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THE EFFECTS OF NESTING ENVIRONMENT ON NEONATAL HYPOXIC-ISCHEMIC INJURY

A Thesis Presented

by

LAURA GRACE ROLLINS, B.S.

Submitted to the Office of Graduate Studies,
University of Massachusetts Boston,
In partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

December 2013

Clinical Psychology Program

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ABSTRACT

THE EFFECTS OF NESTING ENVIRONMENT ON NEONATAL HYPOXIC-ISCHEMIC INJURY

December 2013

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B.S., College of Charleston

Directed by Professor S. Tiffany Donaldson

Term neonates with hypoxic-ischemic (HI) injury are at risk for devastating neurological sequelae. The objective of this study is to determine if altering the early environment for maternal care-taking impacts the immediate and long-term sequelae of HI offspring. The Rice-Vannucci model was used to induce HI in postnatal day (PND) 7 Long-Evans pups. Litters were assigned to a closed nest (CN) or normal standard housing (SH) condition. Neurobehavioral development, cognitive ability, and stress response were assessed to establish any benefits of the CN condition. Finally, postmortem brain tissue was analyzed for morphometric markers of injury.

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LIST OF ABBREVIATIONS

1. HIE- hypoxic-ischemic encephalopathy
2. HI- Hypoxia-Ischemia / Hypoxic-Ischemic
3. CN- Closed Nest
4. SH- Standard Housing
5. CTB- Care taking behavior
6. PND- Post Natal Day
7. GC- Glucocorticoid
8. GR- Glucocorticoid Receptor
9. ICC- Immunocytochemistry
10. LG- Licking and grooming
11. HPA- Hypothalamic-pituitary-adrenal
12. LMA- Locomotor Activity

CHAPTER 1

INTRODUCTION

For every 1000 live births, one to six neonates are diagnosed with hypoxic-ischemic encephalopathy (HIE) (Volpe, 2008). Surviving infants with severe HIE are at high risk for developing cerebral palsy, learning disabilities, intellectual disability and mental illness. Neurologically high-risk infants from low-income, stressful environments tend to have worse outcomes (Resnick, et al., 1990). Adverse outcomes may be associated with elevated maternal stress in impoverished environments which together have been shown to affect the quality and quantity of mother-infant interaction (Ivy, Brunson, Sandman, & Baram, 2008). We hypothesize that mother-infant interaction is key in the disparity of outcome in high-risk infants. Currently, there is no available intervention for HIE that addresses mother-infant interaction or environmental factors. Therefore, the goal of this study is to determine the impact of an early environmental intervention (closed nesting environment) on the long-term neurobehavioral and physiological sequelae of hypoxic-ischemic (HI) injury through altering maternal care taking behavior. To establish a controlled rearing environment and to obtain molecular, cellular, and neurochemical measures, an animal model of HI was employed. Hypoxic-Ischemic injury was induced using the Rice-Vannucci model of unilateral right common

carotid artery ligation in the offspring of 3 Long Evans dams (3 litters, 30 pups). Litters were assigned to a closed nest (CN) condition or standard housing (SH) condition. Neurobehavioral development, cognitive ability, and stress response were assessed until 60 days of age at which time animals were sacrificed and brain tissue was assessed for morphometric markers of injury.

1.1 Specific Aims

Specific Aim 1: To examine the effects of the closed nest (CN) versus standard housing (SH) conditions on the stability of pups' weight gain, as an indicator of frequency of maternal care-taking behavior (CTB).

Litters were assigned to either the CN or SH condition. In the CN condition, a nesting box was provided 10 days prior to expected parturition, which remained in place until weaning on postnatal day (PND) 21. Weights were obtained for pups from PND 7 to 21.

Hypothesis 1: It was expected that pups in the CN condition would exhibit more stable weight gain before and after HI than pups in the SH condition, with weight gain as an index of maternal CTB.

Specific Aim 2: To examine the relationship between CTB and neurological sequelae of HI.

Hypothesis 2a: It was expected that pups with HI in CN would demonstrate less functional impairment on neurobehavioral tasks than pups with HI in SH.

Functional Impairments were assessed with the following measures:

I. Development of early reflexes/physical characteristics (PND 8-21 daily)
   a. Eye opening
   b. Ear unfolding

c. Ear/eyelid twitch

d. Incisor eruption

e. Fore/hind-limb grasp

f. Startle

g. Body weight

II. Negative Geotaxis (PND 8-21 daily)

III. Locomotor Activity (PND 17, 21, 24)

IV. Rope Suspension (PND 17, 21, 24, 28, 31, 35, 42)

V. Water Maze (PND 35-50)

Hypothesis 2b: It was expected that animals in the CN condition, compared to those in the SH condition, would show evidence of less cell death, as quantified by disparities in hemispheric area, in the ipsilateral hemisphere of carotid artery ligation in a sex-dependent manner, with males exhibiting more damage than females.

Measures: Brains were prepared according to protein analysis. The hippocampus was blocked for analysis with anterior and posterior sections to be used for immunocytochemistry (ICC) and morphometric comparison. Serial coronal sections 60 μm thick were compared for differences in morphometry and in presence of necrotic damage.

1.2 Background and Significance

Hypoxic-Ischemic brain injury occurs in approximately 1-6 of every 1000 term live births (de Vries & Jongmans, 2010; Lindstrom, Lagerros, Gillberg & Fernell, 2006). Of these infants, many are at risk of developing severe injury resulting in death or devastating neurological disability, while others develop mild to moderate injury and present with varying degrees of cognitive impairment, learning disabilities, motor abnormalities, and social-emotional difficulties. Even with the best available treatment of moderate systemic hypothermia in the early phase of injury, mortality rates have been reported to be between 31% (Eicher et al., 2005) to 37% (Shankaran et al., 2008). In a study by Eicher et al., incidence of severe outcome (defined as death or severe developmental outcome) at 12 months was 52% in infants who received treatment and 84% in untreated infants. Another study found that among surviving infants at 18 months, 38% had a severe disability and 30% had developed disabling cerebral palsy (Shankaran et al., 2008). Neonatal HI injury continues to evolve as inflammation persists for weeks and months after injury. However, full manifestation of the injury's effects on cognition and social behavior may not be evident until older childhood or adolescence. When children reach school-age, they are faced with new cognitive and social challenges. In comparison to their peers, many children with HI lag in their neurodevelopmental trajectory and are therefore less well equipped to meet the mounting challenges that are expected of them. This disparity between children with HI and their peers becomes increasingly apparent during this time, which feeds into further problems with

psychosocial adjustment. Many children with HI also have trouble meeting major educational milestones due to high rates of learning and attentional difficulties. Children with moderate injury frequently meet criteria for attention deficit hyperactivity disorder. This is true even for those children with no evidence of motor disabilities (Lindstrom, Lagerros, Gillberg & Fernell, 2006). The effects of HI injury continue to create various behavioral and learning difficulties throughout development. One study found that 70% of teenagers with a neonatal diagnosis of moderate HI showed some form of cognitive impairment and 50% required special school placement (Lindstrom, Lagerros, Gillberg, & Fernell, 2006). In the same sample of adolescents, many reported problems with social adjustment and peer-relations.

While researchers continue to gather important information about HI in clinical research settings, animal models provide an opportunity to study neurophysiological effects and environmental manipulations in a way that would not be possible in human samples. Researchers have been developing and refining widely used rodent models of neonatal HI which map well onto the neurological and behavioral developmental trajectory of human neonates with HI. Rat models utilize a bilateral or unilateral carotid artery ligation followed by exposure to hypoxia (Northington, 2006), which creates neurobehavioral profiles similar to those seen in clinical populations. In addition, experimenters such as Lubics et al. (2005) have developed reliable methods for assessing neurobehavioral developmental sequalae of HI in rats, which correspond to neuropathology in rats and clinical pathology in humans. Data gathered from rodent

models have illuminated the clinical picture of HI by providing detailed information about the injury pathway, mechanisms, and developmental progression. This is a valuable tool for understanding the naturally occurring effects of HI as well as the effects of interventions and manipulations.

Using a rodent model, researchers have found that damage resulting from HI injury evolves from the initial insult at birth into adolescence and early adulthood (Mishima et al., 2005), which may indicate a possibility for intervention throughout development. Psychological stress and environmental factors could play an important role in the continuing evolution of this injury as well as in repair. Tsuji et al. (2010) demonstrated a functional improvement after introducing a rehabilitative protocol to adolescent rats subjected to neonatal HI. Studies have also found that environmental enrichment during pre-adolescent and adolescent periods can result in better cognitive functioning for female rats (Pereira, Strapasson, Nabinger, Achaval, & Netto, 2008).

While environmental enrichment may help to decrease injury and facilitate recovery, stress and hardship during these critical periods may exacerbate the effects of HI. Families of children with disabilities often experience high levels of stress. Parents of children with cognitive disabilities have reported stress and apprehension about their child's ability to cope with the demands of everyday life in the future (Floyd & Gallagher, 1997). In addition to fears and anxiety about their child's well-being, parents experience the stress of greater caregiving demands for a longer period of time than is typically experienced with a healthy child. A study of stress and well-being in parents of

children with cerebral palsy found that greater caregiving demands and lower SES predicted greater stress level and poorer mental and physical health in children (Raina et al., 2005). The association between poverty and poor developmental outcome in neurologically high-risk children may be, in part, due to the effects of stress on parental well-being.

Care-taking behavior in rat dams has been shown to be directly related to environmental stress. Repeated exposure to stress during gestation has dramatic effects on maternal licking and grooming (LG) behavior, significantly decreasing care-taking behavior in dams who had exhibited a high frequency of LG with previous litters (Champagne & Meaney, 2006). The lack of a secure nesting environment can be particularly stressful for nesting dams. This stress can negatively impact the dam's care-taking behavior and subsequently affect the development of offspring. Ivy et al. (2008) compared LG activity and arched back nursing of dams with adequate nesting material to those with no nesting material. The dams deprived of nesting material displayed inconsistent LG activity compared to those with nesting material. When nesting material was provided to the deprived dams, care-taking behavior was restored to the full extent observed in the dams in the control condition. This care-taking behavior may act as a buffer between the individual with HI and the environment. The frequency and duration of maternal care-taking behavior has been found to have profound effects on neurodevelopmental processes of the offspring. The effects of handling and parental care-taking behavior have also been demonstrated in the human literature. Research has shown

that parenting behavior mediates the adverse effects of pain and stress on cognitive development in high-risk infants in intensive care (Grunau, et al., 2009). Environmental stress poses additional challenges to parenting high-risk infants, yet within these constraints care-taking may provide a buffer.

As mentioned previously, nesting environment has a dramatic effect on care-taking behavior in dams. Standard lab conditions, in which a rat is housed in Plexiglas cages near other rats in a semi-open environment, are far from conditions preferred by rats in the wild. Research has shown that housing rats according to standard laboratory housing protocols can be stress-inducing due to the lighting conditions that interfere with the instinct to seek shelter from predators (Manser, Broom, Overend, & Morris, 1998). Studies have also shown that nesting in this unnatural environment encourages abnormal adaptation to these conditions, which affects stress response (Wurbel, 2001). A small nest box made of semi-opaque plastic provides an enclosed space for nesting dams, which reduces stress (Wurbel, 2001). If reducing environmental stress and providing a secure nesting environment increase care-taking behavior in dams, the provision of a nest- box should elicit more of this behavior.

In rats, the offspring of dams that display high levels of licking, grooming, and arched-back nursing show greater neural proliferation and synaptic density in the hippocampus, higher density of hippocampal glucocorticoid receptors (GR), and a less reactive hypothalamic-pituitary-adrenal (HPA) axis response (Bredy, Grant, Champagne, & Meaney, 2003) (Liu, et al., 1997). On the other hand, maternal deprivation has been

shown to decrease neural proliferation (Chocyk, Dudys, Przyborowska, Majcher, Mackowiak, & Wedzony, 2011) and accelerate the rate of glial and neuronal apoptosis in white matter tracts (Zhang, et al., 2002). These processes can dramatically alter the developmental trajectory for the normally developing brain. However, to date, few investigators have studied the effects of maternal care-taking behavior on the development of the injured brain. It can be reasoned that, if care-taking behavior affects the rate of apoptosis, synaptogenesis, and gliogenesis, it may affect the severity of damage from resulting from HI, as well as the capacity of the brain to rebuild and to develop adaptively with intervention.

Handling and tactile stimulation, which are thought to exert effects by increasing maternal care-taking behavior, have been shown to buffer the effects of HI on cognitive ability (Chou, 2001), hippocampal volume (Rodrigues, et al., 2004), and overall infarct volume (Davis, et al. (2011). One possible mechanism by which maternal care-taking behavior may influence HI injury is through action on glucocorticoids (GC) and glucocorticoid receptors (GRs). In excitatory injuries, such as ischemia, GCs can exacerbate neuronal loss in the hippocampus, a region implicated in plasticity as well as learning and memory (Reagan & McEwen, 1997). Studies have demonstrated that chronically high levels of GC may increase the vulnerability of neurons to excitotoxic injury in HI (Sapolsky & Pulsinelli, 1985) implicating the regulation of GCs in the neuronal death seen in this injury. However, GCs are not always harmful. As with many neuromodulatory factors, GCs have a dual, context-specific role in injury and repair,

instigating apoptosis as well as neurogenesis (Reagan & McEwen, 1997). In fact, dexamethasone, is a synthetic GC commonly used clinically in preterm labor to stimulate lung maturation in the fetus, and offers antenatal neuroprotection in infants born prematurely. Dexamethasone has also been found to be an effective method of neuroprotection in animals with HI (Tuor, 1997). Tuor (1997) found that when GRs were blocked, this neuroprotective effect was no longer found, indicating that GR density and function contribute to both injury and repair (Tuor, 1997). Other studies have found that the effects of GCs in excitotoxic injury are dose-dependent, in which the amount of GC has a U-shaped association with cell death (De Kloet & JoeEls, 1996), such that apoptosis is most severe when GC concentration is on the lower or upper end of the distribution. Research has also shown that GCs have a U-shaped association with behavioral development, with low amounts of GC activity being necessary for stimulating neuroplasticity as well as learning and memory and higher amounts resulting in behavioral inhibition (Dua et al., 2009). Glucocorticoid receptors are important for modulating the activity of glucocorticoids. Studies have shown that licking, grooming, and arched-back nursing increases stores of hippocampal GRs in the offspring by demethylating glucocorticoid-receptor promoter genes in hippocampal tissue (Weaver, et al., 2005). Therefore, it is possible that creating greater opportunities for maternal care may in fact increase and/or stabilize GRs through care-taking behavior, and this may in turn encourage neuroprotection. That is, more stable GRs may permit more glucocorticoid action thereby aiding in repair and neurogenesis.

