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Supplemental Information

Enhanced Dentate Neurogenesis after Brain Injury Undermines Long-Term Neurogenic Potential and Promotes Seizure Susceptibility

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Supplemental Information

Supplemental Figures

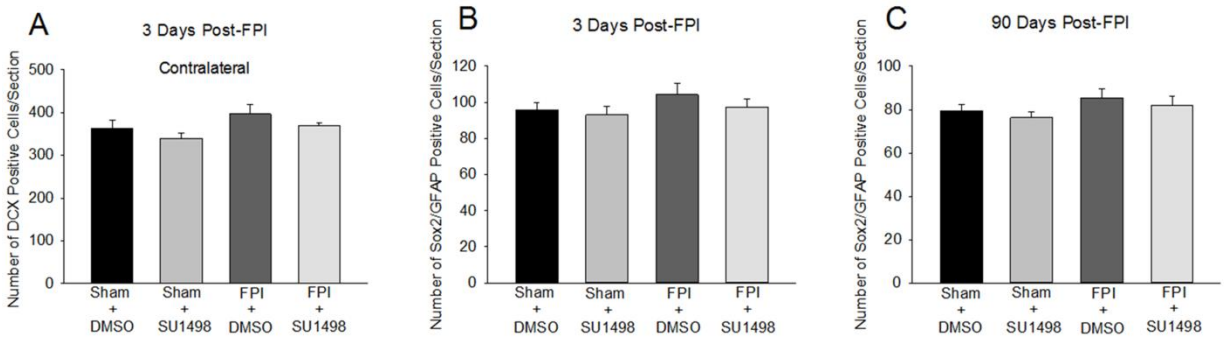

Figure S1: Effect of VEGFR2 antagonist on neurogenesis in contralateral hippocampus and Type I stem cells on the side of brain injury. (A) Stereological quantification of DCX labeled cells in contralateral sections from rats sacrificed 3 days after FPI are compared with data from age-matched sham injured controls. DCX-cells/section, sham-DMSO: 364±18, n=4 rats, sham-SU1498: 339±13, n=4 rats; FPI-DMSO: 397±23, n=4 rats, FPI-SU1498: 369±8, n=4 rats, $p>0.05$ by TW-ANOVA. There were no significant main effects on DCX cell counts as evidenced by the main effect of injury ($F_{(1, 12)} = 3.611$; $p=0.082$). and antagonist treatment ($F_{(1, 12)} = 2.494$; $p=0.140$). Interaction between injury and antagonist was also not significant ($F_{(1, 12)} = 0.00876$; $p=0.927$). (B-C) Stereological quantification of putative Type I stem cells co-labeled for Sox2 and GFAP in sections from rats sacrificed 3 (B) and 90 (C) days after FPI and compared to age-matched sham injured controls. 3 days: Sox2/GFAP-cells/section, sham-DMSO: 96±4, n=4 rats, sham-SU1498: 93±4, n=4 rats; FPI-DMSO: 104±7, n=4 rats, FPI-SU1498: 97±5, n=4 rats, $p>0.05$ by TW-ANOVA. 90 days: Sox2/GFAP-cells/section, sham-DMSO: 79±3, n=4 rats, sham-SU1498: 76±3, n=4 rats; FPI-DMSO: 85±4, n=4 rats, FPI-SU1498: 82±4, n=4 rats, $p>0.05$ by TW-ANOVA. There were no significant main effects on the total number of co-labeled cells as evidenced by the main effect of injury ($F_{(1, 12)} = 1.530$; $p=0.240$). and antagonist treatment ($F_{(1, 12)} = 0.948$; $p=0.350$). Interaction between injury and antagonist was also not significant ($F_{(1, 12)} = 0.179$; $p=0.680$). Data are shown as mean±SEM.

