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Effects of Neonatal Hypoxic Ischemic Brain Injury on Spatial Working Memory

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Effects of Neonatal Hypoxic Ischemic Brain Injury On Spatial Working Memory

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ABSTRACT

Children born prematurely or at very low birth weight (VLBW) have an increased risk for hypoxic ischemic brain injury (HI). HI refers to a lack of adequate blood and oxygen flow in the brain. HI can also occur in the term infant due to birth complications such as prolonged labor, placental dysfunction, or cord prolapse. In both populations (though exact patterns of neuropathology vary) brain damage is likely to occur in the form of decreased hippocampal and cortical volume, and enlargement of the ventricles (Kesler et al., 2004, Nagy et al., 2009). Resulting neuropathology can in turn lead to cognitive and behavioral deficits as children age. For example, children who suffer from HI are more likely to be diagnosed with developmental disabilities such as ADHD, as well as language and memory impairments throughout childhood.

Fortunately, hypoxia ischemia can be modeled in neonatal rats via carotid artery ligation, followed by a period of induced hypoxia. This animal model can produce brain injuries that roughly equate to functional injuries seen in term infants with HI when performed on P7, and also leads to similar cognitive and behavioral impairments. Using this P7 HI model to assess behavioral outcomes, our lab has previously reported deficits in HI males on a Morris water maze task (a measure of simple spatial learning and memory). Based on those findings, the current study sought to assess HI males on a more difficult memory task: the eight arm radial water maze, in which performance requires a heightened reliance on spatial working memory. This paradigm was modified to make the task progressively harder throughout 8 testing weeks, by way of opening additional arms every 2 weeks. Thus, testing proceeded as follows: Condition 1 (Weeks 1 and 2) -- 3 arms open; Condition 2 (Weeks 3 and 4) --5 arms open; Condition 3 (Weeks 5 and 6) --

8 arms open; and Condition 4 (Weeks 7 and 8) --8 arms open, with a 1 hour delay between sample and test trials.

Results revealed that HI animals performed close to Sham levels in the earlier weeks of testing (Condition 1-3). However, during Week 2 of Condition 4, when a retention interval of 1 hour was introduced, we saw an emergent deficit in HI animals with more errors made. Further analysis of Weeks within each Condition revealed that both HI and Sham animals were learning the task as testing progressed. This combination of findings indicates that though both groups could learn the memory task, HI animals showed a deficit on the hardest Condition (implementation of an hour delay). Interestingly, though HI animals were making more errors than Shams in the later weeks of testing, they also were taking significantly *less* time to make an arm choice--suggesting HI animals may act more impulsively. Taken together, our findings indicate a spatial working memory impairment in HI animals, as well as a potential attentional deficit that might be associated with the characteristics of ADHD in clinical populations. These results build a framework for additional studies assessing memory and attentional impairments in HI animals and provide further insight into the consequence of HI within the clinical population.

INTRODUCTION

According to recent reports, premature births account for 12 percent of U.S. live births per year, with an average annual increase hovering around 1 percent (March of Dimes report, 2009). Though there have been significant improvements in the survival of preterm infants, there is also evidence that disability rates in this population have increased. A major factor leading to disabilities seen in preterm infants is their high susceptibility to hypoxic ischemic brain injury (HI), which refers to a critical reduction in blood and oxygen to the brain. In the premature brain, this type of injury is most often due to the immature and fragile nature of the developing neurovascular system, which can lead to bleeding within and surrounding the ventricles if this system is ruptured (Volpe et al., 2001). Such an injury depletes the tissue of oxygen in the subpendymal germinal matrix and is termed intraventricular/periventricular hemorrhage (IVH/PVH). Another common injury underlying HI is reperfusion failure, which reflects immature autoregulation. Resulting ischemia can then lead to periventricular leukamalacia (PVL), a non-hemorrhagic ischemic HI injury (Volpe et al., 2001; Jensen F.E., 2006; Selip et al., 2011). This results in the death of tissue (and in particular, loss of white matter) surrounding the ventricles. This injury is also similar in mechanism to that seen in term infants with hypoxic ischemic injury. Finally, in the preterm population, HI can be caused by chronic lung dysfunction and hypoxia which leads to generalized HI and tissue loss (Barrett et al., 2007; Peterson, 2003; Krageloh-Mann et al., 1999). Due to the patterns of vulnerability of the developing brain at this time, all of these causes of HI lead to a high degree of white matter damage in the premature brain (Barrett et al., 2007; Volpe et al., 2001; Scafidi et al., 2009). This can be attributed to the susceptibility of

preoligodendrocytes (preOLS) to oxidative stress following HI injury very early in development (Segovia et al. 2008, Back et al., 2002, Haynes et al., 2003). This cellular stress reflects a cascade of events consisting of excitotoxic, oxidative, and inflammatory incidents that lead to cell death in the particularly vulnerable immature brain (Volpe et al., 2001, du Plessis & Volpe, 2002, Holopainen & Lauren, 2012, Johnston, 2005). In severe cases of injury, necrosis occurs immediately, while apoptosis (usually a highly regulated cell process) progressively leads to cell death over a longer period of time (Golan & Huleihel, 2006, Northington et al., 2001, Nakajima et al., 2000, see below for review; Volpe et al., 2001). Similar injuries are seen in term infants that experience intrapartum or perinatal complications leading to hypoxia or ischemia, such as cord prolapse, placental abnormalities, and birth trauma (McLean & Ferriero, 2004, Barrett et al., 2007, Fatemi et al., 2009; see Vannucci et al., 2004 for review). Such injuries typically lead to diffuse brain neuropathology termed hypoxic ischemic encephalopathy (HIE ; de Vries & Cowen, 2009, Volpe et al., 2001, Fatemi et al., 2009). Whereas preterm HI injury predominantly leads to white matter damage, HIE in the term infant is characterized by more gray matter damage in the form of hippocampal and cortical volume reduction (Vannucci et al., 2000, Volpe et al., 2001, Huang et al., 2008, Jyoti et al, 2006, Fatemi et al., 2009). Though reflecting different etiologies, both forms of HI ultimately lead to cell death and tissue damage in the brain (Huang et al., 2008, Nosarti et al., 2008), and also lead to similar patterns of behavioral deficits (Volpe et al., 2009).

Mechanisms of HI in preterm and term populations

As noted above, apoptosis is triggered by brain hypoxic ischemic exposure that can occur in both preterm and term populations (see Volpe et al., 2001 for review). In

both populations, brain hypoxia ischemia (and associated loss of critical oxygen) causes a switch from oxidative phosphorylation to the highly inefficient process of anaerobic metabolism. This in turn causes depletion of energy (in the form of ATP), lactate accumulation within cells and finally, loss of normal cellular membrane function (Huang et al., 2008). Moreover, the accumulation of glutamate through the depolarization of presynaptic neuronal membranes triggers an increase in glutamate receptor (NMDA, AMPA, and kainate) activation (Gewies, 2003). Sodium influx through AMPA and kainate receptors leads to cell swelling and rapid necrotic cell death. Additionally, in immature brains, glutamate predominately binds to NMDA receptors. Activation of the NMDA receptor results in an influx of calcium as well as release of protein bound calcium that leads to acute cell death. This also triggers cytotoxic responses, such as activation of membrane phospholipases and production of free radicals (i.e. nitric oxide) from nitric oxide synthase (NOS ; Huang et al., 2008, Alvarez-Diaz et al., 2007). This causes damage to cell membranes as well as potential damage to mitochondria, causing further loss of ATP production and energy depletion (Huang et al., 2008). In one pathway of apoptosis, nicotinamide adenine dinucleotide (NAD+) is reduced by way of poly (ADP-ribose) polymerase-1 (Parp-1) activation, leading to the release of apoptosis inducing factor (AIF). This results in cell death. Through another pathway, cytochrome c is released from distressed mitochrondria due to the increase of the free radical, nitric oxide (NO). Cytochrome c then triggers the activation of caspases, such as caspase 3, that lead to fragmentation of DNA and cell death (Johnston et al., 2001, Huang et al., 2008).