In contrast to the beneficial long-term effects of maternal care-taking behavior on brain structure and stress response, repeated periods without maternal contact can have a detrimental impact on outcome in HI. McPherson et al. (2009) demonstrated that regular short periods of maternal separation was associated with a spike in corticosterone levels in pups with HI, which exacerbated damage in the internal capsule and thalamus.

Given the evidence discussed above, we hypothesized that nesting environment would affect maternal care-taking behavior which would impact the severity of insult and impairment of functional outcome in offspring with hypoxic-ischemic injury. Specifically we proposed that (1) the provision of a nest-box in the home cage would increase maternal care-taking behavior and that (2) pups reared in the nest-box environment as compared to standard housing environment would exhibit less tissue damage and cell death as well as a greater capacity for adaptation and functional improvement after exposure to hypoxic-ischemic insult.

CHAPTER 2

RESEARCH DESIGN AND METHODS

2.1 Animals

Twenty-six Long-Evans rat pups and 3 dams were used. A power analysis (80%, alpha at $p<0.5$ for 9 animals for behavioral measures) was performed for the proposed studies to determine the adequate number of animals in each housing/sex group. In keeping with findings in the current literature, an average of 6-8 animals per sex and treatment group were used for biochemical analyses, which is slightly more than is standard for publication. In order to prepare the tissue for morphometric analysis, ICC, and Western-Blot, animals were sacrificed using live decapitations.

Table 1.

A descriptive table of animals used in the study by housing condition and sex.

	Standard Housing	**Closed Nest**
Female	7	6
Male	6	8
Total	**13**	**14**

2.12 Justification for Species. The rat model of term neonatal hypoxic-ischemic injury has been the predominant animal model for studying the neurological, physiological, and behavioral sequelae of clinical term newborn HIE. Clinical studies of newborn HIE presents many confounding factors, such as timing of injury, degree and duration of oxygen deprivation, length of time to diagnosis and intervention, as well as variable post-injury conditions. The use of a rat model allows for a standardized, validated induction of injury, via unilateral carotid artery ligation and hypoxia, as well as substantial control of the environment in which the individual recovers and develops. Studies with humans have found that neurologically high-risk infants reared in poverty have poorer developmental outcome. A rodent model provides an opportunity to create controlled environmental stress and enrichment in order to test the interaction of environmental conditions and hypoxic-ischemic injury on a level which is not feasible in a clinical population. There is a great deal of literature on the procedure for injury induction, neurobehavioral testing, and physiological measures that have been validated for this model in order to avoid duplication or unnecessary procedures.

There are no appropriate *in vitro* and/or computer modeling available to mimic the full neurobehavioral profile observed in the rodent model. Other models used for hypoxic-ischemic injury include large animal models such as sheep, swine, and primates. Due to the duration of gestation and rate of development, large animal models require investment of significantly more time and resources. Also, the facilities at UMass Boston

are not equipped for large animal testing and housing. In addition, most large animal models, with the exception of primates, are less adept at performing the fine-motor tasks for behavioral testing than rats. The rat model, though it produces a range of injury severity, maps quite well onto the neurological and behavioral developmental trajectory of individuals who survive neonatal HIE. The variability of severity seen in this model serves to increase its validity rather than work against it, in that it accurately reflects the variability seen in the clinical presentation of the injury (Barks et al., 2010).

2.2 Procedures

2.21 Hypoxic-Ischemic Injury Model. The Rice-Vannucci model of unilateral right common carotid artery ligation followed by 90 minutes of exposure to 8% Oxygen balance Nitrogen was used to induce HI injury in rat pups. On day 7, pups weighing between 12 and 16 grams were anesthetized using isoflurane gas while the right common carotid artery was tied in double ligation and sectioned. During the surgical procedure, the pups were sedated and gently secured with surgical tape on a sterile surgical board. The skin over the incision site was disinfected by swabbing with 70% alcohol followed by painting with 2% povidone iodine solution. Aseptic surgery was be performed using sterile instruments to make a midline incision over the trachea, blunt dissection laterally to the right carotid artery bundle, isolating the carotid artery from the jugular vein and vagus nerve and double ligating the artery with sterile surgical silk. The double ligated artery was then severed between the ligations being sure not to cut any of the ligatures. The tissues were then replaced in their natural anatomical positions and the skin closed with two non-capillary, non-absorbable sutures. The surgical site was cleaned with 70%

alcohol, allowed to dry and medical tape applied. Post-operatively, pups were observed for signs of recovery from anesthesia (rolling onto sternal recumbancy, leg movements and ambulation, blinking, normal ventilation rate, pink color to ears and eyes, etc.). After, animals were returned to the dam upon recovery form anesthesia, for approximately 2 hours. Pups that did not recover from anesthesia adequately were humanely euthanized by carbon dioxide overdose and cervical dislocation. When returned to the dam, the animals continued to be monitored to ensure maternal acceptance of the pups, post-surgically. Any pup rejected by the dam or traumatized was either humanely euthanized by carbon dioxide overdose with cervical dislocation or transferred to a same-treatment dam. After a 2-h rest period with dams, pups were placed in an air-tight, Plexiglas chamber with humidified 8% Oxygen balance Nitrogen for 90 minutes (Ikeda, et al., 2001). After the hypoxia exposure was complete, pups were returned to their home cage and dam.

2.22 Nest box. A nest box was constructed from a commercially available plastic box made of opaque, durable plastic. A smoothed, rounded hole was made in the front side of the box for entry. The bottom and rear-facing side of the nest box was open to allow for behavioral observation. *See Figure 2.*

2.23 Euthanasia and Tissue Preparation. After the culmination of behavioral testing on PND 50, animals were sacrificed according to the type of analysis used for brain tissue. All animals underwent live decapitation for brain extraction in order to prevent any complications with protein analysis. All brains were flash-frozen, and stored

at -80˚C. Spleens were removed and weighed after post-mortem and discarded with the remaining tissue. At the time of sacrifice, trunk blood was collected, separated, and frozen for later analysis of corticosterone levels.

2.24 Live decapitation for Western blot analysis. When conducting biochemical analyses in rodents, it is sometimes necessary to examine samples of the tissues and fluids (organs, blood, cells, etc.) in the absence of anesthetics, analgesics or other drugs that would modify the biochemical activity of these tissues and biological fluids. These drugs would contaminate the samples, and obscure the effects of the experimental manipulations. When these circumstances arise, the most rapid and efficient method for terminating the life of the rodent and collecting the samples is decapitation. This method provides only momentary distress to the animal. It is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association, and it is conditionally acceptable in a laboratory setting when performed by trained professionals.

2.25 Immunocytochemistry Protocol. Before sectioning, brains were thawed to room temperature in 4% paraformaldehyde for four days. For cryoprotection, brains were then transferred to a 20% sucrose 40% paraformaldehyde solution for 48 hours followed by a 10% sucrose 40% paraformaldehyde solution for 48 hours before being placed on a sectioning stage in a cryostat. Sixty-micron sections were cut frozen and placed in wells in sequence in preparation for free-floating immunocytochemistry. Tissue was flushed in successive washes and endogenous perioxidase activity lowered to prepare for primary

antibody incubation. Twenty-four to forty-eight hours later, tissue sections were rinsed three times with 95%, 70%, and 50% ETOH and then rinsed with dH_20 twice, then incubated for 1 min in Cresyl Violet (Vectastain Elite kit, Vector Laboratories, Burlingame, CA), rinsing again with dH_20, followed by 50% ETOH, 70% acit ETOH, 95% ETOH, and 100% ETOH. The tissue is then incubated in Histoclear for 5 min. Stained tissue was mounted onto glass slides and cover-slipped using permount. SPOT monochrome camera and software were used to grab light microscope images of brain areas of interest and ImageJ software (NIH) was used to analyze the immunoreacted cells. The extent of cortical damage was determined by calculating the area of each hemisphere as well as the area of the hippocampus. The area of the ipsilateral hemisphere was then compared to the area of the contralateral hemisphere to estimate the size of the lesion in the ipsilateral hemisphere. The area of the hippocampus was calculated using the same procedure.

2.3 Measures

2.31 Development of physical characteristics. From PND8- PND21 pups were checked daily for changes in incisor eruption, eye-opening, and ear unfolding. Results were recorded either as positive if present or negative if the characteristic has not developed. Body weight was recorded on PND 5, daily from PND 7-21, and on testing days after weaning.

2.32 Early Neurobehavioral Developmental Battery

1. Reflexes. Early Reflex development was tested and recorded from the day after injury until weaning (PND8-PND21) according to Lubics neurodevelopmental battery (Lubics, et al., 2005). The following reflexes were tested:

A. Sensory reflexes. Using the refined (by twisting and pulling the cotton) tip of a cotton swab, the edge of the pup's ear was lightly stroked from the outer edge of the ear moving inward. In a similar fashion, each upper eyelid was gently stroked. The response was recorded as present if the muscles of the ear/eyelid contract in response to the stimulation.

B. Grasp. Grasp reflex was assessed by placing the wooden end of a Q-tip on the pad of each paw while the experimenter held the pup firmly by the nape in an upright position. Grasp was recorded as positive (qualified as weak or strong) or negative. The response of each limb was recorded.

C. Auditory Startle Reflex. Pups were individually separated from the litter and placed on a flat surface. If the pup flinches in response to a clapping sound, the result was recorded as positive.

2. Negative Geotaxis. A 30 cm padded board was placed at a 45˚ incline. At the time of testing, pups were placed head-down in the center of the board. The first day that the pup turned around and placed its fore-limbs

on the upper ledge of the board within 30 sec was recorded. If the pup failed to turn around and reach the top of the board within 30 sec, the trial was recorded as negative. The time in sec was also recorded for trials in which the pup reached the top of the board in under 30 sec. Negative Geotaxis was tested daily from PND 8 to PND 21. *See Figure 3.*

2.33 Locomotor Activity. Animals were assessed in an automated locomotor activity chamber for 5 min trials on PND 17, 21, and 24. Pups were placed in the center of a (L: 17.5in W: 17.5in H: 12in) chamber at the beginning of the testing period. The ambulatory distance traveled, ambulatory counts, and vertical counts were recorded over a 5 min observation period using MED-PCIV motion tracking system. *See Figure 4.*

2.34 Motor Coordination. Animals underwent motor coordination tests twice a week from PND 17 to PND 42. These measures were adapted from the neurobehavioral battery described by Lubics et al. (2005) to test motor impairment in a rat model of neonatal HI.

Rope Suspension. To test bilateral for-limb strength, animals were suspended over a cushion by both fore-limbs on a horizontally suspended nylon rope. To test the strength of the affected forelimb contralateral to the injury, the paw of the ipsilateral forelimb was taped with medical tape in order to restrict it's use and animals were suspended by the contralateral forelimb. In both tests, the time up to 30 seconds was recorded. *See Figure*

2.35 Morris Water Maze. Morris Water Maze testing took place in a round pool (dimensions) filled with room-temperature (± 25 °C) water, which was made opaque by mixing small amounts of non-toxic acrylic white paint (See Figure 6). The pool was marked on the rim and on the internal wall with a large, differently colored shapes as cues at the North (N), East (E), South (S) and West (W) points on the pool. Animals were tested in the Morris Water Maze task daily from PND 35 to PND 43. On the first testing day, the rat was placed on the platform and will remain on the platform for 30 sec before retrieval and testing. Each testing day consisted of four consecutive trials with 10 minute inter-trial intervals. Each trial began by placing the rat into the pool facing the wall at a different location each time. Each trial allowed for a latency of up to 60 seconds. If the rat was unsuccessful in acquiring the platform within the 60 second testing period, the experimenter guided the rat to the platform. Once on the platform, the rat was left on the platform for 10 sec before retrieval, whether the rat acquired the platform on its own or with the help of the experimenter. Between each trial, the animals were dried and warmed. Animals' performance was video recorded using a digital video camera (Hitachi DVDcam) to later analyze swimming speed, latency to reach platform, and total distance for each trial.

1. *Visible Platform Training:* visible platform (VP) training took place on testing days 1 through 3. For the VP testing phase, a platform rising 1 inch above the water surface was placed in the SE location in the tub and

remained there for the remainder of the VP trials. For each of the 3 testing days, animals were placed in the tub, facing the tub wall, in the following order for the 4 trials: S, W, E, and N.

2. *Invisible Platform Training:* An invisible platform (IP) testing took place on testing days 4-9. For this phase, the transparent platform was submerged 1 inch below the opaque water's surface so that it was no longer visible. The platform remained in the SE location of the tub for the duration of IP testing. For IP testing, animals were placed in the N, N, NW and NW areas of the tub for the four trials.

Figure 1.

Timeline of study measures.

