Therapeutic hypothermia and hypoxia–ischemia in the term-equivalent neonatal rat: characterization of a translational preclinical model

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BACKGROUND: Hypoxic–ischemic encephalopathy (HIE) is a major cause of morbidity in survivors. Therapeutic hypothermia (TH) is the only available intervention, but the protection is incomplete. Preclinical studies of HIE/TH in the rodent have relied on the postnatal day (P) 7 rat whose brain approximates a 32–36 wk gestation infant, less relevant for these studies. We propose that HIE and TH in the term-equivalent P10 rat will be more translational.

METHODS: P10-11 rat pups were subjected to unilateral hypoxia–ischemia (HI) and 4 h recovery in normothermic (N) or hypothermic (TH) conditions. Brain damage was assessed longitudinally at 24 h, 2 wk, and 12 wk. Motor function was assessed with the beam walk; recognition memory was measured by novel object recognition.

RESULTS: Neuroprotection with TH was apparent at 2 and 12 wk in both moderately and severely damaged animals. TH improved motor function in moderate, but not severe, damage. Impaired object recognition occurred with severe damage with no evidence of protection of TH.

CONCLUSION: This adaptation of the immature rat model of HI provides a reproducible platform to further study HIE/TH in which individual animals are followed up longitudinally to provide a useful translational preclinical model.

Perinatal asphyxial brain damage resulting in hypoxic–ischemic encephalopathy (HIE) is a major cause of acute mortality and chronic neurological morbidity in infants and children (1,2); 20–50% of infants with HIE die within the newborn period; up to 25% of survivors exhibit permanent neuropsychological handicaps. As there are no interventions to prevent perinatal asphyxia, the focus is on developing therapeutics to limit cerebral injury progression and promote normal brain growth and development (3).

Therapeutic hypothermia (TH) has become standard therapy for full-term neonates with moderate-to-severe HIE, with significant neuroprotection supported by multiple randomized clinical trials demonstrating reduced risk of death or neurologic disability at 18–24 mo of age (4–6). However, protection is incomplete, and long-term outcome is not well defined. TH may not improve outcome in neonates with very severe HIE (1,5). Additionally, at 6–7-year follow-up, there was no significant difference in IQ scores between the TH and control groups, suggesting a failure to support cognitive function (4). Thus, additional preclinical studies are needed to explore the benefits and limitations of TH and define adjunct therapies to enhance long-term outcome.

TH safety studies and clinical trials in the neonate were derived from experimental studies in newborn piglets (7) and fetal sheep (8), which demonstrated histopathological neuroprotection with moderate hypothermia. The immature rodent model of unilateral hypoxia–ischemia (HI) has been used extensively since a larger number of animals can be studied and longitudinal assessments and functional outcome can be measured (9). There have been multiple investigations into TH, alone and with other therapies, in neonatal rats and mice, with quite variable results. Studies in the rat have used the P7 animal as originally described (10); however, the P7 rat brain more closely approximates a 32–36 wk gestational age (GA) infant, potentially making it less relevant to the full-term newborn (11).

The purpose of this study was to define and characterize a reproducible, preclinical model of HI plus TH in the immature rat. We utilize rats of both sexes at P10-11, as representative of the term infant brain, and introduce a standard period of hypothermia following HI. Each pup is followed up longitudinally with magnetic resonance imaging (MRI) and behavioral analysis to assess short- and long-term effects. We demonstrate here significant reduction of brain damage and improved motor, but not object recognition, function with this TH paradigm.

RESULTS

Hypothermic Protection Is Temperature Dependent

Following unilateral HI, pups were recovered in either a normothermic (35.5 °C) or hypothermic (30 °C) environment for 4 h. Hypothermic chamber temperature was chosen to achieve a target rectal temperature of 32 °C; rectal temperatures were monitored hourly. Temperatures in normothermic (N) pups ranged from 36.5 to 38.1 °C; hypothermic (TH) pups were 31–34 °C. In the initial studies, comparison of temperature with damage at 2 wk showed that TH was most effective at <33.0 °C; pups that remained >33 °C were significantly more damaged ($P \leq 0.01$; **Supplementary Figure S1** online). Pups that did not reach 33 °C were excluded from subsequent analysis.

Hypothermia Provides Long-Term Protection in Moderate and Severe Injury

Preliminary studies assessed damage at 24 h in hematoxylin and eosin–stained sections; we did not observe a significant effect of cooling (**Figure 1**). However, cell death following HI can continue for days to months; long-term outcome in a developing brain represents a balance between cell death and growth of viable tissue. Subsequent experiments included longitudinal analysis, with MRI and behavior through 12 wk.