The regional expression of glutamate leading to apoptosis, along with the relative presence of glutamate receptors in different gray matter areas, is a major factor that distinguishes which areas are damaged as a result of HI (Huang et al., 2008). This reflects the fact that hypoxia ischemia impairs the function of astroglial glutamate transporters, causing glutamate to accumulate in the synaptic cleft (as mentioned above ; Johnston et al., 2001). Though it has been suggested that the sites in the brain with the highest concentration of glutamate receptors are the most vulnerable to HI injury, the maturity of the brain and the severity of the insult also play an important role. Behaviorally, this massive accumulation of glutamate has been thought to lead to symptoms associated with developmental disorders such as ADHD (Lou et al., 1996). In all cases, the cell death caused by HI leads to tissue loss, with long-tem implications for cognitive as well as motor outcome.

HI and Long-Term Outcomes in Pre-Term Infants

As noted above, in the preterm infant, glutamate predominantly binds to NMDA receptors, flooding the membrane with excitotoxic calcium (Huang et al., 2008). It is important to note, however, that not all preterm infants that undergo HI insult have identical patterns of injury. This translates to a high variability of outcomes in children following preterm birth. However, typically, smaller volumes of the cortex, corpus callosum, hippocampus and increased volumes of the ventricles have been observed in this population when compared to age matched controls (Johnston et al., 2005). Moreover, research has continually shown that these developmental abnormalities in the brain correlate with later cognitive and behavioral deficits in preterm infants as they age (Beauchamp et al., 2008, Woodward et al., 2009, Allen 2008). A common disability seen

in the preterm population upon school age is abnormalities in executive functioning, which includes poor planning and problem solving, impaired working memory and disinhibition (Luu et al., 2011, Edgin et al., 2007). Of particular importance to the current study are the working memory deficits seen in this population, which are proposed to be linked to executive functioning. Research has shown that learning disabilities and behavioral deficits are associated with deficits in executive functioning, as well as lower IQ scores in the VLBW population (Aarnoudse-Moens et al., 2009, Isaacs et al., 2004, Luu et al., 2011). The hippocampus, striatum and their targets are particularly involved in executive functioning, and impairments in these brain areas are due to injury follow preterm birth such as the repetitive episodes of hypoxia due to lung dysfunction and repeated episodes of apnea associated with immaturity of the respiratory center in the brain stem. (Luciana et al., 1999, Gadian et al., 2000). In regards to memory impairments, studies also suggest that the dorsolateral prefrontal cortex in humans, which can also be compromised due to HI, is likely linked to spatial working memory performance (Luciana et al., 1999, Espy et al., 2002). Finally, it has been proposed that spatial working memory relies on the integrity of the right hemisphere in accordance with the neural networks that link cortical and subcortical regions (Baron et al., 2011). Hence, if this area is damaged due to premature birth, spatial working memory deficits may emerge in the preterm population as they age.

In further support of this evidence, adolescents who were born prematurely display poor performance on tests of reading, spelling, intellectual ability, phonological processing, and working memory when compared to typically developing children who were born at term. (Downie et al., 2002). Also, later diagnoses of ADHD, autism and

other developmental disorders have been attributed to the negative effect of prematurity on memory and attention (van Handel et al., 2007). A major component of the attentional deficits seen include impulsivity, or hyperactive behavior, and are apparent in many preterm children that are diagnosed with ADHD (Galera et al., 2011, Espy et al., 2007, Lou et al., 1996). Other studies have shown that working memory deficits and hyperactive behavior are related to one another (Mulder et al., 2010). In particular, in a study of preterm children that tested executive functioning, poor performance on a working memory task was associated with increased rates of inattention and overactive/impulsive behavior (Mulder et al., 2010). On the other hand, in regards to working memory, ELBW (<1000 g) children displayed a spatial working memory deficit when compared to term birth children on a spatial location recall task (Baron et al., 2011). The authors suggest that damage to the prefrontal cortex, which is extremely sensitive to neuromodulatory factors, can alter working memory and spatial location memory and contribute to these effects (Baron et al., 2011). Consistent with these findings, Espy and colleagues also found reduced performance in preschoolers born prematurely on a spatial working memory task. In this study, a delayed-response-type paradigm was used in which subjects had to maintain information from the previous trial in order to guide subsequent responding on the next trial (Espy et al., 2002). Finally, children with brain damage similar to HI have been shown to perform worse than age matched controls on a Digit Span Subtest, another task used to assess working memory (Briscoe et al., 1998, Frisk & Whyte, 1994).

HI and Long-Term Outcomes in Term Infants

While the incidence of behavioral impairment is more common in preterm populations, term children who suffer from a hypoxic and/or ischemic event also show cognitive impairments. In this population, HI injury may be caused by cord prolapse, placental dysfunction or fetal blood loss and leads to associated neuropathology. Resulting deficits include memory impairments observed in this population, much like those seen in the preterm population. For example, term infants suffering a severe HI insult display deficits on "everyday memory tasks" when tested in adolescence and compared to children suffering a mild HI insult upon birth (Marlow et al., 2005). Term children who suffer from HI injury also showed decreased language skills and decreased verbal and performance IQs (Badawi et al., 2001). Though literature on behavioral outcomes in term HIE is scarce, it is evident that behavioral deficits seen in term children with HIE are generally similar to those of preterm children.

Rodent Models of HI

Not only is there an abundance of clinical literature on the behavioral and anatomical outcomes of HI, but there is also a copious amount of animal research on the subject. While most clinical studies focus on preterm infants that undergo an HI event, the majority of animal literature uses a P7 model of HI injury, which predominantly models term injury in the infant and HIE. The most common model of HI in a rodent is the Rice-Vanucci model. This model allows for the study of short term cellular events following HI, as well as evaluation of long term behavioral impairments. In this model (adapted from Rice-Vanucci method –see (Rice et al.,1981)) - the right common carotid artery is cauterized in P7 rat pups, followed by a period of hypoxia in a chamber with

reduced oxygen (8%) for 120 minutes. Following this surgical procedure, rats exhibit decreased volumes of the cortex, hippocampus (predominantly in the CA2 and CA3 regions (Towfighi et al., 1991)), corpus callosum, and increased volumes of the ventricles (Towfighi et al., 1991, Bhutta et al., 2001). Though the Rice-Vanucci method produces a more focal injury than the diffuse anatomic injury seen in human infants, rats subjected to surgery on P7 display fairly similar pathology to HI term infants, with damage predominantly seen in gray matter areas (Rice et al., 1981). However, when surgery is performed on P1-3, more white matter damage is seen, which is more similar to the pathology of preterm HI injury (Sheldon et al., 1996; Delcour et al., 2012; Olivier et al., 2005).