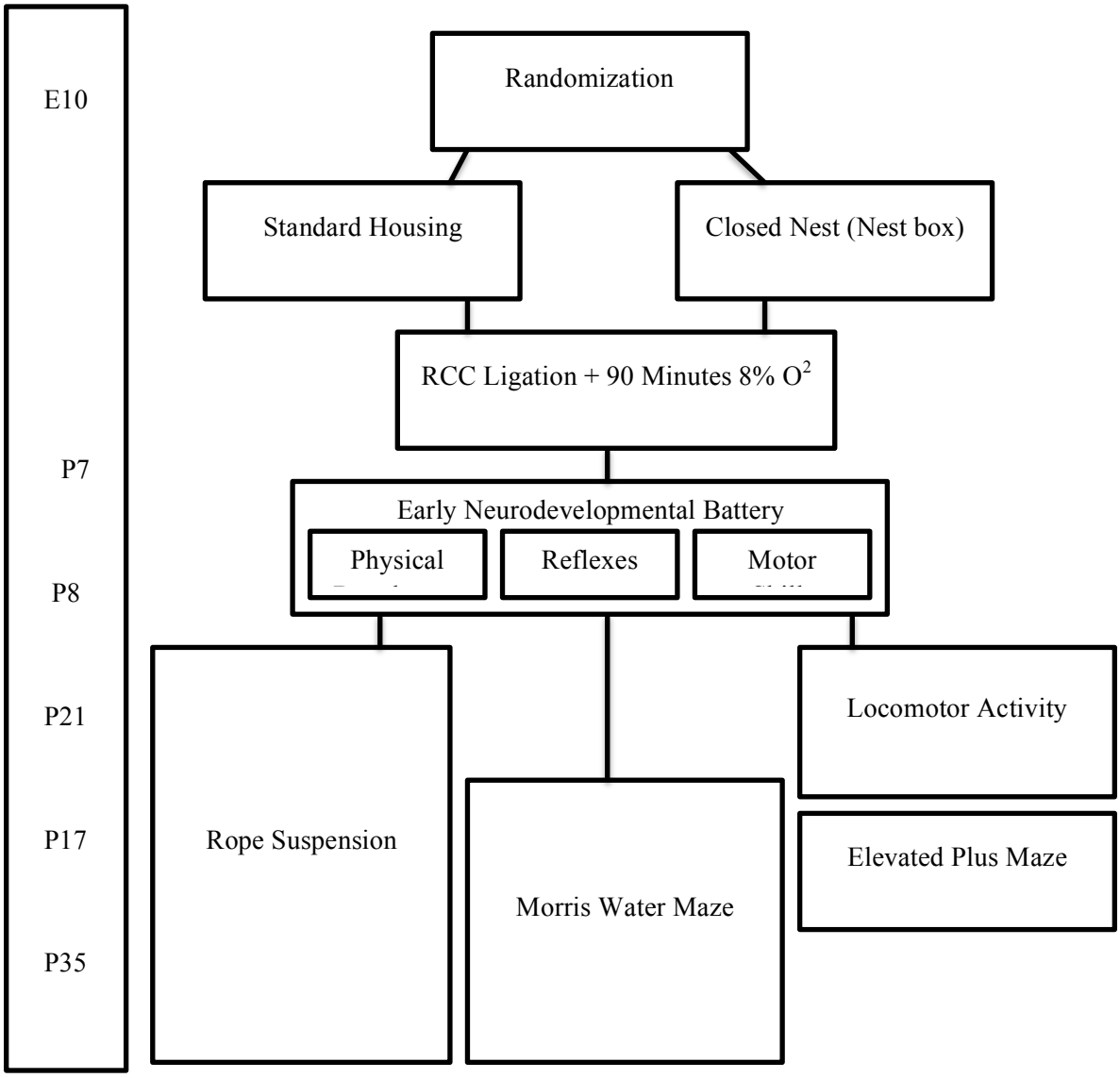


Figure 2.

Standard Housing (Left) Closed Nest (Right)

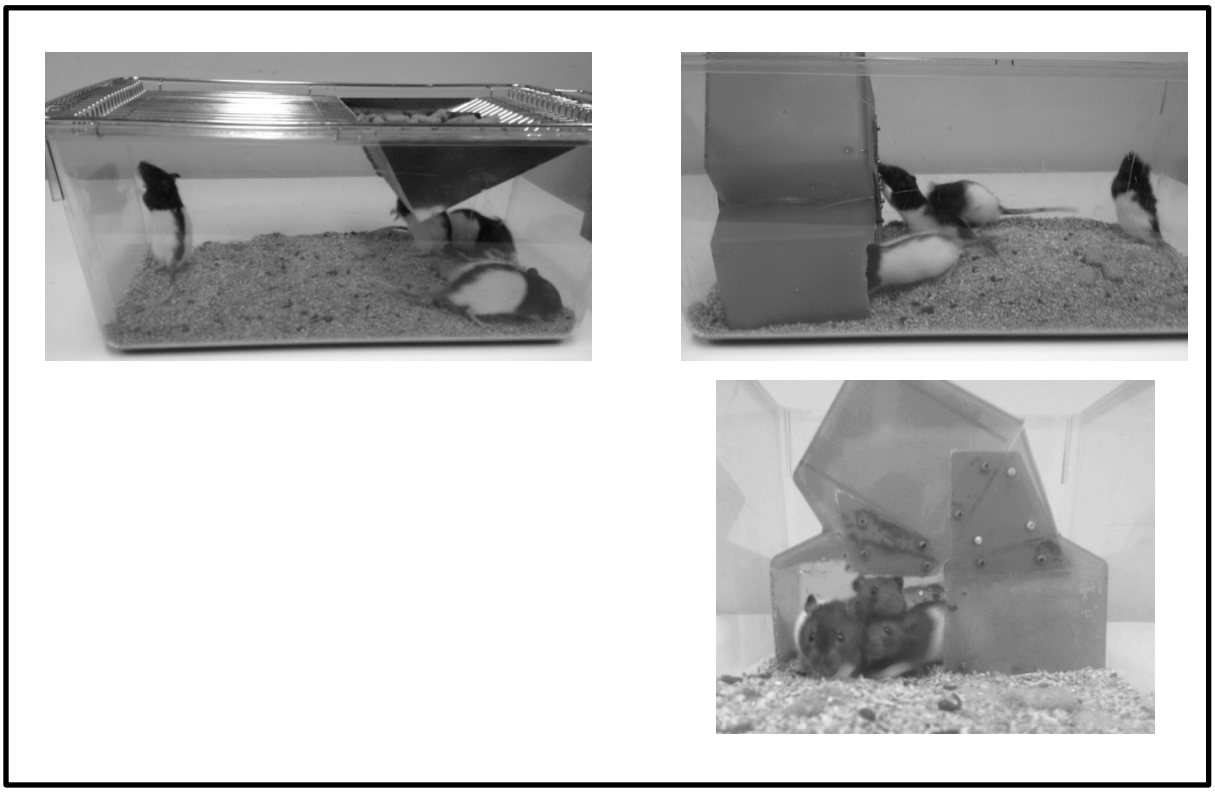


Figure 3.

Negative Geotaxis

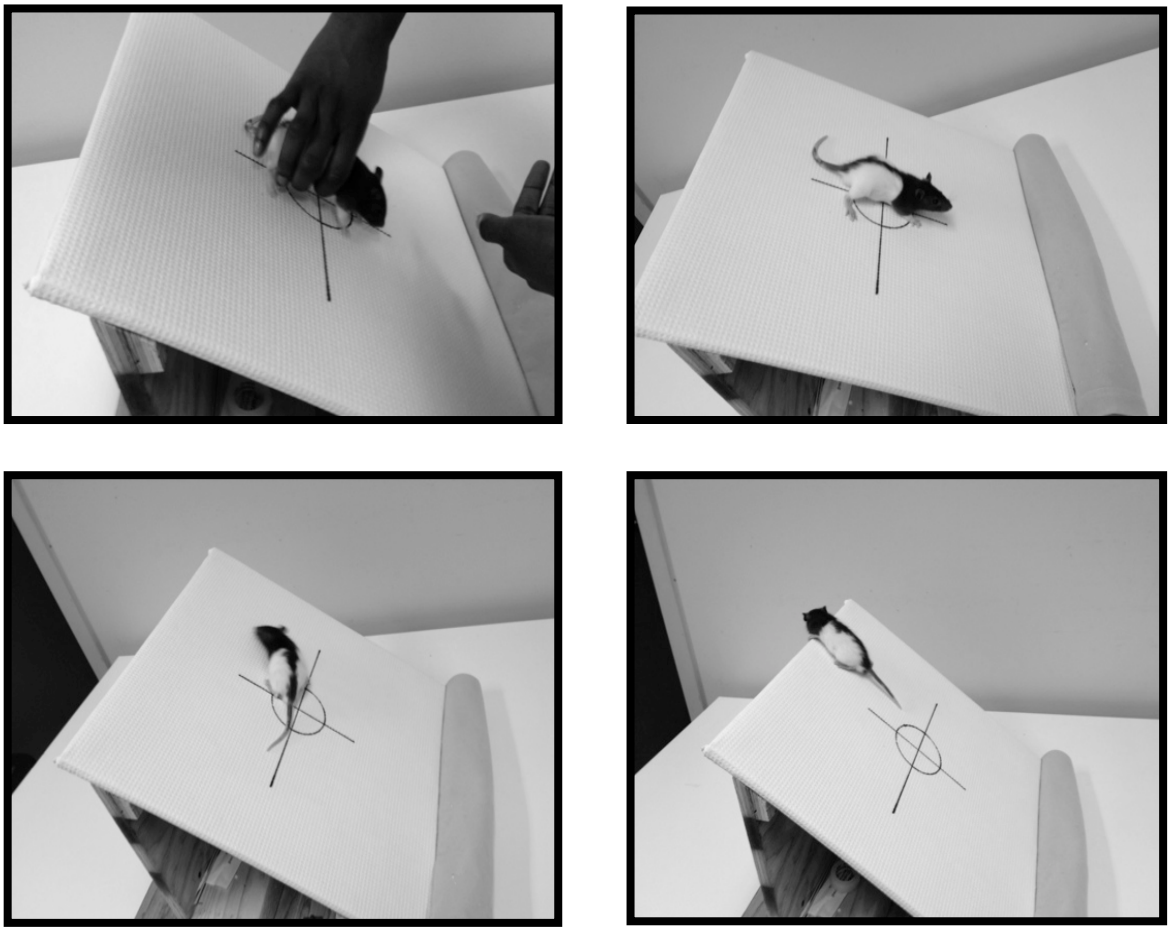


Figure 4.

Locomotor Activity Chamber

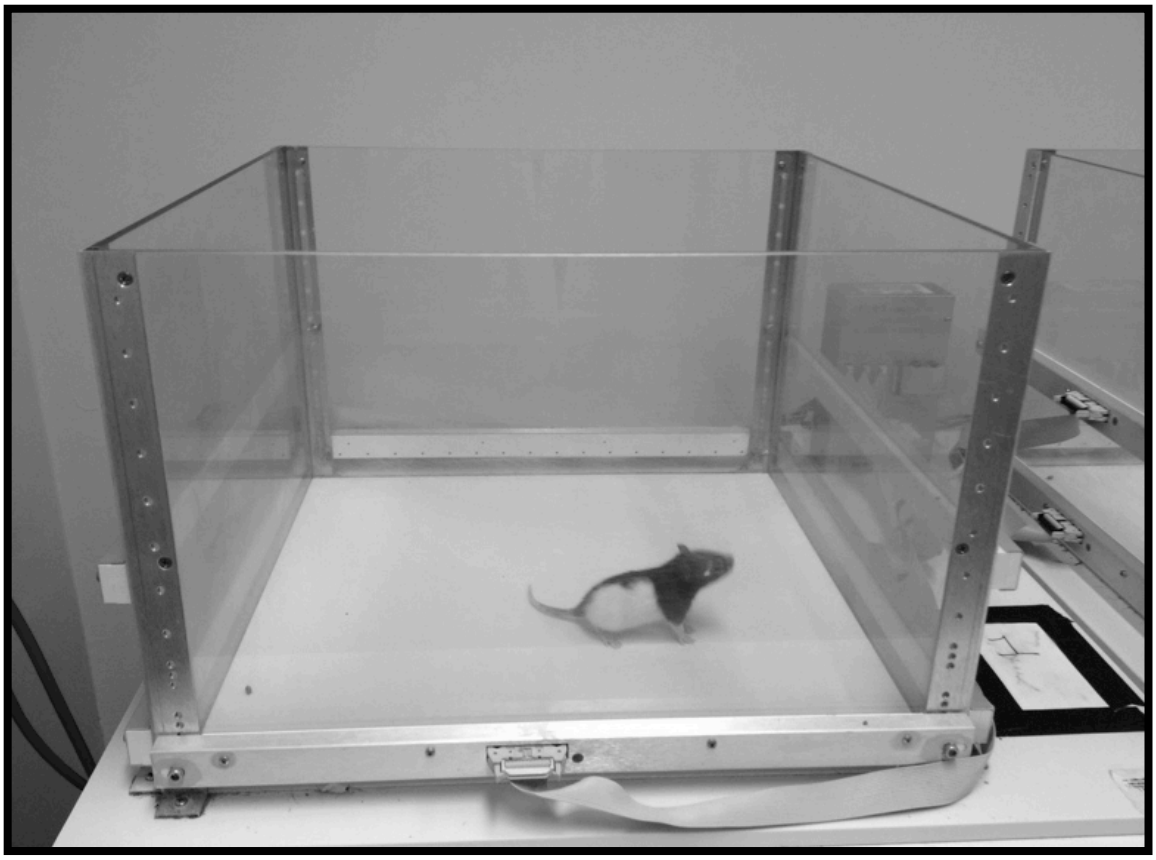
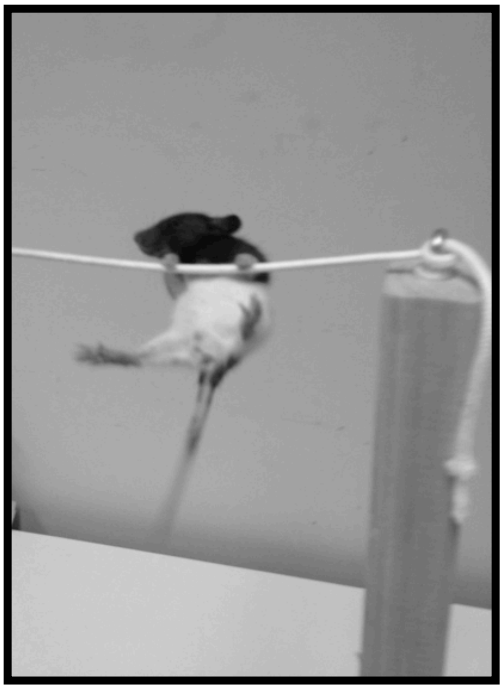


Figure 5.

