracrine effects or replenishing damaged neurocircuitry.

1 Paxinos, G. & Franklin, K. B. J. *The mouse brain in stereotaxic coordinates*. Compact 2nd edn, (Elsevier Academic Press, 2004).

9) For any of the functional assessments, are there any extremes that would cause the researcher to exclude an animal from a study? For instance, if the lesion was too severe, even though initially it was determined to be within the range of consistent injury, is there a cutoff score for the assessments that an animal should not reach during the first testing period (basically the animal performs too well)? This may only be identified after all assessments are completed, as a researcher doing the assessments should be blinded. However, such information is helpful and could be put in the "Representative Results" or appropriate Discussion section for the test.

The only extremes in motor behavior we have observed in several iterations of this protocol have been repetitive myoclonic movements suggesting seizure-like activity. These mice were excluded from behavioral studies. Indeed, after euthanasia these mice were shown to have severe cortical lesions. On the other hand, several other mice which were later revealed to have severe cortical lesions did not show seizure-like activity and were able to remove adhesive strips from both forepaws. Behavior assessment has proven to be a less reliable metric of unintended excessive brain injury than intraoperative evaluation of intact cortical vasculature in this mild CCI model. Please refer to Reviewer 2 comment 4 above for commentary on evaluating cortical vasculature preservation.

Responses to Editor's comments on JoVE manuscript 59561, Furmanski *et al.*

We greatly appreciate the Editor's attention to detail on our manuscript. Here, we summarize the Editor's margin comments on the previous draft text using green highlights. Our responses, and changes to be found in the newly revised draft, appear below in italics.

(Line 2)
Controlled Cortical Impact Model of Mouse Brain Injury with Therapeutic Transplantation of Human Induced Pluripotent Stem Cell-Derived Neural Cells

Ed. - How is the transplantation of hiPSC therapeutic?

Please see below for the response to the following Editorial comment.

(Line 44)
INTRODUCTION:

Ed. - Introduction doesn't contain any details about the hiPSC transplantation and its significance. Please bring out the goal of the protocol very clearly.

We regret the lack of clarity regarding the nature of the transplanted cells in this protocol. We have added a citation regarding hiPSC technology. We have also added a passage to the introduction that ties together existing literature on neural cell therapy for CNS injury and this laboratory's efforts to develop hiPSCs for therapeutic transplantation after experimental brain injury.

(Line 80)
This protocol outlines a complete workflow for performing an experimental brain injury, therapeutic transplantation of cultured cells, and behavioral and histologic analysis of experimental outcome measures.

Ed. - Significance of the same?

As stated above, we have added additional information regarding hiPSCs their potential to aid recovery from brain injury.

(Line 84)
PROTOCOL:

Ed. - Presently the highlight is more than 2.75 pages including headings and spacings. Please consider combining small steps into one so that there are 2-3 actions per step. Also please ensure that the highlights form a cohesive story. I have highlighted some additional steps for clarity.

We have taken the Editor's suggestion to condense multi-part steps to economize on space. We appreciate the cues given by the Editor to make the highlighted sections flow as a distinct narrative. The highlighted sections have been extensively revised to conform to 2.75 pages with 12 point font and 1-inch margins.

(Line 174-175)
1.4. Lavage with saline and suture the scalp incision using simple interrupted stiches.

Ed. - What is the volume used? What kind of suture is used?

No specific volume is used when rinsing the surgery site. Wording has been changed to "as needed", and our suture material is now specified.

(Line 220)
2.4.2. Prepare cell cultures in advance according to standard conditions defined for the cell type.

Ed. – Citation?

We have added a citation that readers may refer to for our specific cell type.

(Line 241)
2.4.5.3. Allot ~5 x 10^5 cells per mouse if performing multiple cell transplantation procedures in a day.

Ed. - In what volume?

Step 2.4.4. specifies that the cells must be diluted to 5 x 10^4 cells/µL. We have clarified that 10 microliters of suspension should be allotted per mouse.