With regard to behavioral outcomes, when hypoxic ischemic brain damage is induced on postnatal (P) day 7 in rat pups, subsequent cognitive and behavioral impairments are evident later in life. For example, research has shown that HI animals perform worse than sham animals on a spatial learning task, and show a slower acquisition curve on a memory task compared to shams (McClure et al., 2005, 2006, Ikeda et al., 2001). Our lab in particular has previously reported deficits in HI animals on a Morris water maze task used to assess spatial learning and memory (McClure et al., 2005, 2006). On other learning tasks such as the plus maze, eight-arm radial maze, and choice reaction time (CRT) task (which is also an assessment of attention), HI rats also show impairments when compared to sham animals (Ikeda et al., 2000, Mishima et al., 2004, Arteni et al., 2010). This suggests both a reference memory impairment and a working memory impairment associated with perinatal HI damage. In the animal literature, and as it pertains to the current study, working memory is defined as “a short

term memory for an object, stimulus, or location that is used within a testing session, but not typically between sessions" (Dudchenko, 2004). This definition originates from experiments by Olton and Honig in which they devised a radial eight arm maze to assess memory in the rodent. Other than memory impairments, animal research using the HI model has also shown deficits in rapid auditory processing (RAP). Specifically, studies in our lab have shown impairments on a prepulse inhibiton paradigm to assess RAP, where HI animals show deficits on difficult RAP tasks, but perform comparably to sham animals on simple RAP tasks (McClure et al., 2005, 2006, Hill et al., 2011). Finally, though research on attentional deficits in HI animals is scarce, there is some evidence of hyperactivity due to chronic hypoxia on a FI (fixed interval) – EXT (extinction) task, where animals had to press a lever at a specific time during an extinction period (Oorschot et al., 2007). In this particular study, hypoxic rats performed more lever presses than sham animals indicating hyperactivity, rather than strictly an attentional deficit.

The current study sought to provide a characterization of working memory impairments associated with P7 HI injury in a rat model (noting that the P7 HI model roughly equates to term injury and/or HIE in the clinical literature). To assess working memory, a modified radial arm maze task was used to compare performance of HI and sham animals under increasing memory demands. Since this task is seemingly more challenging than the Morris water maze task, in which we previously reported HI deficits, it was hypothesized that the demands of the eight arm radial water maze might be too difficult for HI animals during initial stages of testing. Therefore, the task was gradually made harder as testing sessions went on, with the very last week of testing implementing

a delay to assess spatial working memory. Given prior evidence that HI animals show impairments in simple spatial learning/memory on the MWM, we hypothesized that HI animals would be impaired as compared to sham animals on some or all difficulty levels of the eight arm radial water maze task.

MATERIALS AND METHODS

Subjects:

Subjects were 24 male Sprague-Dawley rats, born to time-mated dams (Charles River Laboratories, Wilmington, MA) at the University of Connecticut. Dams were shipped to the University of Connecticut on embryonic day 5 (E5) and were housed in the University of Connecticut OARS facility in a 12-h light/dark cycle. Pups were born on E22 and culled into litters of 10 (8 males and 2 females) on P1. Pups were also housed in the University of Connecticut OARS facility in a 12-h light/dark cycle and were weaned on P21.

Surgical Procedure:

Pups were subjected to hypoxic-ischemic or sham procedure on P7 (n=24). On P7, male pups were randomly assigned to HI or sham groups (n=12 for each group and balanced within each litter). Only males were used based on evidence that behavioral impairments associated with HI are more robust in males as compared to females (Hill et al., 2011). HI pups were anesthetized with isoflourane (2.5%), and an incision was made vertically in the neck. The right common carotid artery was located, separated from surrounding tissue and cauterized to restrict blood flow to the right hemisphere of the brain. Sham animals received a similar surgical procedure with the exception of artery

cauterization. Animals were sutured, all pups were given footpad ink injections for identification, and then placed under a warming lamp which allowed for recovery from anesthesia. Pups were then returned back to their dams for approximately 2 hours to feed. Animals were then separated by litter into 2 containers in which HI animals were subjected to 8% oxygen (balanced with nitrogen) for 120 minutes, while shams were subjected to room air for 120 minutes. Following the hypoxia, animals were returned to their dams, and were later weaned and pair housed on P21.

Eight Arm Radial Water Maze Task:

Testing was conducted in a round 122 cm. diameter black Plexiglas pool which housed a black metal radial arm water maze. The radial maze consisted of 8 removable steel arms that could be opened or blocked off for testing. The pool was filled with room temperature water, and a removable black plastic platform was submerged beneath the water that acted as the invisible escape platform. The platform could be positioned at the end of any arm but could not be seen by the animal. The pool was positioned in a room with spatial cues consisting of a small floor lamp in one corner, a table on the opposite side, 2 empty walls, and a cage rack opposite of one of the empty walls.

On P30 and P31, animals started water maze training. The task was performed between the hours of 12 pm and 3pm each day. The purpose of the training was to get the animals accustomed to the testing procedure, and to make sure they were able to swim and navigate through the maze. There was no memory assessment during this portion of testing. Training consisted of 2 days in which 4 trials were given to each animal each day. During a trial, the subject was placed in the water at the end of a start arm. The platform was submerged at the end of another arm, deemed a "goal arm." All

other arms (except for the start and goal arms) were blocked off. Each animal swam out of the start arm and navigated to the open goal arm, and mounted the submerged platform. The animal was then immediately removed from the water, dried off with a towel and returned to a "holding cage" on a table in the room. After the first animal completed the first trial, its cage mate was given the same test with the same goal and start arm. This procedure was repeated for a total of 4 trials for each animal per day. The same goal and start arm were used for each of the 4 trials, and latencies to the goal were recorded for each trial. On the second swim training day (P31), different start and goal arms were used but the same procedure as the previous day was followed.

Weeks 1 and 2

On P34, working memory assessment began using an adapted radial arm water maze procedure. Subjects were again tested between the hours of 12 pm and 3pm. During a "forced sample" trial, the same procedure as the training test was used; only a start and goal arm were open, "forcing" the animal to go into the goal arm and allowing the animal to keep a representation of the goal arm in its working memory. After the first animal reached the platform and was placed in a holding cage, the second animal was given the "forced sample." Once both cagemates were run on a "forced sample" trial, the first animal was then given a test trial in which another choice arm was opened, the goal arm stayed the same and a different start arm than the sample was used. The same start position was never used between the sample and the test trial, to ensure navigation based on memory rather than turn angle. A series of 4 test trials per animal followed the forced sample trial, with the goal arm remaining the same but the alternate choice and start arm changing. Each rat was tested using this procedure over 4 days of a five day testing

week, and was weighed at the end of testing on the fifth day. Interspersed throughout testing, on the fifth day, a control test was administered. This test consisted of placing the animal in a start arm and allowing them to find the goal arm with all other arms open. The animal was not given a forced sample so it was not expected to know where the goal arm was located. These trials served as a baseline index similar to water escape, ensuring that HI animals were equally able to swim and locate the platform when there was no memory requirement. Sequences of goal, start, and alternate arms changed each day of the 10 days of week 1-2 testing. Latencies to goal and, total number of errors, and latencies per choice were recorded for each animal for each trial.

Weeks 3 and 4

On P48, two more choice arms were opened (total 3 possible goal arms), along with the start arm. This provided a harder task for the animals. A "forced sample" was given to each animal with only a start and goal arm open. The 4 subsequent test trials consisted of the same three possible goal (choice) arms, but different start arms for each trial. This procedure was performed 4 days a week for 2 weeks, with the same control test previously described administered on the fifth day. Latencies to goal, number of errors, and latencies per choice were again recorded for each animal for each trial.