Rope Suspension



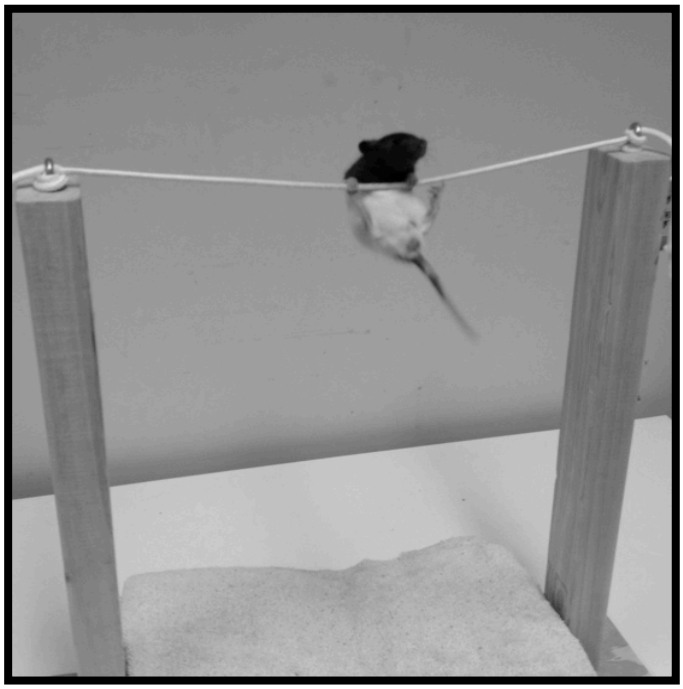
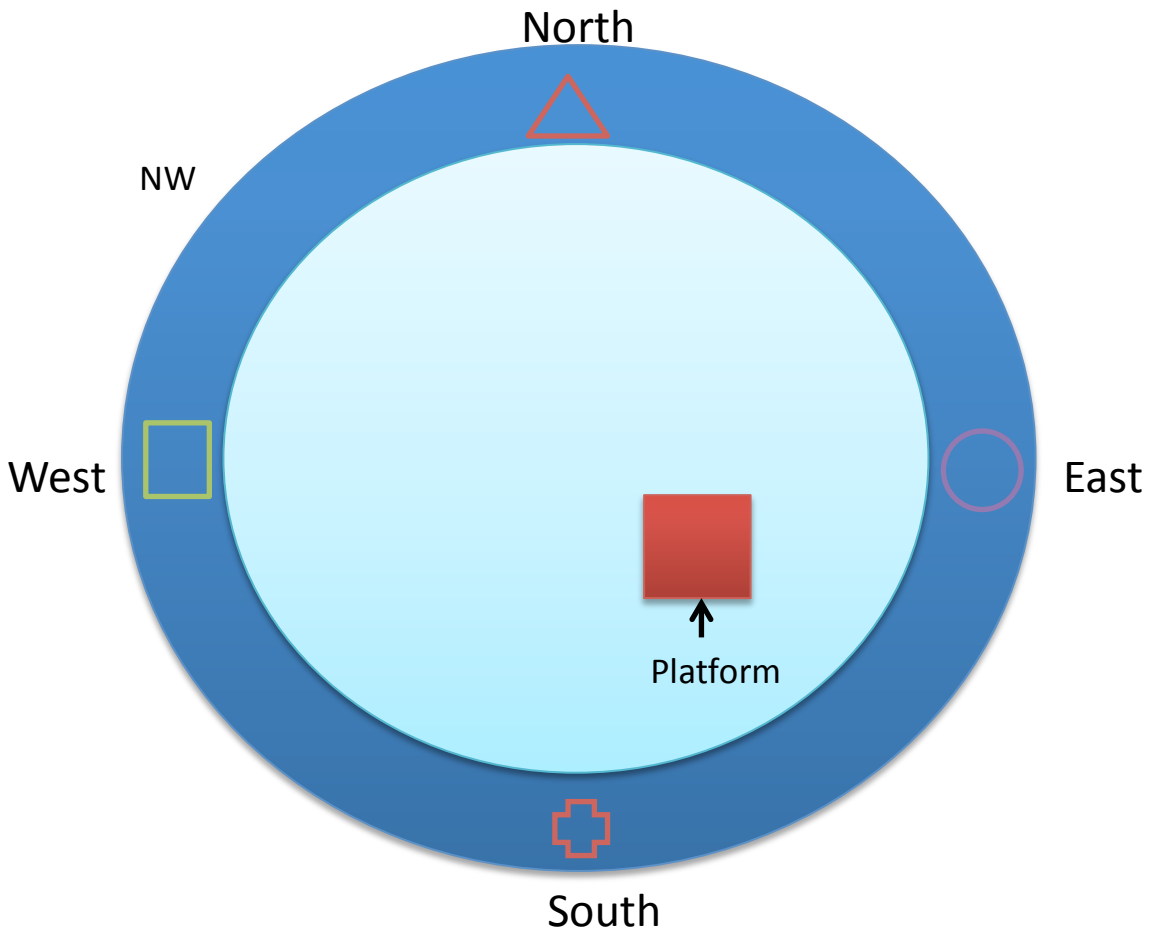


Figure 6.

A diagram of the Morris Water Maze showing the four initiation points indicated by symbols on the inside wall of the pool and location of the platform in the South East quadrant.



2.4 Analytic Plan

All analyses were done using statistical packages (SPSS 20). When multiple comparisons were necessary, a post-hoc test was run. An alpha value of 0.05 was established as the criteria for significance for all tests. Before running final analyses, the distribution of the data was assessed for normality using SPSS according to housing condition and sex. If data were normally distributed within these groupings and sufficient power was found, analyses included housing condition and sex as factors.

In order to address the hypotheses of the study, the following analyses were performed.

Hypothesis 1: It was expected that pups in the CN condition would exhibit more stable weight gain before and after HI than pups in the SH condition, with weight gain as an index of maternal CTB.

In order to compare weight gain over time between NB and SH conditions, weights recorded from postnatal day 7 to 21 were analyzed according to nesting condition using a Linear Mixed Regression model with Repeated Measures.

Hypothesis 2a: It was expected that pups with HI in CN would demonstrate less functional impairment on neurobehavioral tasks than pups with HI in SH.

All behavioral data were analyzed according to nesting condition. The inclusion of sex and severity of injury in the analysis of behavioral data were determined according to the previously stated criteria of normal distribution and sufficient power. The development of early reflexes and physical characteristics were analyzed according to the

first day that the reflex or characteristic is recorded as positive for three consecutive days using a Multivariate General Linear Model. Negative geotaxis was analyzed according to the first day the rat achieved a recorded time of at least two seconds less than the thirty second baseline time (t<29 seconds), and was analyzed using an Independent Samples t-Test. Changes in performance on negative geotaxis and rope suspension over time were analyzed according to nesting condition and previously determined factors using a Linear Mixed Model with repeated measures.

Data collected in locomotor activity were analyzed for total distance traveled, vertical counts, and average velocity according to housing condition and sex using Linear Mixed Models.

Data from the Morris Water Maze task were analyzed using a General Linear Mixed Regression Model with Least Squares Determined post-hoc. In the model, Rat ID was specified as Subject ID; trial was nested within day and specified as the repeated measures variable; and factors were specified as Housing Condition*Trial(Day); and Housing*Sex*Trial(Day). Latency to reach the platform was analyzed. The time it took (up to 60 seconds) to reach the platform on the first trial of each training day was analyzed as a measure of long-term memory. The change in latency to reach the platform on successive trials each training day was analyzed as a measure of short-term memory.

Hypothesis 2b: It was expected that animals in the CN condition, compared to those in the SH condition, would show evidence of less cell death, as quantified by

disparities in hemispheric area, in the ipsilateral hemisphere of carotid artery ligation in a sex-dependent manner, with males exhibiting more damage than females.

Brains were prepared according to protein analysis. The hippocampus was blocked for analysis with anterior and posterior sections to be used for immunohistochemistry (ICC) and morphometric comparison. For morphological analysis, coronal sections 60 μm thick in 300 μm intervals were compared for differences in morphometry and in presence of necrotic damage according to the protocol put forth by Rodrigues et al. (2004). Images of the sections were captured using high-resolution microscope camera and displayed on a high-resolution monitor for analysis. Cortical area, and overall hemispheric measurements were calculated from slices at level -3.30 mm from Bregma. Hemispheric area was calculated using the methods described above by collecting measurements of the space between the outer boundaries of the cortical area and subtracting the area of ventricular space. Hippocampal area was measured in the same fashion.

CHAPTER 3

RESULTS

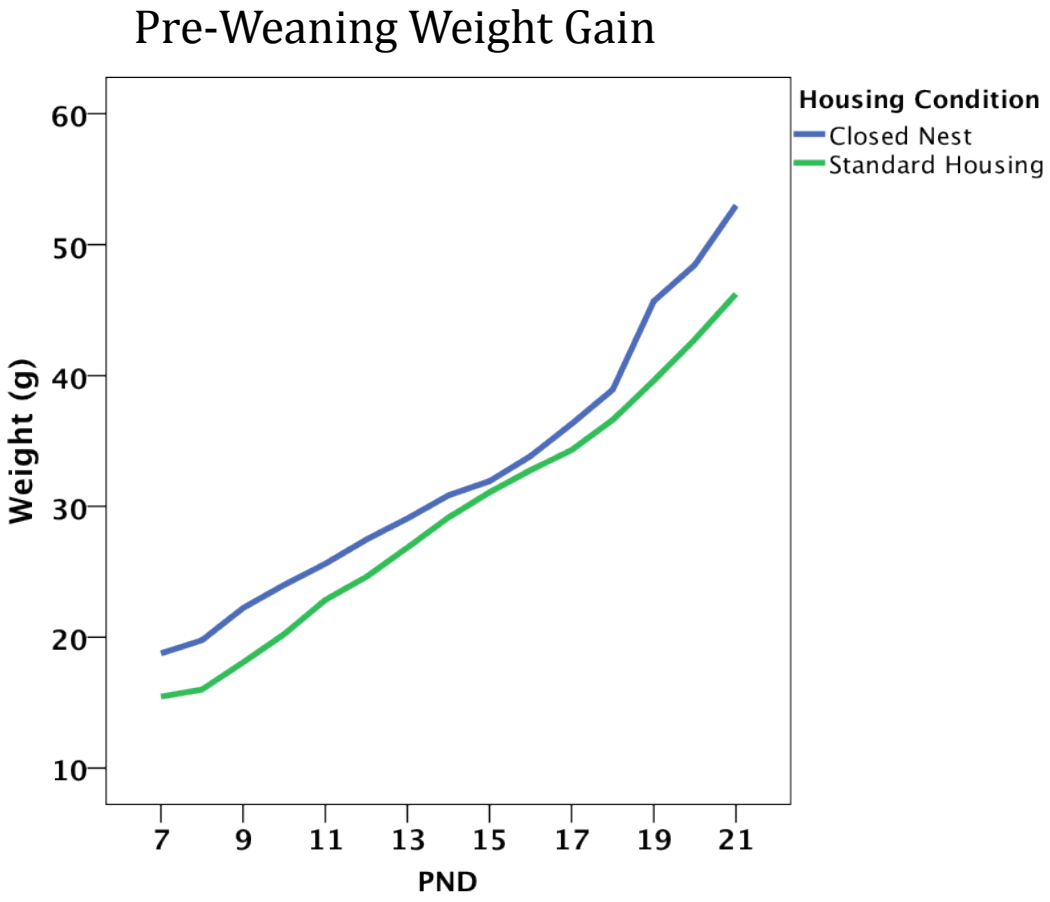
3.1 Neurobehavioral Tests

3.11 Aim 1 To examine the effects of the closed nest (CN) versus standard housing (SH) conditions on the stability of pups' weight gain, as an indicator of frequency of maternal care-taking behavior (CTB).

1. Weight gain. A Linear Mixed Model with repeated measures was used to test whether pups in the CN condition exhibited greater weight gain than those in the SH condition, as an indication of greater frequency of maternal CTB. Body weight data was collected daily from the time of surgery at PND 7 until weaning at PND 21. In support of this hypothesis, pups in the CN condition gained significantly more weight than SH pups, over the 14-day period between the time of the surgery and weaning ($p<0.001$, See Figure 7).

Figure 7.

Weight gain from PND 7 to PND 21.



3.12 Aim 2 To examine the relationship between CTB and neurological sequelae of HI.

3.12a Hypothesis 2a: It was expected that pups with HI in CN would demonstrate less functional impairment on neurobehavioral tasks than pups with HI in SH.

1. Day of appearance of physical characteristics and reflexes

In preparation for a Linear Mixed Model analysis, a preliminary analysis was performed in order to assess for normality of distribution. Data from the following measures in the battery were found to have too little variability to perform valid analyses and were removed from the final analysis. The measures removed included a) fore-limb and hind-limb grasp, b) incisor eruption, and c) eye twitch. The timing of right and left ear unfolding were found to be identical for every subject and were therefore combined into a single variable. For the final analysis, a Multivariate General Linear Model was performed according to housing condition to assess for the day of appearance of ear unfolding, right ear twitch, left ear twitch, right eye opening, left eye opening, and auditory startle.