We performed 10 experiments of HI plus TH; animals were evaluated with MRI at 2 wk. Damage was assessed with an ordinal damage score (DS) and calculation of regional volumes, as described in Methods (**Supplementary Figure S2a,b** online). There was a tight correlation between these two measures (**Supplementary Figure S2c** online). When all the data were considered together, there was a range of damage in both N and TH groups, with a trend toward lower DS with TH (**Figure 2**). On further examination, we observed that the experiments fell into three significantly different outcome groups based on the mean DS in the normothermic pups, reflecting mild ($n = 3$), moderate (N-M, $n = 4$), severe (N-S, $n = 3$) injury (**Figure 3**).
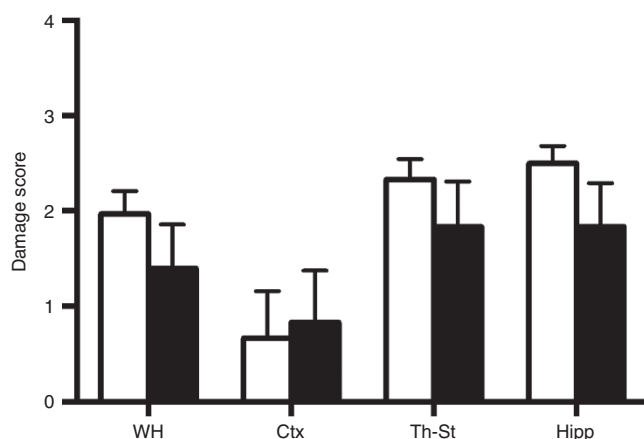
TH did not significantly reduce damage in the mild group, and these animals were not followed up further. When moderate and severe experiments were analyzed separately, TH resulted
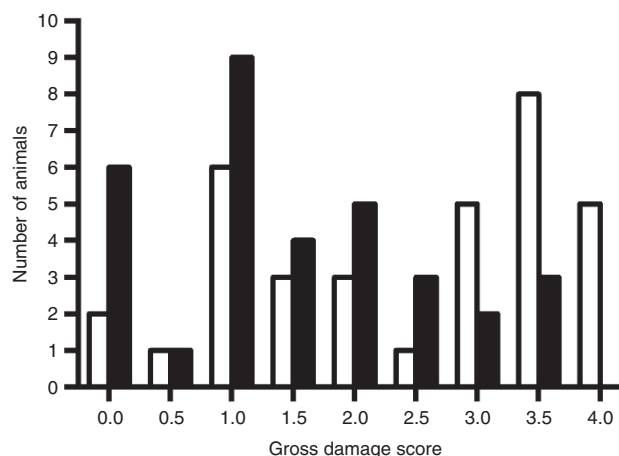

Figure 2. Effect of TH at 2 wk. DS was assessed in all animals from T_2 images demonstrating more animals with lower DS following TH (black bars) relative to normothermic (white bars) recovery. DS, damage score; TH, therapeutic hypothermia.
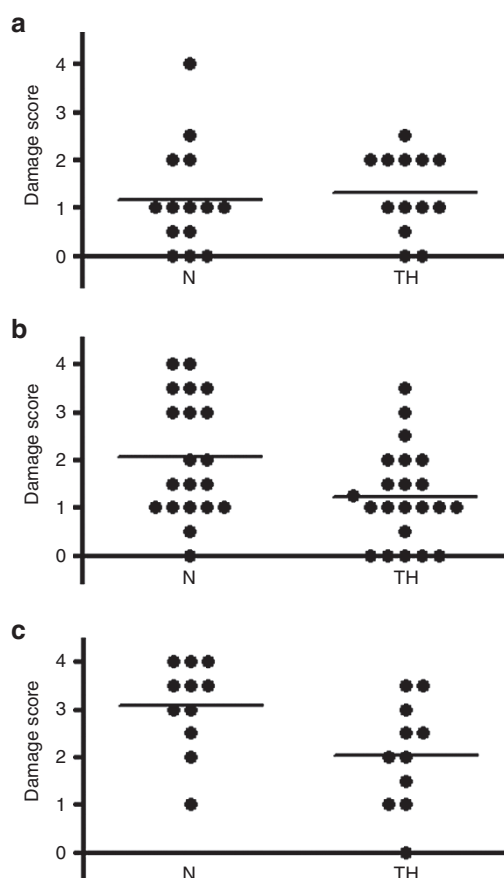
Figure 3. Effect of TH on mild, moderate, and severe damage. Experiments fell into three groups based on mean DS in normothermic (N) animals: (**a**) mild; (**b**) moderate; (**c**) severe ($P < 0.01$, ANOVA). DS in the moderate and severe groups was reduced with TH ($P < 0.01$ for both, unpaired *t*-test). DS, damage score; TH, therapeutic hypothermia.

Figure 1. TH is not protective at 24 hours after HI. P10-11 rats were subjected to HI and recovered in normothermic (white bars) or hypothermic (black bars) chambers, $n = 6$/group, and killed 24 h later. Hematoxylin and eosin–stained cryosections were analyzed for DS (ipsilateral) by two reviewers for whole hemisphere (WH), cortex (CTX), thalamus-striatum (Th-St), hippocampus (Hipp). HI, hypoxia–ischemia; TH, therapeutic hypothermia.

in a significant degree of protection in both groups (TH-M, TH-S, $P \leq 0.01$).