Weeks 5 and 6

On P60, each test trial after the "forced sample" had all arms open, providing 7 choice arms and a goal arm. A total of 4 test trials were given as previously described, with the same goal arm, a different start arm, and all other choice arms open. This testing procedure was performed 4 days a week for 2 weeks, with the control trials again

administered on the fifth day. Latencies to goal, amount of errors, and latencies per choice were recorded for each animal for each trial.

Weeks 7 and 8

On P76, the test trials were administered 1 hour after the "forced sample." All arms were open during test trials. Latencies to goal, number of errors, and latencies per choice were recorded for each trial. This procedure occurred 4 days a week for 2 weeks. The control trial was given on the fifth day of the testing week, and was administered exactly like the previous control trials.

It is important to note that throughout the 8 week testing period, arm sequences were systematically varied so the same start and goal arm weren't used between days.

Histological Analysis:

When behavioral testing was complete (P90), all animals were perfused at the University of Connecticut. Animals were anesthetized using an i.p. injection of a ketamine (100mg/kg) and xylazine (10 mg/kg) mixture. An incision was made and a 26 gauge needle was inserted into the heart, after which subjects were flushed with .9% saline solution followed by 10% formalin. Brains were then removed, weighed, and placed in a 10% formalin solution until they were sliced. A vibratome was used to slice the fixed brains at 60 um each section. Every 5^{th} section was mounted on a chrom-alum subbed slide in preparation for staining. Each section was stained for cell bodies using a Nissl stain in order to take volumetric measurements.

Stained sections were then analyzed for right and left volumes of the cortex, hippocampus, and ventricles using Stereo Investigator Microbright field software on an

Axio 2 Zeiss Microscope. Using 100x magnification with Cavalieri's Estimator software and a grid overlay, volumes were then quantified.

Statistics:

Throughout testing weeks, a control trial was administered (total=6). Analysis of latency and errors for these control trials allowed us to assess for potential Treatment differences when the location of the goal was unknown and no memory demands existed.

Next, trial 1 errors were analyzed using the following variables: Treatment (2 levels; HI and sham), Condition (4 levels), Week (2 levels) and Day (4 levels). We note here that significant effects of Day were ultimately seen for both HI and sham subjects, with the expected learning curve (errors decreasing over Days within Weeks). However, since Day did not interact with Treatment or Condition, trial 1 errors were pooled across 4 days within each week to obtain mean values for Week 1 and Week 2 within each Condition, for each subject. Thus, Day is not further included as a variable in results. Also, note only trial 1 errors were analyzed because these trials were the most sensitive to errors.

Repeated measures ANOVAs were also calculated to compare Week one trial 1 errors and Week two trial 1 errors within each Condition for each individual Treatment group (HI and sham separately). This comparison allowed us to assess how well each group was able to learn the task at a given Condition (as indicated by fewer errors in the second week). Thus, repeated measures ANOVAs were also calculated for Week three versus Week four (Weeks within Condition 2), Week five versus Week six (Weeks within Condition 3), and finally, Week seven versus Week 8 (Weeks within Condition 4) for each Treatment group (HI and sham).

A series of t-tests were then used to analyze the average trial 1 errors within each Week separately, using Treatment as a between variable (and trial 1 errors as the dependent variable). Thus, we examined potential HI effects at each Week separately. Since we have previously reported a deficit on memory tasks in HI male rats (McClure et al., 2005, 2006), p-values are reported as 1- tailed unless otherwise noted.

Next, average latency per choice (including the goal arm) for trial 1 was calculated by taking the average latency to the goal arm divided by the number of errors, plus 1. A two-tailed ANOVA was calculated comparing each Treatment groups' average latency per choice on trial 1 at each week of testing. Thus, Treatment was a between subjects variable (HI and sham; 2 levels), and Condition (4 levels) and Week (2 levels) were within-subjects variables, with average latency per choice (sec) as a dependent variable. Again, t-tests comparing HI and sham latencies were performed at each Week separately.

ANOVAs were also used to compare volumes of the right and left hippocampus, cortex, and ventricles, between HI and Sham animals (using Hemisphere as a repeated measure with 2 levels; R, L). Another series of ANOVAs were computed to assess Hemisphere as a within variable (right vs. left side) for each brain structure (hippocampus, cortex and ventricles), within both HI and sham groups separately.

Following histological analysis, ratios of right versus left cortical volume were also be calculated for all HI animals to assess cortical atrophy following the unilateral injury. Specifically, the volume of the right side was divided by the volume of the left side to get a raw ratio in mm^3. Cortical ratios from the HI animals were then organized in ascending order and split up into 2 groups (Mild and Severe damage). Ratios at or above

1, indicating minimal or no cortical asymmetry, were categorized as a Mild HI injury. Ratios below 1 (with atrophy of the right cortex) were categorized as a Severe HI injury. Sham animals were kept in their own separate group. Using this categorization, and to further validate our sub-grouping, additional t-tests were performed to assess right hippocampal and right ventricular volume in Severe and Mild HI animals (based on our cortical atrophy categorization) as compared to Shams.

Finally, average trial 1 errors were re-analyzed at each Week using t-tests to compare trial 1 errors for Severe and then Mild HI subjects versus Shams (separately). Again, based on prior data, p values were reported as 1-tailed unless otherwise noted. This analysis was performed to asses whether patterns of performance were consistent within the heterogeneous HI group, and specifically whether patterns of errors on difficulty levels of the task were affected by severity of injury.

RESULTS

Control Trials Results

Six sample control trials performed over 6 days interspersed in testing confirmed a lack of group differences in errors made finding the goal when all arms were open and when the goal was in an unknown location. Moreover, these control samples showed that errors stayed high across all days of testing under these conditions (unknown goal location, mean 3-4 errors), confirming that animals were not using some other cue (visual, odor) to find the goal. Moreover, Treatment differences were not seen on control trials at any time.

Overall Effects

Day (4 levels) were analyzed as a function of Treatment (2 levels), Condition (4 levels), and Week (2 levels) and no interactions with Day were found. Therefore, mean Trial 1 errors across 4 days (testing Week) within each Week were used for further analysis. We also found no overall effects of Treatment and no Treatment x Condition interaction ([$F(1,22)=.434$, $p>.05$]; [$F(3,66)=.487$, $p>.05$], respectively). We did find significant effects of Condition [$F(3,66)=4.335$, $p<.05$], but based on *a priori* hypotheses, further planned comparisons were conducted as described below to further interpret these effects.

Analysis of trial 1 errors within each Condition (1-4)

Repeated measures ANOVAs were used to assess each Week (2 levels) within each Condition, for each Treatment group (HI and sham) separately. For HI subjects, these analyses revealed a significant effect of Week within Condition 2, specifically with decreasing errors in the second week ([$F(1,11)=5.770$, $p<.05$], see Figure 1). Thus, HI animals were able to show learning within Condition 2. A repeated measures ANOVA comparing trial 1 errors across Weeks within Condition 4 also revealed a significant Week effect in HI animals [$F(1,11)=5.074$, $p<.05$], again indicating subjects were learning the task and made significantly less errors over time. Further analyses of HI animals' performance within remaining Conditions (1 and 3) revealed no significant effects of Week ([$F(1,11)=2.137$, $p>.05$]; [$F(1,11)=1.233$, $p>.050$], respectively). Trial 1 errors were also analyzed by Week (2 levels) within each Condition for Shams. A significant effect of Week ([$F(1,11)=11.547$, $p<.05$]) at Condition 1 indicated the sham animals were in fact able to learn the task at that Condition. Within Condition 4, a

significant effect of Week was also found ([F(1,11)=7.237, P<.05]), again indicating learning (Figure 1). All other Week comparisons (Condition 2: [F(1,11)=.805, p>.05] and Condition 3 [F(1,11)=1.530, p>.05]) did not show a significant effect.

