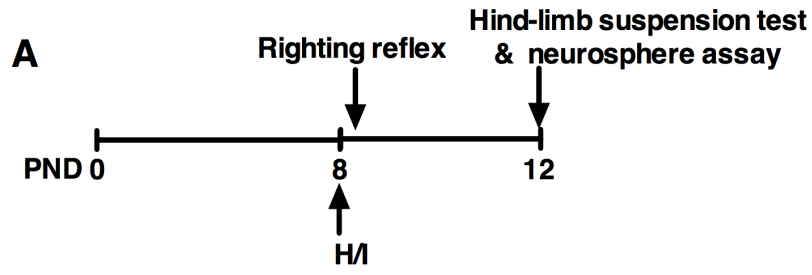


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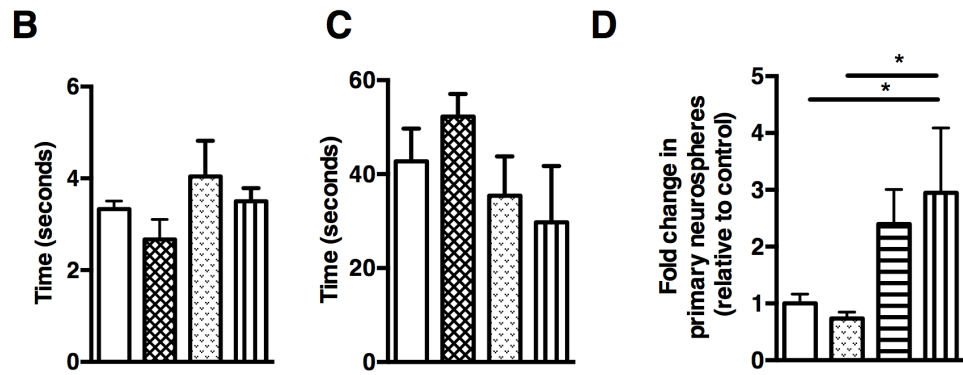
Supplemental Information

**Activating Endogenous Neural Precursor Cells Using  
Metformin Leads to Neural Repair and Functional  
Recovery in a Model of Childhood Brain Injury**

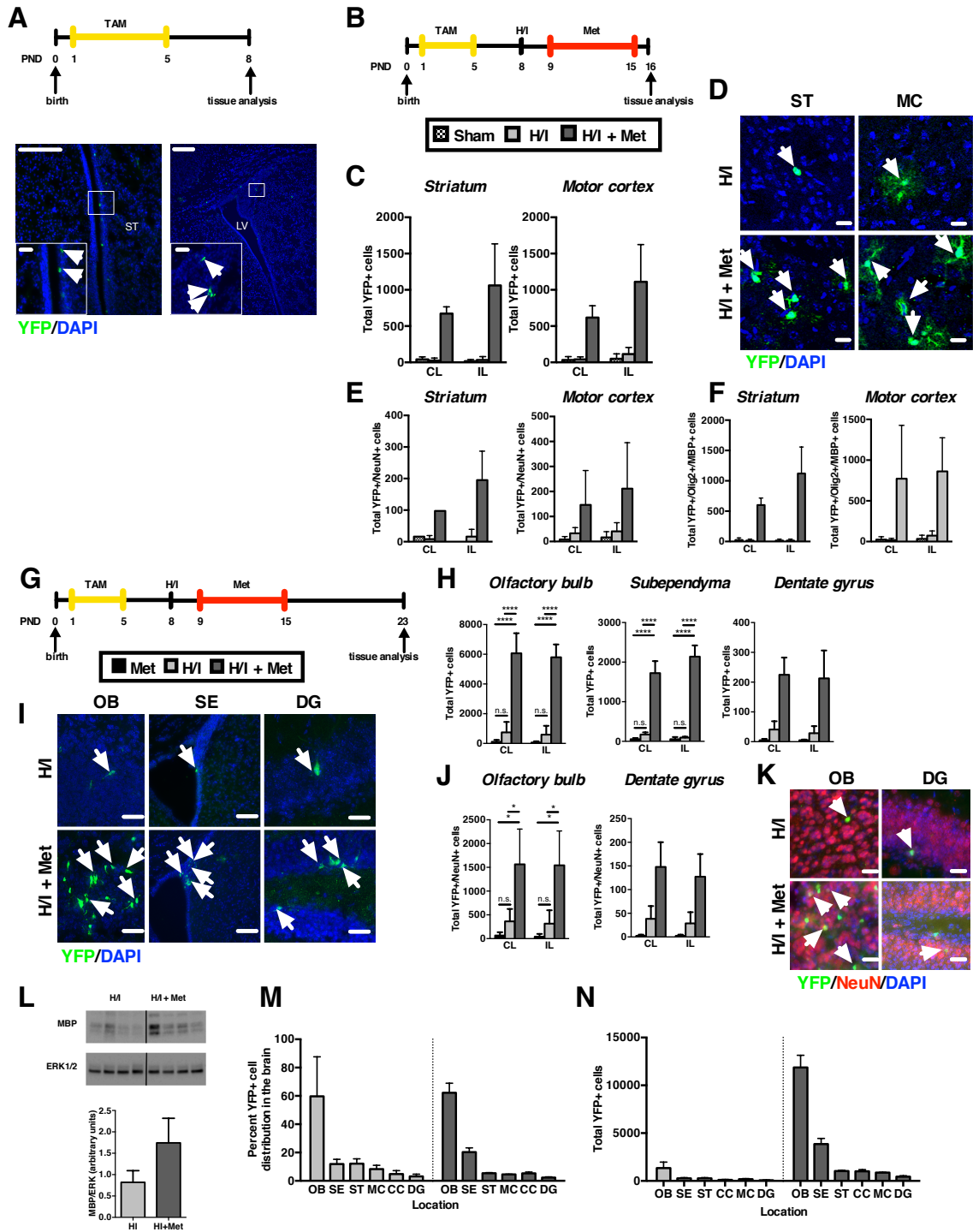
Parvati Dadwal, Neemat Mahmud, Laleh Sinai, Ashkan Azimi, Michael Fatt, Fredric E. Wondisford, Freda D. Miller, and Cindi M. Morshead



