Spring 5-1-2015

Behavioral And Neuroanatomical Outcomes Of Early And Late Preterm Hypoxic-Ischemic Injury And The Neuroprotective Effects Of Whole-Body Hypothermia: An Animal Model

Haley C. Garbus
University of Connecticut - Storrs, haley.garbus@uconn.edu

Behavioral and neuroanatomical outcomes of early and late preterm hypoxic ischemic injury and the neuroprotective effects of whole-body hypothermia: an animal model

Haley Garbus

An Honors Thesis
Submitted in Partial Fulfillment of the
Requirement of the University Scholars Fellowship and
Honors Program
At the
University of Connecticut
2015

Table of Contents

I. Central Aims …… 3
II. Introduction …….. 4
III. Methods …… 6
A. Subjects …… 6
B. Induction of Injury …… 7
C. Behavioral Testing …… 7
1. Auditory Testing …… 7
i. Startle Reduction …… 7
ii. Apparatus …….. 8
iii. Single Tone Procedure ……. 8
iv. Silent Gap 0-100 Procedure …….. 9
v. Silent Gap 0-10 Procedure …… 9
vi. Frequency Modulation (FM) Sweep Procedure ……. 9
2. Learning and Memory Testing…… 10
i. Morris Water Maze…….. 10
ii. Non-spatial Water Maze …… 11
3. Visual Attention Testing …… 11
4. Sensorimotor Testing…….. 12
5. Social Preference Testing…… 12
i. Sociability Task …… 13
ii. Social Novelty Task ……. 13
6. Histology …….. 13
7. Cavaleri's Estimator …… 14
8. Statistical Analysis …….. 14
IV. Specific Aim 1- HI P3/P7…… 15
A. Introduction ……. 15
B. Methods ……..…… 16
C. Results ……. 17
1. Sensorimotor……..17
2. Rapid Auditory Processing …… 18
3. Learning and Memory …….. 21
4. Visual Attention …… 23
5. Histology ……. 25
i. Medial Geniculate Nucleus……28
D. Discussion ……. 29
V. Specific Aim 2- HI Hypothermia …… 32
A. Introduction……. 32
B. Methods…….. 33
C. Results ……. 34
1. Surgery Temperatures …… 35
2. Rapid Auditory Processing …….. 36
3. Visual Attention …… 38
4. Sociability ……..…… 38
D. Discussion…….. 40
VI. Discussion and Future Directions …… 44
VII. References ……. 46

Central Aims

The overarching aims of this research were: first, to characterize the anatomical and behavioral effects of preterm hypoxic-ischemic (HI) injury in rodent models; and second, to explore the method of passively induced whole-body hypothermia as a neuroprotective intervention following HI. Clinical research of preterm injured infants has shown a large range of behavioral deficits, including delays in language learning, spatial and non-spatial memory, visual attention, and motor coordination. The current studies focused on these behavioral abnormalities, using HI rodent models. Specifically, we explored a model for early preterm HI (HI induced on postnatal day (P) 3 in rats), and compared it to the previous models we have used for late preterm HI (HI induced on P7), specifically using behavioral testing to characterize long-term outcomes. Additionally, the current studies sought to better characterize the language deficits associated with HI injury in humans, but using a rodent model to test the "magnocellular theory" of rapid auditory processing deficits. Research indicates that the use of cooling (both head-selective and whole body) has been shown to help reduce HI injury in both human populations and animal models. We set out to investigate how whole-body hypothermia might be used to ameliorate the behavioral deficits in an HI model, and specifically designed our focus on early preterm HI by using a P3 animal model. The overall goal of these studies was to expand upon knowledge regarding the behavioral and anatomical deficits associated with HI, and neuroprotective strategies following HI specifically, passively-induced whole-body hypothermia.

Introduction

Hypoxic ischemic (HI) injuries result from compromised blood and/or oxygen flow to the brain. Such injuries can lead to mortality as well as long-term neurologic abnormalities in early preterm (gestational age < 34 weeks)/very low birth weight (VLBW < 3 lbs, 4 oz) infants (Annibale & Hill, 2008; Volpe, 2001; Volpe, 2009), late preterm (GA < 37 weeks) and full-term infants with birth complications (Fatemi et al., 2009, Volpe, 2001). Preterm/VLBW infants are vulnerable to HI injuries due to their fragile, underdeveloped cardiovascular systems and poor cerebral autoregulation. Fluctuations in blood pressure can result in ruptures of capillaries, resulting in bleeding within or around the ventricles (Volpe, 2009; McClure, Threlkeld, Rosen, & Fitch, 2006). Drops in blood pressure can also cause the collapse of capillaries and ischemic reperfusion failure, leading to non-hemorrhagic HI, and ultimately periventricular leukomalacia or PVL (Volpe, 2001). PVL is often seen in injured preterm infants, and is associated with cyst formation and the damage or destruction of white matter along the ventricles of the brain (Sizonenko, 2003; Paneth, 1990; Follett, Rosenberg, Volpe, & Jensen, 2000; Takashima et al., 2009; & Huang & Castillo, 2008). White matter necrosis affected fifteen out of fifteen premature babies in one study, and eight out of fifteen were affected by PVL (Follett, Rosenberg, Volpe, & Jensen, 2000). HI injuries can also arise in term infants following birth complications such as cord prolapse, placental disruptions/failure, or cord asphyxia (de Vries & Cowan, 2009; Johnston et al., 2001; Lai & Yang, 2011; Volpe, 2001). Full-term infants with HI injury typically exhibit gray matter damage in the cortex, hippocampus, basal ganglia, and/or thalamus (Martinez-Biarge et al., 2011 & Vanucci, R, 2000).

Unsurprisingly, both populations of HI-injured infants experience a wide range of cognitive and behavioral impairments. Although there are differences in timing, manner, and locations of HI injury between preterm/VLBW and full-term infants, both populations develop deficits in language (Steinman et al., 2009; Briscoe, Gathercole, & Marlow1998). Additionally, they experience impairments in learning and memory (Giminez et al., 2004; Luu et al., 2011; Luu et al., 2009; Vicari et al., 2004; Fazzi et al., 2009), visual attention (Mercuri et al., 1997; Mercuri et al., 1999; Cho et al., 2013), and motor coordination (Kono et al., 2011; Mercuri & Barnett, 2003; Mercuri et al., 2004; van Haasert et al., 2004).

A strong negative correlation exists between birth weight and/or gestational age and prevalence of psychiatric abnormalities (Volpe, 1997). One of these abnormalities is attention deficit hyperactivity disorder (ADHD), and its subtype attention deficit disorder (ADD). Both are attentional disorders that involve white matter abnormalities (Volpe, 2007). Such disorders in preterm/VLBW infants are not surprising due to the sensitivity of developing white matter at the time of the injury. Additionally, white matter damage leads to motor deficits in premature/VLBW HI injured children (McClure, Threlkeld, Rosen, & Fitch, 2006), and 5-15% of premature babies weighing less than 1,500 grams will be affected by cerebral palsy (Nanba et al., 2007). The deleterious attentional and motor outcomes have a significant impact on these children's development, and ability to perform in school, as well as to work later as adults. Meanwhile, the lesions on the cortex, basal ganglia, hippocampus, and/or thalamus in full-term HI injured infants have a stronger impact on the individual's information processing, problem solving abilities, and working memory. The degree of global brain injury that full-term infants with HI

injury experienced was negatively correlated with verbal IQ scores when tested at 4 years old (Briscoe, Gathercole, & Marlow, 1998).

HI injuries can be modeled in rodents via a surgical procedure, followed by hypoxic exposure. When rodents are given this injury early in life (P1-5), the pattern of injury is analogous to that which occurs in early preterm human infants. Later in life (P7-12), HI injuries in rats result in a pattern similar to that which appear in late preterm to full-term human infants. In human clinical trails, it has been found that whole-body or head-specific cooling following a full-term HI injury ameliorates the subsequent deleterious effects. However, cooling has not been investigated as a neuroprotective method in preterm infants with HI injury. Therefore, the aims of these studies were to: establish the behavioral differences between rodent models given a HI injury at P3 and P7; and to determine whether whole-body cooling is an effective method for preventing the adverse behavioral and histological outcomes of a P3 HI injury. If rodents with HI at P3 were found to be a suitable model for preterm/VLBW injury in human infants, we could use this model to assess the efficacy of hypothesized neuroprotectants against the injury. Furthermore, the model could be used to understand the differential outcomes for neonatal HI injury in preterm versus full-term infants. Moreover, if cooling is found to be an adequate neuroprotectant against preterm HI injury in the P3 rodent model, it could be used in humans to prevent the deleterious effects of HI injuries.

Methods

A. Subjects: Time-mated female Wistar rats from Charles River Laboratories arrived no later than embryonic day 8 (E8) for both studies. Dams were housed at the University of Connecticut Bousfield animal facility in a 12-hour light/dark cycle (7 AM lights on),

where they gave birth. Following birth, pups were culled into litters of eight, with two females for every eight females. Only male rats were used for both studies.

B. Induction of Injury: On either P3 (Specific Aims 1 and 2) or P7 (Specific Aim 1), pups were randomly selected for sham or HI procedure (treatment balanced within a litter). For both studies, surgeries were performed by graduate students in Dr. Fitch's lab, who have had training and expertise in the surgical procedure. On surgery day, HI pups were anesthetized with isoflurane (2.5%) and a mid-line incision was made in the neck. The right carotid artery was located and cauterized. The incision was sutured and each animal was given a footpad injection indicating its identity number. Sham animals were also anesthetized with isoflurane and given a mid-line incision in the neck, but with no manipulation of the carotid artery. All pups were then placed under a heating lamp and allowed to return to their dams for two hours to recover from anesthesia. Then, the HI animals were placed in a hypoxia chamber containing about 8% humidified oxygen (balanced with nitrogen), while the sham animals were exposed to open air; for 120 minutes. All procedures were approved by UConn's IACUC committee.

C. Behavioral Testing

C.1. Rapid Auditory Processing

C.1.i Startle Reduction: The startle reduction paradigm utilizes the subject's acoustic startle reflex (ASR). The ASR is a relatively large motor response to a startle-eliciting stimulus (SES; in this study, a 150 dB white noise burst). When the SES is coupled with a benign acoustic stimulus just prior to the burst, the animal exhibits pre-pulse inhibition (PPI) or startle reduction. This procedure provides an indirect measure of the animal's ability to detect the cue based on the magnitude of the startle attenuation elicited by the

prepulse cue (see Fitch et al., 2008 for review). Using this procedure, the animal's startle response on cued versus uncued trials as a function of cue properties (duration or complexity) can provide an indirect measure of detection of the pre-SES cue. Age-adjusted variations in the acoustic tasks were used in juvenile (P 21-50) and adulthood (P60+) periods to evaluate auditory processing abilities in HI, HI plus hypothermia, and sham animals (Specific Aims 1-2). Cues proceeding the SES include a 75 dB, 2300 Hz pure tone (Normal Single Tone); gaps of varying duration (0-100 ms) in a 75 dB broadband white noise background (Silent Gap); and the reversal of a variable duration frequency modulated sweep (FM Sweep). For all tasks, the temporal stimulus parameters (ISI duration) are manipulated in an increasingly demanding sequence to assess processing thresholds at different ages and in different groups.

C.1.ii. Apparatus: During auditory testing, each subject was placed on a Med Associates PHM-250 load cell platform in a black polypropylene cage in a quiet testing room.

C.1.iii Normal Single Tone: The single tone task consisted of 103 cued and uncued trials. Animals were tested in one session, and trials were presented in random order. The single tone task was used to determine any hearing or processing deficits before more complex auditory processing tasks take place. Uncued trials consisted of a silent background and then the SES. On cued trials, a 75-dB, 7-ms, 2300-Hz tone was presented 50 ms before the SES. Analysis by treatment groups is preformed to make sure all animals can demonstrate prepulse inhibition, and that there are no confounds such as hearing impairment or lack of startle.

C.1.iv. Silent Gap (SG) 0-100 Procedures: The SG 0-100 procedure consisted of 299 cued or uncued trials per session. All animals were tested on one session/day for four to five days as juveniles. As adults, animals in the P3/P7 study were tested on one day. For all trials, a background broad-band white noise (75 dB) is played. On uncued trials, there was no interruption of the white noise before the SES. Cued trials featured a pause in white noise 50 ms before the SES with a 2, 5, 10, 20, 30, 40, 50, 75, or 100 ms duration. Attenuation score (Att) was calculated for each subject and gap and this measure was used for analysis.

C.1.v. Silent Gap (SG) 0-10 Procedure: This procedure was conducted in the same way as the SG 0-100 task, but cued trials consisted of a break in the white noise 2-10 ms long before the SES.