(Line 433)
4.1. Collect sections of formaldehyde-fixed tissues using standard methods.

Ed. - Before this step, please include how euthanasia step is performed. After how many days was this was performed. Then move on to the collection of tissues and then sectioning.

We have added a detailed protocol for euthanasia, transcardiac perfusion fixation, and tissue collection as requested by the Editor.

(Line 440)
4.3. Perform antibody labeling by standard methods.

Ed. - Citation? Or provide the concentration, time and temperature for secondary antibody incubation

The text now refers to a paper from this laboratory which describes antibody labeling methodology after CCI in mice. Incubation time, temperature, and antibody dilution have been added as requested by the Editor.

(Line 448)
4.3.1.1. Use a *mouse IgG neutralization* kit (see **Table of Materials**) to reduce cross-reactivity with endogenous antibodies.

Ed. - How do you perform the blocking? For how long?

We have changed the wording to avoid using the manufacturer terminology for the mouse IgG neutralization kit. The manufacturer includes directions for using the kit, which we followed verbatim. The IgG neutralization process adds several additional incubation steps that we felt were sufficiently described in the manufacturer's protocol.

(Line 451)
4.3.2. Perform a horseradish peroxidase (HRP) labeling step using secondary or tertiary conjugate method of choice.

Ed. - Citation? Or provide the concentration, time and temperature for secondary antibody incubation.

We have added details regarding the secondary antibody incubation conditions requested by the Editor.

(Line 454)
4.3.3. Perform DAB-peroxide reaction by standard methods to reveal immunolabeling.

Ed. - For how long?

We have added detail to describe a 5 to 10 minute time window which should give satisfactory DAB color development.

(Line 458)
4.5. Quantify numbers of labeled cells using method of choice.

Ed. - E.g method? Citation if any?

We have specified the use of the unbiased stereology method of quantification. Two relevant citations have been provided, and parameters unique to our analysis have been specified.

(Line 462)
Craniectomy surgery facilitates experimental brain injury and therapeutic cell transplantation

Ed. - What was the control in this case?

Craniectomy without CCI injury (sham) controls for mild injury which may occur due to surgery alone. This system is designed to show influences of host brain injury severity on graft survival. Step 5 under Section 1 explains the procedures to omit when performing sham controls.

(Lines 466 to 474)
5 mm diameter craniectomy schematics to uncover primary somatosensory and motor cortices (**Figure 1A**). At 24 h after craniectomy, a second surgery is performed to inject human iPSC-derived neural cell suspension into deep layers of the cortex (**Figure 1B**). Some cerebral edema is normal on the first day following craniectomy, and particularly after CCI. However, cerebral vasculature sparing during all phases of this procedure is crucial for survival of the cortex. Disruption of cortical blood vessels is likely to result

in excessive lesioning of the cortex and is grounds for excluding the animal from the experiment. **Figure 2** illustrates the cell transplantation procedure in a mouse with minimal cerebral herniation and extensive cortical vascularization. These features are good prognostic indicators of a successful surgery.

Ed. - Please include a table to show what was observed after the first surgery and second surgery. Please also include any complications if any. Also include the number of mice undergoing this surgery protocol.

We address several potential surgical complications in the "Mild CCI…" section of the Discussion. However, for clarity we have moved the passage on "Disruption of cortical blood vessels" in the section above to its more appropriate place in the "Mild CCI" Discussion.

This Representative Results section is intended to be illustrative only. We are describing general details about the surgeries. Specific numbers of mice are now included for the experimental results depicted in Figures 3 and 4, as requested by the Editor in subsequent comments.

(Lines 483 to 484)
Behavior tests in this study were performed on postoperative days 1, 3, 5, 7, 10, 14, 21, 28, 35, and 42. **Figure 3** shows results from a pilot experiment in which forelimb function in mice with craniectomy alone (sham) and with CCI injury were compared to forelimb function in naïve mice.