□ Control
▣ Anesthesia alone
▤ Hypoxia alone
▥ Ischemia (IL)
▧ Ischemia (CL)



Supplemental Figure 1



Supplemental Figure 2

## Supplemental Figure Legends

### **Figure S1. Behavioral assessment and examining endogenous NPCs in hypoxia**

**injured and ischemia injured mice. (Related to Figure 2)** (A) Experiment paradigm for H/I model. (B) Righting reflex was tested on mice one hour after injury ( $n \geq 4$  mice/group, one-way ANOVA) (C) Four days after H/I, the hind-limb suspension test was performed ( $n \geq 4$  mice/group, one-way ANOVA). (D) Pups that received ischemia alone had an increase in the numbers of neurospheres on the ipsilateral (ischemic) hemisphere when compared to uninjured controls and hypoxic mice following four days of recovery ( $n \geq 4$  mice/group, one-way ANOVA). Error bars represent mean  $\pm$  s.e.m.  $*P < 0.05$

**Figure S2. Lineage tracing *in vivo*. (Related to Figure 3)** (A) *Nestin-creER<sup>T2</sup>/R26R-YFP* dams received tamoxifen in food chow and pups obtained tamoxifen through the mother's milk prior to H/I. At PND 8, the majority of the YFP+ cells (nestin expressing) were found in the lining of the lateral ventricles, with a few cells in the parenchyma, including the olfactory bulb and rostral migratory stream, in tamoxifen fed pups. Scale bars = 100  $\mu$ m and insets are 20  $\mu$ m (B) Experimental paradigm immediately after metformin treatment (PND 16). (C) Quantification of YFP+ cells in striatum and motor cortex ( $n = 2$  mice/group) at PND 16. Sham-operated controls underwent anesthesia and incision but no artery occlusion or hypoxia. (D) SE-derived YFP+ cells (green) and Hoechst (blue). Quantification for (E) YFP+/NeuN+ neurons and (F) YFP+/MBP+ and YFP+/Olig2+ in striatum and motor cortex at PND 16 ( $n = 2$  mice/group). (G) Experimental paradigm at the time of functional recovery (PND 23). (H) Quantification