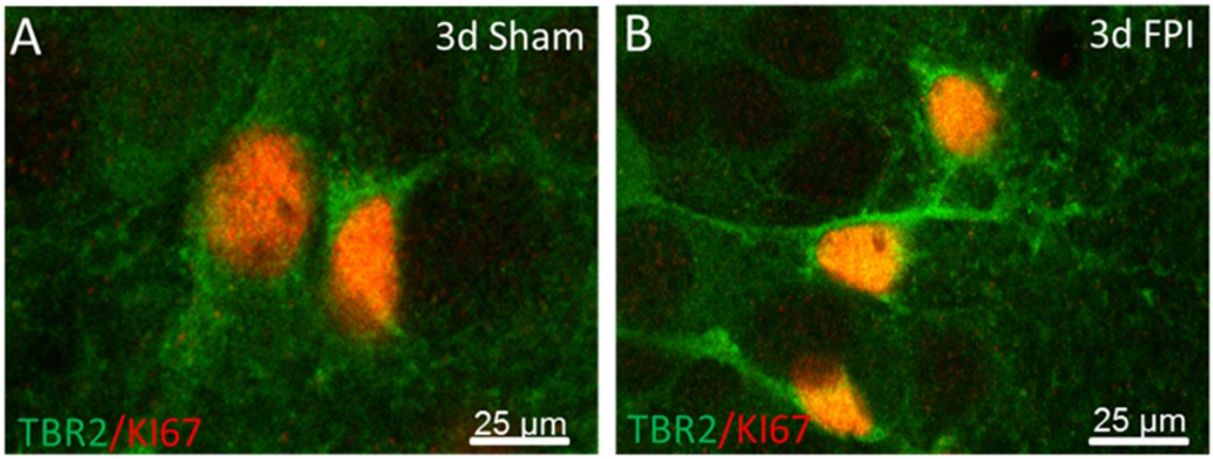

Figure S2: NPC Proliferation 3 days post-TBI

60x high magnification confocal images of sections from rats 3 days after sham (A) or FPI (B) shows NPCs co-labeled for Tbr2 and Ki67.

