



Nesting Environment Provides Sex-Specific Neuroprotection in a Rat Model of Neonatal Hypoxic-Ischemic Injury

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Hypoxic-ischemic (HI) encephalopathy is a devastating injury that occurs when the fetal brain is deprived of oxygen and blood to a degree that may lead to neurological damage, seizing and cerebral palsy. In rodents, early environmental enrichment that promotes maternal care-taking behavior (mCTB) can improve neurobehavioral outcomes and protect against neurological decline. We hypothesized that an enhanced nesting environment would improve mCTB as measured by pup weight gain, and support greater HI recovery in developing rats. Pregnant dams (E15-16) were introduced to either control Standard Facility (SF) housing or closed nestbox (CN) conditions and maintained in larger cages through pup weaning. On postnatal day (PND) 7, male and female Long-Evans rat pups ($N = 73$) were randomly sorted into one of two surgical conditions: control and HI. HI pups received isoflurane anesthesia and right carotid artery ligation, a 2-h rest followed by 90 min exposure to a moist hypoxic (92% N, 8% O₂) chamber. Pups (PND 8) were weighed daily, and tested on the Morris Water Maze (MWM) task (PND 35-50). Results demonstrate significant differences afforded to male and female pups based on weight measure, where CN-rearing modifies pre-weaning adolescent weights in females and increases post-weaning weights in males and females by an average of 10 g. Following successful MWM training and acquisition (PND 35-37), both male and female CN-raised animals demonstrated faster latency to find the hidden platform (HP) during HP trials (PND 38-42) and appeared to freely explore the MWM pool during an additional probe trial (PND 43). Moreover, after sacrifice (PND 60), CN rearing created sex-specific alterations in brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF) immunopositive cell staining of the dorsomedial striatum and CA1 of the hippocampus. CN-rearing afforded HI males higher BDNF levels in the striatum and produced greater GDNF levels in the hippocampus of HI-injured females. These results suggest that early life environmental enrichment positively modifies nesting environment, increases weight gain, as well as spatial learning and memory in a sex-specific directionality. Our findings also implicate correlative changes in corticolimbic neurotrophin protein levels in the CN-reared animals that may contribute to these benefits.

Keywords: Rice-Vanucci P7 HI model, Long Evans rats, environmental enrichment, Morris water maze, neurogenesis, neonatal hypoxic ischemic injury, hippocampus

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Received: 23 May 2018

Accepted: 03 September 2018

Published: 02 October 2018

Citation:

Mason B, Rollins LG, Asumadu E,
Cange C, Walton N and
Donaldson ST (2018) Nesting
Environment Provides Sex-Specific
Neuroprotection in a Rat Model of
Neonatal Hypoxic-Ischemic Injury.
Front. Behav. Neurosci. 12:221.
doi: 10.3389/fnbeh.2018.00221

INTRODUCTION

Neonatal Hypoxic-ischemic encephalopathy (HIE) is a serious neurological injury resulting from oxygen and glucose deprivation during birth. Current estimates predict that 1–2 of every 1,000 full term neonates develop HIE (Volpe, 2009). Surviving infants may go on to develop long-term disabilities, including reoccurring seizures (Silverstein and Jensen, 2007; Björkman et al., 2010), motor impairments (Martinez-Biarge et al., 2011) and attentional and learning deficits (Lou, 1996; Perez et al., 2013).

Cognitive deficits are a hallmark of neonatal HIE, with some studies estimating 70% of adolescent HIE survivors demonstrate some degree of cognitive difficulty and 50% require special educational services (Lindström et al., 2006). Using the Rice-Vannucci method (Vannucci et al., 1999), the most prevalent rodent model of neonatal HIE, the injury is modeled in rats at postnatal day (PND) 7 by ligation of the right common carotid artery followed by oxygen deprivation. This model simulates both morphometric and functional sequelae of term neonatal hypoxic ischemic (HI) injury, including spatial and working memory deficits (Smith et al., 1991; Balduini et al., 2000; de Paula et al., 2009). The bilateral hippocampus and frontocorticolimbic system are highly vulnerable to HI injury, with studies suggesting that these areas are damaged in an estimated 90% of HI models and infarcted in another 56% of subjects (Rice et al., 1981). As a result of HI, early cell death occurs in the neocortex, and a second wave of cell death occurs from 12 h to 24 h after oxygen deprivation, targeting subcortical structures including the hippocampus, striatum and thalamus (Azzarelli et al., 1996; Northington et al., 2007).

Early environmental enrichment with large cages, social peers and sensorimotor stimulation has been shown to somewhat improve the functional memory outcomes of adolescent but not adult female rats with neonatal HI injury, without modifying the hippocampal or striatal damage (Pereira et al., 2008). Maternal care-taking behavior (mCTB) provided by rat dams in the first 2 weeks of life has also been shown to alter the stress response of offspring through direct modifications of the hypothalamic-pituitary-adrenal axis (Meaney, 2001) and neurotrophin levels in the central amygdala (Berman et al., 2014). During the pre-weaning period, greater licking and grooming, and arched back nursing result in the regulation of emotional as well as neural systems (Caldji et al., 1998; Roth and Sweatt, 2011). Pre-weaning environmental enrichment has been successfully applied to promote typical development in animal models (Koo et al., 2003; Szabadfi et al., 2009) and to improve learning and memory through alterations in hippocampal proteins and synapse formation (Venable et al., 1989; Bredy et al., 2003).

Early handling (Chou et al., 2001) and environmental enrichment during early life (Pereira et al., 2008) are neuroprotective in HI injured animals working via morphological changes to dendritic spine density and synaptic branching (Rojas et al., 2013; Zhao et al., 2013). In addition, this neuroprotection involves changes in the expression of

neurotrophins like brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF; Lin et al., 1993; Skaper, 2012). Treatment with exogenous BDNF reduces HI-induced spatial deficits in rats trained on a Morris Water Maze (MWM) task (Almli et al., 2000). GDNF is a neurotrophin present in dopaminergic neurons that has been characterized as a marker of neuronal survival (Wang et al., 2004; Bakshi et al., 2006). Collectively, BDNF and GDNF are thought to act as endogenous neuroprotective agents (Kiprianova et al., 1999; Allen et al., 2013; Chen et al., 2013). The outcome of children who have experienced a brain injury has been correlated to levels of these two proteins, such that decreases in BDNF and GDNF indicate greater working memory deficits (Chiaretti et al., 2003). These findings indicate that environmental interventions may be a potential avenue for prevention or treatment of the dramatic cognitive deficits that often follow HI. Environmental enrichment has been shown to benefit individuals suffering with neurodegenerative diseases such as Huntington's, Parkinson's, or Alzheimer's diseases, through the utilization of the brain's plastic nature (for review, see Laviola et al., 2008). Although environmental enrichment has been well studied in terms of neural plasticity and recovery in many models of neural injury and disease there are few studies that investigate the effects of the early environment on developmental trajectory after HI, and fewer still that seek to understand the interplay between the quality of early environment and cognitive sequelae.