Animals reared in the CN condition exhibited significantly earlier development of several physical characteristics compared to animals in the SH condition, exhibiting ear unfolding 2.23 days earlier ($F(1,24)=67.280$, $p<0.001$) and left eye opening 1.8 days earlier ($F(1,24)=64.0$, $p<0.001$). There was a statistical trend towards earlier right eye opening in CN pups as well, opening an

average of 1 day earlier ($F(1,24)=3.618$, $p=0.069$). Performance on the early neurodevelopmental battery also differed between housing groups in the timing of reflex development. CN pups displayed significantly earlier development of a left ear twitch by 1.9 days ($F(1,24)= 4.851$, $p=0.037$), however, the development of right ear twitch was not significantly different between housing groups ($F(1,24)=2.151$, $p=1.55$). CN pups also exhibited an auditory startle response an average of 1.5 days earlier than SH pups ($F(1,24)=9.757$, $p=0.005$).

Table 2.

Timing of reflex development.

Measure	Standard housing	Closed Nest
Eye opening	15.4 ± 0.2	13.5 ± 0.1*
Ear unfolding	15.5 ± 0.2	13.2 ± 0.2*
Incisor eruption	8.3 ± 0.1	8.4 ± 0.4
Eye twitch reflex	8.3 ± 0.8	8.5 ± 0.9
Ear twitch reflex	15.0 ± 0.7	13.1 ± 0.5*
Auditory startle	14.5 ± 0.3	13.1 ± 0.3*

* $p<0.05$

Figure 8.

Mean postnatal day of first appearance of reflexes and physical characteristics (error bars represent ±1 standard deviation from the mean).

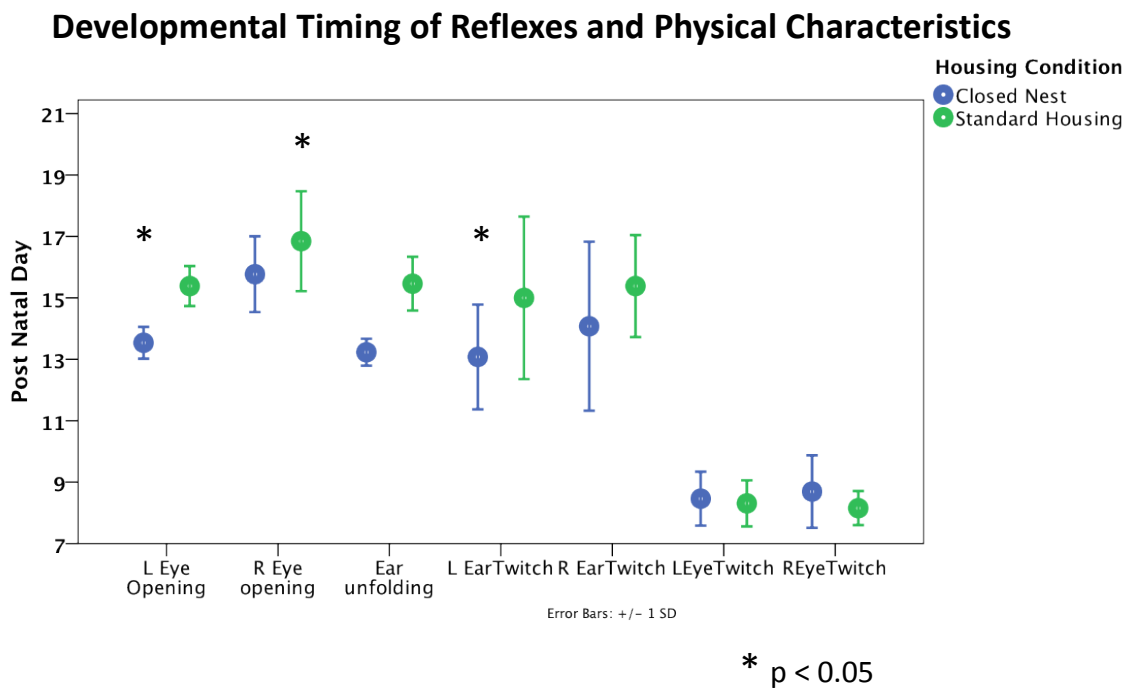
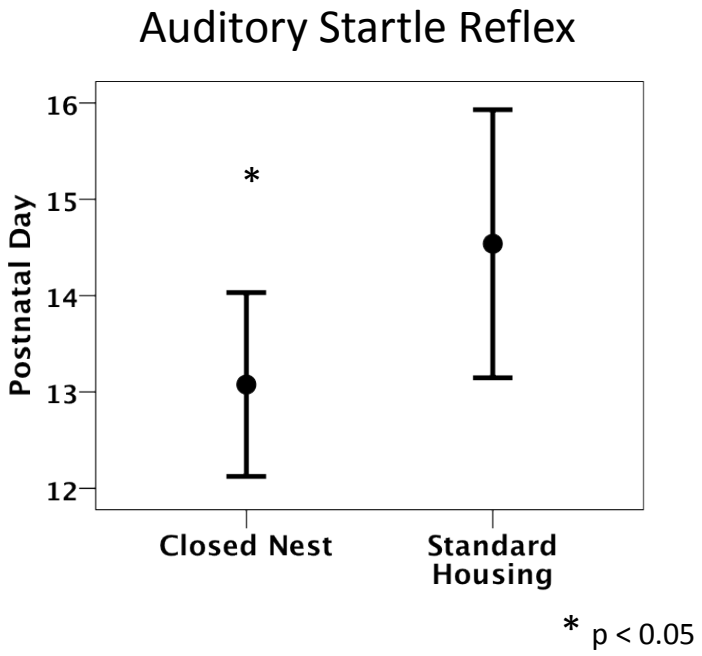


Figure 9.

Mean postnatal day of appearance of the auditory startle response (error bars represent ± 1 standard deviation from the mean).



2. *Negative Geotaxis*

A Linear Mixed Model with repeated measures was used to analyze data from 14 trials of Negative Geotaxis. Over the 14 day testing period, animals in the SH condition reached the top of the board significantly faster in Negative Geotaxis than those in CN condition ($F(1, 27)=9.536$, $p<0.001$). An Independent Samples t-Test was used to assess first day at which animals reached the top of the board in under 30 seconds for three consecutive days. There was a trend for animals in the CN condition to reach the top of the negative geotaxis board in under 30 seconds earlier in development (PND 14.7) than those in the SH condition (PND 16.8) ($p=0.086$).

Figure 10.

Mean time required for animals to reach the top of the Negative Geotaxis board over the 14 day testing period.

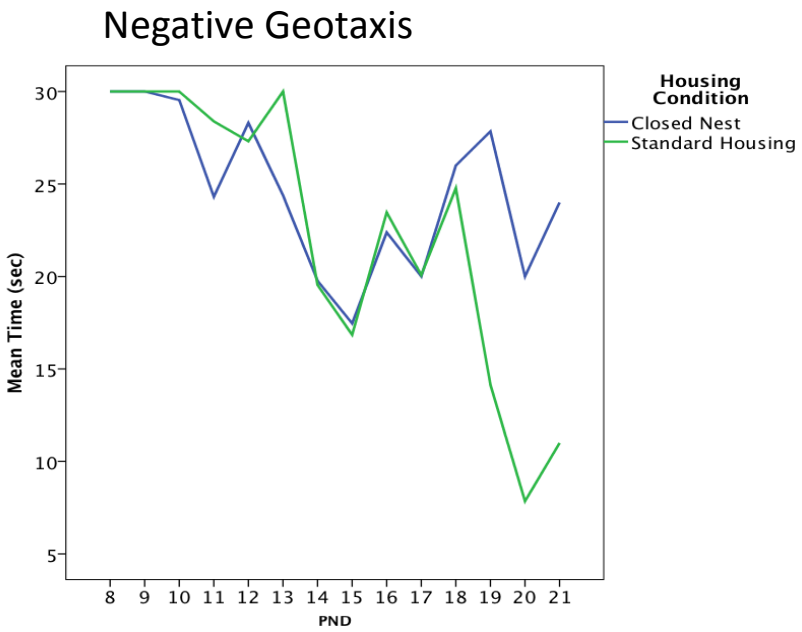
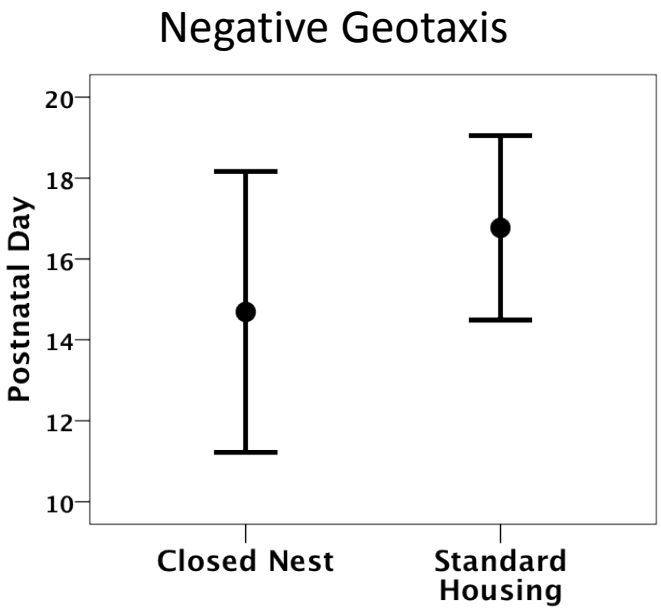


Figure 11.

Mean PND at which animals first reached the top of the Negative Geotaxis board in under 30 seconds.

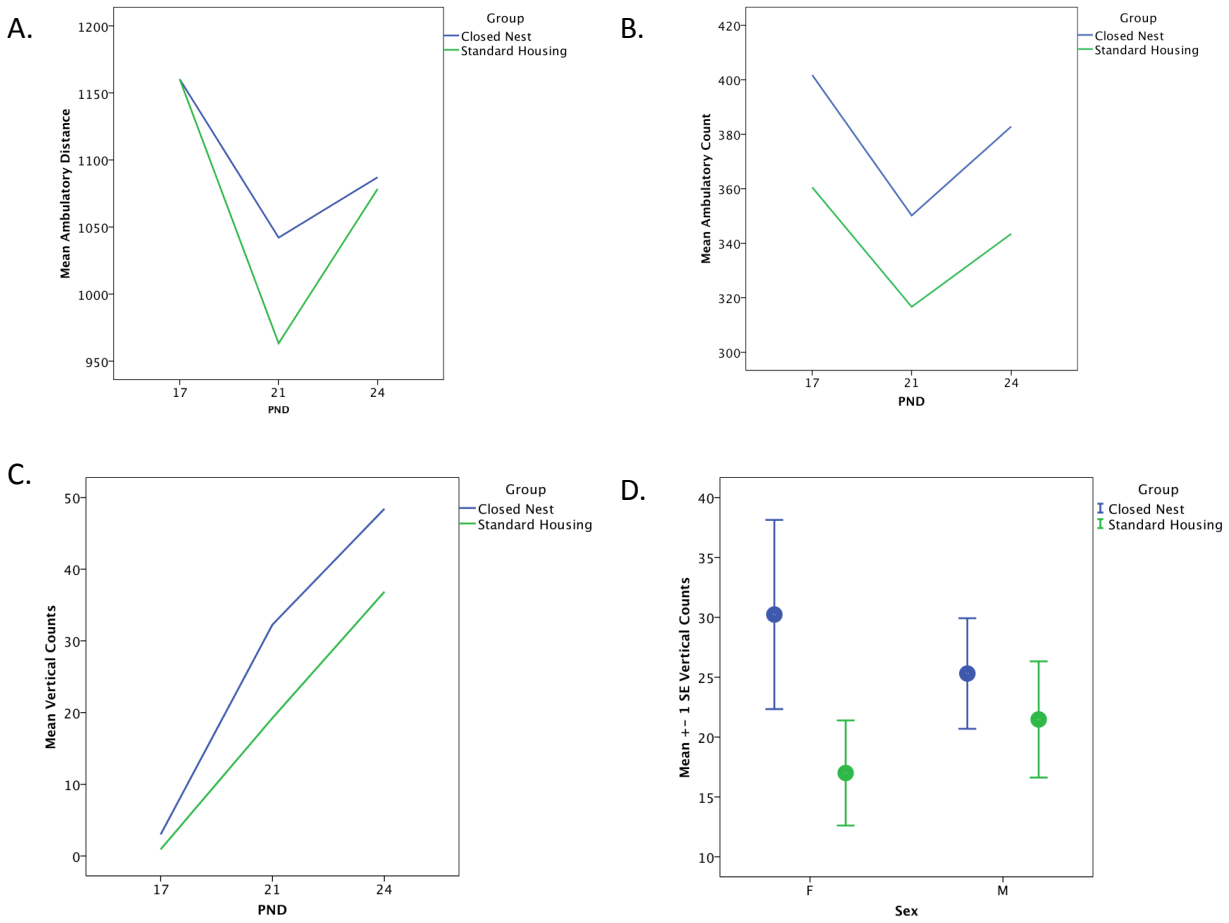


3. *Locomotor Activity*.

There was a significant difference between housing conditions in vertical counts in the LMA task, with animal in the CN condition exhibiting more vertical counts over the three testing periods, with an average of 28.6 counts, than those in SH which had an average of 19.36 counts ($p=0.044$). These results demonstrate less fear avoidance and more exploratory behavior in animals reared in the CN environment. There were no significant differences according to housing condition or sex in ambulatory distance or ambulatory counts.

Figure 12.

These figures show performance in LMA. A) Mean ambulatory distance over 3 testing days, not statistically significant. B) Mean Ambulatory Count over 3 testing days, not statistically significant. C) Mean Vertical Count over 3 testing days, significant difference between CN and SH conditions, p=0.044. D) Mean Vertical Counts by housing condition and sex, not statistically significant.