Analysis of Trial 1 Errors for Each Week (Treatment effects) (Fig.1)

Results of a series of 1-tailed t-tests comparing trial 1 errors for each Week of testing as a function of Treatment revealed no effect for Weeks 1 through 7, with HI and Sham performing comparably ([t(22)=-.631, p>.05]; [t(22)=.129, p>.05]; [t(22)=1.676, p>.05]; [t(22)=.580, p>.05]; t(22)=.322, p>.05]; t(22)=1.093, p>.05]; t(22)=.310, p>.05], respectively; see Figure 1). However, during the last Week of testing, with the implementation of a one-hour delay, an independent samples t-test did reveal a significant effect of Treatment, [t(22)=1.774, p<.05, 1-tailed] with HI animals making significantly more errors than sham animals.

Analysis of latency per choice for trial 1 (Treatment effects) (Fig.2)

An overall ANOVA using Treatment (2 levels; HI and sham), Condition (4 levels) and Week (2 levels) as factors, was used to assess average latency per choice for trial 1 errors. Initially, we found a Condition x Treatment interaction, as well as main effects of Condition and Week ([F(3,66)=4.279, p<.05]; [F(3,66)=15.131, p<.05]; [F(1,22)=111.040, p<.05], respectively). However, given the difficulty in interpreting these complex effects, we further examined Treatment effects within each Week separately.

With regard to individual Weeks, using a series of two-tailed t-tests, we found a significant effect of Treatment with HI animals taking *more* time to make an arm choice on trial 1 as compared to shams during the first Week of testing (Condition 1, Week 1)

[t(22)=2.414, p<.05]. Interestingly, on Weeks 2 (Condition 1, Week 2) and 7 (Condition 4, Week 1), a two tailed one-way ANOVA revealed HIs were taking significantly *less* time to make an arm choice than shams ([t(22)=-2.305, p<.05], [t(22)=-2.295, p<.05], respectively). Analysis of Week 8 also revealed a trend for significance in which HI animals were making arm choices faster (less time) than shams [t(22)=-1.620, p=.119]. All other Weeks (3-6) did not yield significant Treatment effects ([t(22)=-1.165, p>.05]; [t(22)=.729, p>.05]; [t(22)=-1.310, p>.05]; [t(22)=-.342, p>.05], respectively).

Analysis of anatomical volumes (Treatment effects)

Volumetric measures of the cortex were also analyzed as a function of Treatment and Hemisphere. An overall ANOVA assessing total cortical volume in mm^3 as a function of Treatment (HI versus Sham) and Hemisphere (within 2 levels; R, L) revealed no significant effect of Treatment ([F(1,22)=.551, p>.05]), or Hemisphere [F(1,22)=.395, p>.05], nor interaction of Hemisphere x Treatment [F(1,22)=.089, p>.05]. Similarly, an ANOVA comparing hippocampal volume in mm^3 as a function of Treatment (HI and Sham) and Hemisphere (within, 2 levels; R, L) also revealed no significant effect of Treatment [F(1,22)=1.148, p>.05], nor Hemisphere [F(1,22)=.484, p>.05], nor Hemisphere x Treatment interaction [F(1,22)=.067, p>.05]. When analyzing Ventricular volume, 2 animals were dropped (1 HI and 1 Sham) because their tissue did not provide reliable anatomic boundary measures. An ANOVA comparing Ventricular volume in mm^3 in HI and sham animals also revealed no significant effect of Treatment ([F(1,21)=.084, p>.05]), Hemisphere [F(1,20)=.007, p>.05], nor Hemisphere x Treatment interaction [F(1,20)=.525, p>.05].

Overall, no anatomic analyses were significant for any of the above 3 measures, including Treatment on a given Hemisphere, or Hemisphere within a Treatment. However, because we observed a high degree of variability in amount of damage in the HI group (observation), we decided to further break the group down based on right cortical atrophy (which is expected with the unilateral right HI injury). In particular, we looked at the volume of the right cortex as compared to the left, and divided the HI's evenly (n= 6 and 6) into those with a ratio at or greater than 1 (no cortical atrophy) versus those with a ratio below 1 (indicating atrophy with the R smaller than L), calling these Mild and Severe HI respectively. As expected, an analysis of Cortical volume as a function of Hemisphere and Treatment using 3 levels was now significant ([$F(2,21)=.5.667$, $p<.05$]), reflecting the right cortex being smaller than the left in Severe HI's ([$F(1,16)=3.318$, $p<.05$], one-tail –see Figure 3). This effect -- and the lack of a right cortical volume difference between Mild HI and sham -- was expected, since animals were segregated specifically by severity of injury to right cortex. However, we further used this categorization to examine ventricular volume and hippocampal volume. Though no significant differences were seen for ventricular values, we did find that Severe HI's (as categorized by cortical measures) had a significantly smaller right hippocampus as compared to Shams ([$F(1,16)=3.960$, $p<.05$], one-tail; see Figure 4). The R versus Left comparison within Severe HIs did not reach significance. Also, the hippocampus in Mild HI's did not differ from Shams, or between Hemispheres. Nonetheless, this anatomic pattern supports the validity of our sub-grouping into Severe and Mild HI subjects.

Sub-analysis of trial 1 errors as a function of injury

Based in part on prior literature studying severely damaged children with HI injury (Luu et al., 2011, Baron et al., 2011), HI animals were split into two categories of HI damage as noted above: a Severe HI group (n=6) and a Mild HI group (n=6). Our purpose was to ascertain whether examining behavioral data from a sub-group of more injured animals might reveal deficits on the easier versions of the task, thus suggesting our null results at those Conditions could have been driven by a lack of damage in some animals. Trial 1 errors were re-examined at all 4 Conditions, as well as at each Week, using a Mild vs Sham, and Severe versus Sham, comparison (t-test). Results showed that the only Condition to reveal an effect under re-categorization was the final delay Condition (4), and specifically at Week 8. Here we found that Mild HI's had near-significantly more trial 1 errors than Sham [$t(16)=-1.730$, $p=.052$, one-tail], and Severe HI's had a trend towards more errors than Shams [$t(16)=-1.322$, $p=.1$, one-tail]. Interestingly, we did find a significant effect at Week 3 (Condition 2), but this was seen in the Mild versus Sham, and not Severe versus Sham, comparison [$t(16)=-1.787$, $p<.05$, one-tail]. Overall, this re-analysis supports our results by showing that deficits on easier Conditions do not appear to emerge even when examining a sub-set of animals chosen for higher right cortical atrophy, and also with higher right hippocampal atrophy. These findings support our null results at easier Conditions and suggest that even with a higher n, robust processing deficits would be unlikely to emerge among HI subjects at the easier Conditions. This result will be further important for future studies in which HI performance starting initially on a harder (delay) version of the match to sample task will be employed. Overall, the pattern of errors seen when HI animals were split into two

groups was similar to the effects seen when examining the HI group as a whole, thus confirming that our null results for Treatment effects during the easy Conditions of the task was not due to a lack of deficits in a subset of minimally injured animals in the HI group.