C.1.vi. Frequency Modulation (FM) Sweep Procedure: The FM sweep procedure consisted of 102 trials per session. All animals received one session per day of testing for five consecutive days. The background was comprised of a repeated presentation of continuous 75-dB downward frequency sweeps (2,300-1,900 Hz). The sweep could vary in duration (275, 225, 175, 125, or 75 ms) in order to adjust difficulty. Each sweep duration was administered separately within one session and on different days, progressing from easiest to most difficult. Each day of testing, the sweep duration decreased by 50 ms. Sweeps were separated by a between-stimulus ISI, always 200 ms greater than the sweep duration (faster). Uncued trials consisted of the same downward sweep presented 50ms prior to the SES. Cued trials consisted of a reversal of the previous sweep (low to high instead of high to low) but identical in timing, presented 50 ms prior to the SES. All auditory testing took place in both juvenile and adult periods.

C.2 Learning and Memory Assessment:

Before any memory testing took place, all animals were tested on a one-day water escape task to insure no motor deficits existed that might confound the results. The water escape task used an oval tub (40.5 in x 21.5 in) filled with room temperature water and a visible escape platform at one end. Each animal was placed at the end of the tub opposite of the escape platform, and the latency to the platform was collected and used for analysis. All animals were tested in adulthood.

C.2.i. Morris Water Maze (MWM): Spatial ability was evaluated using the MWM. Testing took place for five consecutive days in a 48-inch diameter hard plastic tub with a six-inch escape platform submerged below the water line so the platform was invisible. For each trial, the escape platform was located in the same quadrant in the tub. The tub was surrounded by various extra-maze cues in the room (painted shapes on the wall, the experimenter, lights, etc.), and there were no intra-maze cues. The start position never repeated in one day, and the animal's start position varied randomly between testing days. Each testing day consisted of four trails. The animal's trajectory was recorded with a Sony Digital 8 video camera, which was connected to a Dell Dimensions E521 computer. Installed on the computer was SMART Version 2.5 tracking software, which recorded the animal's velocity (measured in cm/sec), total distance swum (measured in cm), and latency to platform (measured in seconds). For each trial, the animal was given 45 seconds to locate and reach the submerged platform. If the animal failed to reach the platform in 45 seconds, it was gently guided to the platform and allowed to sit for 5 seconds before being removed from the maze.

C.2.ii. Non-spatial Water Maze: Non-spatial ability was evaluated using the non-spatial water maze. Testing took place for five consecutive days in a 48-inch diameter hard plastic tub with a six-inch escape platform submerged below the water so that the platform was invisible to subjects. Four different black and white striped panels (vertical, horizontal, diagonal left, diagonal right) were placed on the interior walls of the maze, and the platform was always placed in front of the panel with vertical stripes. The locations of the stripe cues, which were randomly selected at the beginning of each testing day, changed with each trial (as did the location of the platform). Thus, the internal cues shifted relative to the room across trials, but the target cue indicating the platform remained constant. The starting place for the subjects remained constant through out all trials. Thus the task required the animals to associate the vertical stripes with the platform, and retain that information through out the task. Latency, path length, and swim speed was collected and used for analysis.

C.3. Visual Attention: Animals were tested on a five-choice serial reaction time task to assess visual attention. For a week prior to testing, animals were food restricted (5 grams of chow per 100 grams of weight per day) in order to reach 85% of their initial body weight. The testing chamber consisted of a curved wall with five square windows and a food pellet dispenser on the opposite wall. Animals had to attend to all five holes and correctly identify the illuminated hole with a nose poke. Correct identification of the window resulted in a reward of a sugar pellet (45 mg, BioServe).

Animals were gradually trained on progressively harder tasks. Training began with a day of habituation and a day of nose poke training. Attention training tasks included (listed in order of difficulty): 60-second stimulus duration, 30-second stimulus duration, 10-second stimulus duration, 5-second stimulus duration, and one half-second stimulus duration. All animals were moved onto the next raining task together when all group averages (mean HI performance, mean sham performance) reached 70% correct. In the variable inter-trial interval (VITI) task, the position of the illuminated holes and the inter-trial interval were varied and the stimulus duration was either 10 seconds or 5 seconds (depending on the difficulty of the task). All visual attention tasks were performed in adulthood.

C.4. Sensorimotor Task: Rota-Rod: Animals were tested on a Rota-Rod task to assess motor coordination. Four animals at a time were placed on a rotating drum that slowly accelerated over the course of five minutes, from four to forty-four rotations per minute. Subjects were given two trials per day for five days, and the latency for the subjects to fall (in seconds) was recorded and averaged for each day. These averages were then used for analysis.

C.5.Social Preference: Animals were habituated to a large, metal, oblong tub for five minutes one day before testing. Animals were habituated for another three minutes prior to the tasks on the day of testing. During testing, the tub was divided into three sections of equal size. For each testing and habituation period, animals were placed in the central chamber, which did not contain anything. In each of the side chambers was an inverted plastic storage crate with holes. A video camera was used to record the activity of each rat, which included counts of rearing, grooming, and amount of time spent in each region of the apparatus.

C.5.i. Sociability Task: Following habituation, each animal was returned to its home cages to rest. Then, the rat was placed in the central section of the apparatus for three minutes, but this time, the crate in one of the side chambers contained an inanimate object (stuffed animal) while the crate on the other side contained a rat of the same sex. Animals were recorded for five minutes, and the number of approaches to the object, approaches to the rat, amount of time with the rat, and amount of time with the object were measured.

C.5.ii. Social Novelty Task: Following the social preference task, animals were returned to their home cages to rest for ten minutes while the apparatus was cleaned. Then, each rat was placed in the center of the apparatus, this time with the rat from the social preference task in one of the side chambers, and a novel rat in the opposite chamber. The amount of time the subject spent with each rat was measured for analysis.

C.6. Histological Analysis: Once behavioral testing was completed, all animals were transcardially perfused. Animals were weighed and anesthetized with an intraperitoneal (IP) injection of ketamine (100 mg/kg) and xylazine (15 mg/kg). Rats were perfused with .9% saline and 10% buffered formalin. Following perfusion, brains were extracted and placed in a 30% sucrose solution twenty-four hours prior to slicing on the cryostat. Brain tissue was sliced coronally at sixty micrometers. Every third slice was saved and mounted on a slide in sequential sections through the whole brain. Tissue was stained using cresyl violet procedures or with parvalbumin procedures.

C.7. Cavaleri Estimator: StereoInvestigator software on Cavaleri Estimator was used to quantify brain damage. Specifically, volumes of the left and right volume of cortex, hippocampus, lateral ventricles, and striatum (as a measure for the basal ganglia) were taken for each brain. The same software also measured the area of the corpus callosum. Every other mounted section was counted so that there were sixteen to eighteen representative sections throughout the brain.

C.8. Statistical Analysis: Statistical analysis was performed using SPSS 15.0 software and an alpha criterion of 0.05. Two-tailed analyses were used unless otherwise stated. Initial analysis compared scores for the two sham groups on the P3/P7 study (P3 and P7). In the P3 cooling study, however, there were not enough shams to compare the heated and cooled sham groups, so they were pooled with no differences assumed. For each behavioral task, groups were compared using analysis of variance (ANOVA) with multiple levels of treatment. Based on specific *a priori* hypotheses, planned comparisons were performed (as a function of Treatment) between paired groups. In the P3/P7 study, P3 HI versus sham, P7 HI versus sham, and P3 HI versus P7 HI were compared. During the P3 HI cooling study, P3 HI Normal Temperature versus sham and P3 HI Hypothermia animals were compared.

Repeated measures ANOVA were used to analyze each behavioral task. For rota-rod, variables included Treatment and Day. For the rapid auditory processing task, variables included Treatment, Day, and Gap. Both MWM and NSWM maze tasks used variables that included Treatment and Day. For the visual attention task, variables included Treatment and Day. For the social novelty task and social preference tasks, independent samples t-tests were used, where the variable was Treatment. One-way ANOVA used to

analyze volume of histological structures for both left and right hemispheres. For the corpus callosum, total area was measured using StereoInvestigator software. A two-sample Kolmogorov-Smirnov test was used to analyze cell size distribution in the medial geniculate nucleus.

Location of research facilities: All research took place in the Bousfield psychology building at the University of Connecticut under the supervision of Dr. R. Holly Fitch.

Specific Aims

Aim 1- HI P3/P7:

A. Introduction

The object of Aim one was to characterize the differential behavioral outcomes of HI injury on two different models, specifically at P3 and P7. Many clinical studies exist characterizing the outcomes of HI injury in early preterm and late preterm injured infants separately, and pre-clinical studies using a P1-3 or P 7-10 rodent HI model. However, there are few human or animal studies that directly compare and contrast profiles for the two injuries in terms of behavioral and/or neuroanatomical outcomes. One study, however, found that the inflammatory profile following HI injury in P1 versus P12 HI injured rats differs greatly (Brochu, Girard, Lavoie, & Sebire. 2011). In terms of the clinical literature that characterizes the effects of HI as a function of time, a strong negative correlation exists between birth weight and/or gestational age at birth and psychiatric abnormalities (Abel et al., 2010). Such abnormalities include deficits in attention, language, and motor ability. Attention deficit hyperactivity disorder is a common disorder found in premature and low birth weight children, and is associated with damage to the striatum, which occurs during premature HI (Bhutta et al., 2002).

Additionally, VLBW children show difficulties in standardized language tasks and language comprehension, and language production at four years old (Ortiz-Mantilla et al. 2008 and Jansson-Verkasalo et al. 2004).

In a study comparing P1 and P7 HI injured pups (from our lab), P1 HI injured rats were found to show deficits in RAP as juveniles, but these deficits were ameliorated by adulthood (McClure et al., 2006). Furthermore, P7 HI injured animals showed decreased right cortical, right hippocampal, and corpus callosum volumes, whereas P1 HI injured animals showed no significant alterations in neuroanatomical volume as compared to shams (McClure et al., 2006). For this study, a P3 HI animal model was chosen to model HI of early prematurity. P7 rodents have been used in previous studies in Dr. Fitch's laboratory to study the effects of late preterm HI injury, and therefore, they were used in this study as well.

It was hypothesized that animals given HI at P3 would show less severe deficits than P7 animals on all tasks. It was also hypothesized that the white matter in the P3 brain would be affected more than grey matter (Volpe, 1997 and Volpe, 2001). Meanwhile, it was hypothesized that animals given HI on P7 would have overall worse behavioral outcomes in tasks that require the use of gray matter structures.

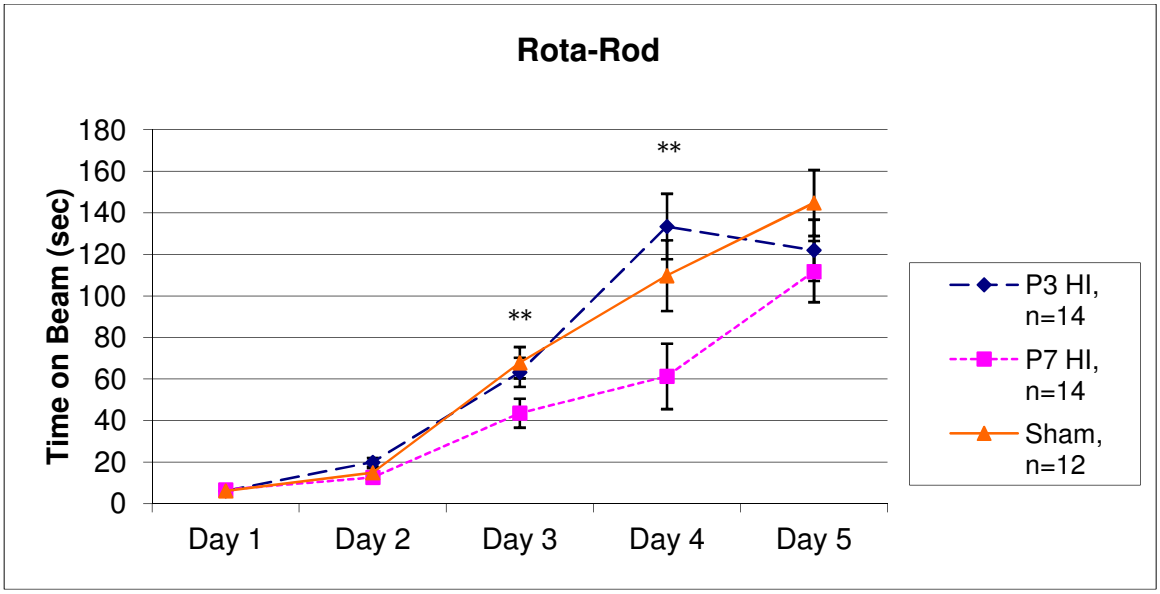
B. Methods for Aim 1: Procedures for Study One are described in the general methods section above. For the induction of HI injury, a subset of animals had HI induced on P3 (n = 14), and a subset of animals had HI induced on P7 (n = 14). As a control measure, a subset of shams received the sham surgery on P3, and another subset of shams received the sham surgery on P7. Only males were used in this study. Rota-Rod testing took place in the juvenile period (P28-32). Animals were tested every other day for five days.