of YFP+ cells in granule cell layer of the OB ( $n \geq 3$  mice/group with the exception of metformin alone where  $n=2$ , two-way ANOVA), subependyma ( $n \geq 3$  mice/group, two-way ANOVA) and dentate gyrus ( $n \geq 3$  mice/group, two-way ANOVA), 2 weeks post H/I. (I) Coronal sections were immunostained for SE-derived YFP+ cells (green) and Hoechst (blue). (J) Quantification of YFP+ cells that were co-labeled with NeuN indicating newborn neurons in olfactory bulb ( $n \geq 3$  mice/group with the exception of metformin alone where  $n=2$ , two-way ANOVA) and dentate gyrus ( $n \geq 3$  mice/group, two-way ANOVA). (K) Coronal sections were immunostained for SE-derived YFP+ cells (green), NeuN (red) and Hoechst (blue). (L) Western blot (*top*) and densitometric analysis (*bottom*) of MBP expression in the ipsilateral motor cortex on PND 23 following either H/I or H/I with metformin treatment (H/I + Met), as outlined in G ( $n = 4$  mice/group). (M) Distribution of SE-derived precursors at PND 23. Graph depicts, the relative YFP+ cell distribution in the brain as a percentage of total YFP+ cells. Metformin treatment did not change the location of where newborn cells are found in the brain. IL and CL hemisphere were pooled together ( $n \geq 4$  mice/group/area with the exception of OB and SE counts for H/I+ Met where  $n = 3$ ; two-way ANOVA). (N) Total YFP+ cells labeled at PND 23. IL and CL hemisphere were pooled together ( $n \geq 4$  mice/group/area with the exception of OB and SE counts for H/I+ Met where  $n = 3$ ). Arrows denote labeled cells. Scale bars = 20  $\mu\text{m}$ . Met = metformin, CL = contralateral hemisphere, IL = ipsilateral hemisphere, OB = olfactory bulb, SE = subependyma, DG = dentate gyrus, ST = striatum, MC = motor cortex, CC = corpus callosum. Error bars represent mean  $\pm$  s.e.m. with the exception of (H, olfactory bulb) and (J, olfactory bulb) where error bars represent mean  $\pm$  s.d. \* $P < 0.05$ , \*\*\*\* $P < 0.0001$

## **Supplemental Experimental Procedures**

**Mice.** C57BL/6 pregnant mice were purchased from Charles River. In a pathogen free facility, a dam was housed in a single cage with a red rectangular/dome house, one nestlet, with food and water available *ad libitum*. Neonates at PND 8 and PND 12 (both males and females) were sacrificed and used for the neurosphere assay. CBPS436A mice, a kind gift from Dr. Miller's lab, were genotyped and used for the neurosphere assay at PND 8 and PND 12 (both males and females). For lineage tracing experiments, *Nestin-CreER<sup>T2</sup>/R26R-YFP* mice were bred in animal facility and offspring genotyped. At time of birth, *Nestin-CreER<sup>T2</sup>/R26R-YFP* neonates (both males and females) were cross-fostered to a CD1 lactating mother (Charles River) and provided with a red rectangular/dome house and one nestlet per cage. Foster mothers received tamoxifen in food chow (approximately 5 pellets) and this was delivered to *Nestin-CreER<sup>T2</sup>/R26R-YFP* neonates (through the mother's milk) to induce Cre recombination from PND1 until PND 5. Briefly, tamoxifen (Sigma-Aldrich, St. Louis, MO) at a concentration of 250 mg/kg was mixed with high fat food (2019, Harlan), 0.5% sucrose and green food coloring and manufactured as pellets by Harlan laboratories. Food chow was then changed to high fat diet for the remainder of the experiment. The brains of these animals were perfused on PND 8, 16 and PND 23. All mice were kept on a 12-hour day/night cycle. All procedures were performed in accordance with institutional guidelines and approved by the Experimental Animal Committee at the University of Toronto.

**Hypoxia/Ischemia injury model.** Briefly, PND 8 pups (2 – 4 g) were anesthetized (isoflurane, 5% induction and 1.5% maintenance) and under a surgical microscope (Möller Hi-R 900) the left common carotid artery was ligated using sutures (Sofsilks, VS889). Bupivacaine (0.25 mg/mL) was administered once intra-operation for local

analgesia. The duration of anesthesia was <15 minutes. Following surgical procedure, neonates were kept under the heat lamp for 15 minutes and then returned to the dam for 1-2 hours of recovery. Neonates were then placed in a plastic container, submerged in a water bath and subjected to hypoxia (8% O<sub>2</sub> in N<sub>2</sub>) for 1 hour at a temperature of 36 °C. Pre-established endpoints for exclusion from the study were mice that demonstrated hunched or abnormal posture, lethargy or reluctance to move, hypothermia, persistent anorexia and dehydration. Injured mice demonstrating typical H/I lesions (i.e. striatal atrophy and enlargement of the ipsilateral ventricle) at PND 23 time point were included in analysis, as described previously (Plane et al., 2004).