This study was designed to address the complexity of functional outcomes, while simultaneously examining potential proteomic changes as they reflect differences in recovery. We introduced rat dams to a closed nestbox (CN) condition as a protected environment as compared to the standard facility (SF) conditions, hypothesizing that the sheltered environment would provide positive enrichment for dams and pups during the pre-weaning period (Mychasiuk et al., 2012). We further hypothesized that the CN environment would improve phenotypic and proteomic features of HI in male and female pups in a sex-dependent manner. This potential benefit was theorized based on several sex-specific effects observed in environmental enrichment, pup weight gain and HI injury. The pattern of damage following HIE is thought to be different between males and females, with females displaying significantly less injury than males in physical and cognitive domains (Zhu et al., 2006; Hill and Fitch, 2012; Smith et al., 2014). Sex differences may also determine the effectiveness of treatment for HIE, to which end pre-weaning environmental modification may be useful (Fan et al., 2011; Nie et al., 2016).

MATERIALS AND METHODS

Animals

All procedures utilized in this experiment were approved by the University of Massachusetts Boston Institutional Animal Care and Use Committee and closely followed applicable portions of the Animal Welfare Act and the U.S. Department of Health and Human Services' "Guide for the Care and Use of Laboratory Animals." Pregnant Long Evans rats ($N = 7$; embryonic day 10)



FIGURE 1 | Picture of Closed Nestbox (CN) inside standard cage with bedding. CN was added to the dam's cage at embryonic day (ED) 10 (E10-15) and remained until weaning on postnatal day (PND) 21.

were purchased from Charles River (Wilmington, VA, USA) and upon arrival, were singly housed and randomly assigned to SF or CN (**Figure 1**) conditions in the animal vivarium in a light- and temperature-controlled environment at 22°C with lights on at 07:00 h and off at 19:00 h. Dams were kept in a Plexiglas cage with dimensions 31.75 cm × 41.7 cm × 17.8 cm. CN conditions were similar, but also included a small (7.75" L × 6" W × 4.5" H), opaque plastic shelter that contained one entrance placed in the center of the Plexiglas cage. Plastic shelters were inserted in the nesting environment of pregnant dams on embryonic day 10 and left undisturbed until time of delivery. After the dams gave birth, litters were sexed and humanely culled to 10–12 pups with even distribution of males and females in order to limit dramatic variations in maternal care (Champagne et al., 2003). Litters were also relatively stable in male to female ratio, never exceeding more than 1:3 females to males. Individual pups within litters were excluded from the research paradigm if they failed to reach a weight standard of 11 g at PND7 during weight check-in during randomization. Ten litters with a total of 73 pups were randomized within litters into one of two surgical conditions: control ($n = 17$ females, $n = 18$ males) and hypoxia ischemia ($n = 19$ females, $n = 19$ males; **Table 1**). The study timeline is depicted in **Figure 2**.

Surgery

On PND 7, male and female pups in each housing condition were randomly sorted within litters into one of two surgical conditions: HI and control groups. Animals in the HI condition underwent carotid artery ligation and hypoxia according to the Levine model of HIE, using the Rice-Vannucci modification

TABLE 1 | Total number of subjects, separated by sex and housing condition.

Subject Grouping	Standard Facility		Closed Nestbox (CN)	
	Male	Female	Male	Female
Control	$n = 11$	$n = 11$	$n = 7$	$n = 6$
Hypoxia-Ischemia	$n = 7$	$n = 9$	$n = 12$	$n = 10$
Total	18	20	19	16

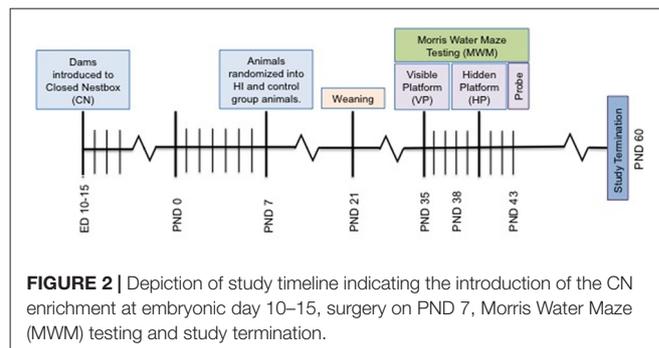


FIGURE 2 | Depiction of study timeline indicating the introduction of the CN enrichment at embryonic day 10–15, surgery on PND 7, Morris Water Maze (MWM) testing and study termination.

(Rice et al., 1981). Pups were anesthetized with 3%–5% isoflurane and maintained on 1%–2% for the duration of the surgical procedure. We performed double ligation and severed the right common carotid artery followed by a 2 h rest period with the dam. HI pups were then removed from the litter and placed into a plastic container and supplied exclusively with 8% Oxygen and 92% Nitrogen for 90 min and then returned to their dams. Within this chamber, temperature was kept at a steady 36°C to mimic normative nesting conditions (Mortola and Dotta, 1992; Cameron et al., 2000). Animals in the control group remained in their home cage and were only removed from their dams to be marked for identification. In the current study, the mortality rate following right carotid artery ligation and post-operative 90-min hypoxia was 5% (two out of 40 total animals; one male and one female). This mortality rate is well within reported standards following HI induction in animals (Nakajima et al., 2000; Demers et al., 2005; Pereira et al., 2008). On PND 21, animals were weaned and housed by sex with littermates, with 2–3 animals per cage.

Body Weight

Animals were weighed daily (grams) from PND 8–21 and every 3–4 days from PND 22–60. From PND 8–14, animals were weighed on a small plastic Sterilin weighboat (ThermoFischer, Cambridge, MA, USA), as appropriate for their size and physiological condition. Upon their maturation as denoted by the full appearance of fur at PND 15, animals were weighed in a closed-lid metal weight cage zeroed on an electronic scale (Basic Electronic Scale, Mettler Toledo International, Inc., Billerica, MA, USA).

Morris Water Maze—Visible Platform

All testing for the MWM trials took place within a pool (120.65 cm in diameter, 176.25 cm in height at its deepest point) filled with water heated to room temperature (± 25 – 27°C) and colored with non-toxic milk powder. Around the pool, key shapes were pasted for internal orientation to the surrounding directions of North (N), West (W), East (E) and South (S) as reference cues for the rats.

PND 35 was the first day of testing, selected based on previous research suggesting that rats are able to perform with mature cognition by PND 30 (Ikeda et al., 2001; Arteni et al., 2003). On the initial test day, animals were placed on the

fixed, visible platform (VP) that was marked by bright green tape in the SE corner of the pool for 15 s, and were allowed to swim freely for up to 20 s. Rats were tested in four trials each day from PND 35–42 and were given up to 60 s to find the VP on each trial. If rats did not find the platform within 60 s they were guided to the platform and were left on the platform for 10 s to familiarize them with its location. Each rat was dried and warmed between each trial and given a 10 min inter-trial interval throughout the four-trial testing period. The latency to reach the platform was recorded from trial to trial. Tests were conducted in a low light setting, with the experimenter standing at a distance out of view of the animals. Ethovision XT11 software and a digital camera (Microsoft Webcam, 12.0; Microsoft, Redmond, Washington, USA) were used to video record information for 78 of the 104 subjects.