DISCUSSION

Hypoxia ischemic injury is a common form of brain damage often resulting from premature birth and associated complications, as well as term birth complications such as cord prolapse, placental dysfunction, and prolonged labor. Neuropathological patterns of HI injury tend to be extremely variable, and can lead to different kinds of cognitive and behavioral deficits later in life. For example, hypoxic ischemic brain damage has been show to lead to language disabilities in school-age children, as well as working memory impairments, which in turn affect IQ and school performance (Aarnoudse-Moens et al., 2009, Isaacs et al., 2004). Moreover, children born prematurely display increased incidence of cerebral palsy, autism, ADHD, and other developmental disorders (Anderson et al., 2008; Woodward et al., 2009; Joyti et al., 2006, Lou et al., 1996). Additionally, preterm or VLBW children display anatomical abnormalities in a variety of brain areas (Peterson, 2003; Bhutta et al., 2001; Beauchamp et al., 2008).

Previous research has explored the etiology of these deficits, at both the behavioral and anatomical level, using a P7 rodent model of HI. This model predominantly mimics term injury seen in the clinical population. Findings reveal that male animals with neonatal HI injury display memory impairments on a Morris water maze task (MWM ; McClure et al., 2005) and slower acquisition curves when compared

to shams on a dry eight arm radial maze task (Ikeda et al., 2001). To our knowledge, an eight arm radial water maze task to assess spatial working memory has not previously been used to assess working memory deficits following early HI. The current study sought to assess such working memory deficits in the HI rat model, using a modified version of the eight arm radial water maze task. Specifically, the task gradually became more difficult over 4 Conditions (every two weeks of the eight week testing period). It was hypothesized that HI animals would have deficits on all difficulty levels when compared to shams, since deficits were previously reported on a seemingly easier assessment of memory using the MWM (McClure et al., 2005). We hypothesized that this particular behavioral assessment would provide a characterization of the HI injury on a working memory task over a more prolonged learning period. Furthermore, we hypothesized that HI animals would be impaired compared to sham animals on some or all difficulty levels of this task.

Overall, the current study showed that following HI on P7, male rats could perform close to Sham levels on most levels of the working memory task, and particularly the early (easier) levels. This may reflect the fact that the behavioral task was initially easy from the start, thus HI animals had the opportunity to learn the task at a gradual rate (Figure 1). However, when the HI animals were tested on the most difficult version of the task (the implementation of a one-hour delay in Condition 4 (Week 8)), a deficit did emerge in the form of more trial 1 errors -- indicating a deficit in this more demanding form of working memory (Figure 1). Since working memory in rodents has been defined as "a delay-dependent representation of stimuli that are used to guide behavior within a task" (Dudchenko et al. 2004) and further explained as short term

memory that should be forgotten once used (Dudchenko et al. 2004), we can confidently interpret our current evidence to indicate a working memory deficit.

Post hoc analyses of trial 1 errors at each week showed that both groups of animals seemed to perform very well when the task was the easiest (Fig. 1 showing both groups hovering around 1 error in Condition 1). This finding is further supported by evidence from other memory studies in which early trials reveal similar performances between HI injured animals and Sham animals (Arteni et al, 2003). For example, previous studies analyzing latency to goal on a water maze task show similar performance for Sham and HIs during initial testing periods (Arteni et al., 2010).

As one caveat, due to the logistics of our behavioral paradigm where 4 test trials followed a forced sample trial, all animals received numerous amounts of repetition and were able to learn the task at a slow pace. Given this gradual increase in difficulty, both HI and sham animals were able to hover around 1-1.5 errors for testing during Conditions 2 & 3 (although a trend toward more HI errors during the first Week of 5-arm testing is evident, this was not significant, nor was it significant when only the performance of the Severe sub-group of HI animals was compared to Shams). Interestingly, both groups showed a jump in errors during the first week of the delayed version of the task (Condition 4), but whereas Sham animals showed robust learning by the second week (Week 8), HI subjects did not, and this effect was significant. This illustrates validation of our paradigm in that as the task became harder and more dependent on working memory, deficits in memory emerged in the HI group. This suggests that the lack of behavioral deficits in Weeks 1-7 in HI animals was not due to lack of brain damage but rather, due to the simplicity of the task (or supported repetition and "training") in the

earlier weeks of testing. The interpretation is consistent with our behavioral analyses of errors using sub-grouping of Mild and Severe HI subjects, wherein Severe HI subjects also showed more errors than shams only in Week 8.

Initially, the lack of HI deficits on the eight arm radial water maze may appear discouraging, but the analyses reported are beneficial in showing that HI animals can perform comparably to sham animals on a working memory task when given the opportunity (i.e. training). This is particularly apparent when looking at Weeks within Condition for each Treatment group. The significant differences seen in Week 3 versus Week 4, and Week 7 versus Week 8, in HI animals indicate that within these two Conditions, HI animals were able to make significantly less errors and thus show learning. This indicates that they can, in fact, learn the task within a testing Condition. Similar analyses of Sham animals showed that Sham animals also made significantly fewer errors across Conditions 1 and 4, again indicating learning. In translating to a clinical setting, these findings suggest that intervention for children with similar HI injury may be beneficial in ameliorating working memory deficits. That is, it is not that impaired children can never learn a memory task, but rather, if they receive appropriate supported intervention in early development, this memory deficit may be able to be attenuated. The current study supports clinical literature showing that memory in preterm children is impaired relative to control children only when the task difficulty increases (Luciana et al., 1999). That same study also suggests that this memory impairment is due to the decreased probability of preterm children to maintain a complex working strategy and thus, to perform worse than control children on a difficult memory task. This difficulty in recruiting search strategies in a memory task is reflected in a high degree of

memory errors at a harder "condition", much like the memory errors reported in the current study (Luciana et al., 1999). Another explanation for the lack of deficits in the earlier weeks of testing could be the age of the animals. Other clinical studies have reported larger memory deficits in preterm children with increasing age (Edgin et al., 2007), and thus assessments in older HI subject (these subjects were P34 at the start of testing) might reveal more robust deficits.

As another interesting finding, we saw that in examining average latency per choice for trial 1 during the first week of testing, HI animals were taking significantly more time to make an arm choice than sham animals (Figure 2). This result seems intuitive, as one would think HI animals would take longer to make an arm choice in parallel to any behavioral deficits. Yet HI animals were not making significantly more errors during the same period. We further found that latency to trial 1 choice decreased from first to second Week in all 4 of the Conditions for Shams, as might be expected with learning. HI subjects also showed a big latency drop across Condition 1, but these effects were not as marked in Conditions 2-4. In fact, during Condition 4, HI animals were initially taking significantly less time than Shams (Figure 2), and into the second Week at Condition 4 were still taking significantly less time, despite the fact that here they were making more errors. This pattern could reflect impulsivity seen once the demands of the task became too challenging. Moreover, this finding might be related to prior ADHD studies using a choice reaction time (CRT) task, specifically showing that on later trials with a delayed reward, hypoxic rats made significantly more lever presses than control rats (Oorschort et al., 2007). It can also be suggested that the significant effect for HIs to be faster at making an arm choice may be related to hyperactivity seen in children with

ADHD, which is a common behavioral deficit following hypoxic ischemic brain injury (Lou et al., 1996, van Handel et al., 2007, Galera et al., 2011, Espy et al., 2007).