**Metformin administration.** For the neurosphere assay, a stock solution of metformin (0.167 mg/mL, Sigma-Aldrich, D150959) was made by dissolving in serum free media (SFM) and subsequently adding to various concentrations in culture. In our *in vivo* studies, a daily dose of metformin at a concentration of 20 mg/kg/day was dissolved in 1x PBS and delivered by implanting a subcutaneous micro-osmotic one week pump (Alzet, 10070) to lactating mothers. Briefly, mothers were anesthetized using isoflurane and injected with ketoprofen i.p. (0.1 mg/10 g body weight) for analgesia. A small incision was made and the pump was implanted on the side and sutured (Sofsilks, 2613-SS683G). Mothers then recovered under the heat lamp and were returned to their litter. For the PBS/metformin injection studies, a daily record of the weights was kept. Pups were injected subcutaneously, once daily from PND 9-15 using a 30G ½ needle with a maximum volume of 50 µL.

**Neurosphere assay.** Neural stem cells were isolated by dissecting the neonatal SE of PND 8 and PND 12 animals. Briefly, tissue was digested with enzymes (1.33 mg/mL

trypsin, 0.67 mg/mL hyaluronidase, and 0.2 mg/mL kynurenic acid, (Sigma- Aldrich) for 10 min at 37°C. Enzyme activity was inhibited with trypsin inhibitor (0.67mg/mL, Roche Diagnostics). Tissue was mechanically dissociated into a single-cell suspension and plated at clonal density (5 cells/ $\mu$ l)(Coles-Takabe et al., 2008) in 24-well polystyrene plates (VWR Scientific) with serum-free medium (SFM) containing 1% penicillin/streptomycin (Invitrogen) and supplemented with epidermal growth factor (20 ng/mL, Sigma-Aldrich), basic fibroblast growth factor (10 ng/mL, Sigma-Aldrich), heparin (7.35 ng/mL, Sigma-Aldrich). Neurospheres  $\geq 80 \mu$ m in diameter were quantified 7 days later. For differentiation assays, individual neurospheres were collected from each condition (approximately 150-200  $\mu$ m), gently triturated, and plated onto laminin (L2020, Sigma-Aldrich) coated 48 well plates (VWR Scientific) in the presence of 10% fetal bovine serum (10082139, Invitrogen) and in the presence and absence of metformin for 7 days.

**Cortical assay.** The SE was carefully dissected and discarded to ensure no contaminating SE-derived neurospheres were grown in culture. Portions of the striatum and motor cortex were then dissected and processed as described above. Neurospheres were counted on day 7.

**Immunofluorescence and antibodies.** Animals were sacrificed with an overdose of sodium pentobarbital, perfused with 4% paraformaldehyde at different time points (PND 8, 16 and 23) and cryoprotected in 20% sucrose overnight at 4°C. Coronal, cryostat sections (20  $\mu$ m) were mounted on Superfrost Plus slides and stained. Briefly, brain sections were rehydrated for 5 minutes and permeabilized using 0.3% triton in PBS for 20 minutes, rinsed, and then blocked with 1% BSA containing 0.3% triton in PBS at



room temperature. Brain sections were incubated with primary antibodies overnight at 4°C in 1% BSA with 0.3% triton in PBS. The next day slides were washed and incubated with an appropriate secondary antibody for 2 h at room temperature, washed and mounted with DAPI mounting media. The primary antibodies used were O4 (1:1000 mouse monoclonal IgM, R&D Systems, MAB1326),  $\beta$ III-tubulin (1:1000, rabbit polyclonal, Covance, PRB-435P-100), GFP (1:500, chicken IgY, Aves Lab, GFP-1020), Olig2 (1:200, rabbit polyclonal, Millipore, AB9610), MBP (1:50, rat monoclonal, Abcam, AB7349), NeuN (1:100, mouse monoclonal IgG, Millipore, MAB377), NeuN (1:500, rabbit monoclonal IgG, Cell Signaling, 12934). The secondary antibodies used were AlexaFluor 488, AlexaFluor 568, AlexaFluor 594, AlexaFluor 647 conjugated goat anti-mouse IgM, goat anti-rabbit IgG, goat anti-chicken, donkey anti-rat IgG, goat anti-mouse IgG (Invitrogen).