Auditory testing took place in both juvenile (P 34-38) and adulthood (P60) periods. MWM testing, NSM (P80-95) and visual attention testing (P100-140) took place in adulthood. Perfusion techniques, slicing, and CV staining procedures are detailed above in the General Methods Section.

P3/P7 Study Results

Rota-Rod (P28-32): A 5 (Day) x 3 (Treatment) repeated measures ANOVA revealed a significant overall Treatment effect [($F(2,37) = 5.247$, $p < .05$] (figure 1a). Upon closer examination, a Day x Treatment interaction was revealed [$F(1,8) = 2.719$, $p < .05$]. There was no difference between P3 HI animals and sham ($p > .05$), but P7 HI animals and shams were significantly different ($p < .05$). There was a significant treatment effect between P7 HIs and shams [$F(1,24) = 12.776$, $p<.01$], with P7 HI subjects performing worse. Independent t-tests revealed specific differences on Day 3 of testing [$t(24 = 2.461$, $p<.05$], and on Day 4 of testing [$t(24) = 3.199$, $p<.05$], with P7 HI subjects performing worse. A 5 (Day) x 2 (Treatment) repeated measures ANOVA revealed a significant Treatment effect between P3 HI and P7 HI groups [$F(1,26) = 7.261$, $p <.05$], with P7 HI subjects performing worse. Additional independent t-tests revealed differences on Day 2 [$t(26) = 2.334$, $p<.05$], on Day 3 [$t(26) = 2.132$, $p<.05$], and Day 4 [$t(26) = 2.985$, $p<.05$] of testing.

Figure 1a



Rapid Auditory Processing:

Normal Single Tone (NST) (P30 & P58): There was a significant Treatment effect found in juveniles [$F(2,37) = 3.753$, $p<.05$] and adults [$F(2,39) = 3.812$, $p<.05$] on the normal single tone task (figure 1b.). A difference in body weight across the three groups of animals may have been the most likely cause of this difference, with HI P7 animals showing significantly lower body weights than the other two groups. As a result, all further auditory measures were covaried with individual scores from the Normal Single Tone (NST) data.

Figure 1b

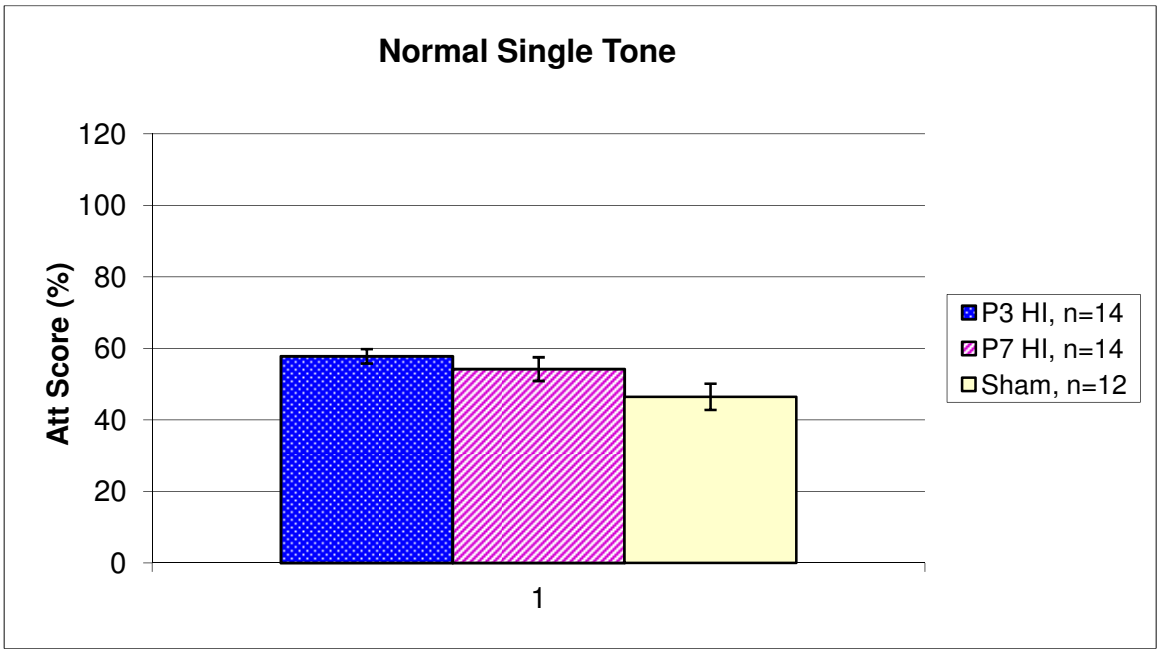
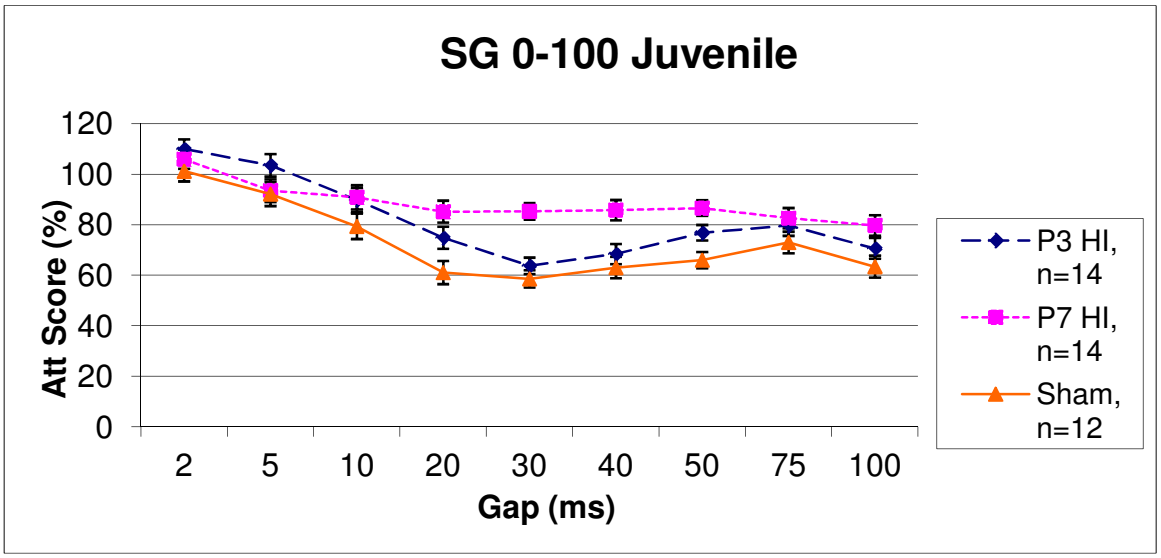


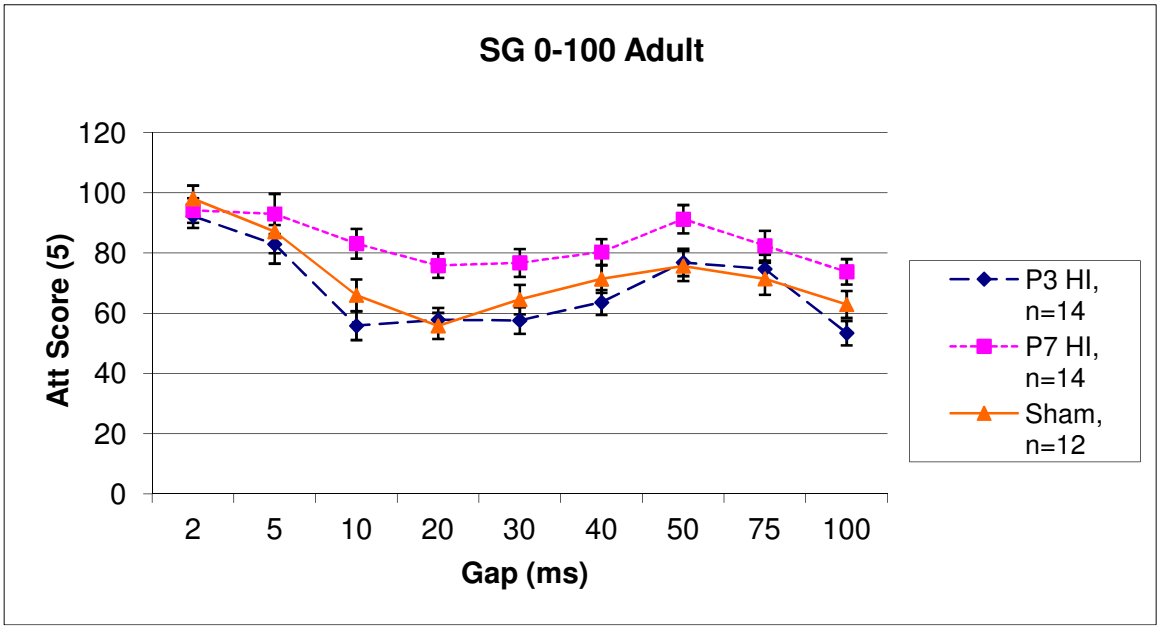
Figure 1c



Silent Gap 0-100: For juvenile RAP testing, on an occasional test day, one or even two data files were compromised due to unpredicted computer buffer overflows. As a result of this need to drop that data, there are slight variations in degrees of freedom for behavioral analyses. We found an overall Treatment effect in juveniles [$F(1,2) = 7.48$, $p < .05$] in the Silent Gap 0-100 task (figure 1c). Further analysis revealed a significant Gap x Treatment interaction [$F(1,16) = 3.03$, $p < .05$]. Upon closer analysis, P3 HI were trending to perform worse than shams [$F(1,1) = 3.93$, $p = .07$], and HI P7 were performing worse than shams [$F(1,1) = 14.749$, $p < .05$]. There was no difference between P3 HI and P7 HI groups [$F(1,9) = 4.405$, $p > .05$].

As adults, animals were retested on Silent Gap 0-100, and a 9 (Gap) x 3 (Treatment) repeated measures ANOVA revealed an overall Treatment effect [$F(2,36) = 5.799$, $p < .05$] (figure 1d). There was no longer a significant difference between shams and P3 HI [$F(1,23) = .201$, $p > .05$], but the difference between P7 HI and sham animals remained [$F(1,23) = 3.699$, $p < .05$, one-tailed). There was also a difference found between P3 HI and P7 HI groups [$F(1,25) = 10.085$, $p < .05$], with P7 HI performing worse compared to both shams and P3 HI animals.

Figure 1d

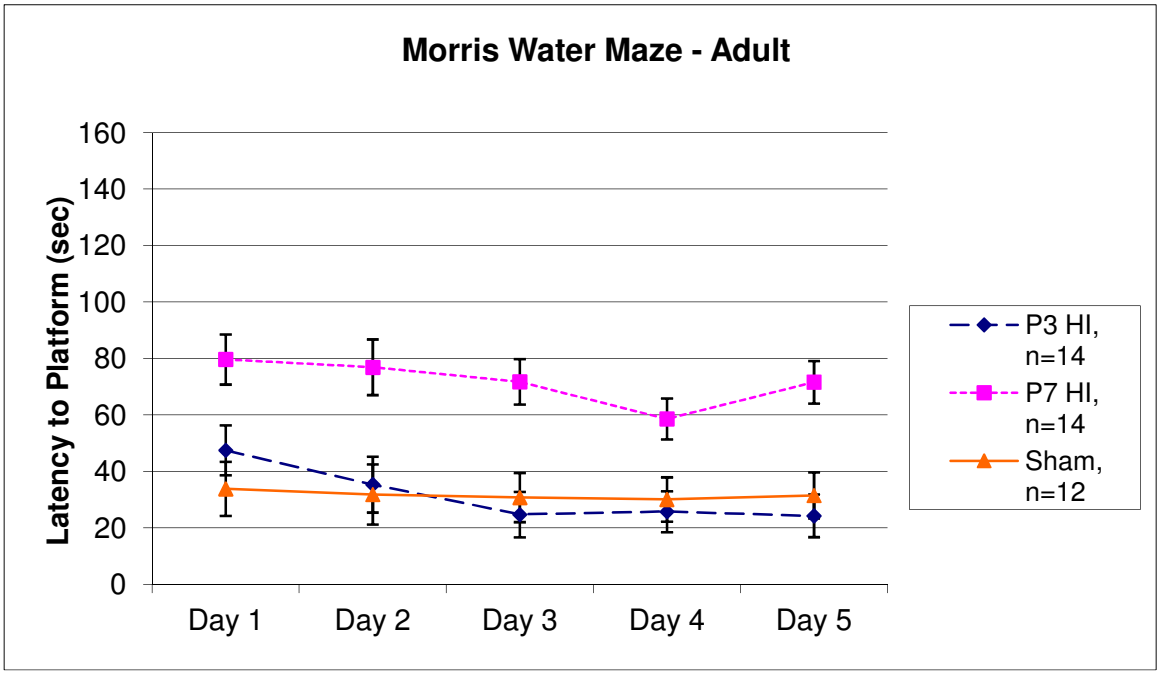


C.3. Learning and Memory (P80-95)

Water Escape (P80): A Univariate ANOVA revealed no differences among groups on the water escape task [$F(2,37) = 2.024$, $p > .05$]. Therefore, we concluded that there were no differences among groups in swimming ability (data not shown).