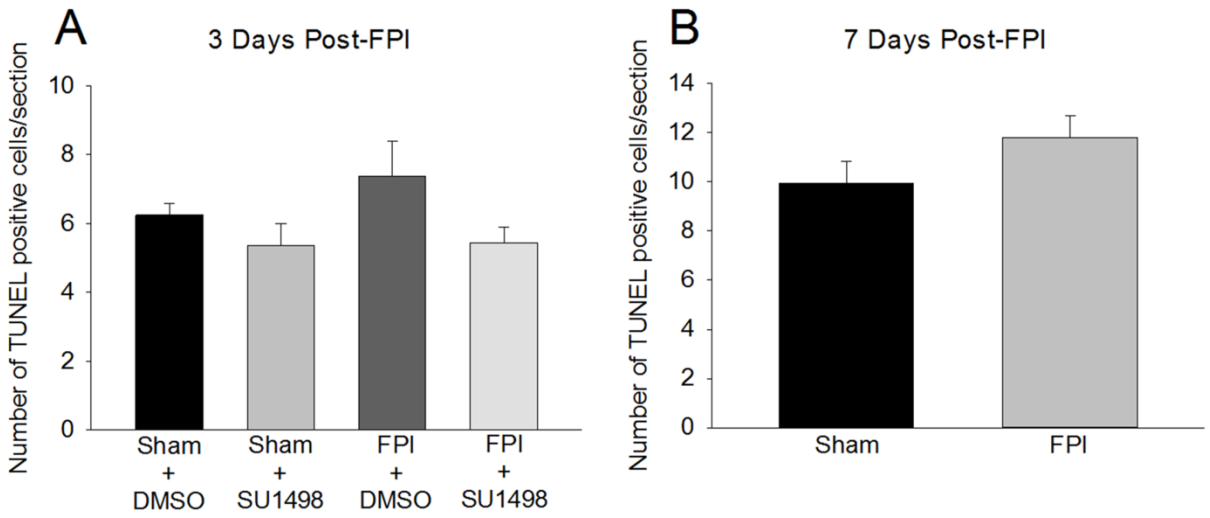

Figure S3: Lack of effect of FPI and VEGFR2 antagonist on number of TUNEL positive cells in the dentate subgranular zone. (A) Stereological quantification of TUNEL positive cells in sections from rats sacrificed 3 days after FPI are compared with data from age-matched sham injured controls. TUNEL-cells/section, sham-DMSO: 6±0, n=4 rats, sham-SU1498: 5±1, n=4 rats; FPI-DMSO: 7±1, n=4 rats, FPI-SU1498: 5±1, n=4 rats, $p>0.05$ by TW-ANOVA. (B) Stereological quantification of TUNEL positive cells in sections from rats sacrificed 7 days after FPI are compared with data from age-matched sham injured controls. TUNEL -cells/section, sham: 10±1, n=4 rats; FPI: 12±1, n=4 rats, $p>0.05$ by Student's t-test. Data are shown as mean±SEM.