Ed. – How many mice per group? What is the difference between the two? Please bring out this clarity in the protocol section. Also, what happens when you perform the hiPSC transplantation? Do you perform any behavioral studies on transplanted mice? Please also include the details in the discussion section. Point out where the distinction between craniectomy control and CCI is indicated. Also point out that in these pilot studies, engrafted animals had not been enrolled in behavior testing studies.

Sample size n = 12 has been specified as requested by the Editor.

Step 5 under Section 1 describes the difference between craniectomy/sham control and CCI. We use "sham" as a shorthand term for craniectomy-only controls.

We have performed follow-up experiments which combined all of the methodologies in the pilots presented here. Behavior testing was performed for 56 days after surgery, and tissues were collected for histologic analysis. Further analysis is ongoing, but preliminary data show persistence of transplanted cells after 8 weeks. We have moved the brief mention of this graft survival observation from the second paragraph of the "Mild CCI" Discussion to "Refinement to DAB" Discussion. We also have added a statement that these experiments do not combine behavior testing with cell transplantation, but that ongoing studies in our laboratory will do so.

(Line 498)
Transplants of each of the three neural cell phenotypes were tested in our CCI model of traumatic brain injury using the procedure described above and depicted in **Figure 2.** The mice were euthanized for histologic analysis at 7 days after transplantation.

Ed. - Again need a number of mice in this case.

Numbers of mice for all 6 experimental groups (3 transplants x 2 surgery conditions) have been included as requested by the Editor.

(Line 500)
Human cell grafts could be clearly distinguished from host tissue in sham surgery and CCI brains (**Figure 4**).

Ed. - Again please bring out clarity with respect to sham and CCI.

Step 5 under section 1 was revised to more clearly describe the methodologic difference between sham and CCI surgeries.

(Line 500 to 502)
Astrocyte grafts showed poor survival compared to NSCs and neurons and were not considered for future experiments.

Ed. - How do you differentiate between Astrocyte, neuron and NSC in the histological section of the brain?

The cell types were not concealed at the time of transplantation – only the injury group enrollment (craniectomy/sham vs. CCI) is blinded.

(Line 522)
Figure 3: Behavioral evaluation of sensorimotor integration after brain injury.

Ed. - Please include what does error bar represent. How many mice per group?

Error bars refer to Standard Error of the Mean (SEM). Error bars and group mean data are now clarified in the text. The number of animals in each experimental group has been updated.

(Line 534)
Figure 4: DAB immunohistochemistry for human cell grafts in mouse brains.

Ed. – Please include scale bars in the figure.

A 1000 micron scale bar is now included in a revised Figure 4 as requested by the Editor.

(Line 553 to 555)
Neither edema nor small cortical vessel rupture are necessarily negative indicators. Hematomas and abnormal coloration due to ischemia are clearer indicators of surgical complication. Documenting intraoperative events and correlating complications with histopathologic outcomes are crucial to refining good craniectomy technique.

Ed. – Citation?

This description is based on personal observations and informal discussions with our colleagues over 2 years while performing these surgeries.

(Line 603 to 604)
Pretreatment with hydrogen peroxide is crucial for reducing non-specific staining in brain tissue due to endogenous peroxidases in erythrocytes. Non-specific staining in these experiments was further reduced using ~~the Vector Laboratories Mouse On Mouse (MOM) pretreatment~~ a mouse IgG neutralization kit (refer to Table of Materials).

Ed. - Please remove the commercial name from the manuscript and use a generic term instead.

We have changed the wording in the passage to a more general description of the antibody neutralizing kit.

(Line 614 to 616)
~~Although not a radical departure from established methods,~~ this protocol could be useful for other preclinical studies of human cells transplanted into the mouse central nervous system.

Ed. - This needs more discussion.

We are unsure what the Editor desires in terms of extended discussion. We have removed the statement shown above in strike-through font, as it is confusing and does not contribute value to the text.