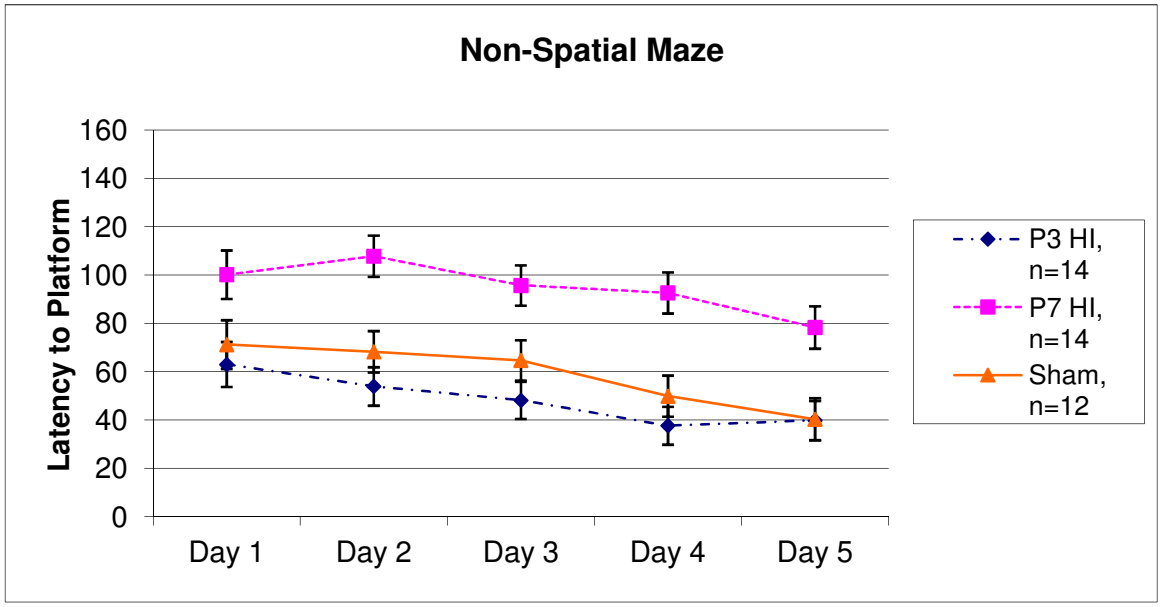
MWM: A 5 (Day) x 3 (Treatment) repeated measures ANOVA showed no significant differences in swim speed [$F(2,37) = .502$, $p > .10$] across groups. Again, this suggests that there were no underlying differences in either HI groups in swimming ability or vision that would confound further results. An overall 5 (Day) x 3 (Treatment) repeated measures ANOVA did reveal a significant Treatment effect [$F(2,37) = 9.959$, $p < .05$] for latency to reach the platform (Figure 1e). A follow up repeated measures ANOVA revealed no difference between P3 HI and shams [$F(1,24) = .00003$, $p > .10$]. A significant difference was found between P7 HI animals and shams [$F(1,24) = 10.098$, $p < .01$], with P7 animals performing worse. Finally, there was a difference between P3 HI and P7 HI groups [$F(1,26) = 10.985$, $p < .05$], with P7 HI performing worse.

Figure 1e



Nonspatial Water Maze (P91-95): A 5 (Day) x 3 (Treatment) repeated measures ANOVA revealed no differences in swim speed among groups [$F(2,37)= 2.223$, $p > .10$]. A 5 (Day) x 3 (Treatment) repeated measures ANOVA revealed an overall Treatment effect [$F(2,37) = 10.10$, $p < .01$] for latency to reach the platform (Figure 1f). Follow-up repeated measures ANOVAs revealed no difference between the P3 HI group and shams [$F(1,24) = 3.385$, $p > .10$]. There was, however a difference between P7 HI and shams [$F(1,24) = 9.232$, $p < .01$] as evidenced by a 5 (Day) x 2 (Treatment) repeated measures ANOVA, with HI P7 animals performing worse. Further analysis also revealed a difference between P3 HI and P7 HI animals [$F(1,24) = 16.834$, $p < .01$], with P7 HI animals again performing worse.

Figure 1f



Visual Attention (P100-140):

One sham was dropped from visual attention testing due to time constraints in the testing day. Therefore, the sham group was n = 11 for visual attention analyses.

5-second stimulus duration: On the 5-second stimulus duration task, a 6 (Day) x 3 (Treatment) repeated measures ANOVA showed an overall effect of Treatment [$F(2,26) = 3.122$, $p < .05$] (figure 1g). Additional repeated measures ANOVAs showed a significant difference between P3 HI and shams [$F(1,23)= 5.845$, $p < .05$], with P3 HI performing worse. No differences were found between P7 HI animals and shams [$F(1,22) = .570$, $p > .10$] or between P3 HI and P7 HI [$F(1,25) = 2.64$, $p > .10$].

Figure 1g

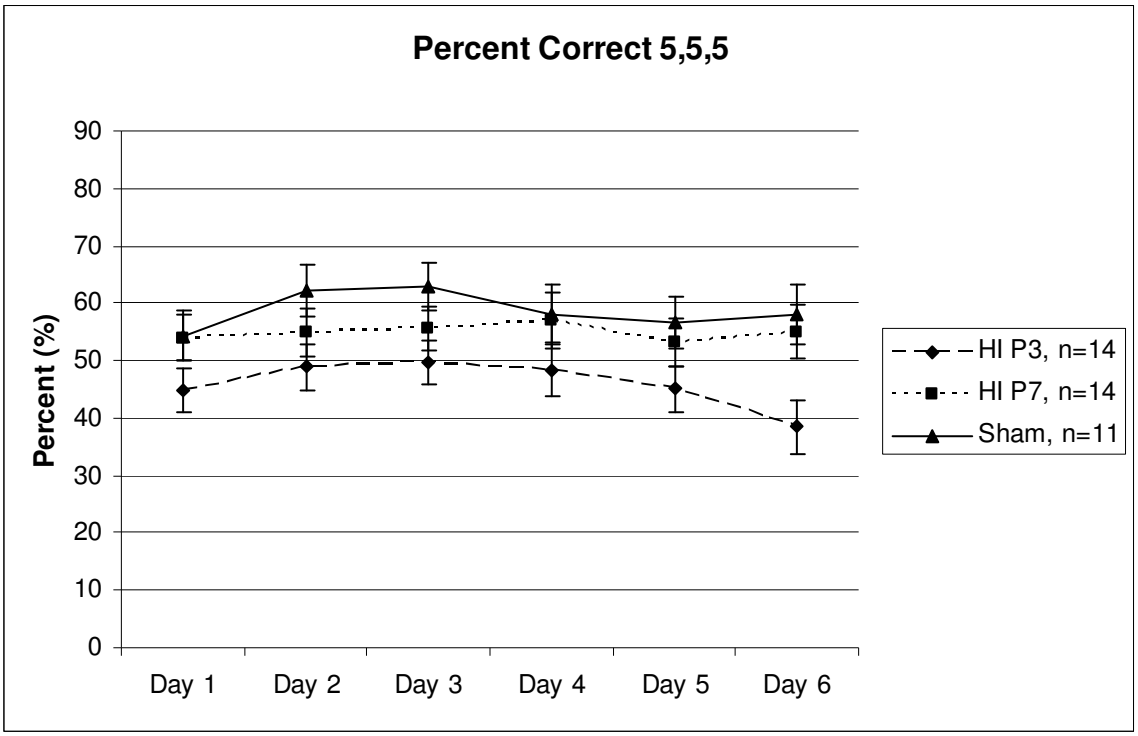
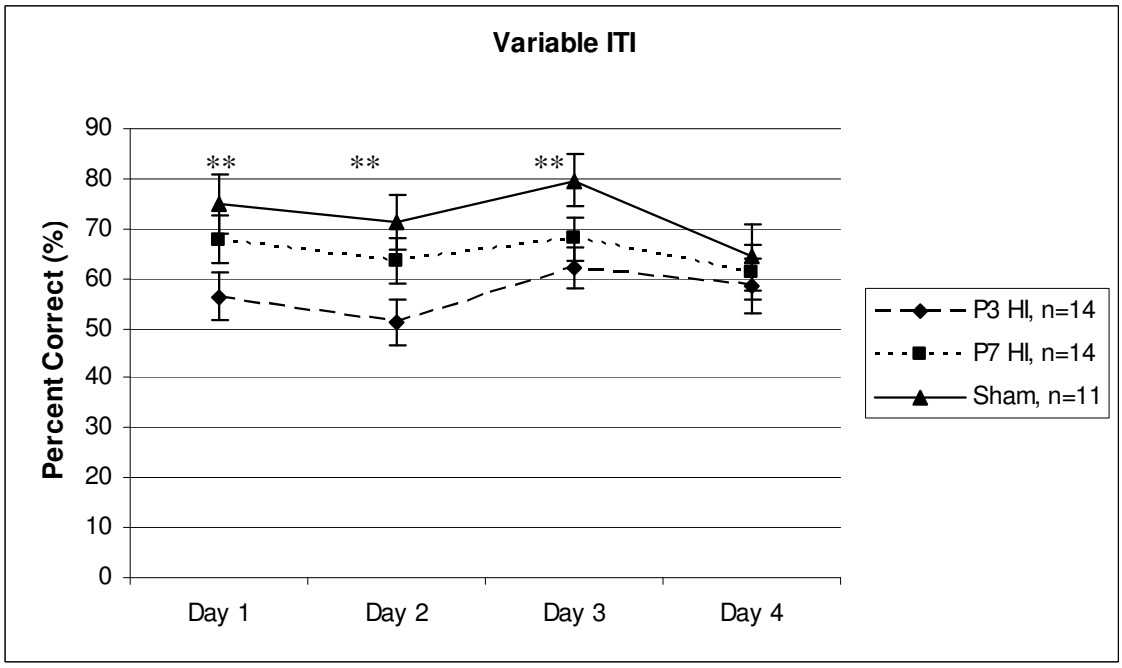


Figure 1h



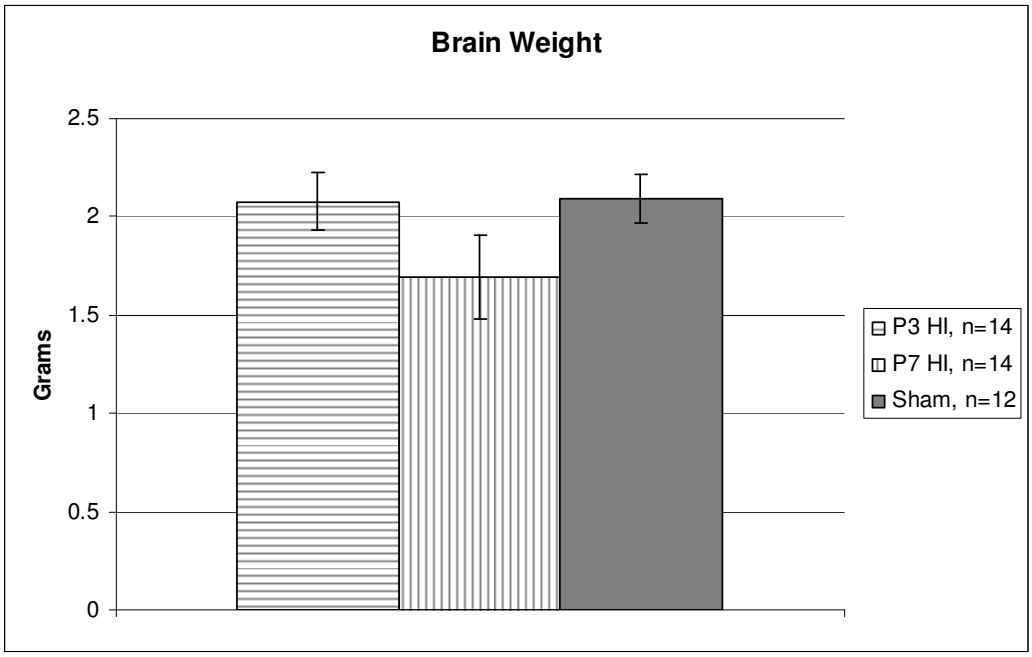
Variable Inter-Trial Interval (VITI): A 4 (Day) x 3 (Treatment) repeated measures ANOVA showed a trend towards an overall Treatment effect [$F(2,29) = 2.569$, $p = .09$] (figure 1h). There was a significant Day x Treatment interaction [$F(6, 87) = 2.287$, $p < .05$], suggesting that Treatment effects were more robust during initial testing. A repeated measures ANOVA revealed a Treatment effect between P3 HI and shams [$F(1,18) = 5.49$, $p < .05$], with P3 HI animals performing worse. Further examination also revealed a significant Day x Treatment interaction [$F(2,54) = 3.056$, $p < .05$] with P3 HI performing worse. An independent samples t-test revealed differences on Day 1 [$t(23) = -2.562$, $p < .01$], Day 2 [$t(23) = -3.010$, $p < .01$], and Day 3 [$t(23) = -2.431$, $p < .05$] between P3 HI and sham animals, with P3 HI animals performing worse. A repeated measures ANOVA also revealed a Day x Treatment interaction between P3 HI and P7 HI groups [$F(3,66) = 2.68$, $p < .05$], suggesting that the difference between groups diminished over testing. Follow-up independent sample t-tests revealed a group difference on Day 1 [$t(26) = -2.199$, $p < .05$] and a trend on Day 2 [$t(22) = -1.780$, $p = .08$].