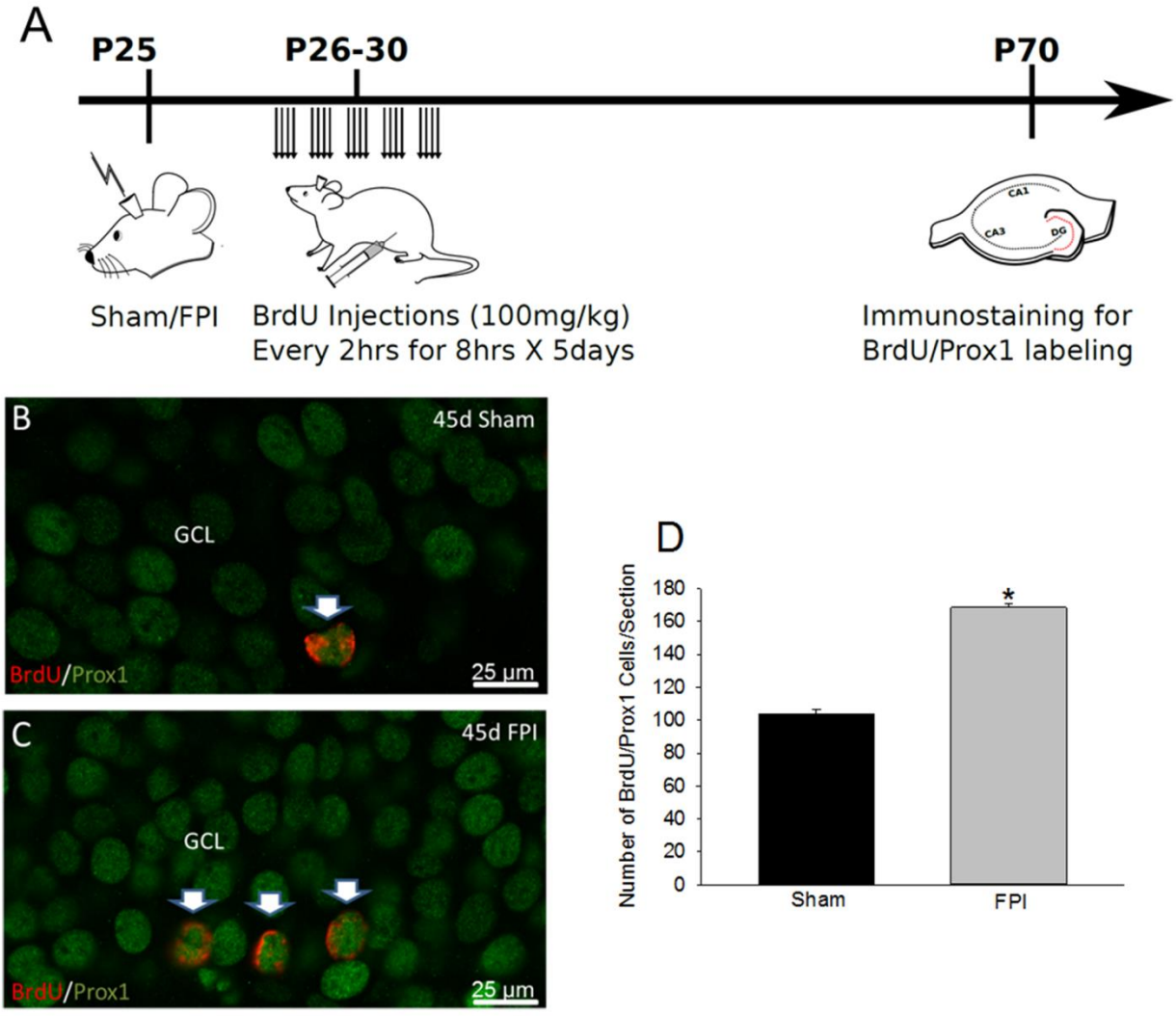

Figure S4: IGCs generated after FPI survive and mature into granule cells. (A) Schematic illustrates the timeline of FPI followed by BrdU treatment and euthanasia for histology. (B-C) Confocal images of sections from (B) control and (C) FPI rats co-labeled for BrdU and Prox1. Note the increase in neurons co-labeled for BrdU and Prox1 after FPI. (D) Summary plot shows quantification of the BrdU and Prox1 co-labeled cells 45 days after injury. BrdU/Prox-1 positive cells/section, sham: 103±3, n=4 rats, FPI: 169 ±3, n=5 rats, * indicates $p<0.05$ by Student's t-test. Data are shown as mean±SEM.

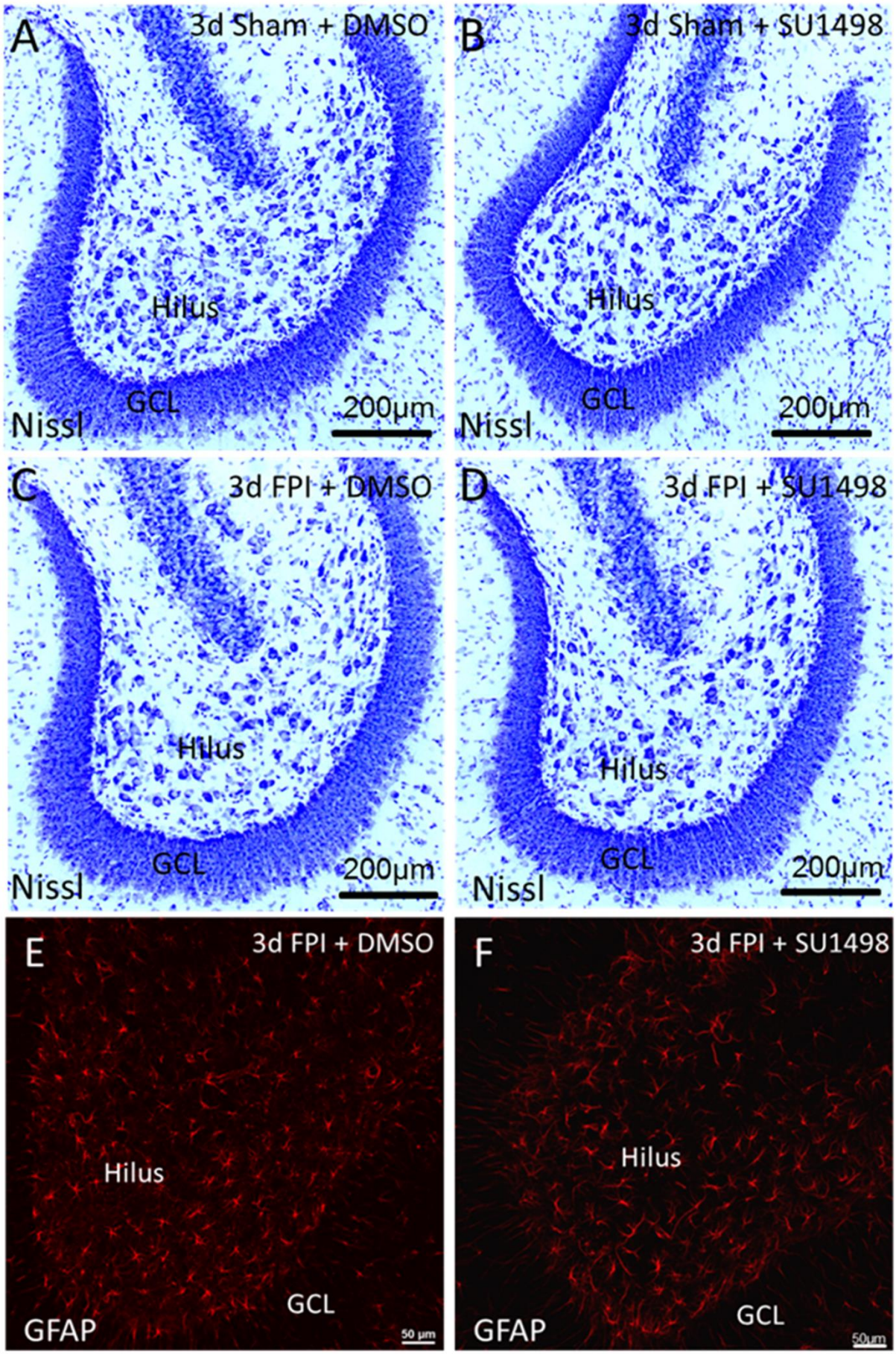
A
3d Sham + DMSO
Hilus
GCL
Nissl
200μm
B
3d Sham + SU1498
Hilus
GCL
Nissl
200μm
C
3d FPI + DMSO
Hilus
GCL
Nissl
200μm
D
3d FPI + SU1498
Hilus
GCL
Nissl
200μm
E
3d FPI + DMSO
Hilus
GCL
GFAP
50 μm
F
3d FPI + SU1498
Hilus
GCL
GFAP
50μm

