Therapeutic Hypothermia Provides Variable Protection against Behavioral Deficits after Neonatal Hypoxia-Ischemia: A Potential Role for Brain-Derived Neurotrophic Factor

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Abstract

Background: Despite treatment with therapeutic hypothermia (TH), infants who survive hypoxic ischemic (HI) encephalopathy (HIE) have persistent neurological abnormalities at school age. Protection by TH against HI brain injury is variable in both humans and animal models. Our current pre-clinical model of hypoxia-ischemia (HI) and TH displays this variability of outcomes in neuropathological and neuroimaging end points with some sexual dimorphism. The detailed behavioral phenotype of this model is unknown. Whether there is sexual dimorphism in certain behavioral domains is also not known. Brain-derived neurotrophic factor (BDNF) supports neuronal cell survival and repair but may also be a marker of injury. Here, we characterize the behavioral deficits after HI and TH stratified by sex, as well as late changes in BDNF and its correlation with memory impairment. **Methods:** HI was induced in C57BL6 mice on postnatal day 10 (p10) (modified Vannucci model). Mice were randomized to TH (31°C) or normothermia (NT, 36°C) for 4 h after HI. Controls were anesthesia-exposed, age- and sex-

matched littermates. Between p16 and p39, growth was followed, and behavioral testing was performed including reflexes (air righting, forelimb grasp and negative geotaxis) and sensorimotor, learning, and memory skills (open field, balance beam, adhesive removal, Y-maze tests, and object location task [OLT]). Correlations between mature BDNF levels in the forebrain and p42 memory outcomes were studied. **Results:** Both male and female HI mice had an approximately 8–12% lower growth rate (g/day) than shams ($p \leq 0.01$) by p39. TH ameliorated this growth failure in females but not in males. In female mice, HI injury prolonged the time spent at the periphery (open field) at p36 ($p = 0.004$), regardless of treatment. TH prevented motor impairments in the balance beam and adhesive removal tests in male and female mice, respectively ($p \leq 0.05$). Male and female HI mice visited the new arm of the Y-maze 12.5% ($p = 0.05$) and 10% ($p = 0.03$) less often than shams, respectively. Male HI mice also had 35% lower exploratory preference score than sham ($p \leq 0.001$) in the OLT. TH did not prevent memory impairments found with Y-maze testing or OLT in either sex ($p \leq 0.01$) at p26. At p42, BDNF levels in the forebrain ipsilateral to the HI insult were 1.7- to 2-fold higher than BDNF levels in the sham forebrain, and TH did not prevent this increase. Higher BDNF levels in the forebrain ipsilateral to the insult correlated with worse performance in the Y-maze in both sexes and in OLT

in male mice ($p = 0.01$). **Conclusions:** TH provides benefit in specific domains of behavior following neonatal HI. In general, these benefits accrued to both males and females, but not in all areas. In some domains, such as memory, no benefit of TH was found. Late differences in individual BDNF levels may explain some of these findings.

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Introduction

Hypoxic-ischemic (HI) brain injury is a major cause of morbidity and mortality among full-term neonates [1–4]. Therapeutic hypothermia (TH) has become the standard of care for neonates meeting criteria for moderate-to-severe HI encephalopathy (HIE). However, despite the use of TH, [1, 3–9] up to 55% of HIE survivors still have persistent neurological abnormalities at school age, including 43% with significant disabilities [3, 8, 10–14]. While the responses to TH after HI brain injury are variable in humans, details about specific neurobehavioral domains protected by TH are limited. Except for motor outcomes, large randomized clinical trials (RCTs) are not designed to answer questions about specific neurobehavioral end points [7, 12, 15–17]. Nevertheless, RCTs provide evidence that while deficits in motor domains are attenuated by TH after HIE, deficits in memory and learning domains may persist despite TH [18]. Some preclinical mouse models of hypoxia-ischemia (HI), including ours, emulate some facets of the injury produced by HIE and the variable protection afforded by TH in humans [19–21].

Sexual dimorphism documented in preclinical models of HI may contribute to the variable injury and response to treatment [19, 21–24]. However, the importance of sex as a biological variable influencing HIE outcomes has been sparsely studied in humans [25–27]. While males appear to be at higher risk for cerebral palsy and worse development after perinatal brain injury, including HIE, [25, 26, 28–30] sexual dimorphism in response to TH is not characterized. Similarly, growth failure in neonates suffering from HIE has been previously described [31], but the effect of TH to ameliorate growth disturbance is not known, nor is any possible influence of sex.

Neurotrophins, specifically brain-derived neurotrophic factor (BDNF), are essential to promote synaptic plasticity within the hippocampus and prefrontal cortex [32], regions of the brain essential for memory consolidation [33–37]. BDNF also plays a pivotal role in supporting cell survival and tissue repair after HI injury via

binding to tropomyosin receptor kinase B (TrkB) [38–41]. Alternatively, mature BDNF may facilitate cell death via binding to the ubiquitous p75 neurotrophin receptor (p75ntr) [42]. While BDNF deficits in the hippocampus and cortex occur in both sexes by 96 h after neonatal HI in the mouse [39], BDNF changes at late time points after HI and TH are not known. Sex-specific changes in late BDNF levels after HI injury may explain the variability in behavioral responses to TH [19, 21, 24]. Here, we characterize (i) growth and behavioral deficits after HI and TH, (ii) the influence of sex on these outcomes, (iii) the late changes of BDNF in injured and uninjured brain regions, and (iv) the possible individual influence of changes in BDNF on memory at p42 in a postnatal day 10 (p10) mouse model of HI injury.