Histology

Brain tissue was sliced and stained using cresyl violet staining procedures. Volumetric measurements of the hippocampus, cortex, ventricles, corpus callosum, and striatum were taken using MBF StereoInvestigator. Brain weight measurements (grams) were taken following perfusions. A Univariate ANOVA revealed an overall Treatment effect [$F(2,34) = 23.117$, $p < .01$] (Figure 1i). Upon further investigation, an independent samples t-test showed no differences between P3 HI and sham brain weights [$t(22) = -.249$, $p > .05$]. There were significant differences in brain weight between P7 HI and

sham animals [t(23) = -5.441, p <.01] and P3 HI and P7 HI animals [t(25) = 5.338, p <.01], with P7 HI animals showing smaller brain weight than both groups.

Figure 1i



Ventricular Volume: A Univariate ANOVA revealed a Treatment effect for right ventricular volume [F(2,39) = 18.77, p < .001], but no Treatment differences for left ventricular volume [F(2,39) = 2.20, p > .05] (data not shown). Independent sample t-tests revealed a difference between P3 HI and P7 HI [t(26) = -4.529, p < .001] and between P7 HI and shams [t(24) = -4.137, p <.001], with P7 HI animals showing a higher rate of ventriculomegaly. There was no difference between P3 HI and shams in right or left ventricular volume.

Cortical Volume: A Univariate ANOVA revealed a significant Treatment effect for right cortical volume [F(2,39) = 33.9, p <.001] but no effect on left cortical volume [F(2,39) = .923, p > .05] (data not shown). An independent samples t-test revealed a difference between P3 HI and P7 HI [t(26) = 6.089, p <.001], and between P7 HI and shams [t(24) =

6.063, p <.001], with P7 HI animals showing smaller right cortical volumes. There were no differences found between HI P3 animals and shams in right cortical volume.

Hippocampal Volume: A Univariate ANOVA revealed a Treatment effect for right hippocampal volume [F(2,39) = 25.58, p < .001], but no effect on left hippocampal volume [F(2,39) = 1.93, p >.05] (data not shown). A follow-up independent samples t-test revealed a difference between P3 HI and P7 HI [t(260 = 6.531, p <.001], and between P7 HI and shams [t(24) = 4.675, p < .001], with P7 HI showing smaller right hippocampal volumes. There were no differences found between HI P3 animals and shams in right hippocampal volume.

Striatal Volume: A Univariate ANOVA revealed a significant Treatment effect in right striatal volume [F(2,39) = 34.08, p <.001], but no effect on left striatal volume [F(2,39) = 1.19, p >.05] (data not shown). Independent samples t-tests revealed a significant difference between P3 HI and P7 HI [t(26) = 6.021, p < .001], and between P7 HI and shams [t(24) = 7.084, p < .001], with P7 HI showing smaller right striatal volumes compared to both other groups. No differences were found between P3 HI and shams in right striatal volume.

Corpus Callosum Area: A Univariate ANOVA revealed a Treatment effect on corpus callosum area [F(2,38) = 32.364, p < .001] (data not shown). Additional independent sample t-tests revealed a significant difference between P3HI and P7 HI [t(24) = 6.372, p < .001], with P7 HI animals showing smaller corpus callosum area in comparison to both groups. We found no difference between P3 HI and sham animals.

Histological Outcomes on the Medial Geniculate Nucleus:

Number of Cells in the MGN: A one-way ANOVA revealed no Treatment effect on the number of cells in the left MGN [$F(2,39) = .421$, $p > .05$]. There was, however, a Treatment effect in the right MGN [$F(2,39) = 14.423$, $p < .001$]. Specifically, independent samples t-tests revealed a difference between P7 HI and sham animals [$t(24) = 5.445$, $p < .001$], and between P3 HI and P7 HI animals [$t(26) = 4.540$, $p < .001$]. P7 HI animals showed fewer cells per section than either sham or P3 HI animals. There was no difference between P3 HI and sham animals [$t(24) = .104$, $p > .10$]. There was no Treatment effect on size of cells for the left MGN [$F(2,39) = .421$, $p > .05$].

Cumulative Distribution MGN: In the left MGN, a Kruskal-Wallis non-parametric analysis revealed an overall Treatment effect [$H(2) = 10.6555$, $p < .005$]. Follow-up analyses using the two-sample Kolmogorov-Smirnov test revealed a difference between P7 HI and sham groups ($p > .001$), with P7 HI animals showing a shift towards larger cells. There was no difference between P3 HI and sham groups ($p > .05$), nor between P3 HI and P7 HI ($p > .05$).

In the right MGN, a Kruskal-Wallis non-parametric analysis revealed an overall Treatment effect [$H(2) = 86.233$, $p < .001$]. Follow-up analyses using the two-sample non-parametric Kolmogorov-Smirnov test revealed a significant difference between P3 HI and sham groups ($p < .001$), with P3 HI animals showing a shift toward larger cells. Furthermore, results showed a difference between P7 HI and shams ($p < .001$), and between P3 HI and P7 HI ($p < .001$), with P7 HI animals showing a shift toward smaller cells compared to both groups.

Behavioral and Cell Size / Number Correlation: A correlation was found for P7 HI animals between raw RAP scores in the juvenile period (third day of testing), and number of cells per section in the right MGN [$r = -.577$, $p < .05$]. Similar correlations were found for P3 HI and sham animals and were in the expected direction, but those failed to reach significance. There were no correlations found in any group between cell measures and adult RAP scores, although scores reflected a trend for fewer/smaller cells to be related to worse performance.

Discussion for Aim 1:

Behavioral and histological analysis revealed interesting implications about the timing of preterm HI injury. First, the current study replicated a "recovery of function" for RAP in animals given early preterm HI, (P3), but not in late preterm, (P7) injury. Previously, data from McClure et al., 2006 demonstrated that animals with HI injury induced on P1 showed deficits on RAP tasks in juvenile periods, but not as adults (McClure et al., 2006). However, when animals were given late preterm HI injuries on P7, persistent deficits in RAP were seen in both juvenile and adult periods (McClure et al., 2006). Like the McClure et al., 2006 study, the current findings showed that, as was seen in that the P1 HI injured animals, P3 HI animals also had a more transient impairment in auditory discrimination (McClure et al., 2006). Meanwhile, P7 HI animals in both studies showed persistent deficits on the SG 0-100 task compared to shams, as seen throughout the lifespan.

Furthermore, P7 HI animals showed somatosensory deficits (as assessed by the rotarod) in comparison to P3 HI and sham animals, suggesting possible basal ganglia, cortical, and/or cerebellar damage. Human infants with HIE show gray matter damage, including the basal ganglia (Huang & Castillo, 2008; Martinez-Biarge et al., 2011). Additionally, there were no differences seen on Day 1 or Day 5 of rotarod testing between any of the groups, suggesting that P7 HI animals could eventually "learn" the task. This "learning," however, did not occur as quickly in P7 HI animals as it did for P3 HI rodents and shams.

The findings from the current study also show that P7 HI injured animals exhibit deficits on learning and memory tasks in adulthood, specifically on the MWM and NSM. Once again, these findings replicate earlier findings, where P7 HI animals showed deficits on MWM and NSM (McClure, Threlkeld, Rosen, & Fitch, 2006). There was no Day x Treatment interaction for any groups on either of the tasks, arguing against a possible "learning delay" in the P7 HI animals.

Interestingly, P3 HI animals showed worse performance on visual attention tasks than both P7 HI animals and shams. Here, P3 HI animals showed deficits on the hardest (5 second stimulus duration) training task, as well as the VITI task, when compared to shams. On the other hand, P7 HI animals had no deficits on the visual attention tasks as compared to shams. To our knowledge, this current study is the first to demonstrate visual attention deficits in P3 HI injured animals. Preterm birth in humans has been associated with increased risk for attention deficit hyperactivity disorder (ADHD) in school aged children (Lindstrom et al., 2011). Children born prematurely have also been reported to have deficits in visuospatial abilities (Baron et al., 2009). Preterm children

who show deficits in visual object and visual spatial recognition had no changes in cortical areas known to be involved in these tasks, suggesting other underlying mechanisms might lead to these deficits (Fazzi et al., 2009). Identifying these mechanisms would be a potential topic for future research.

P3 HI and sham animals had the same brain weights, but P7 HI animals had smaller brains than both groups. Therefore, P7 HI animals appear to have more global brain injury and loss of tissue, while P3 HI animals either do not have alterations in brain structures per se, or exhibit cellular abnormalities, not detectable by gross volume measures. P7 HI animals showed increased ventricular size compared to P3 HI and sham animals. This could be reflected in the decreased brain weight of P7 HI animals as compared to shams, and also P3 HI rats. Furthermore, the P7 HI rodents showed a reduced cortical volume as compared to P3 HI and sham animals. Finally, decreased corpus callosum and hippocampal volumes in the P7 HI animals may explain their deficits in RAP, motor learning, and/or learning and memory tasks. Fewer cells in the right MGN, larger cells in the left MGN, smaller cells in the right MGN, and a correlation for P7 animals between juvenile RAP testing and cells in the right MGN also point to anatomical abnormalities that led to P7 HI deficits in RAP.

There remains the question of how P3 HI animals showed a robust deficit in visual attention as compared to the other two groups, yet tested comparably to shams in every other task. One implicated structure is the striatum, which has previously been associated with attention and impulsivity; however, it was the P7 HI group that showed smaller right striatal volumes compared to P3 HI and sham animals. Therefore, there may more be cellular or neurochemical alterations in the striatum or other structures that

affected the P3 HI animals' abilities in visual attention that might not be caused by a gross loss of tissue.

In piloting a model for early preterm HI, it has become apparent that the P3 HI animals do not show the same behavioral deficits as their late preterm HI counterparts. Specifically, the P3 HI animals performed comparably to shams on motor learning, adult RAP, and learning and memory tasks. Meanwhile, P7 HI animals showed a deficit in each one of these domains. Interestingly, P3 HI animals did show a "recovery of function" from juvenile to adult RAP, the mechanism of which is still unknown. That said, the P3 HI animals showed a robust deficit in visual attention testing that was not seen in P7 HI animals. Furthermore, the overall brain weight of P3 HI animals was comparable to that of shams, while P7 HI animals had significantly smaller brains and reduced volumes of different brain regions. Overall, the mechanism of protection that might spare some of the behavioral deficits and reduced brain masses of HI in P3 animals is unknown and should be considered a subject for future study. Furthermore, the histological differences between P3 HI and P7 HI brains should be identified at a cellular level in order to determine the cause of visual attention deficit in P3 HI animals.

Aim 2- Passively-Induced Whole Body Hypothermia Following HI

A. Introduction

Clinical human data shows protective effects of selective-head cooling on term infants affected by perinatal hypoxia (Gunn et al, 2005). Furthermore, full term infants who received full body cooling after an HI insult were less likely to die than their uncooled counterparts (Shankaran et al., 2005). While such studies indicate that either selective head-cooling or whole-body cooling has a neuroprotective effect on full term infants with

HIE, no human clinical trials have investigated the effects of cooling in preterm infants with HI. One study using fetal sheep found that cooling had protective effects on brain function, but it did not examine the behavior of treated versus untreated animals (Gunn et al. 1997). Therefore, the aim of the current study was to examine the behavioral effects of passive, whole-body cooling on models of early preterm HI injury, specifically, the P3 rodent that was discussed in Aim 1. From Aim 1, it was established that P3 HI animals show RAP deficits as juveniles and visual attention deficits as adults. Furthermore, low birth weight, young gestational age, and hypoxic conditions have been identified as risk factors for autism (Kolevzon, Gross, & Reichenberg, 2007). Therefore, the purpose of Aim 2 was to determine the effects of passively-induced hypothermia following HI in a P3 animal, specifically whether hypothermia would ameliorate the deleterious effects of injury on juvenile RAP, visual attention, and social behavior. It was hypothesized that while P3 HI animals kept at normal temperature following the injury would show expected deficits in juvenile RAP, visual attention, and social behavior, HI animals kept at hypothermic temperatures would perform comparably to shams on these tasks.