Figure S5: Lack of effect of SU1498 on early post-injury hilar neuron loss and astrogliosis. (A-D) Representative Nissl stained sections from Sham and FPI rats treated with vehicle (DMSO) or SU1498 and sacrificed 3 days after injury illustrate the lack of drug effect on post-FPI hilar cell loss. N=4 sections from 4 rats for each group. (E-F) Semi-quantitative analysis of GFAP labeled cells shows application of SU1498 does not have an effect on astrocyte expression in the hippocampus 3 days after brain injury. N=6-8 sections for 4 rats for each group. Average intensity in A.U /section, FPI-DMSO: 12.02±0.58, n=4 rats; FPI-SU1498: 13.14±0.80, n=4 rats, $p>0.05$ by Student's t-test.

Supplemental Experimental Procedures

All procedures were approved by the Institutional Animal Care and Use Committee of the Rutgers New Jersey Medical School, Newark, New Jersey and are in compliance with the ARRIVE guidelines. Datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Histology and Immunohistochemistry

Immunostaining was performed on free-floating hippocampal sections (40 μm) obtained from the injured and uninjured hemisphere of FPI and age-matched sham-operated rats perfused with 4% paraformaldehyde in phosphate buffered saline 3 or 90 days after injury. Sections were washed in buffer and blocked in 10% normal donkey serum with 0.3% Triton for 1 h. For doublecortin (DCX) staining, contralateral sections were incubated overnight in anti-doublecortin primary antibody (1:100, goat polyclonal; Santa Cruz, sc-8066) in 0.3% Triton and 2% normal donkey serum. Double staining for Sox2 and GFAP was performed by sequential labeling with anti-Sox2 (1:100 goat polyclonal, Santa Cruz, sc-17320) for 1 h followed by addition of anti-GFAP (1:500, mouse monoclonal, Millipore Sigma, MAB360) and overnight incubation.