4. Rope Suspension.

Before analyzing data from the rope suspension task, preliminary tests for normality revealed a problem with the data for one of the three litters on PND 35 due to experimenter error. This error did not effect other tests on this day. Data from PND 35 from all litters were then removed from the final analysis. A Linear Mixed model with repeated measures was used to analyze the data from the one-arm and two-arm rope suspension tasks on PND 17, 21, 24, 28, 31, 38, and 42. The analysis revealed a trend towards the SH group performing better than the CN group on the test of forelimb strength using bilateral forelimbs, which potentially allows compensatory mechanisms to compensate for unilateral HI injury ($F(1,13)= 1.702$, $p=0.069$). Figure 13 shows the different patterns in bilateral suspension for each housing group over the 25 day testing period, with the SH group exhibiting gains in strength until PND 31 followed by a sharp decline in performance between PND 31 and PND 42. The CN group, however, exhibited a sharp decline in performance on PND 21 (day of weaning) with steady improvement until PND 31 and maintenance of this strength through the end of the testing period. CN animals did not have the sharp decline in performance at PND 31, as was seen in the SH group. In contralateral rope suspension, CN group exhibited significantly greater strength in the affected (left) forelimb ($F(1,13)= 6.838$, $p<0.001$). This resilience in CN animals is particularly pronounced after PND 24, at which point the performance of SH animals plateaus while CN animals continue to show improvement.

Figure 13.

Mean time (in seconds) for bilateral rope suspension from PND 17 to 42.

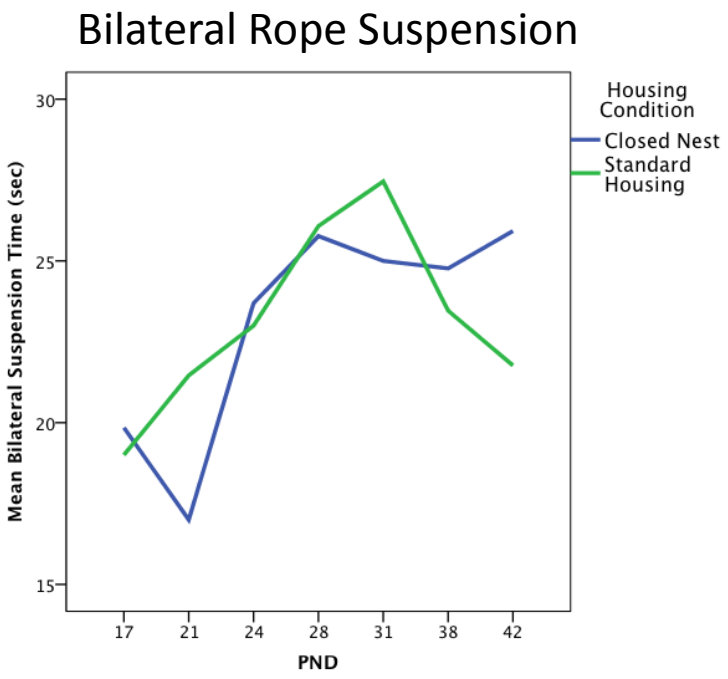
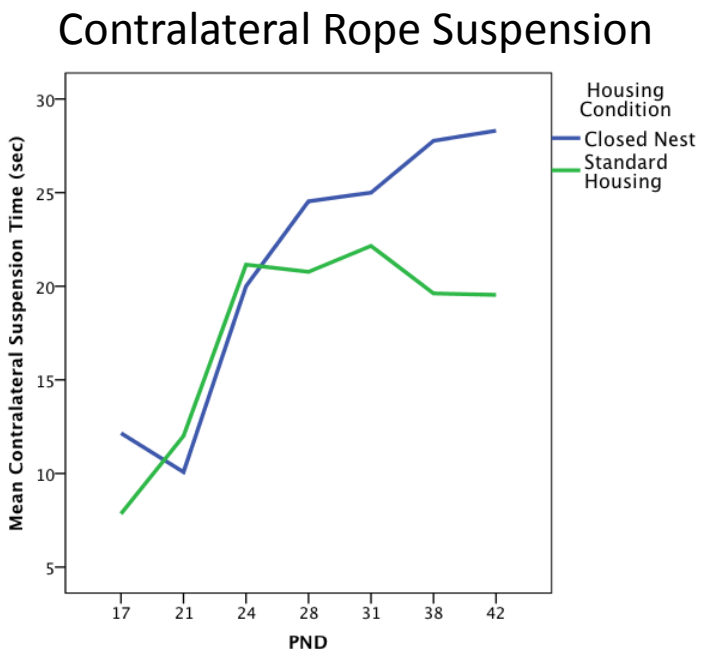


Figure 14.

Mean time (in seconds) for contralateral rope suspension from PND 14-42.



5. Morris Water Maze

A Generalized Linear Mixed Model with repeated measures followed by a Least Squares Determined post-hoc analysis, was used to analyze the data from animals' performance in the Morris Water Maze to test whether animals in the CN condition demonstrated less functional impairment on neurobehavioral tasks than pups in SH.

Visible Platform Training: Animals in the CN condition demonstrated significantly shorter latencies to reach the platform over the four daily trials occurring on three consecutive training days in the visible platform testing period ($F(2,23)=3.873$, $p<0.001$). There was no significant interaction effect of housing condition and sex on water maze performance ($F(3, 24)=1.110$, $p=0.332$). A post-hoc analysis revealed that there were significant differences between housing conditions on platform acquisition on trial 1 for both the first and second training days ($p<0.05$). The first trial on each training day following the introductory training day (day 1), can be used as an indicator of long-term memory functioning. On the first training day, which was the introductory trial for visible platform, SH animals reached the platform significantly faster than CN animals ($p=0.001$). On the first trial of the second day, once animals had been given an opportunity to learn the location of the platform in relation to initiation points from all four directions (S, W, E, N) and utilize this information in so much as it was retained

from 24 hours prior, Animals in the CN condition found the platform significantly faster than SH animals ($p<0.05$). On the first trial of the third training day, animals from the SH group had caught up to animals in CN and there was no significant difference between their performances.

Invisible Platform Testing: Over the five-day testing period, consisting of four trials per day, CN animals found the hidden platform significantly faster than SH animals ($F(2, 39)=1.771$, $p=0.004$). There was no significant interaction effect of housing condition and sex on the time to reach the platform ($F(3, 40)=0.865$, $p=0.706$). Like the findings on the visible platform training, there was a significant difference between housing conditions on the time to reach the platform on the first trials according to a post-hoc analysis. The SH group exhibited a shorter latency to reach the platform on the introductory trial on the first day of invisible platform testing ($p=0.005$). However, on the first trial of each training day following the introductory trial, the CN group located and reached the platform significantly faster than the SH group (Day 2, $p=0.10$; Day 3, $p=0.001$;Day 4, $p=0.002$; Day 5, $p=0.035$).

Figure 15.

Mean latency to reach the platform for each trial on visible platform testing by housing condition.

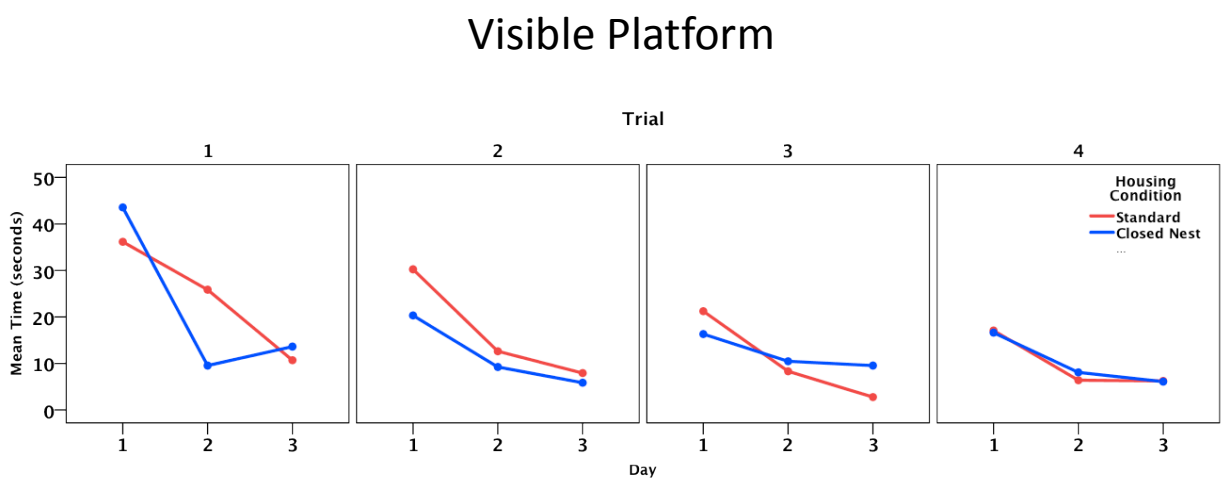


Figure 16.

Mean latency to reach the platform for each day on visible platform training by housing condition.

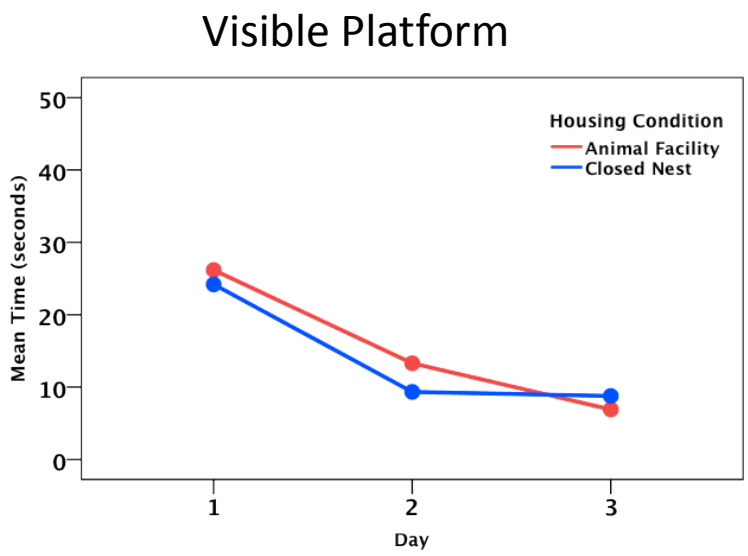


Figure 17.

Mean latency to reach the platform by day on visible platform by housing condition and sex.

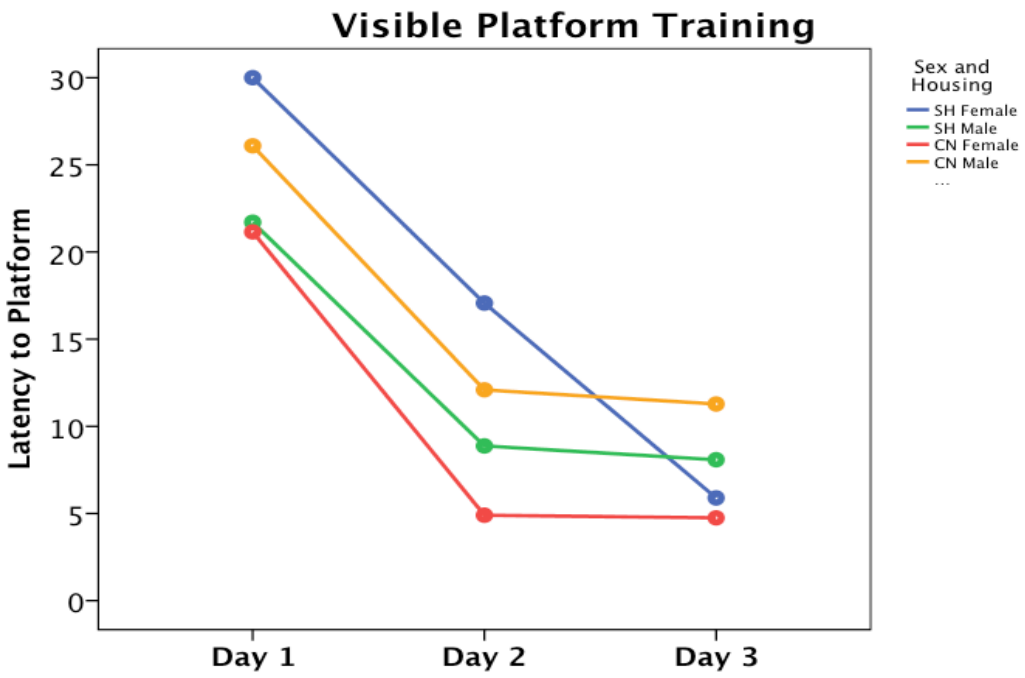


Figure 18.

Mean latency to reach the platform by trial on invisible platform by housing condition.

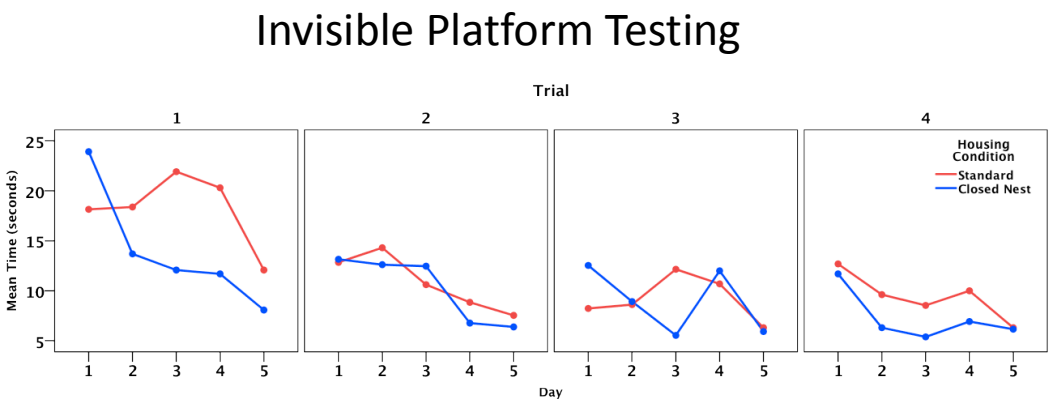


Figure 19.