B. Methods for Aim 2: For Aim 2, all HI procedures were the same as described in the general methods section. All HI and sham surgeries took place on P3. A randomly assigned subset of HI animals was placed under a heating lamp only during the 120 minutes of hypoxia (cooled). In addition, a randomly assigned subset of shams was placed under only a heating lamp while they were exposed to open room air. HI normothermic and sham animals were placed indirectly on top of a slide warmer and under a heating lamp in order to maintain normal body temperature during matched

procedures. As a result, there were four groups of animals: HI Normothermic, HI Hypothermic, Sham Normothermic, and Sham Hypothermic.

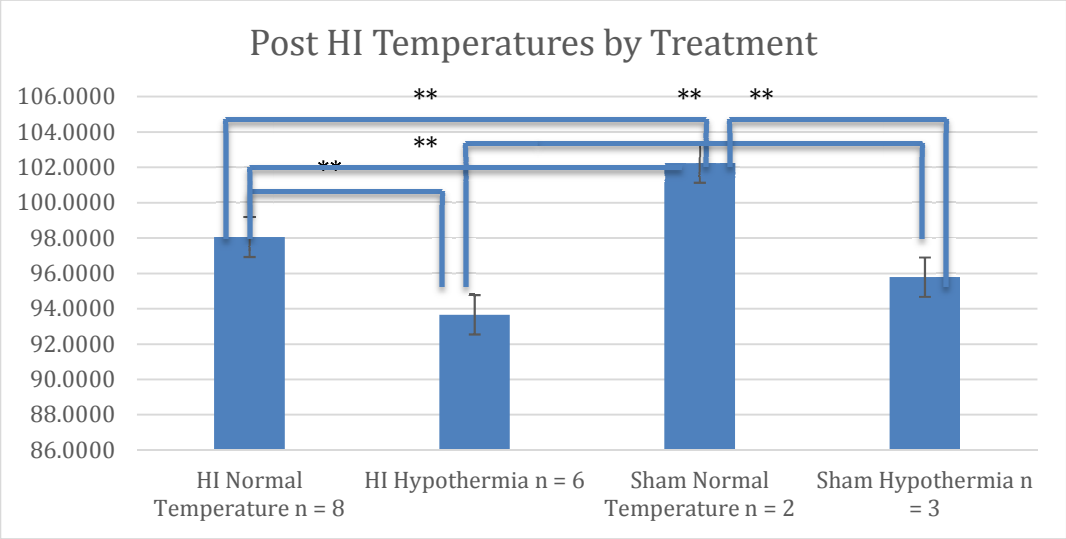
Auditory testing (Silent Gap 0-100, Silent Gap 0-10, FM Sweep) took place in only the juvenile period (P25-40). Visual attention testing and social behavior testing occurred in adulthood (P60-180). Upon completion of behavioral testing (P180), animals were trans-cardially perfused, brains were sliced at 60 micrometers on a cryostat, mounted on slides and stained using parvalbumin procedures.

C. Results

Due to the small number of shams in the normal temperature and hypothermia groups, they were assumed to be equal and were pooled into a single sham group (n=5). The groups were as follows: HI Normal Temperature, n = 9; HI Hypothermia, n = 9; and Sham, n = 5. During the juvenile period, one HI Normal Temperature animal died and was dropped from analyses. Furthermore, three animals in the HI Hypothermia group were not able to learn the visual attention task and were dropped from all analyses. Finally, the HI Hypothermia group and sham animals showed no significant differences in behavioral tasks. Furthermore, if the cooling failed to ameliorate HI, then including the HI Hypothermia group in the Sham group would have reduced any differences between the Sham and HI Normal Temperature group. Finally, the n was small due to unexpected attrition (n = 19). For these reasons, the HI Hypothermia group was pooled together with the Sham group for behavioral analysis, resulting in final groups as HI Normothermic Temperature, n = 8 and Sham/HI Hypothermia, n = 11.

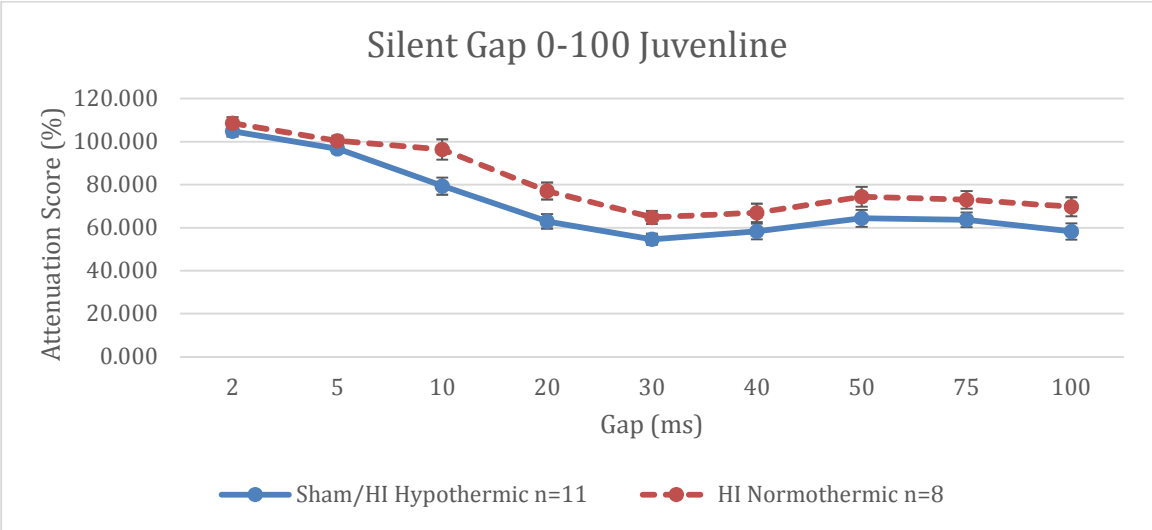
Surgery Temperatures: A one-way ANOVA revealed a significant Treatment effect [$F(15, 3) = 42.552$, $p < .05$] on post HI temperature between groups (see Figure 2a). Follow-up independent samples t-tests revealed a difference [$t(12) = 10.150$, $p = .000$] between HI Hypothermic and HI Normothermic groups, with HI Hypothermic animals at a lower post surgery temperature than HI Normothermic Temperature groups. Additionally, it was found that there was a difference [$t(8) = -11.311$, $p < .05$] between Sham Normothermic and HI Normothermic groups, with the Sham Normothermic group at a higher temperature than the HI Normothermic animals, (probably attributable to differences in surgical duration). There was no difference, however, between the HI Normothermic Temperature group and the Sham Hypothermia group [$t(9) = 3.400$, $p > .10$]. There was also a trend [$t(7) = -2.137$, $p = .070$] for Sham Hypothermia animals to be at a warmer temperature than HI Hypothermia animals. There was also a difference between HI Hypothermia animals and Sham Normothermic Temperature animals [$t(6) = -9.651$, $p < .05$], with Sham Normothermic Temperature animals at a higher temperature than the HI Hypothermia group. Finally, an independent samples t-test revealed a difference [$t(3) = -4.315$, $p < .03$] between Sham Normothermic Temperature and Sham Hypothermia animals, with the Sham Normothermic Temperature animals at a higher temperature than the Sham Hypothermia group.

Figure 2a



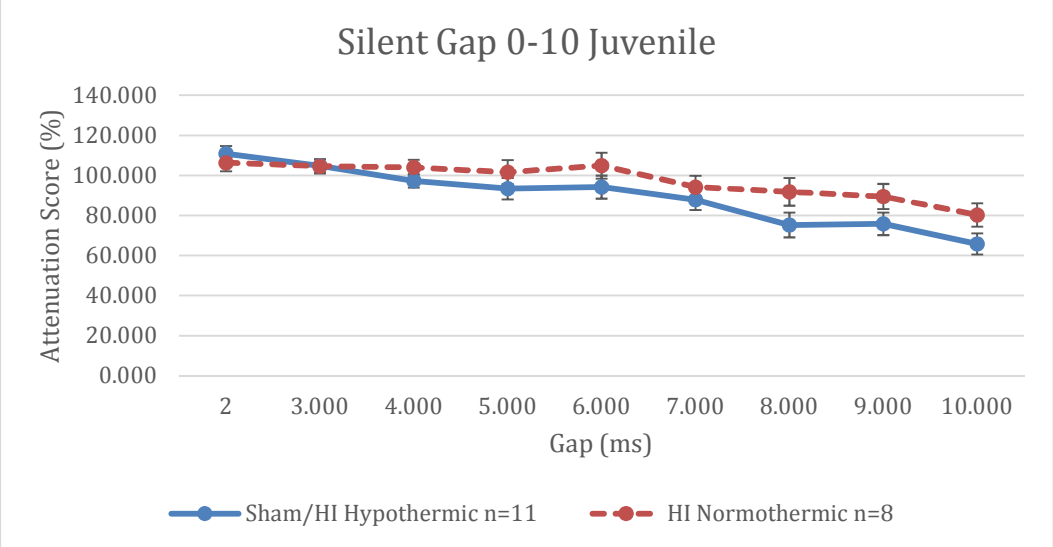
Rapid Auditory Processing SG 0-100: A 5 (Day) x 9 (Gap) x 2 (Treatment) repeated measures ANOVA revealed an overall Treatment effect [$F(1,17) = 5.839$, $p < .03$] with the Sham/HI Hypothermia animals having lower attenuation scores than the HI Normal Temperature group (Figure 2b). Furthermore, a significant Day x Treatment interaction [$F(1,4) = 2.896$, $p < .03$] was also revealed, indicating a slower rate of learning in HI Normal Temperature animals.

Figure 2b



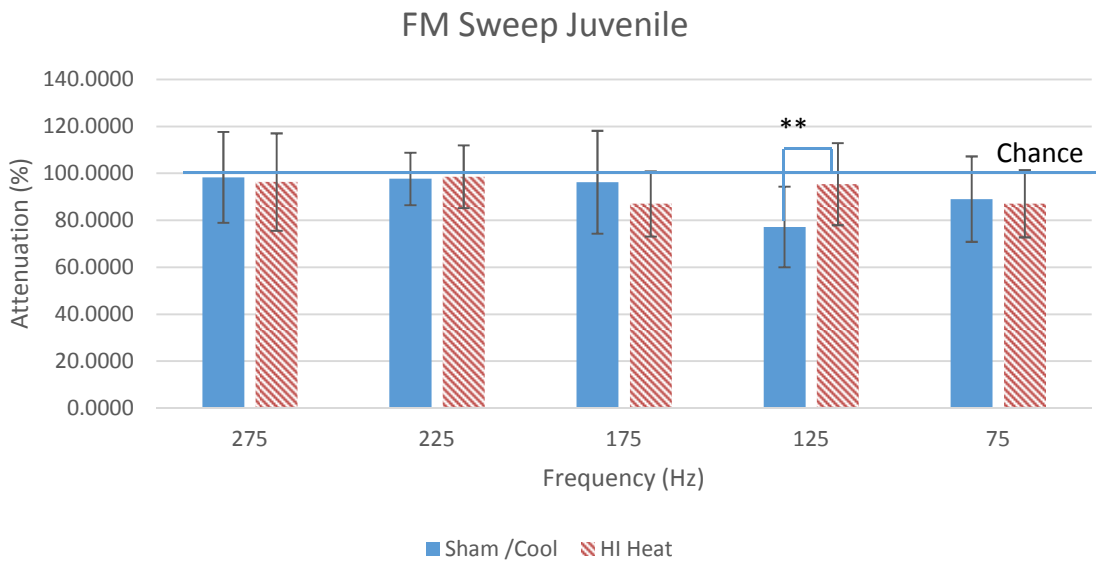
Rapid Auditory Processing Silent Gap 0-10: A 5 (Day) x 9 (Gap) x 2 (Treatment) repeated measures ANOVA failed to reveal a significant overall treatment effect [$F(1, 17) = 1.671$, $p > .10$] (see Figure 2c). There was, however, a significant effect of Day ($p < .05$) and Gap ($p < .001$). Furthermore, there was a Gap x Treatment interaction ($p < .02$). In addition, there was a trend for a difference for Gap 8 on Day 3 of testing ($p = .070$) and for Gap 10 on Day 4 of testing ($p = .084$). Furthermore, there were differences between groups for Gap 8 on Day 4 of testing and for Gap 9 on Day 4 ($p < .02$). For all of these, the Sham/HI Hypothermia group had lower attenuation scores (better performance) than the HI Normal Temperature group. The Gap x Treatment interaction may be due to all subjects' inability to discriminate cues at 1-3ms, while the effect of Day probably reflects the learning occurring over repeated trials of the SG 0-10 task.