To label BrdU sections underwent DNA denaturation by serial incubation in HCL and borate buffer as detailed previously[1]. Double immunostaining for BrdU and Prox-1 was performed by sequential incubation in anti-BrdU (1:200 rat monoclonal, Abcam, AB6326) for 1 h followed by addition of anti-Prox-1 (1:2000, rabbit polyclonal, Millipore, ab-5475) overnight.

Following treatment with primary antibodies, sections were washed and immunostained with fluorescent secondary antibodies: donkey anti-goat Alexa 594 (1:500; ThermoScientific, A-11058) to reveal Doublecortin, donkey anti-goat Alexa-488 (1:500; ThermoScientific, A-11055) to reveal Sox2, donkey anti-mouse Alexa-594 (1:500; ThermoScientific, A-21203) to reveal GFAP, donkey anti-rabbit Alexa-488 (1:500; Abcam, ab150073) to reveal prox-1, donkey anti-rat Alexa-594 (1:500; ThermoScientific, A-21209) to reveal BrdU and mounted using Vectashield (Vector labs). Controls omitting primary antibody were routinely included. Fluoro-Jade C staining was performed as detailed previously [2] and positive controls were routinely included.

Quantification was performed using randomized systematic sampling protocols, selecting every tenth section along the septo-temporal axis with the hippocampus on the injured side. Cell counts were performed with the Optical Fractionator probe of Stereo Investigator V.10.02 (MBF Bioscience) at X100 (oil objective) on an Olympus BX51 microscope. In each section, the subgranular zone was outlined by a contour traced under a X10 objective (Fig. 1F) and the number of labeled cells was estimated based on planimetric volume calculations in Stereo Investigator.

Nissl Staining

Nissl staining was performed on sections from rats fixed with 4% paraformaldehyde 3 days after sham or head injury. Hippocampal sections (40 μm) were mounted on Superfrost Plus slides (Fisher Scientific – 12.550.15) and air dried. Slides were immersed in 100%, 90% 70% and 30% ethanol, and water for 30 seconds each followed by a 1 min incubation in a cresyl violet solution before being dehydrated in 30%, 70%, 90% and 100% alcohol and water for 30 seconds each and then cleared in xylene for 2 min. The sections were then mounted with DPX.

Tunel Staining

Tunel staining (Invitrogen APO-BrdU TUNEL Assay Kit – A23210) was performed on sections from rats fixed with 4% paraformaldehyde 3 days after sham or head injury. Hippocampal sections were mounted on Superfrost Plus slides (Fisher Scientific – 12.550.15), air dried and a pap pen was used to outline the sections in a hydrophobic barrier and in each step 50 uls of solution was applied to each section. First a DNA labeling solution containing a reaction buffer, TdT enzyme and BrdUTP was added to the sections and incubated at 37 degrees C for 120 minutes. Sections were then rinsed for 5 minutes and an antibody solution containing Alexa Fluor 488 dye-labeled anti BrdU antibody was applied to each section and incubated in the dark for 45 minutes. A staining buffer of propodium iodide/RNase A was then added to each section and incubated in the dark for 30 minutes. A wash buffer was then added for each section for 5 minutes for 2 consecutive washes. The sections were then air dried and mounted with Vectashield.

BrdU Labeling

For labeling neurons born after FPI, animals received four injections of 5-bromo-2-deoxyuridine (BrdU) (100 mg/kg, i.p; ThermoFisher, 000103) at two hour intervals for 5 consecutive days immediately following FPI or sham-injury[1].

References

1 Covey, M. V., Jiang, Y., Alli, V. V., Yang, Z. & Levison, S. W. Defining the critical period for neocortical neurogenesis after pediatric brain injury. *Dev Neurosci* **32**, 488-498, doi:10.1159/000321607 (2010).

2 Neuberger, E. J., Abdul-Wahab, R., Jayakumar, A., Pfister, B. J. & Santhakumar, V. Distinct effect of impact rise times on immediate and early neuropathology after brain injury in juvenile rats. *Journal of neuroscience research* (2014).