Methods and Materials

Animals

One hundred and thirty-seven C57BL6 pups were used in this study. The mortality rate for this model was 15% (52% male and 48% female). Sixty percent of these deaths occurred during the hypoxia phase of the experiment, and 40% occurred after the end of the TH/normothermia (NT) phase of the experiment (25% due to the results of brain injury [1 TH/4 NT] and the remaining 15% because of maternal cannibalism).

Animal experimental protocols were approved by the Institutional Animal Care and Use Committee at Johns Hopkins University School of Medicine, and were carried out with standards of care and housing in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, US Department of Health and Human Services 85-23, 2011.

HI Injury and TH

HI was induced in C57BL6 mice (Charles River Laboratories, Wilmington, MA, USA) on p10 using the modified Vannucci model for mice [43] with permanent unilateral right carotid artery ligation, followed by 45 min of hypoxia ($\text{FiO}_2 = 0.08$) 1 h after ligation. During surgery, which lasted up to 5 min, mice were anesthetized with inhaled isoflurane (3% for induction, followed by 1% maintenance) followed by a 5- to 10-min period of recovery until spontaneous ambulation was recovered. Isoflurane combined with nitrous oxide as used in this protocol produces analgesia, sedation, and muscle relaxation. Animals from each litter were randomized to TH (31°C) or NT (36°C) for 4 h after HI as previously reported by our group [19]. Four hours of TH at 31°C following HI brain injury at p10 provides significant cortical, hippocampal, and striatal neuroprotection when assessed by MRI [19]. Following the TH/NT phase, the pups were returned to the dam. Mice assigned to the control group were exposed to inhaled isoflurane on p10 for 5 min at similar concentrations as described above for the TH and NT groups. Age-matched littermates were used as age/sex-matched, anesthesia-exposed sham controls. Mice were monitored daily for at least 45 min to assess their activity, grooming, and ability to access food and water.

Fig. 1. Timeline representation of experimental protocol. IHC, immunohistochemistry; WB, Western blot.

Behavioral Tests

Tests were conducted in a quiet experimental room between 2 and 7 p.m., under low-light conditions (80–110 lm) except for the balance beam test. In this test, a bright light was used as an avoidance stimulus to promote motion along the beam toward the dark box at the other end. Timing and exposure to low lumen light for testing were used to simulate dusk (the first part of the dark phase), the period at which mice initiate their most intense activity [44]. Each behavioral test was performed within similar time frames, and the order of testing of mice within the litters (6 pups/litter and 2 litters/experimental session) was randomly assigned. After 30 min of habituation in the experimental room, mice were weighed on the day of injury, and at the beginning and end of each testing week. Following initial evaluation of reflexes at p16, behavioral testing was performed between p22 and p36 (Fig. 1), based on the minimal age at which the animals were considered developmentally ready to perform the tasks. Most tasks were recorded using video, except for the adhesive removal task and reflex evaluation. Videos were reviewed and scored by 2 team members blinded to mouse groups, and discrepancies were resolved by 2 other team members. Following behavioral testing, the mice were returned to the dam.

Reflexes

The appearance of age-appropriate motor skills (balance, coordination, and strength) develops by p21 in mice. We chose to test animals at p16 during the period when reflexes appear, and at p26 to evaluate for the extinction of primitive reflexes [45]. We assessed (i) negative geotaxis, (ii) air righting, and (iii) forelimb grasp.

(i) Negative geotaxis was assessed to evaluate labyrinthine reflex, strength, and coordination. Pups were placed face-downwards on a 45° incline. Time to right upward on the incline was measured; a time >30s was considered a failure.

(ii) Air righting was assessed to evaluate postural righting and coordination. Pups were suspended in a supine position 13 cm above a soft surface and dropped. Positive air righting was counted if the pup landed prone.

(iii) Forelimb grasp was assessed to evaluate strength. Pups were placed on a wire suspended over a soft surface; the length of time of forelimb grasp was measured.

Open Field

Open field activity evaluates the locomotion and pivoting behavior of rodents [46]. Open field is also used to assess anxiety behavior and is a general assessment of animal basal locomotor activity and exploration [47]. The open field arena consists of a plastic chamber (40 × 30 cm), divided into central and peripheral areas. Animals were placed in the center of the arena, and left to explore freely for a total of 5 min. Parameters analyzed included total time spent in the center and at the periphery, and time spent moving, resting, and grooming.

Balance Beam

Balance beam performance evaluates motor coordination and balance as previously reported [48]. Performance was measured by quantifying the time it took the mouse to cross the beam and the number of paw slips that occurred. The apparatus consisted of 3 different raised wood beams (12- or 6-mm-wide and 6-mm-round beams), 80 cm in length and placed 50 cm above the table top. The finish (safe point) consisted of a black box at the end of the beam filled with nesting material. A bright light placed above the start point was used as an aversive stimulus. Animals were given 3 trials to cross each beam after the training day. A maximum of 60 s was allowed for the cross. The parameters analyzed included: time to cross beam at each trial, and the number and side of hind-paw slips.