For VP training, rats were tested from PND 35–38, and were dropped off from the South, North, East and West for each of the 3 days. The platform remained fixed in the SE corner. For the hidden platform (HP) trials, the platform was made translucent and remained fixed in the SW corner of the pool 0.5 inches under the surface of the opaque water. Each day, animals were placed in the pool in the directions of N, N, NW and NW for a total of four trials. Animals were tested in HP from PND38–42.

After completing VP and HP testing, we conducted probe trials (PND43) and captured video information for these trials with a subset of animals. Probe trials consisted of a 60 s swimming period for each animal, without a platform. For 60 s animals were allowed to freely swim around the pool and swimming behavior was coded for proximity to the most recent location of the platform.

Brain Morphology and Immunofluorescence

Animals were sacrificed at PND60 with the aid of a restraint cone (Harvard Apparatus, Holliston, MA, USA) and live decapitation. Brains were extracted and snap frozen with 2-methylbutane chilled on dry ice, and then stored at -80°C until the time of post-fixation and cryoprotection (increasing 5%–20% sucrose + 4% paraformaldehyde).

Following this, brain tissues were microsectioned at 20–25 μm (Leica CM 3050S; Leica Biosystems, Buffalo Grove, IL, USA) taken at Bregma 4.70–5.60 mm for the striatum (Paxinos and Watson, 2004) and Bregma -3.80 to -0.16 mm for the hippocampus thaw-mounted and placed directly on adhesive slides (SuperFrost Plus; ThermoFischer, Cambridge, MA, USA). Microsections were randomly divided into Nissl, control and positive immunofluorescent groups to include 5–6 animals per treatment and housing group. For Nissl stains, first we removed the fat (95% EtOH, 15 min) then rehydrated (70% and 50% EtOH, 5 min each). Next we ran the sections through dH_2O washes (1–2 min) followed by immersion in Cresyl Violet (Sigma, 0.1% (2–4 min)); this was again followed by dH_2O wash (1 min). After, we returned to dehydration steps (50% EtOH (1 min), 75% acid EtOH, 95% and 100% EtOH (2 min) and the clearing agent, Histoclear (National Diagnostics, Atlanta, GA, USA). In models of HI injury, necrosis and apoptosis

(Northington et al., 2007) are assessed close in time to the injury (see also Cai et al., 2008). Given that we sacrificed the animals \sim PND60, nearly 2 months after the HI injury, we instead think that information about the size of the infarct and relative damage to the hippocampus is important for the data presented in this manuscript (Ikeda et al., 2001).

After, sections were fixed with 4% paraformaldehyde for 30 min at a time and, repeatedly rinsed with phosphate buffered saline (PBS) for 10 min per wash. After five washes, slides were soaked in bovine serum albumin for 30 min, and then were covered with the primary antibodies BDNF (1:1,000; ThermoFischer, Cambridge, MA, USA) and GDNF (1:500, ThermoFischer, Cambridge, MA, USA) overnight. The next day, slides were recovered from the humidity chamber and washed with PBS again for 10 min per wash. Following this, slides were coated with two fluorophore conjugated secondary antibodies for approximately 1 h: goat anti-rabbit AlexaFluor 488 (IgG H&L) and goat anti-mouse AlexaFluor 647 (IgG H&L) at a concentration of 1:600 (Abcam, Cambridge, MA, USA and ThermoFischer, Cambridge, MA, USA). Excess antibody was washed with PBS three times, with 10 min per wash. Fluorescence was enhanced with anti-fade reagent (Prolong anti-fade reagent with 6-diamidino-2-phenylindole (DAPI); ThermoFischer, Cambridge, MA, USA), and slides were coverslipped. To preserve apparent fluorophores, slides were coated with clear nail polish around the edges to prevent oxidation. Slides were imaged within the week, with up to six images taken for each bilateral brain area of interest. Fluorescent images were converted from native high resolution .CZI to .JPEG format and dropped in ImageJ (National Institutes of Health, online). A 500×500 pixel yellow-colored region of interest (ROI; 2.23 μm per pixel) was manually drawn on the center of each image, and positional consistency was maintained through the use of an ImageJ placement macro (six images per subject, two merges in total overlap; 12 images if images were found to be ambiguous).

ImageJ was used to set color thresholds to maintain consistency for each fluorophore type and were subsequently processed using ImageJ to avoid experimenter bias and to increase objectivity. Initial GDNF counts proved inconsistent and variable across groups, measurement was altered to suit the diversity of the stain-type. Measurement analysis of GDNF fluorescent intensity level was restricted to a 500×500 pixel (2.23 μm per pixel) square. Circularity and overall size of GDNF-tagged cells were relatively unrestricted, as the main factor analyzed was mean brightness of areas contained within centered ROIs.

Data Analysis

All data were coded and interpreted with the use of SPSS 22.0 and 23.0 (Windows 10 and Mac 6.1 compatible versions). Body weight was analyzed using a Generalized Linear Mixed Model (GLMM) with repeated measures, with Housing and Surgical conditions. Each analysis was done separately for males and females. MWM trials were analyzed using GLMM with repeated measures. The latency to reach the platform was assessed according to Housing Condition (SF or CN) and Surgical

Condition (Control or HI) across the four trials of each testing day. Each analysis was done separately for males and females.

For the probe trial, collected data were analyzed with the use of the Ethovision XT11 (Leesburg, VA, United States). The MWM area was divided into four quadrants based on location of the symbols placed around the pool (NW, NE, SW, SE) and time spent in each quadrant was analyzed by the tracking system in sec. Videos were randomly coded for each subject and then run through data analysis for the variables of Movement (gauging the swimming style of the animal from its center) and Speed (how quickly an animal moved throughout the pool) as controls based on possible physical deficits between groups (Bona et al., 1997). Swimming speed was determined as also given by the independent variables of Housing Condition, Surgical Condition. The significance was set at probability of 0.05 or less. Confidence intervals were set at 95%.

A two-way between-subjects ANOVA was performed for the factors of Surgical condition (Control or Hypoxia-Ischemia) and housing condition (SF or CN) to compare means in BDNF and DAPI levels, as well as modifications to average GDNF levels based on fluorescent intensity following fluorescent microscopy in the dorsomedial striatum and CA1 of the hippocampus of subjects. All data was tested for normality. For two of the dependent measures, striatal counts of BDNF in males and striatal DAPI levels in females, homogeneity of collected data was not met under Levine's Test. While this was not complete unexpected due to the possible variability in targeted tissue, data were adjusted to reflect normal distributions through the exclusion of BDNF striatal counts in two male subjects and through the exclusion of DAPI striatal counts in one female subject.

RESULTS

Brain Morphology

Due to the severity of the injury sustained in the SH group, there was not enough intact tissue from this group to yield data from measurements of cortical or hippocampal area that could be statistically compared in a meaningful way. However, the lack of tissue is in itself an important finding that distinguishes animals from the two housing conditions. Therefore, the results from the measurements of hippocampal area will be reported in descriptive terms.