Figure 2c



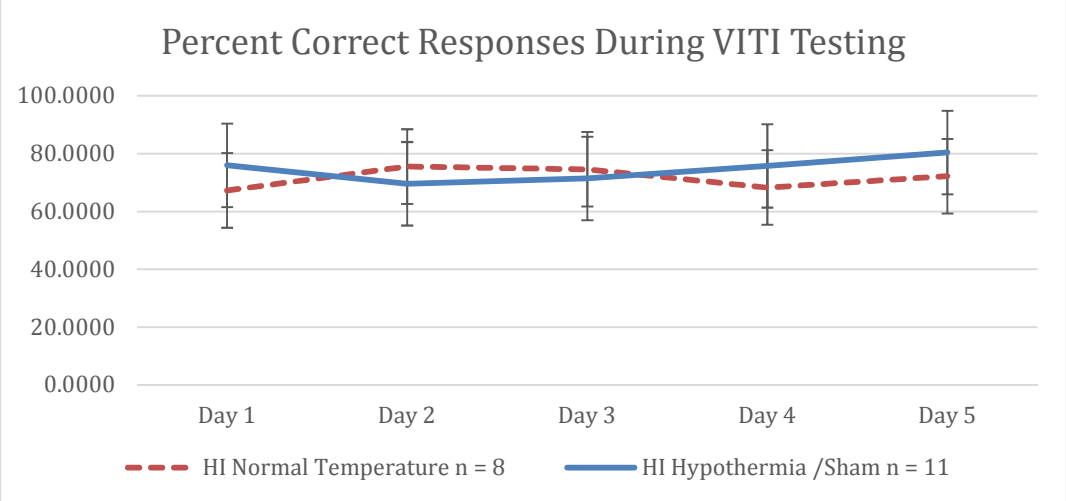
Rapid Auditory Processing FM Sweep: An independent-samples t-test revealed that HI Normal Temperature animals had higher (worse) attenuation scores on Day 4, 125 ms [$t(17) = 5.156$, $p < .05$] (Figure 2d).

Figure 2d



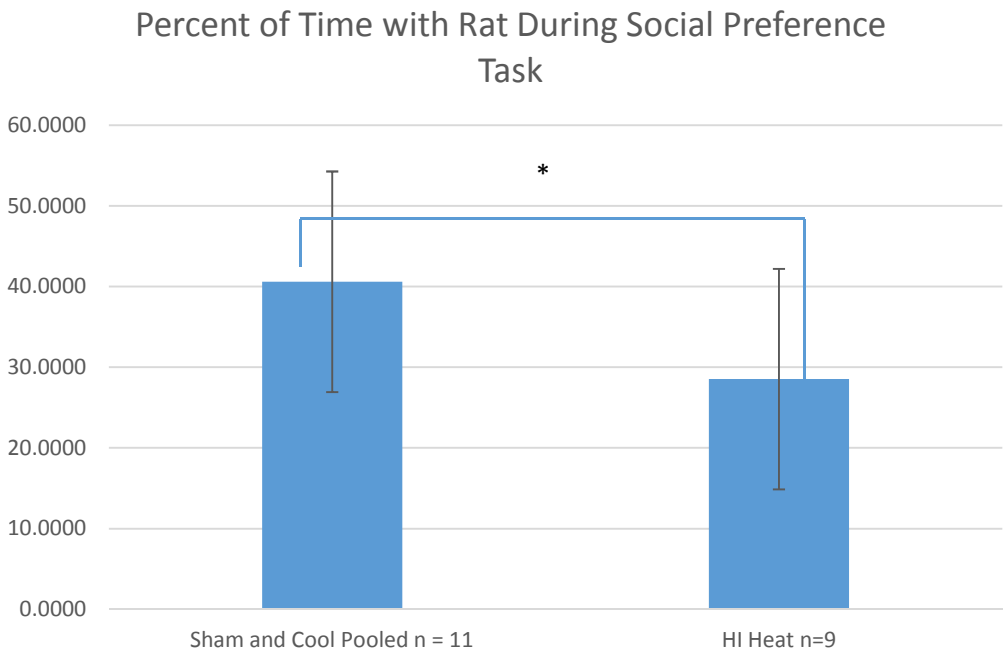
Visual Attention: A repeated-measures ANOVA revealed no difference [$F(1,17) = .204$, $p > .10$] between HI Normal Temperature and HI Hypothermia/Sham animals on VITI (see Figure 2e).

Figure 2e



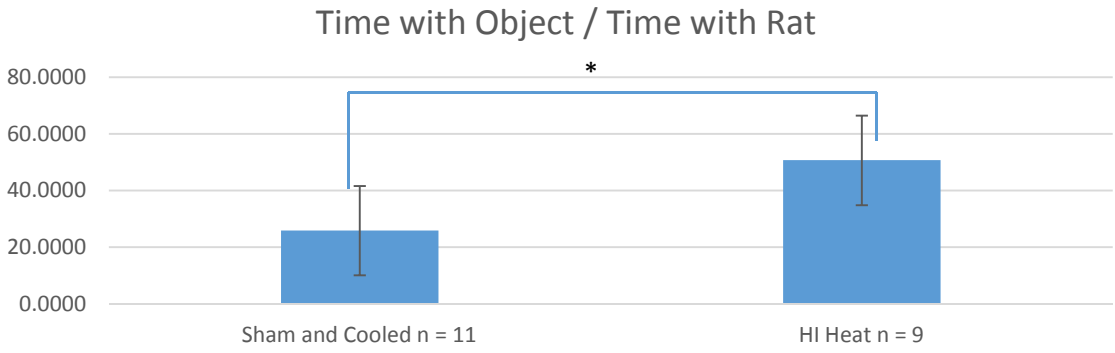
Sociability Task: An independent samples t-test revealed a trend [$t = 1.779$, $p = .093$] for the Sham/HI Hypothermia group to spend more time with a rat of the same sex versus an inanimate toy (Figure 2f).

Figure 2f



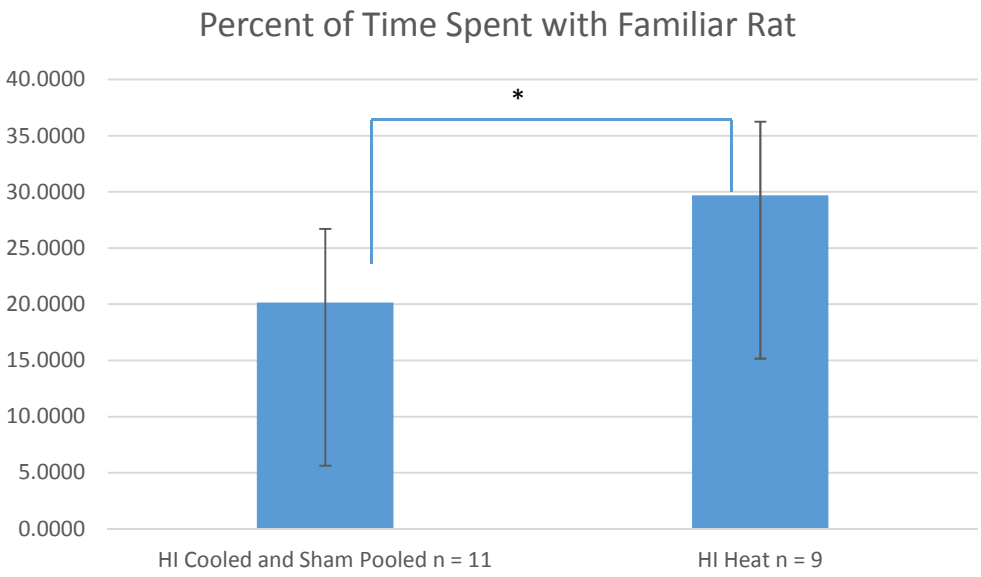
Furthermore, an independent samples t-test revealed a trend [t(17) = -2.012, p = .060] for the HI Normothermic group to have a higher object/rat time ratio than Sham/HI Hypothermic animals (Figure 2g).

Figure 2g



Social Novelty Task: An independent samples t-test revealed a trend [t(17) = 1.938, p = .069] for the HI Normal Temperature animals to spend more time with the familiar rat (see Figure 2h).

Figure 2h



Discussion

In the current study, prior findings were replicated, showing that animals with an "early" (early preterm equivalent) HI injury show juvenile deficits on a RAP task (McClure, Threlkeld, Rosen, & Fitch, 2006; Alexander et al., 2014). Specifically, animals given HI at P3 that were kept at normal temperatures showed deficits on the Silent Gap 0-100 task and during Day 4 of the FM Sweep task. Furthermore, these animals showed a Gap x Treatment interaction on SG 0-10, showing that all animals were most likely unable to discriminate cues at 1-3ms prior to the burst. Interestingly, there were no differences between groups on visual attention testing, with both the HI Normal Temperature animals and Sham / HI Hypothermia animals performing comparably on the VITI task. Such findings contradict the previous study, where P3 HI injured animals showed a robust visual attention deficit (Alexander et al., 2014). However, there were some trends revealed during the socialization tasks, in which the HI Normal Temperature group spent less time with a rat of the same sex than the Sham/HI Hypothermia group

when given the choice to explore the rat or an object. Furthermore, the HI Normal Temperature group spent more time exploring a familiar rat than the Sham/HI Hypothermia group when given the choice between exploring a novel rat and a familiar animal. Such novel findings agree with the clinical literature that puts low gestational age, low gestational weight, and intrapartum hypoxia as risk factors for autism (Kolevzon, Gross, & Reichenberg, 2007).

The neuroprotective effects of passively-induced whole body hypothermia appear to be evident in the current study, as pooling HI animals that were kept at hypothermic temperatures during hypoxia with the sham group still resulted in group differences between the HI Normothermic Temperature and Sham/HI Hypothermia groups.

That said, there are underlying problems with the current study. To begin with, the low n of nineteen total animals made it impossible to compare the two sham groups against each other on behavioral tasks. As a result, it was assumed that the shams that were kept at normal temperatures and the shams that were kept in hypothermic conditions were behaviorally the same.

Furthermore, there were inconsistencies in the temperatures of the groups. For instance, the HI Normal Temperature animals and Sham Normothermic Temperature group had different temperatures post HI. Additionally, there was no difference in temperature between the HI Normal Temperature and the Sham Hypothermia groups. Therefore, the HI Normothermic Temperature group may not have even been maintained at a normal temperature if it was kept at the same temperature as the Sham Hypothermia group, and therefore, it would be difficult to say that there was a neuroprotective effect of cooling if the HI Normal Temperature group was already kept at hypothermic

temperatures. That said, there was a difference in temperature between the HI Hypothermia and HI Normothermic Temperature groups. Furthermore, HI Hypothermia animals were at lower temperatures than the Sham Normal Temperature animals post-hypoxia, so it appears as though the HI Hypothermia animals were actually kept at hypothermic temperatures. As a result, it appears as though any differences between HI Hypothermia and HI Normothermic Temperature animals were due to the reduced temperatures of the HI Hypothermia animals.

For future study, however, several conditions would need to occur. First, there would need to be a larger sample size, with at least six animals in each sham group and twelve animals in each HI group. That way, it would be possible to compare the normal temperature and hypothermia shams on behavioral tasks rather than pooling them into the same group. Additionally, the larger sample size would allow for the HI Hypothermia group to be tested independently of the Shams, which would verify the neuroprotective effect of cooling.

Second, temperatures would be maintained in a more consistent way. A higher accuracy of temperature control would make it easier to determine whether hypothermia actually has a neuroprotective effect following HI.

Third, this study failed to replicate previous findings, in which P3 HI animals showed deficits in visual attention (Alexander et al., 2014). As a result, a larger sample size would be necessary to determine whether there is truly a visual attention deficit in P3 HI animals. One potential problem is that the Sham Normal Temperature group was about 102 degrees F after hypoxia. Perhaps this temperature was out of the normal temperature range, and as a result, modeled a fever instead of a healthy infant kept at

normal temperatures. Furthermore, it is possible that while cooling may be beneficial to injured animals, it has deleterious effects in those that are healthy. As a result, perhaps both temperature manipulations of shams negatively affected their behavior in the visual attention task. Furthermore, shams were observed to have larger body weights than either HI group. As a result, they received more food while being food restricted during visual attention testing, making them less motivated than their HI counterparts to perform the visual attention tasks. With regards to the HI animals, three from the HI Hypothermia group were dropped from the study after the first round of visual attention training because of a failure to learn the task. However, these animals were not recognized immediately, and therefore, delayed the advancement of the subjects to the next round of training. As a result, it is possible that all the groups were over-trained in visual attention, and any deficits that could have been seen in the HI Normal Temperature group were compensated for as a result of this over- training.