Regional volumes were calculated for ipsilateral and contralateral hemispheres (**Figures 4a,b**). TH was protective of neocortex and thalamus/striatum, but not hippocampus in both cohorts. Examination of individual ipsilateral/contralateral ratios (**Figure 4c**) is a measure of extent of growth of the injured, relative to the contralateral, hemisphere; an abnormality in the contralateral hemisphere is determined by comparison with control. Contralateral volumes were not different from controls, except for a significant reduction in hippocampal volume in the severe cohort (**Supplementary Figure S3** online), possibly indicating a

heightened vulnerability of the hippocampus to HI that is resistant to TH. **Figure 5** depicts the evolution vs. resolution of damage in 2 animals (N, TH), from the severe cohort: 24 h and 2 wk T_2 MRI images, and histology of corresponding sections. Interestingly, despite little apparent difference at 24 h, the injury in the N pup evolves into infarction and large cavitation at 2 and 12 wk; this process is reduced in the TH pup. Final DS derived from histological analysis of all brains at 12 wk demonstrated both the evolution of injury and confirmed long-term protection with TH (**Figure 6a,b**). Calculated change in DS for each animal between 2 (**Supplementary Figure S4** online) and 12 wk demonstrated minor evolution of damage in the N-M animals (**Figure 6c**),
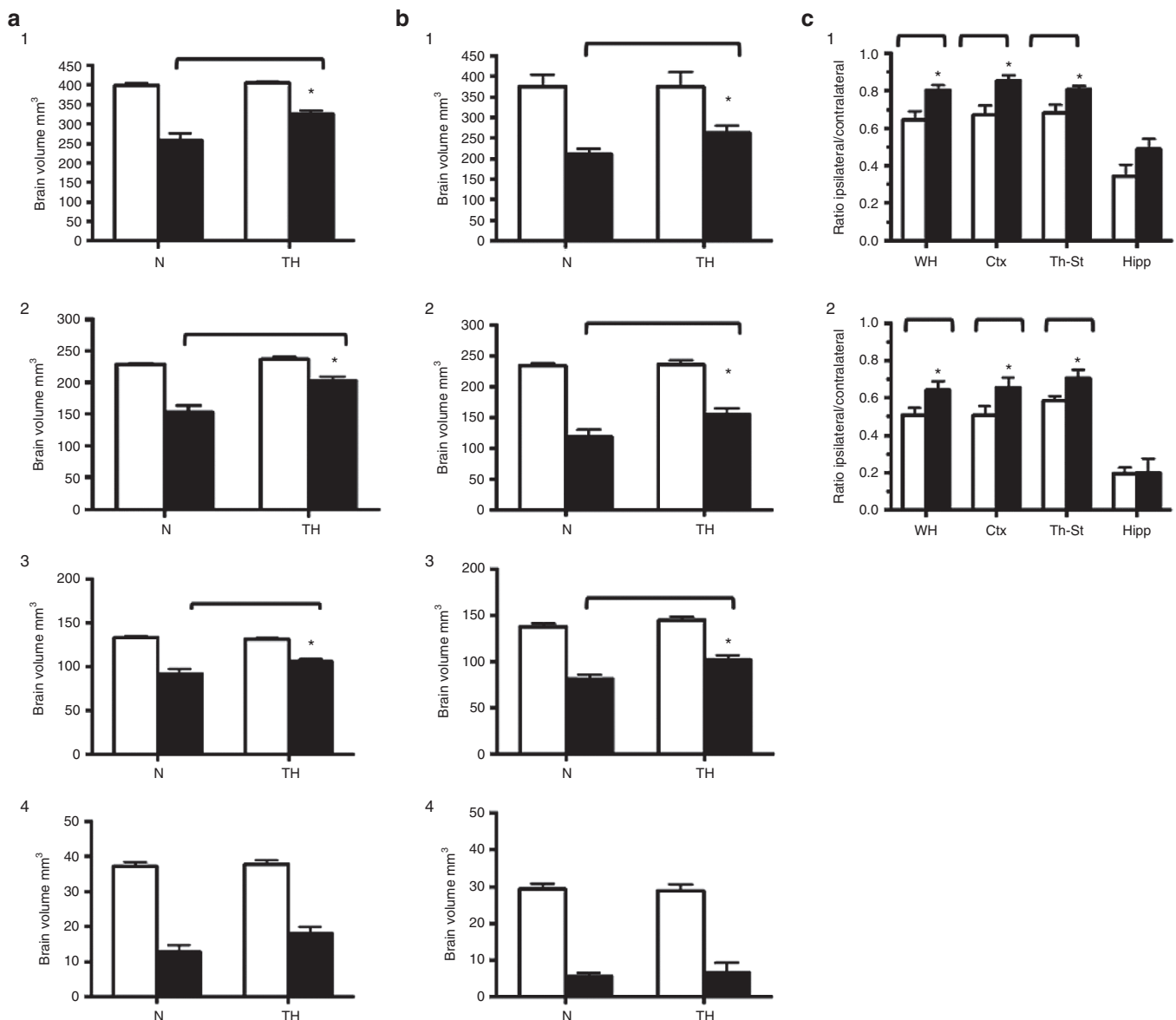