Observations of right and left hippocampal areas revealed that all CN animals retained tissue in both right and left hippocampal areas, whereas there were no SF animals which had a visible right hippocampus (ipsilateral to the injury). For SF animals, there were either large infarcts where the right hippocampus would be in un-injured animals, or the hippocampal area was not present in tissue that was intact. Females in the SF group had some visible left hippocampus tissue (contralateral to the injury), whereas SF males did not have any visible hippocampal tissue in the left hemisphere. In CN animals, all animals had both right and left hippocampi, although each displayed a disparity between right and left hippocampi, with the right measuring smaller than the left. Measurements of the cortical area of the right and left hemispheres showed some

asymmetry between hemispheres, with the right (ipsilateral) hemisphere measuring smaller than the left (contralateral) hemisphere as expected. However, the degree of disparity appeared to differ greatly between groups. There was a dramatic disparity between right and left hemispheres for both males and females in the SF group, but comparatively little disparity in CN animals (Figure 3). This indicates that on average, regardless of sex, CN animals had less damage from infarct or atrophy in the ipsilateral hemisphere than SF animals.

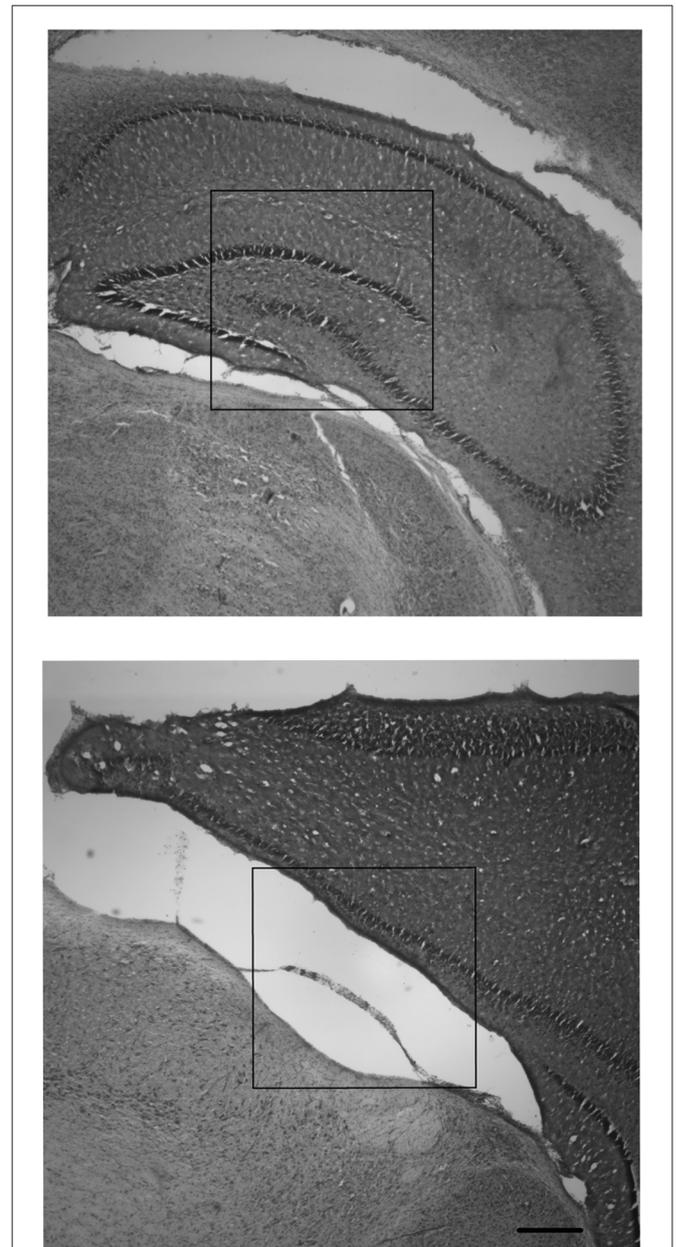


FIGURE 3 | Nissl-stained coronal hippocampal sections (ipsilateral to injury) from representative males exposed to Hypoxic-ischemic (HI) injury and reared in CN (top panel) and Standard Facility (SF; bottom). CN rearing provided enduring neuroprotection in late adolescence following neonatal HI insult. Scale bar = 200 μ m for all images (bottom right); 20 \times magnification.

Body Weight

Pre-weaning weight measurements were collected daily from PND 8 to PND 21 and adolescent weights were collected every 3–4 days from PND 24–49. There were no significant differences between surgical groups or housing conditions for males during this pre-weaning period. However, pre-weaning female weights were significantly different between surgical conditions, with HI animals weighing significantly less than control animals ($F_{(1,34.02)} = 5.80, p < 0.05$). Analysis of adolescent weights yielded significant differences between housing conditions for males and females in the HI condition ($F_{(1,32.00)} = 6.25, p < 0.01$), with CN animals weighing an average of 10 grams more than SF animals. There were no other significant differences between conditions.

Morris Water Maze Performance

Visible Platform Training

VP training allows animals to learn the water maze testing procedure, and also measures spatial memory acquisition. The latency for animals to reach the platform decreased over the 3 days of VP testing indicating successful memory acquisition across study conditions for males ($F_{(2,462)} = 103.20, p < 0.05$), and females ($F_{(2,3478)} = 71.30, p < 0.05$). For females, there were significant differences between surgical conditions in latency to reach the platform regardless of housing condition ($F_{(1,29)} = 8.02, p < 0.05$), with control animals performing significantly better than HI animals. Unexpectedly, there was no significant effect of housing condition for males or females on latency to reach the platform independent of other study factors, and there

were no significant interaction effects between study variables (Figure 4).

Hidden Platform Testing

HP testing with a stationary platform location, requires animals to develop memory strategies using spatial cues without the visual cue of the platform. A decrease in latency across days indicates functional long-term memory. There was a significant decrease in latency by day indicating functional long-term memory across treatment and surgical conditions for males ($F_{(4,800)} = 8.57, p < 0.05$), and females ($F_{(4,522)} = 6.08, p < 0.05$). For males, there was a significant effect of surgical condition on time to locate the platform, with HI animals performing significantly worse than control animals ($F_{(1,800)} = 11.35, p < 0.001$). There were no significant differences for males according to housing condition, or significant interaction effects between study factors. Females demonstrated a significant interaction effect between surgical condition and housing condition ($F_{(1,522)} = 9.37, p < 0.001$), with HI SF females performing significantly worse (12.04 s compared to HI CN (7.58 s), Control CN (6.06 s) and Control SF (6.00 s; Figure 5).