Adhesive Removal

Adhesive removal evaluates somatosensory and motor function or deficits in rodents [49]. Tactile responses were measured by recording the time of initial contact with the adhesive and how much time it took the animal to remove the adhesive from each side. Two small round pieces of adhesive tape (6 ± 0.5 mm) were applied to each of the mouse's front paw with equal pressure, in alternating paw placement order. The mice were then placed in a testing box and allowed a maximum of 60 s to remove the adhesive. The first contact and total time for removal from each paw were recorded.

Y-Maze Test

The Y-maze test evaluates memory in rodents, based on their tendency to explore new environments [50]. Rodents normally prefer to investigate a new arm of the maze rather than returning

to one previously visited. Several brain areas including the hippocampus, septum, basal forebrain, and prefrontal cortex are involved in this task [51]. The apparatus consists of 3 identical Plexiglas arms interconnected at a 120° angle to an equilateral triangular center compartment. Phase 1 of the Y-maze assesses working memory. Mice were placed in the center of the maze and allowed to explore the maze. Arm alternations were counted over a 5-min testing period and calculated as a percentage of the total arm entries. Spontaneous alternations performance (SAP), 3 successive different arm visits, and estimates of good working memory were assessed along with the total number of arm visits. Three days after phase 1, phase 2 was performed to assess spatial and recognition memory. In the acquisition phase, 1 of the arms was closed by a vertical wall (novel arm); thus, animals were only able to explore the 2 open arms for a total of 5 min. After a delay of 30 min, all the arms were opened and the mice were allowed to explore for 5 min. The percentage of entries to the novel arm was analyzed in the retention phase.

Object Location Task

Object location task (OLT) evaluates spatial and working memory, based on the tendency of rodents to distinguish new environments and/or objects [52, 53]. A day of habituation to the arena is followed the next day by a 5-min period to explore 4 objects placed in the arena, and then a delayed phase (30 min later) to explore the objects again after 2 of them have been switched in location. The arena was the same used for open field testing. All objects were of similar size but different in color, texture, and material. Encounters of the mice in closed proximity (<1 cm) to the object were deemed exploratory if the nose of the mouse was pointing towards the object and the mouse was looking or sniffing at the object. Parameters analyzed included percentage time exploring objects in familiar location and percentage time exploring object in novel location. Times in seconds were used to calculate exploratory preference (EP) score in percentage (EP score = time exploring object in novel location $\times$ 100/total time exploring all objects) [54] and discrimination index (DI) (DI = (time exploring the novel object – the familiar object) $\times$ 100/total time exploring all objects) [55]. The scores measure discriminatory behavior in exploratory time between novel and familiar objects. For the EP score, >50% indicates good working memory and <50% indicates impaired memory; for DI, a positive score = more time spent with the novel object, a negative score = more time spent with the familiar object, and zero = no preference for either object [53].

BDNF ELISA

BDNF protein levels were measured from fresh forebrain tissue obtained when the mice were sacrificed at p42. Each sample included tissue from the cortex and hippocampus (forebrain), and samples were taken ipsilateral and contralateral to the injury from NT- and TH-treated HI mice. A similar tissue sample was obtained from p42 anesthesia-exposed control mice. Protein homogenates were prepared as previously described [56], and protein concentrations were determined using the Bradford assay [57]. The mouse sandwich ELISA method was used according to the manufacturer's instructions (Biosensis Pty. Ltd. Thebarton, SA, Australia). The kit consists of a precoated mouse monoclonal anti-mature BDNF capture antibody, with a detection range of 2–2,000 pg/mL and no detectable cross-reactivity with other cytokines. Samples

were clarified by centrifugation at 10,000 g at 4°C for 2 min, then 50 μ l (202 $\pm$ 62.5 μ g protein) were incubated for 45 min at 37°C on the precoated plate, and then exposed to biotinylated anti-mouse BDNF antibody for 30 min at 37°C. Following washes, avidin-biotin-peroxidase complex working solution was applied for 30 min at 37°C. TMB (3,3',5,5' tetramethylbenzidine buffer) developing agent was added to each well after washes and after 8 min of incubation at 37°C, a TMB stop solution was applied. Plates were read at 450 nm in a microplate reader (BioRad, Hercules, CA, USA) within 30 min after application of the stop solution, and analyzed using a linear regression model. Results were adjusted to total loaded protein per well (pg [BDNF]/mg [protein]).