Figure 4. TH improves brain volumes at 2 wk except for hippocampus. Volumes of healthy brain tissue in contralateral (white bars) and ipsilateral (black bars) hemispheres were calculated from 2 wk MRI images as described in Methods. (**a**) Moderate, (**b**) severe; depicting volumes for: 1, whole hemisphere; 2, cortex; 3, thalamus-striatum; 4, hippocampus. TH reduced brain volume loss for all regions except hippocampus, *$P < 0.05$, ANOVA. (**c**) Paired ipsilateral/contralateral ratios, for normothermic (white bars) and TH (black bars) for: 1, moderate; 2, severe cohorts; *$P < 0.05$. MRI, magnetic resonance imaging; TH, therapeutic hypothermia.
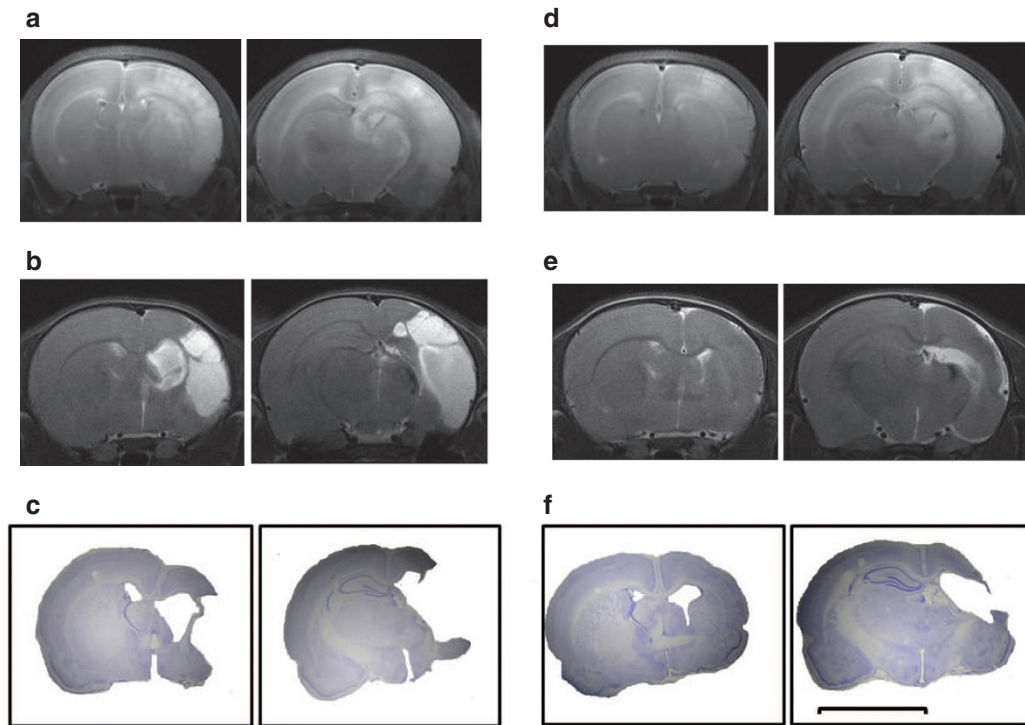
Figure 5. TH reduces progression of injury following HI: sequential T$_2$ MRI and histology. Representative MRI images obtained from 1 N (**a**–**c**) and 1 TH (**d**–**f**) pup at 24 h (**a,d**) and 2 wk (**b,e**) are compared with images from final histology of each animal at 12 wk (**c,f**). Each panel depicts an anterior and posterior coronal section. Bar = 5 mm. HI, hypoxia–ischemia; MRI, magnetic resonance imaging; TH, therapeutic hypothermia.
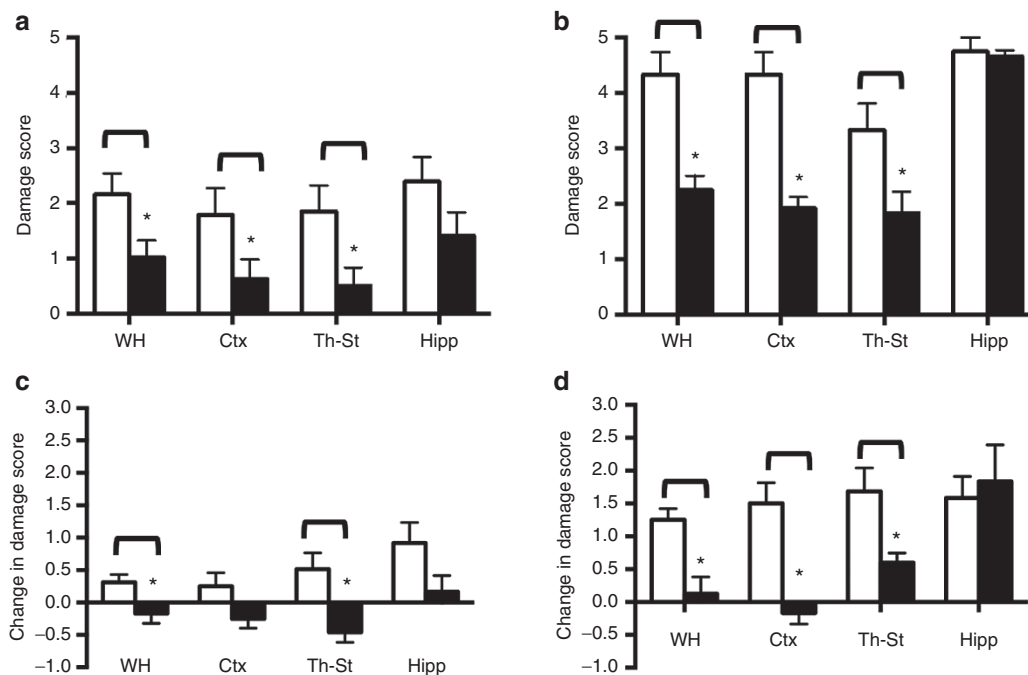