Probe Trial

Probe trials are conducted without a platform after VP training. These trials serve as control conditions to determine swimming speed and distance traveled as measures of locomotor ability, separate from memory function. Male animals displayed no significant differences between surgical condition or housing

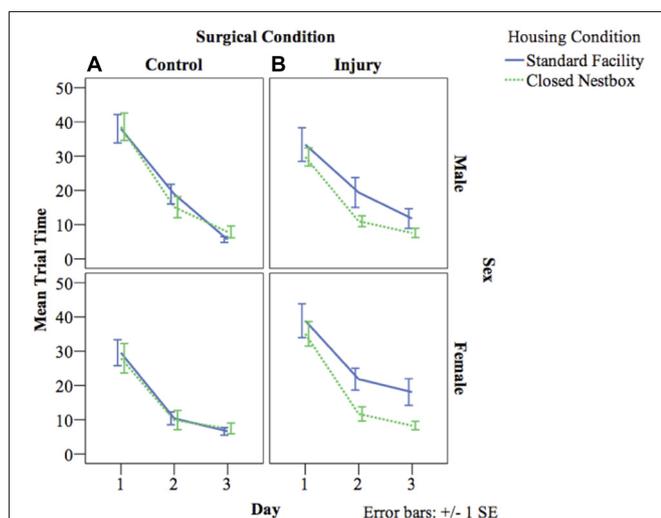


FIGURE 4 | (A,B) Mean latency by study condition in visible platform (VP) training. The following graphs mark the average latency of animals to reach the platform over the course of the four water maze trials each day for three consecutive days. The lines compare average latency for animals in the CN and SF housing conditions according to surgical condition: **(A)** Control, **(B)** HI Injury. Only main effects were observed for latency for males ($F_{(2)} = 103.20, p < 0.05$), and females ($F_{(2)} = 71.30, p < 0.05$) with both demonstrating decreased latency across the three training days indicating successful task acquisition.

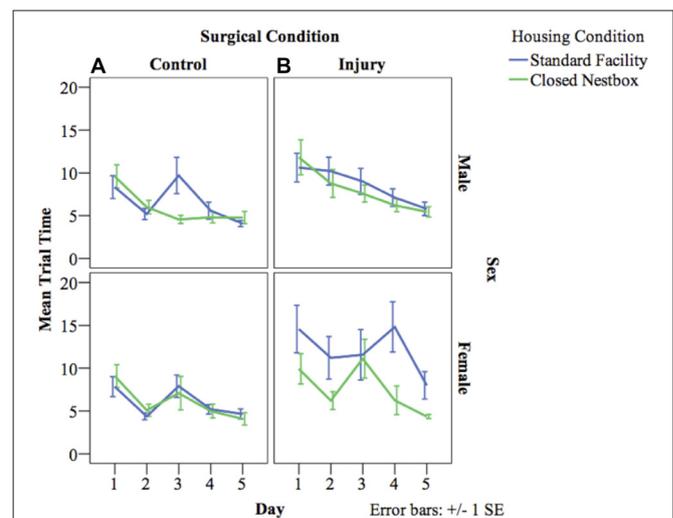
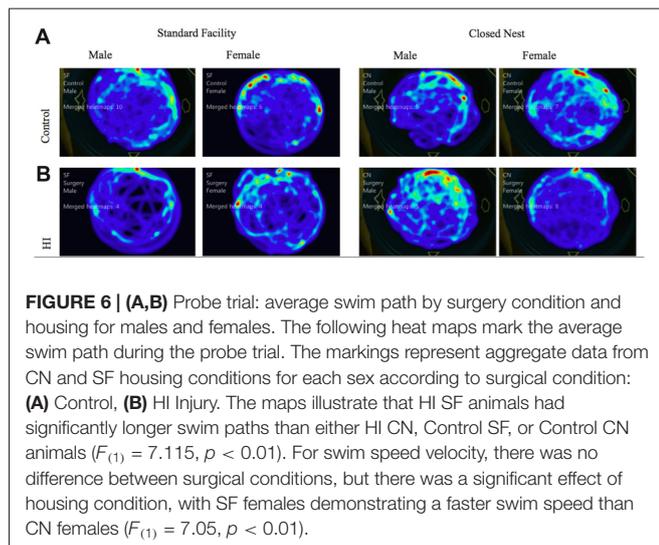


FIGURE 5 | (A,B) Mean latency by surgical and housing condition in hidden platform (HP) testing for males and females. The following graphs mark the average latency of animals to reach the HP over the course of the four trials each day for five consecutive days. The lines compare average latency for animals in the CN and SF housing conditions according to surgical condition: **(A)** Control, **(B)** HI Injury. There were significant differences between surgical conditions regardless of housing condition for males ($F_{(1)} = 11.35, p < 0.001$; HI males relative to Control males). Females demonstrated a significant interaction effect between surgical condition and housing condition ($F_{(1)} = 9.37, p < 0.001$; HI SF as compared to all other groups, same sex), with HI SF females performing significantly worse than all other groups.

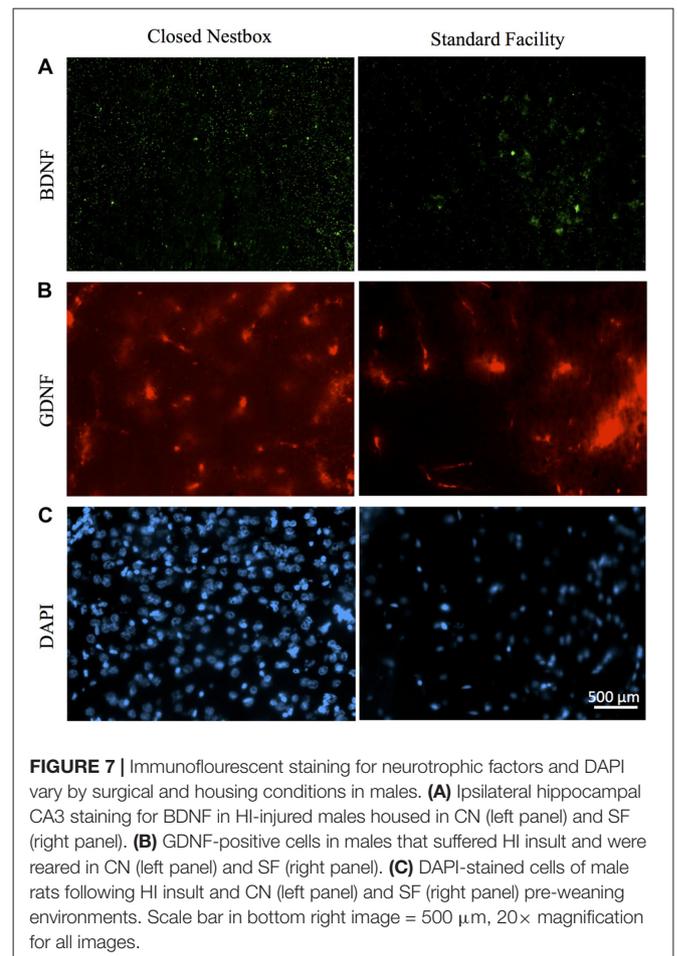


condition in either distance traveled or velocity of movement. Female animals however, displayed a significant interaction effect of housing condition and surgical condition, with HI SF animals demonstrating significantly longer swim paths than either HI CN, Control SF, or Control CN animals ($F_{(1)} = 7.115, p < 0.01$). For swim speed velocity, there was no difference between surgical conditions, but there was a significant effect of housing condition, with SF females demonstrating a faster swim speed than CN females ($F_{(1)} = 7.05, p < 0.01$; **Figure 6**). Male and female HI animals in both housing conditions demonstrated comparable swimming speed velocity and distance traveled in relation to intact animals, indicating that differences in latencies can be attributed to differential learning acquisition, search strategy, working memory and visuospatial memory ability; rather than differences in motor ability due to injury. We also have preliminary data that indicate HI injured pups show sex differences in a rope suspension task with HI-injured females reared in CN holding on to the rope longer (unpublished data). See **Figure 6** for representations of aggregate swim paths.