Western Blots

For Western blot analysis, phospho-TrkB (pTrkB) and TrkB samples of the forebrain were used. Protein homogenates were prepared in homogenization buffer with phosphatase, protease inhibitors, and 20% (w/v) glycerol. Concentrations were determined using the Bradford assay [57]. Aliquots (30- μ g) of homogenized protein were diluted 3:1 (v:v) in 4 $\times$ loading buffer under reducing conditions and loaded into 4–20% mini-protean TGX polyacrylamide precast protein gels (BioRad). Protein was transferred to a nitrocellulose membrane using TransBlot Turbo midi-size (BioRad), stained with Ponceau S, blocked with 2.5% nonfat dry milk with 0.1% Tween-20 in 50 mM Tris-buffered saline (TBST, 50 mM Tris/HCl and 150 mM NaCl, pH 7.4), except for pTrkB (Thermo Fisher PA5-36695) which was blocked in 2.5% bovine serum albumin (BSA). Nitrocellulose blots were incubated overnight at 4°C with primary antibodies at 1:500 (pTrkB) and 1:200 (TrkB). Ten micrograms of human TrkB-transfected 293T cell lysate (sc-113925; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) showing a band at 145 and 95 kDa was used for the identification of TrkB. After exposure to each primary antibody, the membranes were washed with TBST, exposed to secondary antibodies for 1 h, and then developed using enhanced chemiluminescence (Clarity Western ECL Substrate, BioRad). To quantify protein immunoreactivity, optical density (OD) was determined with iVision software adjusted for background. The reliability of sample loading and protein transfer was verified by staining nitrocellulose membranes with Ponceau S before immunoblotting and the use of β -actin as a loading control. The antibody pTrkB was used, i.e. mouse polyclonal antibody raised against phosphorylation site tyrosine 515 (Thermo Fisher PA5-3669). There was no cross-reactivity with other Trk receptors. This antibody detects bands of 145–95 kDa (1 μ g/mL). TrkB was also used, i.e. rabbit polyclonal antibody raised against amino acids 160–340 of TrkB of human origin (sc-8316) with no cross-reactivity with other Trk receptors. The antibody detects TrkB bands of 145–95 kDa (1 μ g/mL).

Immunohistochemistry

Glial fibrillary acid protein (GFAP) immunohistochemistry was used to assess morphological changes in astrocytes, suggesting persistent activation at p42. The mice were killed with an overdose of isoflurane and exsanguinated with 0.1 M cold PBS (pH 7.4) via intracardiac perfusion. Brains were perfusion-fixed with 4% paraformaldehyde/0.1 M PBS for 30 min at 4 mL/min. Tissues were cryoprotected with graded immersion in 15 and then 30% sucrose in PBS until they sank, and were then frozen and stored at –80°C until cut at 50 μ m on a freezing microtome [56]. Floating immu-

nohistochemistry for GFAP was performed as previously described [56] with whole rabbit antisera anti-GFAP antibody (DAKO North America, Carpinteria, CA, Cat. No. Z0334 at 1:1,000). Goat anti-rabbit antibody (1:200) was used as the secondary antibody and DAB as the chromagen.

Statistical Analysis

Statistical analysis was performed using one-way ANOVA with Tamhane post hoc analysis stratified by sex. Significance was assigned to a $p < 0.05$. Correlations between BDNF levels and behavioral outcomes were analyzed using the Spearman Rho. The analysis design only allows comparison within treatment groups of the same sex and age.

Results

Neonatal HI Affected Subsequent Growth

Mice were weighed routinely from p16 to p39 ($n = 18$ –21/group of males and $n = 15$ –17/group of females). At p26, NT mice weighed 13% less than shams (ANOVA, $p = 0.04$; $p = 0.04$ vs. sham; Fig. 2a). Scatterplot of weight versus age showed the greatest differences at p26 (Fig. 2b). However, when stratified by sex, male and female mice at p26 did not differ in weight (Fig. 2c). Differences by treatment groups and sex emerged by p36 after weaning from the dam. Female NT mice weighed 11% less than sham mice at p36 ($p = 0.002$; Fig. 2d) and 9% less than sham mice at p39 ($p = 0.01$; Fig. 2e). There were no differences between sham and TH groups at either p36 or p39. Prior to weaning, significant differences in the rate of growth were also observed in male mice. The NT-treated male mice grew slower (ANOVA $p = 0.01$; $p = 0.02$ vs. sham), 0.2 g/day less than sham mice (Fig. 2f). Female mice treated with TH after HI also grew slower than sham mice up to p26 (ANOVA $p = 0.04$; $p = 0.03$; Fig. 2f). However, after weaning from the dam, the female mice caught up to be no different from the shams when growth was assessed for the entire 23-day time period (Fig. 2g). In contrast, slow growth persisted in HI-injured male mice after weaning, with the growth rate of the NT and TH male mice not comparable to the shams overall (Fig. 2g). Overall growth curves from the male and female mice are shown in Figure 2h, i.

Early Differences in Negative Geotaxis and Later Differences in Open Field Behavior

No differences for forelimb grasp or air righting were observed within the treatment groups with or without stratification by sex at p16 and p26. However, when negative geotaxis was assessed at p16, injured mice treated with NT and TH turned uphill 3 and 2.8 times faster than

shams, respectively (ANOVA, $p < 0.001$; sham vs. NT $p < 0.001$, sham vs. TH $p < 0.001$) (grouped data not shown). At p16, when stratified by sex, female HI mice turned uphill 2.3 to 2.9 times faster than shams (ANOVA $p = 0.001$; sham vs. NT $p = 0.01$; sham vs. TH $p = 0.04$), and male HI mice turned 3.6 to 4 times faster than male shams (ANOVA, $p < 0.001$; sham vs. NT $p = 0.004$; sham vs. TH $p = 0.002$; Fig. 3a). At p26, no differences between injured and uninjured mice were found in either sex or in grouped data; however, a greater degree of variability and longer times exploring downhill emerged in the sham animals (grouped data not shown) (Fig. 3b). Open field performance at p36 unveiled a sex-specific behavior. While no differences were documented on the percentage of time moving, resting, and grooming, overall, HI-injured mice spent more time exploring the periphery than the center of the field (ANOVA $p = 0.001$; $p < 0.05$ vs. sham in all cases, grouped data not shown). Stratification by sex demonstrated that differences between uninjured and injured mice occurred significantly in female HI mice (ANOVA $p = 0.004$; sham vs. NT $p = 0.01$ sham vs. TH $p = 0.02$; Fig. 3c, d). TH did not protect female mice against this behavior. Percent time at the periphery and in the center was similar in male mice regardless of treatment.