Figure 6. Effects of HI and TH on long term DS. Rats from (**a**) moderate and (**b**) severe cohorts were killed at 12 weeks post-HI. Cryosections were stained with cresyl violet and analyzed for hemispheric and regional DS as described. Change in DS between 2 and 12 wk was calculated for each animal in (**c**) moderate and (**d**) severe. White bars, normothermic; black bars, TH. *$P < 0.05$, unpaired *t*-test. DS, damage score; HI, hypoxia–ischemia; TH, therapeutic hypothermia.

with more striking changes in the severe cohort (**Figure 6d**). In both cohorts, DS in the TH groups were either little changed or actually improved, except for the hippocampus.

In these studies, we did not observe a significant effect of gender on extent of damage or degree of protection by TH (**Supplementary Figure S5** online).

Hypothermia Protects Motor Learning and Dexterity but not Object Encoding and Recognition Following HIE

To characterize protection of sensorimotor function conferred by TH, rats were tested as young adults (P50–70) on the beam walk test. This measurement of a rodent's ability to traverse an elevated beam is a robust test of locomotor control and is particularly sensitive to hindlimb deficits (12). Rats were tested on two beams of increasing difficulty. Successfully traversing the beam on at least 3 of 5 attempts was considered a "pass." The highest difficulty level passed differed as a function of group (Kruskal–Wallis test; $P < 0.001$; **Figure 7a**). All of the N and TH animals in the moderate cohort, and all but one of the controls, passed both levels successfully. By contrast, none of the rats in the severe cohort passed both levels and several did not pass either (**Figure 7a**). Overall, the performance of this group was significantly worse than either controls or moderate groups (all P values < 0.05).

For rats that passed level 2, videos were analyzed for left (affected hindlimb) and right foot slips (**Figure 7b**). For left foot slips, the mixed ANOVA revealed an effect of group ($F_{(2,19)} = 3.7$; $P < 0.05$) and an interaction between group and beam difficulty level ($F_{(2,19)} = 4.7$, $P < 0.05$). Planned comparisons at each difficulty level revealed an effect of TH on motor control at the more difficult level ($F_{(2,19)} = 5.9$ $P < 0.05$). N-M rats performed worse than both control and TH-M; the performance of the TH-M rats was significantly better than their noncooled littermates (Tukey honest significant difference; $P < 0.05$) and not different from controls (Tukey honest significant difference p > 0.4). There were no group differences for right foot slips.

Temporal lobe-mediated object memory was investigated with novel object recognition (NOR), a behavioral test of encoding and memory that relies on the animal's innate exploratory behavior and requires no pretraining, and no added stressors (13,14). NOR involves an initial exposure to two identical objects followed, after a delay, by presentation of one familiar object (from the previous sample phase) and one new object. Control animals spend significantly longer exploring the novel object; this is expressed by a discrimination index (DI_{NOR}) calculated as the difference between time spent with the novel vs. familiar object as a percentage of total time.