Mean latency to reach the platform by day on invisible platform by housing condition.

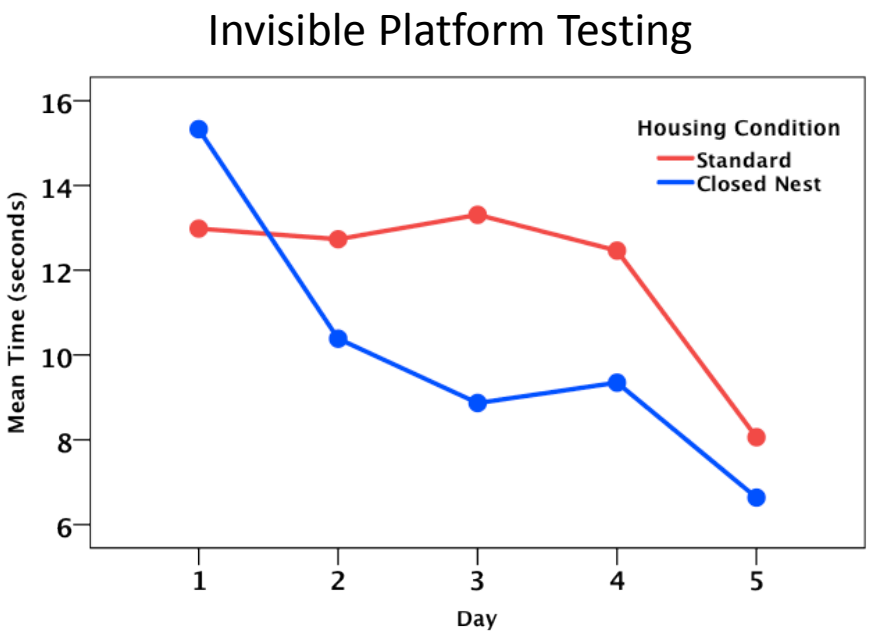
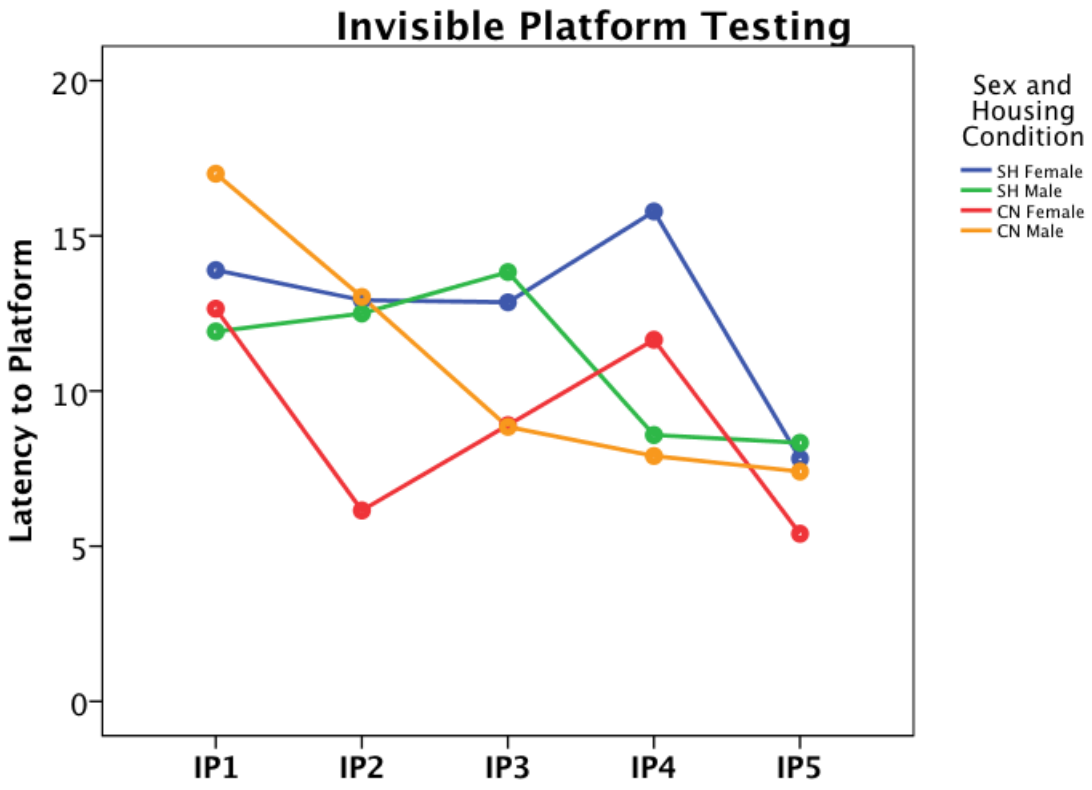


Figure 20.

Mean latency to reach the platform by day on invisible platform by housing condition and sex.

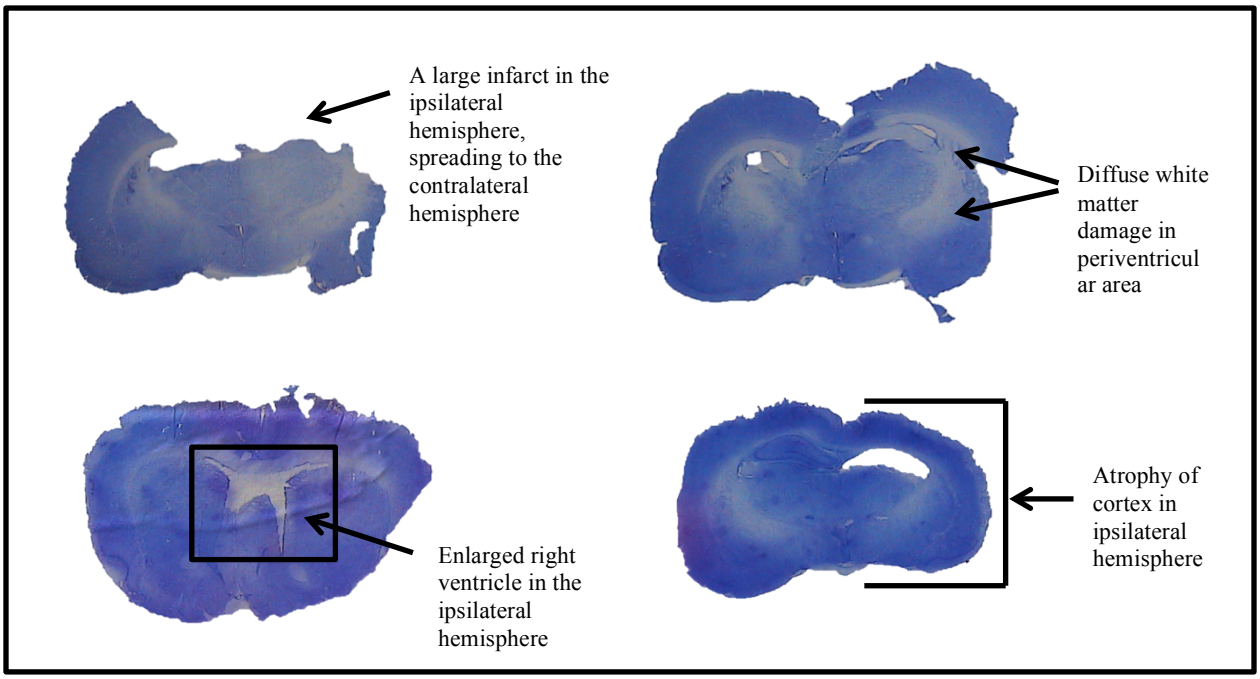


3.12b Brain Pathology

Representative brains were selected so that 2 males and 2 females from each treatment group were represented in the morphological analysis. Observations of gross pathology were used to select one severely injured brain and one grossly normal brain for each sex and group. In HI models that induce injury by unilateral right common carotid artery ligation, it is typically expected that the hemisphere ipsilateral to the injury (right side) will be most severely affected. HI injury occurs through a process of immediate cell death (necrosis) due to oxygen deprivation, followed by waves of programmed cell death (apoptosis) as the injury progresses. The injury can create large cysts in the gray and white matter where cell death has occurred. In addition, the ventricular and periventricular areas are particularly vulnerable to HI insult and enlargement of the ventricles as well as cell death in this area are frequently observed. Observational analysis of the representative brain tissue of 8 animals revealed individual differences in injury patterns, with some animals exhibiting a significant loss of gray and white matter on the ipsilateral hemisphere, some exhibiting an injury of primarily white matter regions emanating from the ventricles, while others only showed slight atrophy in the ipsilateral hemisphere (for a diagram of the types of injuries represented, see Figure 21). The severity of injury was evenly stratified among the 8 brains selected for analysis, with 3 animals exhibiting severe injury, 3 exhibiting moderate injury, and 2 showing mild injury. The patterns of injury are discussed below according to housing group and sex.

Figure 21

Hypoxic Ischemic brain injury patterns.



Descriptions of Injury Patterns

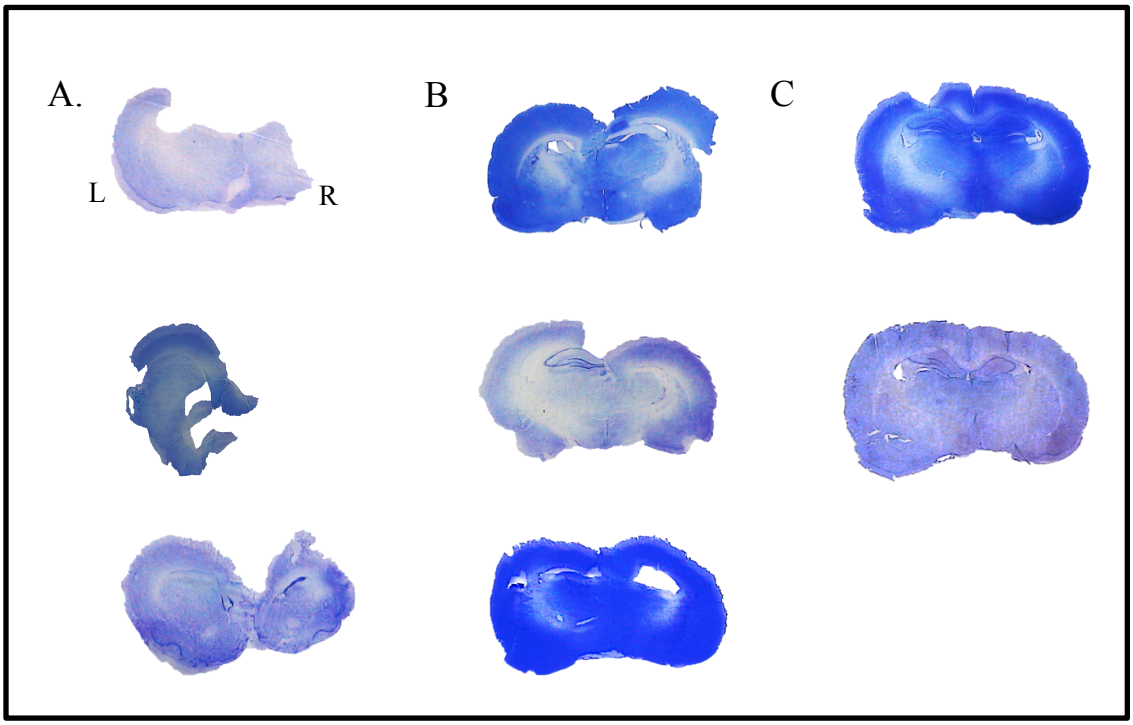
For two animals in the SH group (1 male and 1 female) and one female in the CN group, the tissue integrity was too poor to section successfully. However, pieces of the tissue remained intact and were mounted for analysis. Further inspection revealed large cysts encompassing gray and white matter in the ipsilateral hemisphere. There was also evidence of significant atrophy of the ipsilateral hemisphere in places where the cyst did not destroy the tissue.

The tissue of one male in the SH condition and one female in the CN condition revealed moderate injury which was primarily isolated to the ipsilateral hemisphere. Both demonstrated a similar injury pattern with the slight atrophy, a small cystic infarct in the gray matter, as well as enlarged ventricles and mild periventricular damage observed in the ipsilateral hemisphere. One female from the SH group demonstrated a moderate injury with a different injury pattern, primarily consisting of significant atrophy and tissue loss in the white matter of the ipsilateral hemisphere. While this animal had a relatively intact cortex, large portions of white matter were missing, including the right side of the hippocampus.

The least severe damage that was observed was found in both males from the CN condition, which sustained only mild injury. These animals had grossly normal brain structures, with slight atrophy of the ipsilateral hemisphere as well as a slightly smaller right hippocampus. There were no cysts, enlarged ventricles, or periventricular damage observed in this group.

Figure 22

Nissl-stained cross-sections from representative animals (A) Animals with severe damage: female from SH (top), male from SH (middle), female from CN (bottom), (B) Animals with moderate damage SH male (top), SH female (middle), CN female (bottom), (C) Animals with mild damage: CN males (top and bottom).



Morphometry

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

Observations of right and left hippocampal areas revealed that all CN animals retained tissue in both right and left hippocampal areas, whereas there were no SH animals which had a visible right hippocampus (ipsilateral to the injury). For SH animals, there were either large infarcts where the right hippocampus would be in un-injured animals, or the hippocampal area was not present in tissue that was intact. Females in the SH group had some visible left hippocampus tissue (contralateral to the injury), whereas SH males did not have any visible hippocampal tissue in the left hemisphere. In CN animals, all animals had both right and left hippocampi, although each displayed a disparity between right and left hippocampi, with the right measuring smaller than the left. This points to a greater resiliency of the vulnerable hippocampal area in animals reared in the closed nest environment and may indicate a mechanism for improved spatial learning in the hidden platform test of the Morris Water Maze.