**Microscopy and cell counting.** A Zeiss Observer D1 inverted microscope was used to visualize immunofluorescence using YFP (500 nm excitation; 535/50 emission filter), Texas Red (560 nm excitation; 630/75 emission filter), Cy5 (620 nm excitation; 700/75 emission filter) for differentiated neurospheres and tissue sections. Images were acquired at 20x objective using Axio Vision (version 4.8.1.0). For differentiation of neurospheres, cells were counted within the field of view in five areas [top, bottom, left side, right side (all equal distance from the edge of the well) and center]. Percentages of differentiated cells were calculated to the total numbers of cells in each field of view and plotted as fold increase. In tissue sections, quantification was performed for the ipsilateral and contralateral hemispheres for all analysis. The total number of cells per section was counted in every 5<sup>th</sup> section and multiplied by the total number of sections per region.

Counts for the corpus callosum, motor cortex, striatum and SE began were as described previously (Morshead et al., 1998). The granular cell layer in the OB and the DG in the hippocampus were also counted.

**Western blot.** The motor cortex was dissected from H/I and H/I + metformin treated brains at PND 23. This region was then lysed in Radioimmunoprecipitation assay (RIPA) buffer supplemented with 2 µg/ml Aprotinin, 10 µg/ml Leupeptin, 1mM PMSF, 10mM Sodium Fluoride, and 1mM Sodium Orthovanadate. Equal amounts of protein (15 µg) per sample were run on Mini-PROTEAN TGX Gels (4-20%, BioRad, Hercules, CA), transferred to 0.2-µm nitrocellulose membranes, blocked for 30 min with 5% non-fat dry milk, 0.1% Tween-20 in TBS, and incubated overnight at 4 °C with primary antibody in blocking buffer. After washing several times with 0.1% Tween-20 in TBS, membranes were incubated for 1 h at room temperature with the following secondary antibodies: goat anti-rat (1:5000, Life Technologies) and goat anti-rabbit (1:10 000, Life Technologies). After several more washes, detection was performed using ECL chemiluminescence reagent (GE Healthcare, Buckinghamshire, UK). The following primary antibodies were used: rat anti-MBP (MCA409S, 1:1000, ABD Serotec, Ralieggh, NC) and rabbit anti-ERK1 (K-23, 1:10 000, Santa Cruz Biotechnology, Santa Cruz, CA).

### **Behavioral testing**

**Righting reflex.** One hour after H/I, PND 8 mice were tested on the righting reflex. Animals were placed on supine position and the time to turn into prone position was measured as described previously (Ten et al., 2003). Pups were allowed three attempts to perform the reflex for up-to 60 seconds per attempt and the mean time was recorded.

**Hind-limb suspension test.** Four days post H/I, PND 12 mice were tested on the hind-limb suspension test as described previously (El-Khodori et al., 2008). Briefly, mice are placed head-down, hanging by their hind limbs on the edge of a plastic 50 mL falcon tube with tissue at the bottom to protect the head of the pups upon their fall into the tube. The latency to fall from the edge of the tube was assayed over a 60 s period.

**Cylinder rearing test.** To evaluate functional asymmetry resulting from unilateral brain lesion and consequent hemiplegia, the cylinder test was performed at 22 days of age. Mice were individually placed in a Plexiglas transparent cylinder (7.5 cm x 15 cm height) and video recorded for 4 minutes or until they reached 10 touches per paw. Initial forepaw (left/right/both) preference of weight-bearing touches during a rear was recorded. The relative proportion of left (ipsilateral) forepaw contacts was calculated as:  $(\text{left} - \text{right}) / (\text{right} + \text{left} + \text{both}) \times 100$ . Sham animals showed no preference for either right or left fore-paw initiation but H/I injured animals demonstrated a preference in using the left forepaw depending on the severity of the insult.

**Statistical analysis.** Data were analyzed using Prism Software (Graphpad, Version 6). An unpaired Student's t-test was used for two group comparisons. A one-way ANOVA was used for multiple group comparisons followed by Bonferroni's post-hoc test. For analysis comparing contralateral and ipsilateral hemispheres a two-way ANOVA was performed followed by Tukey's Multiple comparisons test. A statistically significant level was defined as  $P < 0.05$ . Error bars are reported as mean  $\pm$  s.e.m unless stated otherwise.

## **Supplemental References**

Coles-Takabe, B.L., Brain, I., Purpura, K.A., Karpowicz, P., Zandstra, P.W., Morshead, C.M., and van der Kooy, D. (2008). Don't look: growing clonal versus nonclonal neural stem cell colonies. *Stem Cells* 26, 2938–2944.

Morshead, C.M., Craig, C.G., and van der Kooy, D. (1998). In vivo clonal analyses reveal the properties of endogenous neural stem cell proliferation in the adult mammalian forebrain. *Development* 125, 2251–2261.