Expression of DAPI, and Neurotrophins BDNF and GDNF

In males, HI injury resulted in significantly lower average BDNF levels in both the hippocampus ($F_{(1,14)} = 14.672, p < 0.01$; **Figure 7**) and striatum ($F_{(1,12)} = 40.053, p < 0.001$; **Figure 7**) in comparison to control males, regardless of housing condition. We observed partial eta squared of 0.488 and 0.769, with 0.873 and 1.0 power, respectively. CN rearing increased average BDNF counts in the striatum for male subjects compared to SF males ($F_{(1,12)} = 7.232, p < 0.05$); here, we observed partial eta squared of 0.376, with 0.695 power. There was no interaction effect between housing condition and surgical condition on BDNF counts in males.

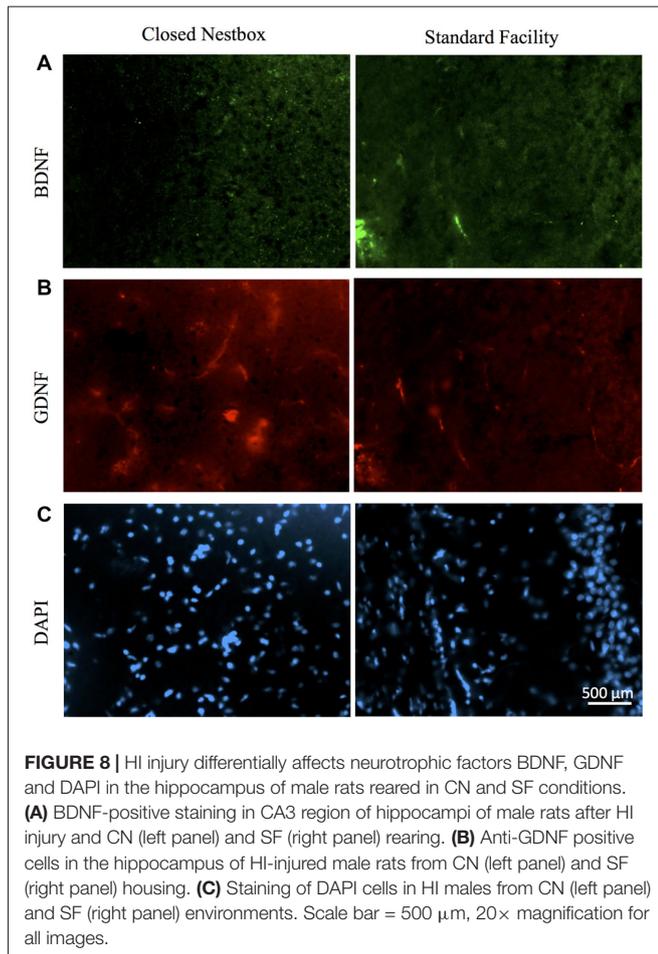
Females with HI did not display a lower hippocampal BDNF levels than control females ($F_{(1,13)} = 0.068, p > 0.05, NS$), however, there was a trend towards higher striatal BDNF counts for control females ($F_{(1,13)} = 4.315, p = 0.058$). Rearing in the CN



condition resulted in higher hippocampal BDNF compared to SF females ($F_{(1,13)} = 4.883, p < 0.05$). There was no significant interaction effect of surgical condition and housing condition ($F_{(1,13)} = 3.588, p > 0.05, NS$). However, there was an observable trend for HI females in the CN condition to exhibit hippocampal BDNF levels similar to that of control females. HI females in the SF conditions possessed lower BDNF hippocampal counts than all other groups (**Figure 8**).

The DNA-binding stain, DAPI (4',6-diamidino-2-phenylindole) was used in order to both verify fluorescent staining but also to evaluate cell populations within each brain ROI. Interaction effects of surgical condition and housing condition indicated that the number of hippocampal cells present were significantly greater in control animals reared in SF conditions versus all other groups ($F_{(1,14)} = 18.938, p < 0.01$). Control males reared in CN conditions possessed lower levels of DAPI-stained cell bodies in the hippocampus as compared to all other groups, including all HI males. No significant differences were observed for males in striatal DAPI levels according to surgical condition ($F_{(1,14)} = 2.650, p > 0.05, NS$) or housing condition ($F_{(1,14)} = 0.327, p < 0.05$; **Figure 9**).

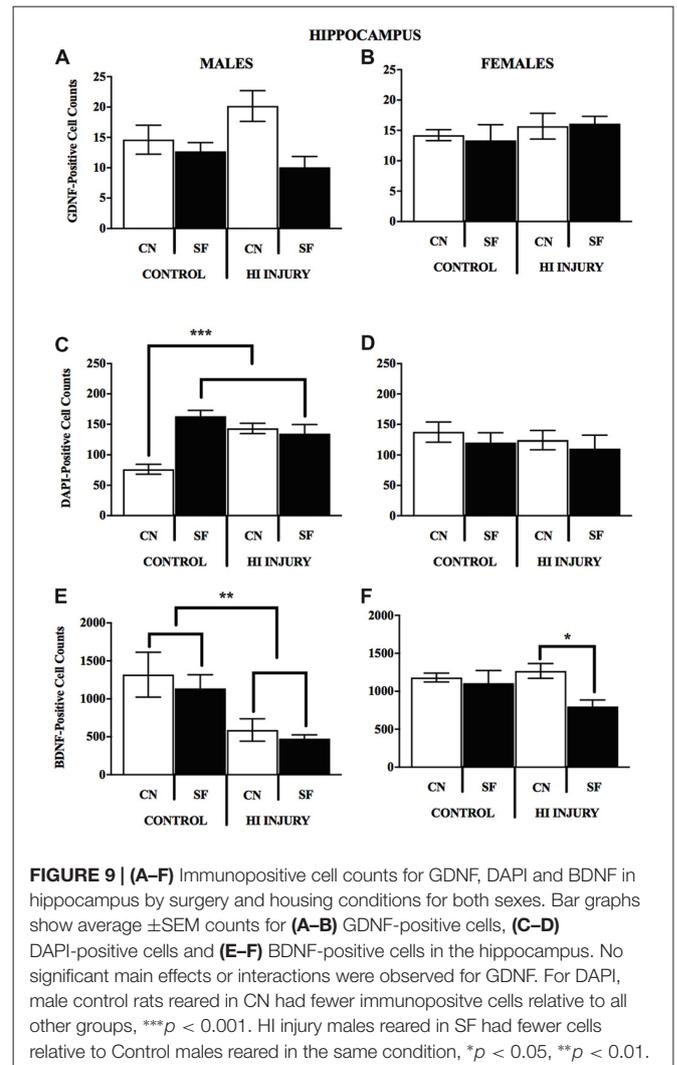
For female animals, two-way ANOVA revealed a significant difference between HI and control females ($F_{(1,12)} = 5.211, p < 0.05$), with control females exhibiting significantly greater



counts of DAPI-stained cells in the striatum than HI females (**Figure 10**). No differences in hippocampal DAPI-stained cells based on surgical condition ($F_{(1,13)} = 1.011$, $p > 0.05$, NS) or housing condition ($F_{(1,13)} = 0.291$, $p = 0.599$). There was also no effect of housing condition ($F_{(1,13)} = 3.345$, $p = 0.090$) on DAPI levels in the striatum of females.