Unfortunately, though it has been previously reported in our lab, anatomical analyses did not yield significant reductions in the hippocampus, cortex, or increases in ventricular volume in HI animals. Additionally, there were no significant differences in right vs. left side of these structures in the HI group, which we typically see in HI animals following this procedure. Notably, although the findings were not significant, patterns were in the expected direction. Moreover, we were able to quantify cortical atrophy using a right/left volumetric ratio for all animals. Prior evidence has pointed toward the cortex being very involved in working memory and decision making, with executive functioning being the main system responsible in preterm infants (Luciana et al., 1999, Gadian et al., 2000). Particularly, the dorsal prefrontal cortex and parietal cortex seem to play an important role in working memory performance (Baron et al., 2011). The cortical atrophy ratio allowed us to separate HI animals into Mild and Severe HI groups (n=6, 6), with more asymmetrical cortical volumes (R<L) categorized as Severe HI. The Severe HI group also showed significant atrophy of the right hippocampus while the Mild group did not. This separation is relevant because clinically, studies typically use only severely injured preterm children, or extremely low birth weight (ELBW) infants (<1,000g ; Luu et al., 2011, Baron et al., 2011). When comparing all three groups (Mild HI, Severe HI, and sham), the pattern of behavioral deficits did not differ from the original analysis between HI and sham animals, specifically in showing that Severe HI animals showed a nearly significant increase in errors only in Week 8. Thus, the failure to see significant

deficits on the easier versions of the eight arm radial water maze task (Conditions 1-3) does not appear to reflect a lack of damage.

The current study builds the framework for future behavioral studies assessing working memory, and potentially attention, in rodents with HI injury. It will be interesting to see if deficits in working memory emerge in initial stages of testing were the task made more difficult to start. Based on the results of the current study and other clinical and animal studies mentioned previously, it can be hypothesized that a clear working memory deficit might be apparent in HI animals from the beginning if the Condition 4 paradigm was implemented initially. Moreover, since we were able to see significant deficits on week 8 of testing after subsequent repetition in previous testing weeks, it can be suggested that testing HI and Sham animals on a delay dependent working memory task would yield significance in regards to trial 1 errors. It can also be noted that even though there was a lack of significant anatomical pathology in HI animals, behavioral deficits still seem to emerge, and the effects seen do not appear to reflect a high-performing sub-group of minimally injured animals. Moreover, anatomical deficits might have been more robust if volumetric measures were performed immediately after the HI insult, rather than in adulthood.

One pitfall of the current study is the amount of test trials each animal was given after the forced sample trial. The other 3 test trials (for which data is not reported) could have facilitated the learning of the behavioral paradigm, thus skewing our behavioral results. Other studies in our lab using a similar eight arm radial water maze task to test developmental disability and microgyric lesions have shown deficits when animals are given only 1 test trial after a forced sample trial (Szalkowski et al., 2012, Fitch et al.,

2008). This leads to a justification for future studies to test animals using the behavioral testing format outlined in Fitch et al. (2008) and Szalkowski et al. (2012).

In closing, the current study can serve as a stepping stone to further assess working memory impairments and attentional deficits in a rodent model of HI. To our knowledge, there are few studies that have investigated these two etiologies together, and we believe it is important to continually assess how these two deficits relate to one another. It will be interesting to assess the performance of HI animals when tested on a task specifically designed to assess attention, rather than utilizing the eight arm radial water maze paradigm. In addition, using the data in the current study, paired with the testing format mentioned above (making the testing procedure hardest from the beginning), we can further validate our claim that HI injured rats can learn as well as sham animals only when the behavioral paradigm is easy to start.

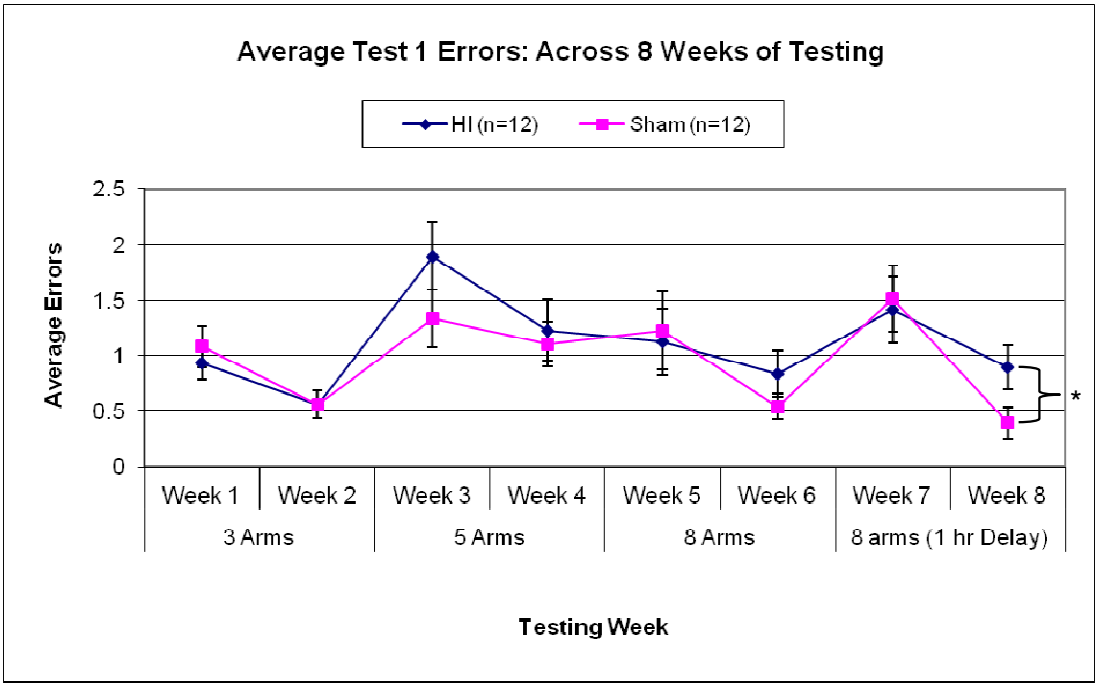

Figure 1: A one tailed independent samples t-test revealed a significant effect of Treatment (* =p<.05), with HI animals making significantly more errors than sham animals on Week 8.