TH Protected against Motor Deficits

Motor outcomes were evaluated with the balance beam and adhesive removal tests. As a whole, injured and uninjured mice performed similarly in the 12-mm, 6-mm, and round beam assessments, with differences only revealed when the results were stratified by sex. No differences were observed in either sex on the 12-mm beam (Fig. 4a). However, male NT mice took approximately twice as long to cross the 6-mm beam (ANOVA $p = 0.05$ and $p < 0.05$ vs. sham; Fig. 4b) and the round beam (ANOVA, $p = 0.02$ and $p < 0.05$ vs. sham; Fig. 4c). TH improved the performance of male mice on both the 6-mm and round beams. Motor deficits were not found in female mice using the balance beam test; they were, however, evident in injured female mice using the adhesive removal test. Once again, no differences in the adhesive removal test were observed when all mice were compared (grouped data not shown). With sex stratification, it was evident that NT female mice took longer than those treated with TH to remove the adhesive from the left paw (contralateral to the injured brain) (ANOVA $p = 0.03$; Fig. 4d, e).

Fig. 2. Neonatal HI, treatment with TH, and growth. Following HI, injured mice grew slower than sham mice. TH ameliorated growth deficits only in injured females. Grouped data for weight from p16 to p39 are presented as a box and whisker plot (**a**) and a scatter plot (**b**). Sex-stratified weight data for p26 (**c**), p36 (**d**), and p39 (**e**), and also growth rates from p16 to p26 (**f**) and to p39 (**g**) are represented as box and whisker plots, while growth curves for males (**h**) and

females (**i**) from p16 to p36 are represented as scatter plots. For box and whisker plots, boxes represent the interquartile range (IQR) limited by the 25th and 75th percentile (lower and upper limit, respectively), the line inside the box indicates the median, and the whiskers extend to 1.5 times the IQR. Outliers are not represented. * $p < 0.05$ vs. one-way ANOVA (grouped or stratified by sex) with post hoc analysis using Tamhane.

Fig. 3. Negative geotaxis and open field behavior. Injured mice regardless of treatment or sex turned uphill faster than sham in the negative geotaxis test. Only injured females spent more time at the periphery of the open field. Sex-stratified negative geotaxis data at p16 (**a**) and p26 (**b**) and open field data for time at the periphery (**c**) and center (**d**) are represented as box and whisker plots, where boxes represent the interquartile range (IQR) limited by the 25th and 75th percentile (lower and upper limit, respectively), the line inside the box indicates the median, and the whiskers extend to 1.5 times the IQR. Outliers are not represented. * $p < 0.05$ vs. sham, one-way ANOVA (grouped or stratified by sex) with post hoc analysis using Tamhane.

TH Was Not Protective against Memory Deficits Found after Neonatal HI

The Y-maze test at p22 (phase 1) and p26 (phase 2) and OLT at p23 were used to evaluate memory. Grouped together, injured mice had a similar number of visits to all arms and percentage of SAP as did uninjured mice during Y-maze phase 1. However, the percent of entrances to the novel arm during phase 2 in injured mice was 10% lower in NT and 17.5% lower in TH mice than in shams (ANOVA $p < 0.001$; both $p < 0.01$ vs. sham, grouped data not shown). When stratified by sex, an additional difference emerged, male NT mice had 43% more visits to all arms of the maze (ANOVA $p = 0.005$ and $p = 0.04$ vs. sham; Fig. 5a) without any increase in the number of SAP (Fig. 5b). Confirming the overall data on percent novel arm visits, the percentage of visits to the novel arm by NT male and female mice were 12.5% ($p = 0.05$) and 10% ($p = 0.03$) lower than that of the shams (ANOVA male $p = 0.002$; ANOVA female $p = 0.001$; Fig. 5c). TH did not improve the percentage of visits to the novel arm, which was 18% lower in males ($p = 0.002$) and 15% lower in fe-

males ($p = 0.002$) than in shams. When evaluating spatial and working memory by OLT, no differences were seen within the entire group. Again, when groups were stratified by sex, injured male mice performed worse than sham. Both NT and TH male mice had a 35 and 25% lower EP score than sham (ANOVA $p < 0.0001$; NT $p \leq 0.0001$; TH $p = 0.0001$; Fig. 5d). No differences were observed among female mice. TH male mice also showed a -19.4 versus $+1.5$ DI in sham mice (ANOVA $p = 0.05$ and $p = 0.003$; Fig. 5e).