The DI_{NOR} differed across experimental groups ($F_{(4,26)} = 3.1$; $P < 0.05$; **Figure 7c**). As expected, naive controls spent more time investigating the novel than the familiar object; DI_{NOR} significantly greater than 0 (one-sample t-test, $t_{(8)} = 3.1$; $P < 0.05$). Performance of moderate-HI animals (N-M and TH-M) was not significantly different than controls (Student's t-tests; P values > 0.15) and indicated reliable recognition of the novel object ($DI_{NOR} > 0$; N-M: $t_{(4)} = 3.5$; TH-M: $t_{(5)} = 3.5$; P values < 0.05). However, relative to controls, moderate-HI groups showed shorter object exploration times during the sample phase, with a trend toward increased exploration time in the TH-M group relative to N-M (**Figure 7d**). Object recognition was significantly impaired in the severe HIE groups, with N-S and TH-S rats performing no better than chance (**Figure 7c**).
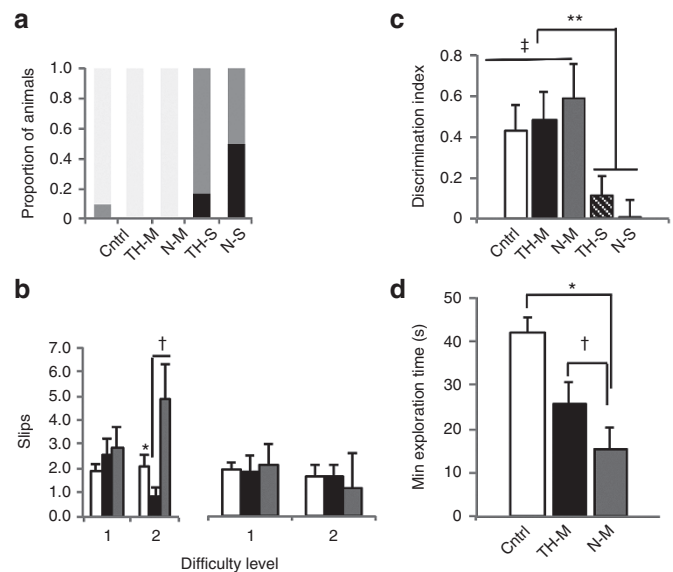

Figure 7. Motor and cognitive function in TH- and N-treated HI rats. TH improves motor performance on the beam test. (**a**) Beam difficulty at first failure. Most rats in control (Cntrl) and moderate (-M) groups pass to level 2 (white bars); severe (-S) groups fail at (gray bars)/before (black bars) level 1. (**b**) Contralateral hindlimb slips (left panel) on the difficult beam are increased in rats with moderate HIE with normothermic recovery (*$P < 0.05$, N-M, gray bars > Cntrl, white bars); TH-M (black bars) rats are less impaired (†$P < 0.05$, TH-M < N-M). No difference in ipsilateral (right) hindlimb slips (right panel). Object encoding and recognition is impaired by HI. (**c**) Cntrl (white bars) and moderate HIE rats (TH-M, black bars; N-M, gray bars) show above-chance recognition of the novel object (†$P < 0.05$ $DI_{NOR} > 0$); severe HIE rats do not (**$P < 0.05$, severe HIE:N-S (hatched bar) and TH-S (dark gray bar) vs. all other groups). (**d**) Object contact time for moderate HIE rats is less than for Cntrls (white bar) during the sample phase. There is a trend for TH (black bar) to mitigate this effect. *$P < 0.05$, moderate HIE vs. Cntrl; †$P < 0.1$, TH-M > N-M (gray bar). DS, damage score; HIE, hypoxic–ischemic encephalopathy; TH, therapeutic hypothermia.

DISCUSSION

In this study, we present a modification of our original model of HI in the P7 rat to more closely model the clinical situation of therapeutic hypothermia following birth asphyxia in the term infant. The major features of this modification are: (i) use of the P10-11 rat pup; (ii) temperature monitoring in all pups; (iii) 24-h MRI analysis to assess initial injury severity; (iv) subsequent 2 wk MRI analysis for evaluation of injury progression; and (v) evaluation of sensorimotor and cognitive outcomes as adults prior to sacrifice and histopathology. We propose that this preclinical paradigm will provide a valuable platform to both further understand the benefits and limitations of hypothermia following HI and to evaluate potential adjunct therapeutics to improve outcome.

This model was originally developed in the P7 rat pup, which approximates the cerebral development of a 32–36 wk gestation human infant (10). In the past 30 y, this model has come to be widely used as a preclinical model of HIE and was the paradigm in which the initial rodent studies of TH were conducted (9,15,16). Subsequent studies investigated delayed hypothermia and combination therapies (17–20) with quite variable results and inconsistent protection with TH. To more

closely model the full-term infant brain we have chosen to investigate the effects of hypothermia in the P10-11 rat pup (11,21,22). An important and potentially clinically relevant difference between the responses to HI at the different ages is the increased vulnerability of the hippocampus in the older rat pup, as previously described (23). The extreme vulnerability of the hippocampus to HI is replicated in the studies presented here, in both moderately and severely damaged animals. Further, the hippocampus was the only region that was not protected by TH, as has been recently reported clinically (24). Severe HI injury resulted in nearly complete loss of the ipsilateral hippocampus, as well as a decrease in the contralateral hippocampal volume, which is a new observation in this model and will be investigated in future studies.