Analysis of GDNF immunoreactive cells revealed a significant interaction effect between surgical condition and housing condition ($F_{(1,14)} = 5.743$, $p < 0.05$) indicating that CN improved the abundance of GDNF in the striatum of both control males and HI males (**Figure 10**). Although no significant differences were found between the factors of surgical and housing conditions on hippocampal GDNF, housing condition trended towards significance ($F_{(1,14)} = 4.337$, $p = 0.056$), with CN reared HI males showing the greatest levels of GDNF intensity compared to all other groups.

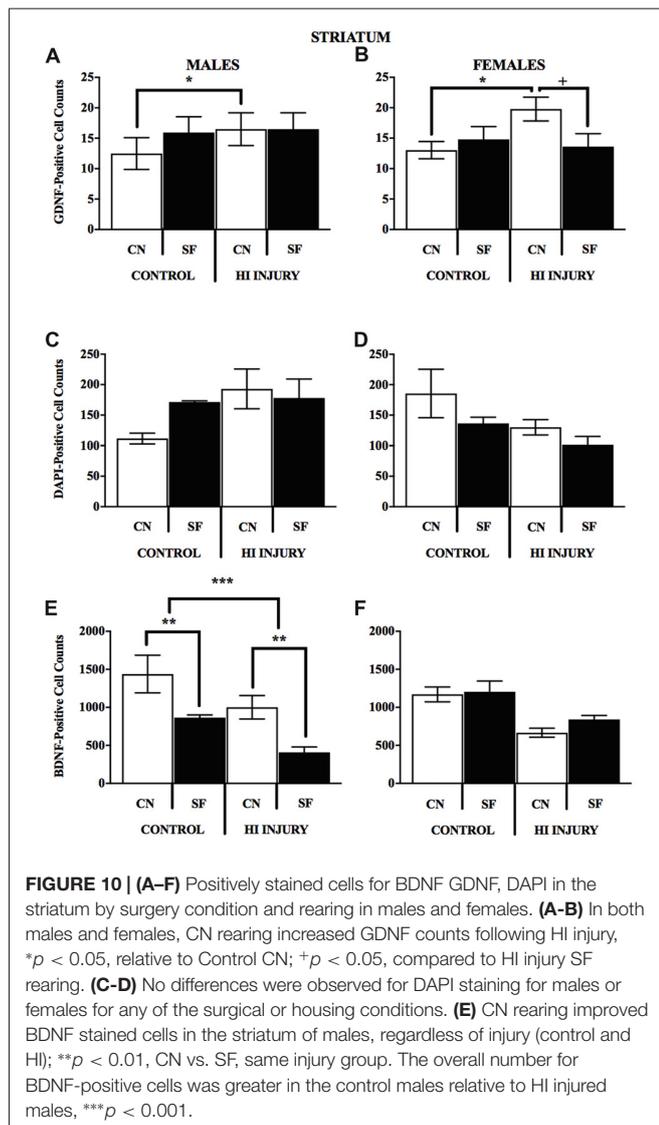
For females, there was a significant interaction effect between surgical condition and housing condition on GDNF counts in the striatum ($F_{(1,13)} = 5.777$, $p < 0.05$), with HI-CN females showing the highest counts of all groups. No significant differences were observed in regard to hippocampal measurement of GDNF for females or for males by housing or surgical condition, although a trend towards significance was observed in males by housing condition ($F_{(1,14)} = 4.337$, $p = 0.056$).



DISCUSSION

Overview of Findings

The present study was designed to assess the potential buffering effects of enrichment in the early pre-weaning environment on the negative neurological and cognitive effects of term neonatal HI injury. Dams were given a CN environment that we posited would provide early environmental enrichment for dams and pups and might promote mCTB, as measured by pup weight gain. Indeed, even though HI injury lowered pre-weaning weights for females, CN rearing protected against weight loss during adolescence following HI insult in males and females. The closed nest environment lead to significant changes in neurotrophin levels after HI injury in females and males suggestive of neuroprotection, namely higher levels of GDNF in the striatum in HI injured animals. The observed trend for HI-CN females to have near-normal levels of hippocampal BDNF may suggest even greater neuroprotection from early environmental enrichment for females.



testing phase, when a HP was located in a fixed position for each testing day, all animals regardless of injury, and housing condition, were able to learn the location of the HP, as indicated by decreased latencies across testing days (Hill et al., 2011). This suggests that all animals demonstrated some degree of intact long-term memory and adaptive search patterns, however, there were significant differences between injury and housing groups in the speed with which they were able to remember and locate the HP. Only female animals with HI injury demonstrated a significant functional benefit from CN housing, showing comparable latencies to those of control females in both housing conditions, and SF HI females demonstrating significant impairment in comparison. Males with HI injury demonstrated significantly longer search times, indicating poorer long-term memory and search strategies than intact males, regardless of housing condition.

Previous studies have found that, in addition to being more vulnerable to neonatal HI (Hill and Fitch, 2012), males are less responsive to some treatments for neonatal HI injury than are females (Pereira et al., 2008; Fan et al., 2011; Nie et al., 2016). Research by Pereira et al. (2008) showed a selective benefit of environmental enrichment on visuospatial memory for HI injured female animals, without having an effect on males. We have unpublished data indicating improve rope suspension performance for HI injured females but not males reared in CN (unpublished data; Mason et al., 2016). There are several mechanisms for sex differences in HI injury and recovery that have been proposed, including sex-dependent cell-death pathways (Zhu et al., 2006), protective effects of estrogen (Gerstner et al., 2009), and structural neurological differences due to early testosterone exposure (Hill et al., 2011).

Other labs have demonstrated that adolescent and adult environmental enrichment leads to improved performance in MWM for mice and rats without injury (Hullinger et al., 2015; Garthe et al., 2016). Interestingly, there were no independent effects of housing condition on water maze performance for control animals. Some research has indicated that environmental interventions may be specifically beneficial for injured or neurologically compromised animals (Pereira et al., 2008; Ravenelle et al., 2014). It may be the case that interventions targeting early nesting environment are specifically helpful for functional repair after injury and less helpful for memory performance in healthy, normally developing animals. This may be particularly true for animals exposed to neonatal HI, due to the vulnerability of hippocampal tissue (Jansen and Low, 1996; McAuliffe et al., 2006).