Late Changes in BDNF Expression Correlated with Memory Outcomes

Since memory outcomes are linked to changes in BDNF [35–38, 58], we evaluated if injury and treatment group correlated with forebrain BDNF levels at p42 ($n = 7$ – 11 /group). In NT mice, BDNF level in the hemisphere ipsilateral to the injury was 2-fold higher than the level in sham mice (ANOVA $p = 0.02$ and $p = 0.03$ vs. sham; Fig. 6a). Similarly, in TH mice, BDNF level in the ipsilateral hemisphere was 1.7-fold higher than sham ($p = 0.02$

Fig. 4. Motor outcomes and protection by TH. TH protects against motor impairment in both male (balance beam performance) and female (adhesive removal test) mice following neonatal HI. Sex-stratified balance beam data for the 12-mm (a), 6-mm (b), and round (c) beams at p24, and adhesive removal data for the right (c) and left (d) paw at p36 are represented as box and whisker plots,

where the boxes represent the interquartile range (IQR) limited by the 25th and 75th percentile (lower and upper limit, respectively), the line inside the box indicates the median, and the whiskers extend to 1.5 times the IQR. Outliers are not represented. * $p < 0.05$ vs. sham, one-way ANOVA (grouped or stratified by sex) with post hoc analysis using Tamhane.

vs. sham). No differences were documented in BDNF levels in the contralateral hemisphere (Fig. 6b). Ratio of BDNF levels in the ipsilateral versus contralateral hemispheres (I/C ratio) of NT mice was 2.2-fold higher than shams (ANOVA $p = 0.004$ and $p = 0.009$ vs. sham; Fig. 6c). When stratified by sex, no differences were seen for ipsilateral or contralateral BDNF level, but male NT mice had a 2.8-fold higher I/C ratio than sham (ANOVA $p = 0.005$ and $p = 0.004$ vs. sham; Fig. 6d). Correlation of BDNF levels with memory outcomes, including percent entries to novel arm (Y-maze phase 2) and EP score (OLT) was performed on an individual basis. Male or female mice with higher BDNF levels in the ipsilateral hemisphere (Fig. 6e, h), lower levels in the contralateral hemisphere (Fig. 6f, i), and greater I/C ratios (Fig. 6g, j) had worse

performance in memory tests. Increased BDNF levels in the hemisphere ipsilateral to the injury in HI mice did not result in increased phosphorylation of TrkB (data not shown).

Astrocyte Activation at Late Stages after HI Brain Injury

Activated astrocytes are a significant source of BDNF after injury [59, 60], so we investigated if astrocyte activation persisted by p42, the time at which BDNF changes were documented. GFAP immunostaining of brain coronal sections from sham mice at p42 revealed quiescent astrocytes with small soma and thin processes dispersed throughout the cortex, mostly in the lacunosum molecular layer of the hippocampus (Fig. 7a). In contrast, GFAP

Fig. 5. Memory outcomes and TH. Following neonatal HI, TH does not protect against memory deficits as assessed by the Y-maze and object location test (OLT) in either sex. Sex-stratified Y-maze data for the total number of visits (phase 1, **a**), percent SAP (phase 1, **b**), and percent visit to the novel arm (phase 2, **c**) at p22–p26, and also OLT data for EP score (**d**) and discrimination index (**e**) at p23 are represented as box and whisker plots, where the boxes

represent the interquartile range (IQR) limited by the 25th and 75th percentile (lower and upper limit, respectively), the line inside the box indicates the median, and the whiskers extend to 1.5 times the IQR. Outliers are not represented. * $p < 0.05$ vs. sham, one-way ANOVA (grouped or stratified by sex) with post hoc analysis using Tamhane.

staining from injured mice treated with NT revealed areas of persistent/late astrogliosis (Fig. 7b). In injured mice treated with NT, large numbers of hypertrophic and densely GFAP-stained astrocytes with thick and abundant processes were visualized throughout the hippocampus, particularly in the lacunosum moleculare and pyramidal cell layers, with areas of glial scarring observed in the radiatum layer (Fig. 7b'). In mice treated with TH, morphologically reactive astrocytes were present but were less abundant, and the area of scarring in the radiatum was smaller (Fig. 7c). GFAP staining of the hemispheres ipsilateral and contralateral to the HI injury are shown (Fig. 7d) again, demonstrating the marked amount of gliosis evident in the ipsilateral hippocampus, even in

this relatively moderately injured animal. Abundant reactive astrocytes in a perivascular location are seen at higher power in the ipsilateral hemisphere of the injured mice (Fig. 7d', d'').

Discussion

In this preclinical mouse model, exposure to HI produced a variable degree of deficits in growth, and motor, memory, and other behavioral domains. Using a TH protocol previously described by our group [19], we showed that TH provided a variable degree of neuroprotection to specific domains of behavior, often in a sex-specific man-

Fig. 6. Late BDNF changes after HI. Higher BDNF levels observed in the forebrain of the hemisphere ipsilateral to the injury correlate with worse memory outcomes regardless of treatment. Grouped BDNF data for ipsilateral (a), contralateral (b), ipsilateral/contralateral (I/C) ratio (c), and sex-stratified I/C ratio (d) are represented as box and whisker plots, where the boxes represent the interquartile range (IQR) limited by the 25th and 75th percentile (lower and upper limit, respectively), the line inside the box indicates the median, and the whiskers extend to 1.5 times the IQR. Outliers

are not represented. a–c White boxes, shams; black boxes, NT mice; grey boxes, TH mice. * $p < 0.05$ vs. sham, one-way ANOVA (grouped or stratified by sex) with post hoc analysis using Tamhane (a–c $n = 14$ per group; d $n = 7$ per group/per sex). Subject-by-subject correlation between BDNF levels in the ipsilateral (e, h) and contralateral (f, i) hemispheres, and I/C ratio (g, j) vs. % entries to novel arm (Y-maze phase 2, group data) and EP score (OLT, male mice). Analysis by the Spearman Rho. r , correlation coefficient. Significance determined by $p < 0.05$.