The rigorous control of temperature during hypothermia is vital to both the efficacy and reproducibility of the protection. Target temperature in clinical practice of TH in asphyxiated infants is 33.5–34.5 °C (25,26), and most rodent studies aim to achieve a rectal temperature of 32–33 °C, as we did in the current study. Rectal temperature has been shown to correlate well with brain temperature in this model (16). Most studies monitor temperature in sentinel pups that are excluded from further analysis, based on the observation that the presence of the rectal temperature probe affects the outcome (27). We monitored rectal temperatures in all of the pups at hourly intervals and observed a range of post-HI temperatures in both normothermic and hypothermic animals. Importantly, we observed that those pups that "resisted" cooling and did not reach the target temperature of < 33 °C demonstrated a variable degree of damage including the most severe, whereas none of the effectively cooled pups were severely damaged. The reason(s) for this variation in response are unknown; however, postischemic hyperthermia is frequently observed consistent with severe damage in preclinical studies and in asphyxiated infants (28). An inability to reach the target temperature during cooling might reflect a more severe insult at the outset, or some other variable in the animal. We employ a cutoff temperature of 33 °C as an exclusion criterion in these studies.

The animal-to-animal variability in this model is well known. Since the original publication in 1981, we, and others, have made numerous technical adjustments to minimize the variability, including controlling litter size, reduced time of anesthesia, prewarming, and prehypoxia. However, as in human infants, there is still a variable response to HI, ranging from none to mild, moderate, or severe. The response to hypothermia is also variable with the greatest benefit observed in the context of moderate injury in both clinical trials and experimental studies. Thus, in order to evaluate the treatment effect, it is important to assess the severity of injury in each animal; MRI provides the tool to stratify experimental animals into mild, moderate, and severe injury groups. Of the experiments included in this study, three resulted in mild injury in normothermic pups with no added protection with TH (**Figure 3**); further analysis of the remaining pups showed that 4 h of TH immediately following HI in the P10-11 rat is protective in both moderate and severe injury.

An effective neuroprotective intervention must include long-term functional improvement, in addition to reduction in brain damage. Defects in locomotor and cognitive function are common in children with HIE and experimental animals following unilateral HI (29). A complete battery of behavioral tests will be needed to evaluate functional outcome. However, in this initial study, we used two simple tasks to evaluate sensorimotor function (beam walk) and recognition memory (NOR). Injury to the ipsilateral sensorimotor cortex is characteristic of this animal model, and the resulting motor impairments can be reliably detected by the beam walk test (12). The results of **Figure 7** reinforce the importance of evaluating experimental animals in the context of severity of injury. Impairment in beam walking was significant in N-M and worse in N-S cohorts. TH cohorts showed improvement of locomotor function on the beam walk, relative to their corresponding normothermic animals. Although we did not fully evaluate improvement of performance with time, a retest of the severely damaged cohort did not improve their scores (data not shown). Future studies will initiate testing at a younger age to follow a potential trajectory of recovery of motor function and possible effects of TH.

The NOR test was chosen for an initial evaluation of cognitive outcome as it relies on neuronal connections within the medial temporal lobe; object recognition has been shown to depend on temporal lobe circuits in both rodents and primates (30–32). The test is suitable to rats across a developmental timeline from weaning to old age, requires no pretraining and does not require use of potential stressors (33). Based on the measurement of the DI_{NOR} alone, it would appear that there was no effect of moderate HI damage in object recognition. However, HI animals did spend significantly less time with the objects in the familiarization/sample phase. Reduced exploration time has been reported before, both for neonatal HI (34) and traumatic brain injury in the adult rat (35). While preliminary, we note a trend for TH to normalize exploration time in the moderately damaged animals. Further testing will utilize an expanded NOR paradigm to tease apart relationships between object exploration and subsequent recognition, and the effects of HI and TH. Severely damaged animals were unable to perform this task at all (**Figure 7c**). The apparent "sparing" of the moderate group might relate to extent of bilateral damage to hippocampal–perirhinal circuits. Recent studies on NOR highlight the importance of the perirhinal cortex (31,32,36) and, although there is hippocampal damage in essentially all of the animals post-HI, the perirhinal cortex is more variably involved, especially in severe injury. It is noteworthy that while moderately damaged animals retained contralateral hippocampal volume, severely damaged animals did not, potentially compromising function in hippocampal–perirhinal circuits. It will be important to more fully characterize injury to hippocampal–perirhinal circuitry, and its relationship to NOR and other recognition memory tasks in this model over an extended timeline.

There are limitations to this study. MRI imaging is being increasingly utilized early in asphyxiated newborns to predict neurodevelopmental outcome (37–39). We have not yet

analyzed our MRI T_2 images to that extent, nor did we conduct a sufficient battery of neurodevelopmental tasks to fully assess outcome. However, this preclinical platform offers the opportunity to pursue such studies as well as include more advanced MRI imaging techniques to further define the trajectory of ongoing neurodegeneration or repair. Thus, we propose that this initial long-term study provides the basis for a useful, reproducible, and translationally relevant experimental paradigm to model the effects of therapeutic hypothermia following HIE in the term infant.