With regards to the sociability tasks, these findings are novel, however they are trends rather than significant effects. This, however, might be due to the low sample size of the study, and therefore, a larger n would be necessary to verify these results. Overall, it appears as though the HI Normal Temperature animals display "autistic" characteristics by choosing to spend a greater amount of time with an object than a rat of the same sex. Furthermore, the inability of the HI Normal Temperature animals to identify a novel rat may reflect "autistic" tendencies and a deficit in social attention. Therefore, these findings are novel, and also consistent with the clinical literature (Kolevzon, Gross, & Reichenberg, 2007).

In order to continue to test the idea that P3 HI animals have a behavioral profile similar to that of a child with ADHD, other behavioral measures must be taken as well. Hyperactivity should be assessed in future studies, using tasks that involve counting rears during an open field task or the number of approaches to an object or rat during the social preference task. Furthermore, a task needs to be developed in order to differentiate between attention and social recognition. The HI Normal Temperature animals spent more time with the familiar rat than the Sham/HI Hypothermia animals, suggesting that they failed to recognize the familiar rat as one they had already seen before. However, it is unknown whether this pattern was due to an inability to discriminate between rats, or a deficit in social attention. Finally, histological studies should take place to determine what cellular or structural abnormalities exist in the P3 HI animal and whether hypothermia ameliorates these.

Discussion and Future Directions:

Taken together, the completed studies presented here add to the literature demonstrating the effects of neonatal HI on both behavioral and anatomical aspects in rodents. First, we demonstrated differences between P3 and P7 HI injured rats on multiple behavioral tasks. Specifically, we found transitory RAP deficits and chronic visual attention deficits in the P3 HI animals, while P7 HI animals showed long-term RAP deficits, delayed motor learning, and impaired spatial and non-spatial memory. Furthermore, we revealed that there is a more global loss in tissue in P7 injured animals, while P3 HI animals do not appear to have such anatomical abnormalities. Additionally, it was found that the MGN cells of both P3 and P7 HI animals have abnormalities in size, which can be correlated with attenuation scores in RAP. Second, we demonstrated the

potential neuroprotective effects of passively-induced whole-body hypothermia following HI in P3 animals. Mainly, P3 HI animals that were kept at normal temperatures showed significant RAP deficits as juveniles and trends towards abnormal social behavior, while cooled HI animals were comparable to shams.

Future studies should examine the effects of different modalities of cooling, specifically active cooling to a particular temperature versus passively induced hypothermia. Furthermore, the location of cooling, particularly whole-body versus head-selective, may have different behavioral outcomes. Furthermore, P3 HI animals kept at normal temperatures and P3 HI animals kept in hypothermic conditions should be directly compared in a study with a larger n. Additionally, vulnerability of specific brain regions following neonatal HI and how these differences are related to the resulting behavioral deficits should be studied. Finally, a study is necessary to replicate the visual attention deficits in P3 HI animals. Better control over temperature among groups is necessary. Another study could also investigate the effects of cooling animals to different temperatures following HI, and the optimal timing of cooling following the injury. These and future studies have important implications for potential research on therapeutic strategies following neonatal HI, and applications of these findings can profoundly affect clinical populations.

References

Abel KM, Wicks S, Susser ES, Dalman C, Pedersen MG, Mortensen PB, Webb RT 2010 Birth weight, schizophrenia, and adult mental disorder: is risk confined to the smallest babies?. *Arch Gen Psychiatry* 67:923–93

Alexander, M.L., Garbus, H., Smith, A., Rosenkrantz, T. & Fitch, R.H. 2014. Behavioral and histological outcomes following neonatal HI injury in a preterm (P3) or term (P7) rodent model. *Behavioral Brain Research,* 259, 85-96.

Annibale, D. J., & Hill, J. (2008). Periventricular hemorrhage-intraventricular hemorrhage. Retrieved from www.emedicine.medscape.com

Baron, I. S., Erickson, K., Ahronovich, M. D., Coulehan, K., Baker, R., & Litman, F. R. (2009). Visuospatial and verbal fluency relative deficits in 'complicated' late-preterm preschool children. *Early Human Development, 85*(12), 751-754.

Bhutta AT, Cleves MA, Casey PH, Cradock MM, Anand KJS. Cognitive and behavioral outcomes of school-aged children who were born preterm: a meta-analysis. JAMA 2002;288:728–37.

Brochu ME, Girard S, Lavoie K, Sebire G: Developmental regulation of the neuroinflammatory responses to LPS and/or hypoxia-ischemia between preterm and term neonates: An experimental study. Journal of Neuroinflammation 2011; 8:55.

Briscoe J, Gathercole, SE, Marlow, N: Short-term memory and language outcomes after extreme prematurity at birth. J Speech Lang Hear Res 1998; 41: 654-666.

Cho HK, Jang SH, Lee E, Kim SY, Kim S, Kwon YH, Son, SM: Diffusion tensor imaging- demonstrated differences between hemiplegic and diplegic cerebral palsy with symmetric periventricular leukomalacia. AJNR Am J Neuroradiol 2013; 34: 650-654.

de Vries, L. S., & Cowan, F. M. (2009). Evolving understanding of hypoxic-ischemic encephalopathy in the term infant. *Seminars in Pediatric Neurology, 16*(4), 216-225.

Fatemi, A., Wilson, M. A., & Johnston, M. V. (2009). Hypoxic-ischemic encephalopathy in the term infant. *Clinics in Perinatology, 36*(4), 835-58.

Fazzi E, Bova S, Giovenzana A, Signorini S, Uggetti, C, Bianchi P: Cognitive visual dysfunctions in preterm children with periventricular leukomalacia. Dev Med Child Neurol 2009; 51: 974-981.

Fitch, R. H., Threlkeld, S. W., McClure, M. M., & Peiffer, A. M. (2008). Use of a modified prepulse inhibition paradigm to assess complex auditory discrimination in rodents. *Brain Research Bulletin, 76*(1-2), 1-7.

Follett PL, Rosenberg PA, Volpe J, Jensen FE. NBQX attenuates excitotoxic injury in developing white matter. J Neurosci 2000;20:9235-41

Gimenez M., Junque C., Narberhaus A., Caldu X, Salgado-Pineda P, Bargallo N, Segarra D, Botet F: Hippocampal gray matter reduction associates with memory deficits in adolescents with history of prematurity. Neuroimage 2004; 23: 869-877.

Gunn AJ, Gluckman PD, Wyatt JS, Thoresen M, Edwards AD. on behalf of the CoolCap Study Group.Lancet. 2005;365:1619–1620.

Gunn AJ, Gunn TR, de Haan HH, Williams CE, Gluckman PD. Dramatic neuronal rescue with prolonged selective head cooling after ischemia in fetal lambs. J Clin Invest. 1997;99(2):248–56.

Huang B, & Castillo M: Hypoxic-ischemic brain injury: Imaging findings from birth to adulthood. Radiographics 2008; 28: 417-39

Johnston, M. V., Trescher, W. H., Ishida, A., & Nakajima, W. (2001). Neurobiology of hypoxic-ischemic injury in the developing brain. *Pediatric Research, 49*(6), 735-741

Kolevzon A, Gross R, Reichenberg A. Prenatal and perinatal risk factors for autism: A review and integration of findings. *Arch Pediatr Adolesc Med.* 2007; 161 (4): 326- 333.

Kono Y, Mishina J, Yonemoto N, Kusuda S, Fujimura M, & NICU Network, J: Neonatal correlates of adverse outcomes in very low-birthweight infants in the NICU network. Pediatr Int 2011;53: 930-935.

Lai, M. C., & Yang, S. N. (2011). Perinatal hypoxic-ischemic encephalopathy. *Journal of Biomedicine & Biotechnology, 2011*, 609813.

Lindstrom, K., Lindblad, F., & Hjern, A. (2011). Preterm birth and attention-deficit/hyperactivity disorder in schoolchildren. *Pediatrics, 127*(5), 858-865.

Luu,TM, Ment L, Allan W, Schneider K, Vohr BR: Executive and memory function in adolescents born very preterm. Pediatrics 2011; 127: e639-46.

Luu TM, Ment, LR, Schneider, KC, Katz, KH, Allan, WC, Vohr, BR: Lasting effects of preterm birth and neonatal brain hemorrhage at 12 years of age. Pediatrics 2009;123:1037-1044.

Martinez-Biarge M, Diez-Sebastian J., Kapellou O, Gindner D, Allsop JM, Rutherford MA, Cowan FM: Predicting motor outcome and death in term hypoxic-ischemic encephalopathy. Neurology 2011; 76: 2055-2061.

McClure Melissa M, Threlkeld Steven W, Rosen Glenn D, Fitch Holly R 2006 Rapid auditory processing and learning deficits in rats with P1 versus P7 neonatal hypoxic-ischemic injury. Behavioral Brain Res 172:114-121

Mercuri E, Atkinson J, Braddick O, Anker S, Cowan F, Rutherford M, Pennock J, Dubowitz, L: Visual function in full-term infants with hypoxic-ischaemic encephalopathy. Neuropediatrics 1997; 28: 1 55-161.

Mercuri E, Barnett AL: Neonatal brain MRI and motor outcome at school age in children with neonatal encephalopathy: A review of personal experience. Neural Plast 2003;10: 51-57.

Mercuri E, Barnett A, Rutherford M, Guzzetta A, Haataja L, Cioni G, Cowan F, Dubowitz, L: 2004. Neonatal cerebral infarction and neuromotor outcome at school age. Pediatrics 2004; 113: 95-100.

Mercuri E, Haataja L, Guzzwtta A, Anker S, Cowan F, Rutherford M, Andrew R, Braddick O, Cinoni G, Dubowitz L, Atkinson J: Visual function in term infants with hypoxic-ischaemic insults: Correlation with neurodevelopment at 2 years of age. Arch Dis Child Fetal Neonatal Ed 1999;80: F99-104.

Nanba, Y., Matsui, K., Aida, N., Sato, Y., Toyoshima, K., Kawataki, M., . . . Oka, A. (2007). Magnetic resonance imaging regional T1 abnormalities at term accurately predict motor outcome in preterm I nfants. *Pediatrics, 120*(1), e10-9.

Ortiz-Mantilla S, Choudhury N, Leevers H, Benasich, AA: Understanding language and cognitive deficits in very low birth weight children. Dev Psychobiol 2008; 50: 107-126.

Paneth N, Rudelli R, Monte W, Rodriguez E, Pinto J, Kairam R, Kazam E 1990 White matter necrosis in very low birth weight infants: Neuropathologic and ultrasonographic findings in infants surviving six days or longer. J Ped 116:975-984

Shankaran S, Laptook AR, Ehrenkranz R, Tyson J, McDonald S, Donovan E, Fanaroff AA, Poole WK, Wright LL, Higgins RD, Finer NN, Carlo WA, Duara S, Oh W, Cotten CM, Stevenson DK, Stoll BJ, Lemons JA, Guillet R, Jobe AH. National Institute of Child Health and Human Development Neonatal Research Network.Whole-body hypothermia for neonates with hypoxic-ischemic e ncephalopathy. N Engl J Med. 2005 Oct 13;353(15):1574–1584.

Sizonenko SV, Sirimanne E, Mayall Y, Gluckman PD, Inder T, Williams C 2003 Selective cortical alteration after hypoxic-ischemic injury in the very immature rat brain. Peditr Res 54:263-269

Steinman KJ, Gorno-Tempini ML, Glidden DV, Kramer, JH., Miller SP, Barkovich, A.J. Ferriero, DM: Neonatal watershed brain injury on magnetic resonance imaging correlates with verbal IQ at 4 years. Pediatrics 2009; 123: 1025-1030.

Takashima Sachio, Itoh Massyuki, Oka Akira 2009 A history of our understanding of cerebral vascular development and pathogenesis of perinatal brain damage over the past 30 years. Neonatal Neurobiology: The Road to Here 16:226-236

van Haastert IC, de Vries LS, Eijsermans MJ, Jongmans MJ, Helders PJ, Gorter JW: Gross motor functional abilities in preterm-born children with cerebral palsy due to periventricular leukomalacia. Dev Med Child Neurolo 2004;50:684-689.

Vannucci R: Hypoxic-ischemic encephalopathy. Am J Perinatol 2000; 17: 113-120.

Vicari S, Caravale B, Carlesimo GA, Casadei, AM, Allemand F: Spatial working memory deficits in children at ages 3-4 who were low birth weight, preterm infants. Neuropsychology 2004;18:673-678.

Volpe, J. J. (1997). Brain injury in the premature infant--from pathogenesis to prevention. *Brain & Development, 19*(8), 519-534.

Volpe JJ 2001 Neurobiology of periventricular leukomalica in the premature Infant. Peditr Res 50:553-562

Volpe, J. J. (2009). Brain injury in premature infants: A complex amalgam of destructive and developmental disturbances. *Lancet Neurology, 8*(1), 110-124.