Fig. 7. Late activation of astrocytes. Representative photomicrographs of sham (**a**), NT (**b**), and TH (**c**) mice and details (**a'**–**c'**) in hippocampal CA1 region. Large numbers of hypertrophic and densely GFAP-stained astrocytes primarily throughout the hippocampus of injured mice treated with NT. Active astrocytes are mostly seen around glial scarring in injured mice treated with TH. **d** Comparative GFAP staining of the hemispheres ipsilateral and contralateral to the HI injury. In contrast to contralateral staining, abundant reactive astrocytes are visualized in the ipsilateral hemisphere of the injured mice (**d'**, **d''**). LM, lacunosum molecular layer; PL, pyramidal cell layer; RL, radiatum layer.

ner. Furthermore, a late surge in mature BDNF in the hemisphere ipsilateral to the injury correlated with deficits in working memory. The source of late BDNF may be from persistently activated astrocytes as previously suggested [60, 61]. Overall, these findings were consistent with evolving data from clinical trials [4–6, 14, 15, 62–64], making this preclinical model a potent tool for the investigation of neonatal HI and TH.

The effect of TH on functional outcomes following neonatal HI is inconsistent among preclinical rodent models. Although rodents injured by HI manifest with

cognitive impairments, attention deficits, and increased impulsivity and compulsivity [65–67], the reported protection afforded by TH is contradictory [19, 21, 24, 68–74]. These contradictions may arise from the lack of proper standardization in behavioral testing in immature rodents, the variable protocols of TH used, and the effects of other biological variables such as sex and growth. Our protocol of TH attenuates cortical and hippocampal injury at 8 and 20 days after neonatal HI at p10 (full-term equivalent), while providing limited protection of memory domains [19], consistent with findings in humans

[18]. Furthermore, the sexual dimorphism documented in our model of HI and TH matches that reported in other experimental models of perinatal brain injury [19, 21, 24, 74].

Because poor growth in the setting of neonatal HI brain injury may be both a cause and a consequence of neurological and behavioral impairments, we analyzed the growth of the mice following HI and TH for a month after injury. A marked variability in weight was observed overall. Males were heavier than females after weaning, adding to this variability. When growth rate was stratified by sex, differences emerged. Both injured male and female mice had poorer weight gain after weaning, and only the females treated with TH recovered. The reason for the difference in growth after HI is unclear, possibly reflecting abnormal suck-swallow patterns often seen in the clinic [31], or other neuromotor, neuroendocrine, or behavioral deficits [75]. The relative protection against growth failure afforded to females by TH may be explained by the difference in outcomes documented using the balance beam and adhesive removal tests to assess motor balance/coordination and sensorimotor processing, respectively [48, 49]. TH after HI preserved sensorimotor processing in female mice, and motor balance and coordination in male mice. Since intact sensorimotor processing is necessary for the development of exploratory behaviors, including food-seeking [76], we hypothesize that these very refined differences in the protection of various motor domains with TH may account for the sexual dimorphism in growth observed in our experiments.

Early assessment of sensorimotor deficit by measuring primitive reflexes in neonates who suffer birth asphyxia is predictive of cerebral palsy and developmental disability [77]. Because sensorimotor reflexes also evaluate behavioral and functional development in neonatal mice, we chose to assess them based on the age of physiologic acquisition [45]. While no difference in air righting and forelimb grasp were observed, in the negative geotaxis test, both male and female HI-injured mice turned uphill faster than shams regardless of treatment. It is controversial but possible that uninjured mice display a more positive geotaxis (downward movement) due to exploratory behavior instead of a true negative geotaxis response with possible anxiety as observed in the injured mice. These findings are in agreement with observations by Motz and Alberts [78], who disagree with the use of negative geotaxis as a measurement of sensory function in rodent pups, and describe the phenomenon of “positive” geotaxis in which rodents normally orient downhill [78, 79]. Additionally, injured female mice in this model display

more anxiety-like behavior in the open field performance, and TH does not provide protection in this testing domain either. Although mice have a natural tendency to explore the periphery more than the unprotected center areas while in the open field, mice with anxiety-like behavior spent even more time at the periphery than in the center than the shams did [74, 80]. Congruent with these findings, a higher prevalence of anxiety disorders and increased impulsivity and compulsivity characterize HIE survivors [81].