METHODS

Animal Model of Unilateral HI Plus Hypothermia

Timed pregnant Wistar rats (Charles River Laboratories, Wilmington, MA) in groups of three were purchased at embryonic (E) day 15, housed individually, and allowed to deliver vaginally. On the day of birth, pups were randomized and reassigned to dams (10/litter, ~ equal male and female).

Unilateral HI was induced in the right hemisphere at P10 according to our standard protocol (10,40), 8%O_2/bal N_2, 65 min. During hypoxia, the temperature in the chamber was maintained at 34.5 °C to achieve an average rectal temperature of 36–36.5 °C (nesting temperature), monitored in two nonligated pups (Physitemp, Clifton, NJ). Pups were randomly assigned to either normothermic or hypothermic recovery for 4 h. Normothermic recovery was in the same chamber as the hypoxia; hypothermic pups were placed in open jars in a water bath set at 28 to 30 °C to maintain a target rectal temperature of 32 °C. Pups were rewarmed and placed back with the dam, weaned at P21, and handled every other day. Following MRI and behavioral analysis, animals were deeply anesthetized with a fatal dose of pentobarbital prior to perfusion-fixation with 4% paraformaldehyde. Brains were removed, cryoprotected, and 50 μm sections were cut on a freezing microtome (Microm, Walldorf, Germany). All animal experiments were approved by the Weill Cornell Medical College IACUC.

MRI Analysis

Acquisition of images:T_2-weighted images were acquired at 24 h and 2-wk post-HI using a RARE sequence on a Bruker Biospec 7.0-T MR scanner with the following acquisition parameters: RARE factor 10, matrix 256×256, FOV 20×20 mm, slice thickness 1 mm, 17 contiguous coronal sections at P11, and 20 contiguous coronal sections at P25; TR_{eff} = 2,191.1 ms at P11 and 2,577.8 ms at P25, TE_{eff} 42.9 ms. A 72 mm Bruker linear coil was used for excitation and a quadrature brain coil was used for signal reception. MRI images from Bregma 2.52 to −5.40 (8 images) were given a gross DS according to the ordinal scale: 0 = no damage, 1 = atrophy alone, 2 = atrophy with ventriculomegaly, 3 = 10–25% infarct, and 4 = 25–100% infarct. Regions of interest (ROI) were traced (**Supplementary Figure 2b** online) using Image J software (NIH, Bethesda, MD). Regional volume measurements were calculated as the product of "intersection distance (1 mm) × area of normal tissue" for whole hemisphere, cortex, thalamus/striatum plus hypothalamus, and hippocampus.

Histochemical Analysis

Coronal brain sections from Bregma 2.52 to −5.40 (1 out of every sixth section) were mounted onto gelatin-coated slides. The sections were stained with a 1% cresyl violet solution (Sigma, St Louis, MO) and evaluated with light microscopy (Olympus, Tokyo, Japan). Using the same scoring system used for the MRI, overall and regional DSs were determined for each section.

Behavioral Analysis

The beam walking test was used to evaluate gross motor function at 6 wk post-HI. Beams were 40 cm long, elevated 32 cm from the table surface. Level 1 beam was flat and 2.5 cm wide; level 2 beam was a cylindrical rod of 2.5 cm diameter. A dark box was at the end of the beam; rats instinctively advance into the box when placed on a beam. Rats were placed on the beam 10 cm from the box to learn the test. Then, each rat was placed 40 cm from the box and given five attempts to cross the beam. Videos were recorded and the fails, i.e., inability to cross, right and left foot slips were recorded by an investigator blinded to the treatment.

The novel object recognition test was performed at 8-wk post-HI. The day before the test, rats were allowed to explore the testing chamber, a polycarbonate box (dimensions 40×40 cm) and allowed to explore for 12 min. The sample phase and recognition test were separated by a 6 min. During the sample phase, two identical objects were placed near the corners of the box, and the rat was allowed to explore both objects for 5 min. The rat was removed for 6 min, wherein the box was cleaned with a chlorine dioxide liquid (Clidox; Pharmacal Research Laboratories, Waterbury, CT), and one of the familiar objects was replaced with a novel object of the same material but a different shape. The rat was returned to the box and allowed to explore for 2 min. Any-Maze video tracking system software (Stoelting, Wooddale, IL) was used to record the time spent with novel and familiar objects. Object exploration was defined as the rat directing its nose toward the object at a distance of <2 cm. The videos were reviewed offline by two investigators blinded to the treatment. The DI_{NOR} was calculated as ((time with novel object − time with familiar object))/(total time spent with objects).

Statistics

All values were expressed as the mean ± SEM. Effects of HI and TH were assessed with Kruskal–Wallis and ANOVA tests for noncontinuous and continuous variables, respectively. Omnibus tests were followed by planned pairwise comparisons. *Post-hoc* tests detecting an effect of TH were corrected for multiple comparisons with Tukey's honest significant difference method. Fisher's exact test and Dunnett's *t* were also used for *post-hoc* analyses for the behavioral studies.

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