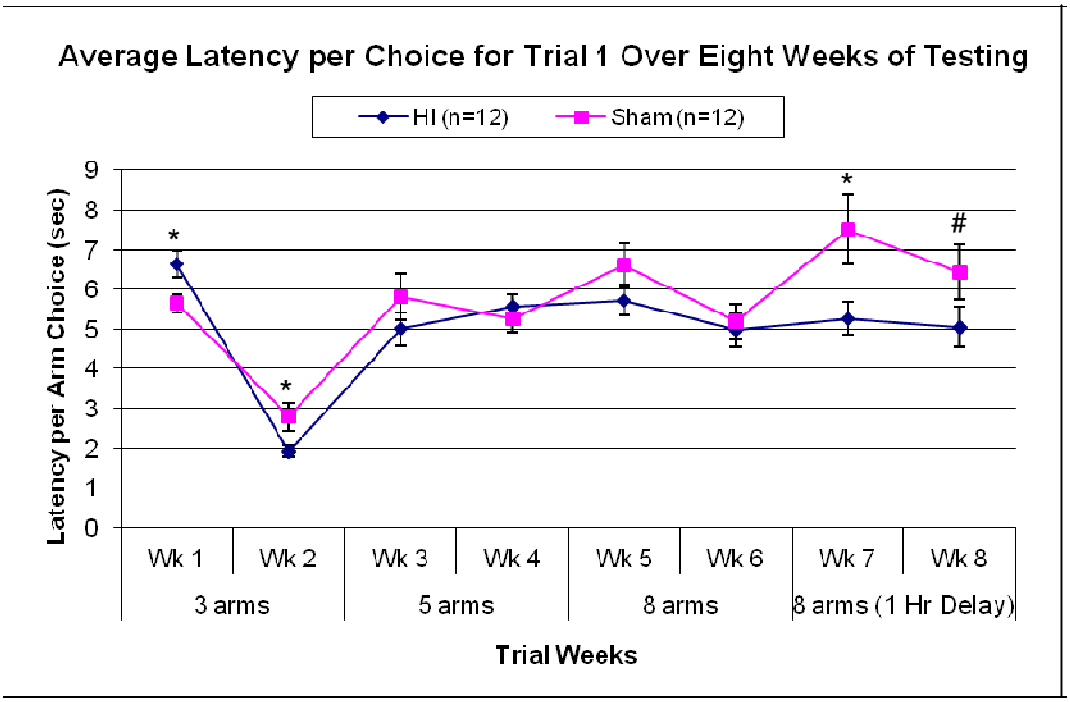

Figure 2: A significant effect of Treatment revealed HIs were taking *more* time to make an arm choice on trial 1 during the first week of testing (* =p<.05]) only. On Weeks two and seven only, another t-test revealed HIs were taking significantly *less* time to make an arm choice than shams (* =p<.05). Analysis of Week 8 revealed a trend for significance for HIs to take less time than shams to make an arm choice (# = p=.119).

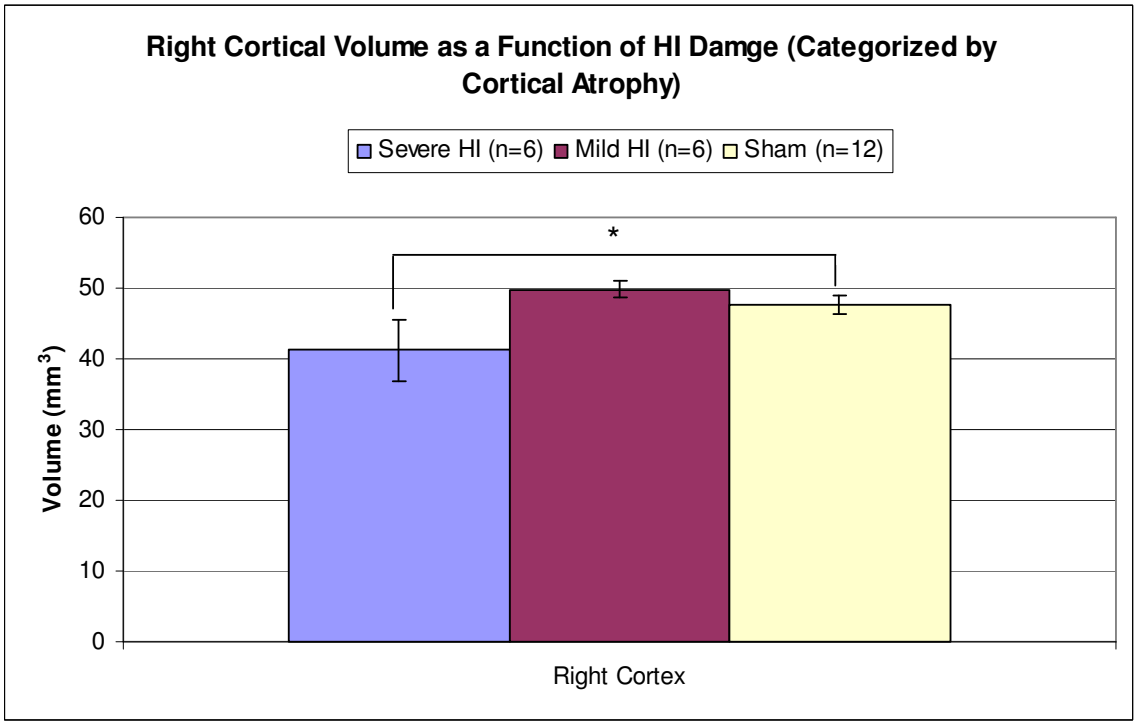

Figure 3: Using sub-groupings based on cortical atrophy, a one tailed ANOVA comparing right cortical volume in Severe HI and sham animals revealed a significant effect of Treatment showing smaller right cortical volume in Severe HIs (*=p<.05).

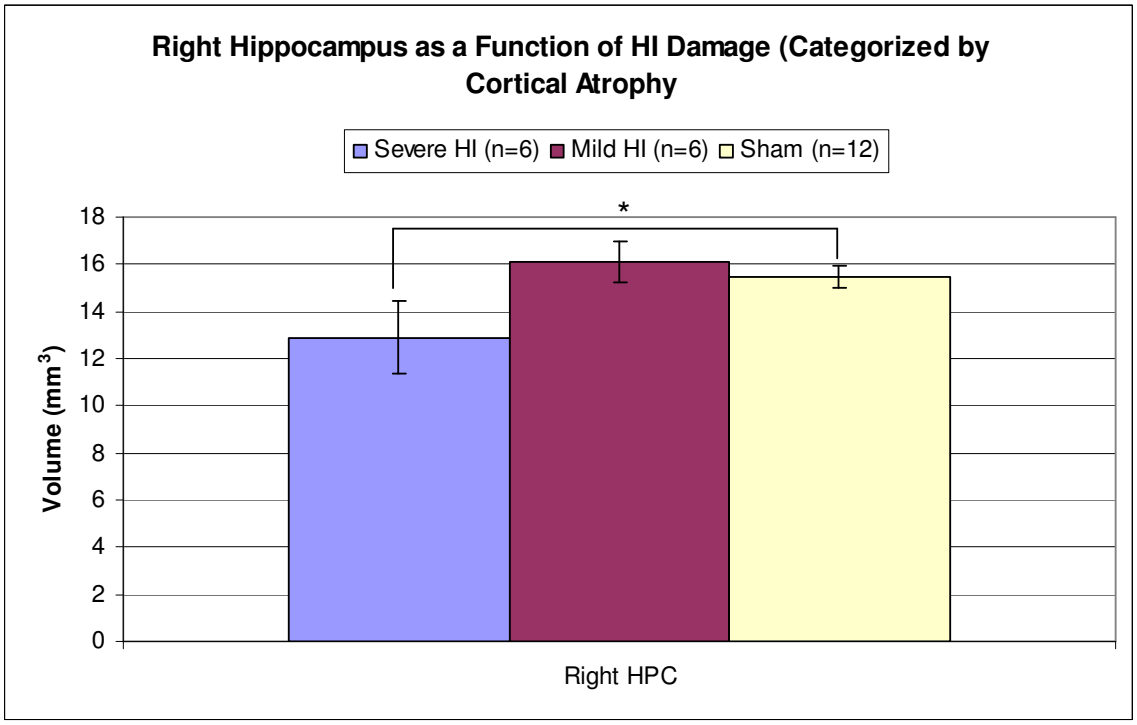

Figure 4: Using sub-groupings based on cortical atrophy, a one tailed t test comparing right hippocampal volume in Severe HI and sham animals revealed a significant effect of Treatment showing a smaller hippocampal volume in Severe HIs (*=p<.05).

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