Clinical trials using TH for birth asphyxia have consistently documented a decrease in the incidence of motor impairments at the age of 18 months and again at 6–7 years [1, 3–5, 62, 82, 83]. Except for motor outcomes, no large RCTs have been designed that answer questions about specific neurobehavioral end points. In agreement with these clinical trial results, our mouse model of 4 h of TH following HI brain injury also showed better motor outcomes in the treated mice than in the NT mice at both p24 and p36. Additionally, we found differences in motor impairment using sex stratification and 2 different tests. While male NT mice took twice as long to cross the 6-mm and round balance beams, female NT mice took almost twice as long to remove the adhesive from the left paw (contralateral to the injured hemisphere). Regardless of the test, TH provided protection against motor impairment in both sexes. Differences in results in the balance beam and adhesive removal tests may provide further understanding of how each sex displays motor deficits. While success in crossing the beam requires motor strength, coordination, and balance [48], success in removing the adhesive also requires somatosensory recognition [49]. It is not known whether differences between sexes in coordination and somatosensory recognition after injury and TH explain differences in the sensitivity of the tests to show motor deficits in males and females.

Despite TH, learning and memory impairment persists 6 or 7 years after birth asphyxia [1, 2, 15, 18]. Similarly, in this study, deficits in spatial recognition and working memory were documented in HI mice of both sexes despite treatment with TH. All the injured mice visited the novel arm of the Y-maze less often than the sham mice. In addition, injured NT male mice had more overall arm visits than shams. The increased number of total arm visits may be indicative of hyperactive behavior previously described in rodents exposed to HI injury [65, 67], but the performance in the Y-maze is not designed to evaluate hyperactivity. Similarly, during OLT performance, injured male mice had a negative DI score compared to shams, implying impaired memory associated with anxi-

ety-like behavior. Sex differences in the ability to discriminate objects has been hypothesized before [84–86] in many species including humans and rodents, with females having a greater ability to recognize different objects. In this study, only injured male mice displayed an impaired ability to recognize differences in object location.

BDNF levels in the cortex and hippocampus (forebrain) acutely increase by 24 h after HI injury in the mouse and then decrease by 96 h [39]. Similar findings are documented in a middle cerebral artery occlusion stroke model in rats [87]. Changes in BDNF occurring at late stages after neonatal HI injury have not been documented previously. Thirty days after HI, BDNF levels in the injured forebrain were 2-fold higher than those in the sham forebrain, and TH did not have any effect on this increase. Neither the source nor the significance of this late elevation of BDNF in the ipsilateral brain is known. BDNF may be used by the neurons to improve their survival and thus promote recovery after injury [58], and may also be a surrogate for persistent ongoing injury or the activation of astrocytes [61, 88]. Although BDNF level in the forebrain contralateral to the injury was unchanged by HI exposure, the lack of compensatory BDNF upregulation and thus a deficit relative to the ipsilateral side may have been detrimental. Altered ipsilateral versus contralateral BDNF levels may signal disrupted interhemispheric connectivity, well-described in models of unilateral stroke [89–91]. Disrupted integrity of the corpus callosum and deficient BDNF levels in patients with memory impairment substantiate the importance of interhemispheric connectivity and the effects of BDNF produced in regions remote from the injury [92]. Our data suggest that a similar pathophysiology may be occurring in this model, given that individual mice with higher I/C BDNF ratios had worse performance in the Y-maze memory test and in the OLT, while those with higher contralateral BDNF had better outcomes. Because these BDNF changes were not associated with changes in TrkB phosphorylation, we speculate that the association with memory impairments may be mediated by the binding of BDNF or pro-BDNF to p75Ntr [39, 42]. These findings coincided temporally with the persistent activation of astrocytes occurring over a month after the HI injury in this model. Whether activated astrocytes were the source for the increased BDNF level measured in the forebrain ipsilateral to the injury is unclear.

We acknowledge several limitations to this study. An imaging correlate was lacking, limiting the assessment of overall neuropathology and white matter integrity, both variables that affect neurobehavior. However, studies by

our group and others have shown that findings on neuropathology do not necessarily predict which subjects will develop neurobehavioral impairments [19, 93, 94]. In this study, no evaluation of the feeding pattern and fluid/food intake was performed, limiting the interpretation of the growth data. However, all animals had access to food and water *ad libitum*, even while in the testing room, and they were monitored daily for at least 45 min to ascertain accessibility despite potential motor impairments. Testing young mice remains a challenge and there is no clear standardization. In fact, the performance of our shams was poorer than expected compared to adult mice in some tests. Nevertheless, we were still able to detect differences attributable to injury and treatment. Lastly, we were unable to compare outcomes between sexes or between time points. Instead, we were only able to accurately document the differences between groups of the same sex, and at the same age or period.

In summary, the variable injury and incomplete neuroprotection by TH presented here are consistent with clinical data from large RCTs [4–6, 14, 15, 62–64] and also several other experimental models of HI and TH [19, 20, 95]. The growth deficits following HI injury and TH were also similar to those described in HIE survivors who developed cerebral palsy [96]. The sexual dimorphism we observed was consistent with that reported in other experimental models of perinatal injury [19, 21, 24, 74]. BDNF changes in mice performing worse after HI, regardless of treatment, may provide information about the importance of BDNF at late time points after perinatal brain injury. Our results confirmed that sex stratification is necessary in some assessments of outcome following neonatal HI. Our findings that abnormalities in certain behavioral domains were unresponsive to TH support the relevance of this model for preclinical testing of adjuvant therapies for neonatal HI.

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Disclosure Statement

The authors declare no conflict of interest related to the data presented in the manuscript.

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