Neurotrophic Factors

BDNF and GDNF likely contribute to neuroprotective effects following HI injury in neonatal rats (Abe, 2000; Jin et al., 2003) and novel treatments can be assessed through changes in these two factors (Miyazaki et al., 2001; Griesbach et al., 2004), as well as through overall number of cell bodies present (Levison et al., 2001). The neuroprotective effects of BDNF are mediated at least in part by ERK1/2 secondary signaling; this signaling blocks the activation of caspases necessary

for apoptotic mechanisms following HI damage and ensuing necrotic stress (Han and Holtzman, 2000; Jones and Bergeron, 2004). Furthermore, GDNF aids in the survival of brain tissue via inflammatory proteins activated during neonatal HI, such as interleukin-6 and caspase-3 (Miyazaki et al., 2001; Kilic et al., 2003).

Overall, average BDNF level was higher in intact animals, and lower in HI animals, as has been reported (Pereira et al., 2008; Chavez-Valdez et al., 2014). CN conditions *reversed* the overall reduction in hippocampal BDNF levels after HI injury for both males and females. HI-CN conditions resulted in non-significant differences BDNF levels in females in striatal tissue. By contrast, HI CN levels were significantly higher than HI SF and control levels were greater than HI injury levels. It is likely that endogenous hormones contribute to this dimorphism in some respect since BDNF levels in intact female rats fluctuate depending on estrous cycle (Franklin and Perrot-Sinal, 2006), and with higher levels of estradiol, female rats have increased recall, general memory and spatial navigation over males (Luine and Frankfurt, 2013).

We quantified the average number of cell bodies using DAPI immunofluorescence. It was surprising that HI insult did not have a significant effect on the number of DAPI-tagged cells in the striatum of study subjects. However, we posit that the lack of specificity in this fluorescent DAPI staining—which produced coloration of all activated, double-stranded DNA-containing nuclei rather than neurons alone—could be one possible explanation for this (Levison et al., 2001). Interestingly, we did find DAPI counts in CN control males were lower than all other groups, implying reduced brain structure following environmental enrichment (van Praag et al., 2000).

No notable distinctions were observed for hippocampal GDNF staining in either males or females. However, striatal GDNF counts were significantly affected by CN rearing conditions for both females and males with HI. In females, HI-CN animals had more striatal GDNF positive cells over HI-SF and control CN animals. In a rodent model of Parkinson's disease, viral vector transfer of BDNF and GDNF into nigrostriatal neurons was no more potent than GDNF alone (Sun et al., 2005), implying that current striatal changes in GDNF alone may underlie MWM sex differences.

Early environmental enrichment improved search patterns in the probe trial following MWM testing, regardless of injury. Despite observing similarities in swimming speed and latency across control and HI conditions, CN conditions appeared impactful for rodent navigation in the MWM. It is unlikely that induced differences between groups are due to physical movement and/or exercise related to the testing protocol. Past research has indicated that it may take 28–31 days to persistently upregulate endogenous BDNF through physical activity in the MWM (Adlard et al., 2004; Griesbach et al., 2004). While physical stimulation may indeed induce transient changes in neurotrophins from baseline (Huang et al., 2006; Ferris et al., 2007), animals in our current study were sacrificed more than 2 weeks following the conclusion of behavioral testing.

Conclusions From Cognitive and Neurotrophic Outcomes

The neuroprotective benefit of a CN environment following HI injury was partly sex-specific. For females exposed to HI, those reared in CN demonstrated cognitive abilities similar to those of non-injured females, in contrast to HI females reared in SF housing that demonstrated significant functional impairment and diminished weight gain. There was a trend for HI females to demonstrate a similar beneficial effect of CN on neurotrophic factors, which may depend on levels of estradiol (Luine and Frankfurt, 2013). In contrast, males with HI injury showed no significant cognitive benefit from CN. However, both males and females in the HI-CN condition did demonstrate higher levels of GDNF in the striatum, indicating that CN may have afforded some neuroprotection to HI males. HI injury most prominently affects cortical matter and hippocampal tissue (Busl and Greer, 2010), and males are more likely to incur severe immediate damage to these regions than females (Liu et al., 2007; Hill and Fitch, 2012; Smith et al., 2014). In terms of recovery period, females are thought to have “depressed” metabolic function in comparison to males (Morken et al., 2014). It is plausible that CN rearing, while theoretically protective from environmental stress in the laboratory setting, was not intensive enough to sufficiently rescue the damage induced by HI for males.

The difference between male and female levels of these neurotrophins could be directly related to the availability of the nest itself from birth. Reduced nesting environment leads to “fragmented,” and stress-provoking maternal behavior in rat dams towards their offspring (Ivy et al., 2008). Adult laboratory rats do not spontaneously build nests (Van Loo and Baumans, 2004), and it has been suggested that researcher-provided materials for nesting may give rats a degree of control over their laboratory cage surroundings (as reviewed by Simpson and Kelly, 2011). Further, a small opaque enclosure similar to our CN can decrease environmental stress (Würbel, 2001). An early study by Manser et al. (1998) showed that rats will preferentially occupy an opaque nesting box with a roof and surrounding walls and a smaller entrance when offered multiple types of nesting, a design style similar to the CN provided here. Adult female rats have been hypothesized to have more complex interactions with their nesting environments as a result of hormones, and so it was expected the pregnant dams would adapt to the CN (Pietro Paolo et al., 2004). Since rat dams demonstrate preferential licking and grooming for male pups, regardless of strain (Moore et al., 1997; McGowan et al., 2011), it is possible that a low-stress nursing environment provided for more equal distribution of maternal care for both sexes, thereby improving care specifically for female pups.

One future direction could include additional enrichment after weaning through the traditional social sensorimotor stimulation in housing animals with peers, toys and physical objects as it may promote further neuroplasticity through experience (Johnston et al., 2009). This may diminish apoptotic changes close to injury (Bondi et al., 2014) that we could investigate with earlier sacrifice days, and, therefore, create additive effects to the benefits presented here. In clinical

populations, most infants with HIE are provided with standard-of-care treatment, therapeutic hypothermia (Eicher et al., 2005), if they can be treated within 6 h of life. Environmental enrichment may provide adjunctive benefits in combination with hypothermia. In future studies, examining the combination of treatment approaches could provide further insight into clinical applications.

AUTHOR CONTRIBUTIONS

LR conceived the original pilot study and worked with SD to develop a full experiment. BM supported the expansion of the work and contributed novel insights in developing the experiment, particularly molecular targets. LR and BM completed surgeries and behavioral testing with support from EA, CC and NW. LR, BM, EA, CC, NW and SD completed the terminal procedures including animal sacrifice, brain extraction and tissue preparation for immunohistochemical analyses. BM completed all immunohistochemical work. SD, LR, BM,

EA, CC and NW contributed to writing the Methods and entered data for analyses. BM and LR completed statistical analyses and wrote the Results. LR and BM both contributed equally to all aspects of the manuscript writing with support from SD.

FUNDING

LR and BM were supported by UMass Boston's internal Master's student grants (Spayne Grant).

ACKNOWLEDGMENTS

We gratefully acknowledge Dr. Dorethea Jenkins for her advice supporting the work and development of the protocols (Committee Member, LG's Master's thesis). We also extend gratitude to Rebecca Ravenelle and Elizabeth Boates for assistance with animal maintenance and husbandry, technical help and support with behavioral testing.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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