Measurements of the cortical area of the right and left hemispheres showed some asymmetry between hemispheres, with the right (ipsilateral) hemisphere measuring smaller than the left (contralateral) hemisphere as expected. However, the degree of disparity appeared to differ greatly between groups. There was a dramatic disparity between right and left hemispheres for both males and females in the SH group, but comparatively little disparity in CN animals (see Figure 25). This indicates that on average, regardless of sex, CN animals had less damage from infarct or atrophy in the ipsilateral hemisphere than SH animals. This points to a possibility that a CN environment in the early pre-weaning period provides a form of neuroprotection for animals exposed to neonatal HI.

Figure 23

Examples of disparity between right and left hippocampal areas: A) Slight R/L disparity, B) Moderate R/L disparity, C) Intact L hippocampus and absent R Hippocampus despite intact tissue, D) Intact L hippocampus and absence of R hippocampus due to infarct, E) Incomplete L hippocampus and absence of R hippocampus due to infarct.

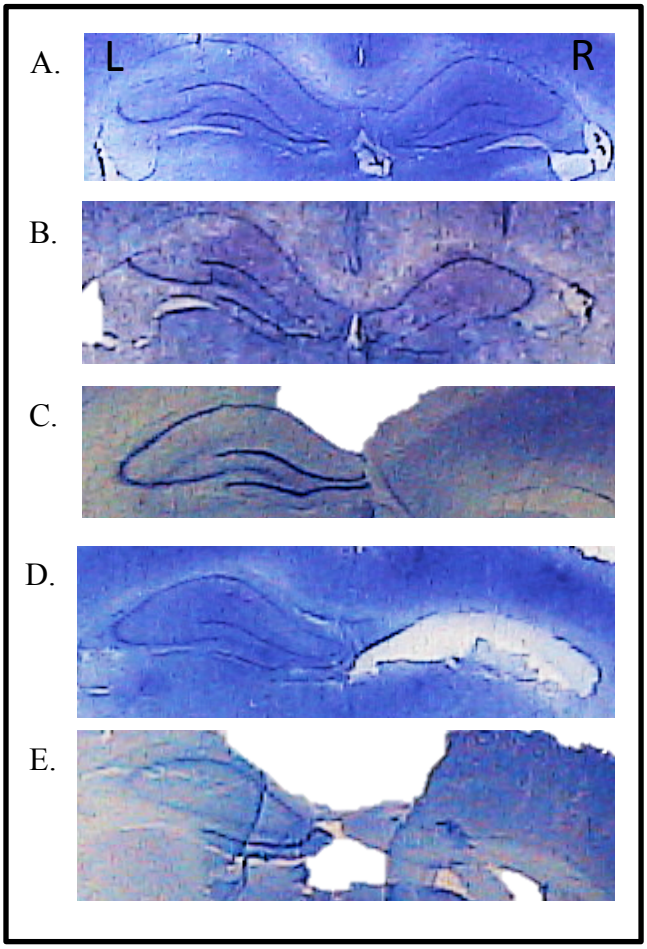


Figure 24

Mean area of the right and left hippocampus.

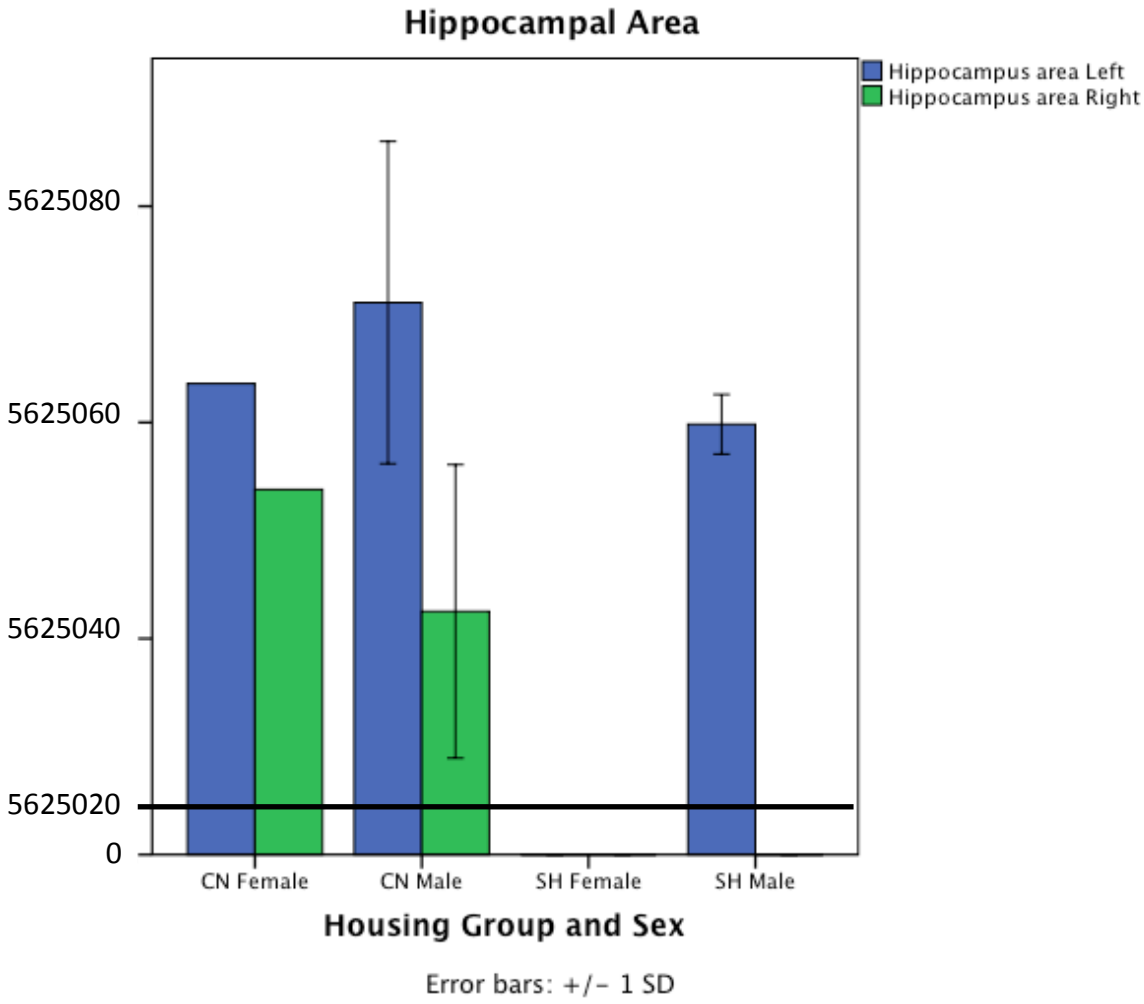


Figure 25

Mean cortical area of the right and left hemispheres.

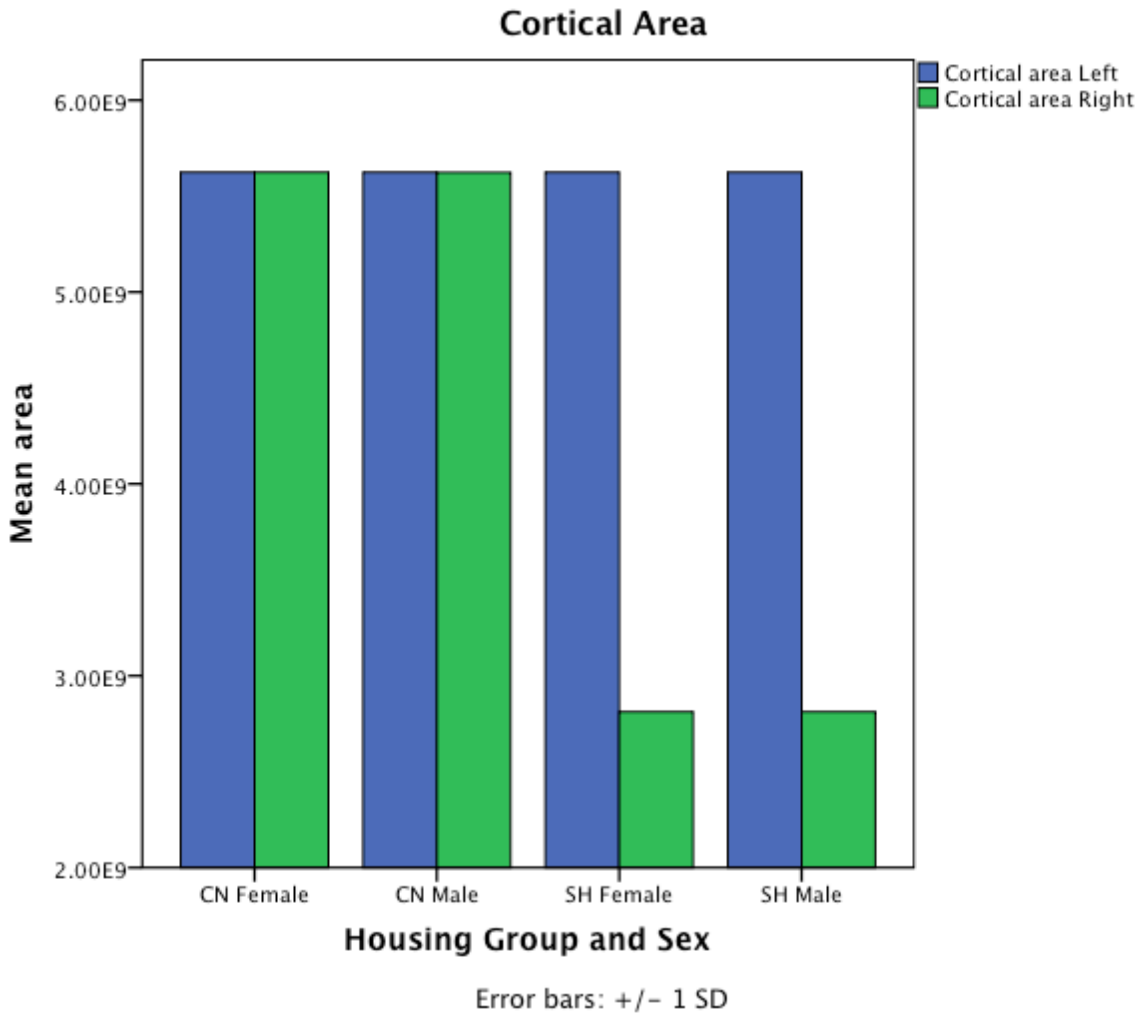


Table 3.

Support for study hypotheses.

Hypothesis	Description	Support
Hypothesis 1	It was expected that pups in the CN condition would exhibit more stable weight gain before and after HI than pups in the SH condition, with weight gain as an index of maternal CTB.	**Supported**
Hypothesis 2a	It was expected that pups with HI in CN would demonstrate less functional impairment on neurobehavioral tasks than pups with HI in SH.	**Partially supported**
Hypothesis 2b	It was expected that animals in the CN condition, compared to those in the SH condition, would show evidence of less cell death, as quantified by disparities in hemispheric area, in the ipsilateral hemisphere of carotid artery ligation in a sex-dependent manner, with males exhibiting more damage than females.	**Partially supported**

CHAPTER FOUR

DISCUSSION

This study was designed to determine whether early, enriched pre-weaning environment, through increased maternal care-taking behavior, could buffer the deleterious effects of neonatal HI. In clinical studies and animal models of neonatal HI, the injury has been found to negatively impact neurodevelopment (Shankaran et al., 2008; Lubics et al., 2005), functioning in cognition and memory (Lindstrom, Lagerros, Gillberg, & Fernell, 2006; Pereira, Strapasson, Nabinger, Achaval, & Netto, 2008), as well as motor coordination and strength (Lubics et al., 2005). The findings from the current study point to a marked functional resiliency after neonatal hypoxic- ischemic injury in animals reared in a closed nest environment, compared to those reared in standard housing conditions. Animals reared in a closed nest environment during pre-weaning gained weight after surgery at a faster rate than those in standard housing, indicating higher frequency of nursing behavior. Pups reared in CN also exhibited earlier development of some physical characteristics and reflexes compared to pups reared in standard housing. Closed nest animals demonstrated more exploratory behavior in LMA and better motor strength and coordination in the affected forelimb. In MWM, CN animals performed significantly better than SH animals overall and demonstrated stronger long-term memory functioning. The functional advantages of the CN

environment, which were demonstrated by the data from behavioral tests, were supported in the results of the brain pathology as well. Closed-nest animals had less hippocampal and cortical damage from HI than animals in standard housing. Animals in CN and SH conditions did not differ on some measures which have been found to be impacted by HI injury, such as the development of the grasp reflex and ambulatory activity in the LMA task. In negative geotaxis, the data revealed results contrary to the stated hypothesis and other behavioral data, with SH animals performing Negative Geotaxis significantly faster than CN animals over the 14 day testing period, even though CN animals developed this skill earlier than those in SH. Some possible explanations for these seemingly contradictory findings are discussed below. All of these findings are discussed in relationship to the aims of the study, with consideration to the related literature as well as limitations and future directions.

The first aim of the study was to examine the effects of the closed nest (CN) condition and the standard housing (SH) condition on frequency of maternal care-taking behavior (CTB), including licking and grooming behavior and nursing, as interpreted by stability of pups' weight gain. We hypothesized that dams in CN condition would exhibit more frequent CTB than dams in SH as indicated by stability of offspring weight gain. These data supported this hypothesis, with pups in CN exhibiting significantly higher daily body weights over the period of PND 7 until weaning at PND 21, than pups in the SH condition. Animals reared in the CN environment also demonstrated significantly earlier eye opening and ear unfolding, which are indicative of physical maturation.

Neonatal HI injury has been associated with lower daily body weights in rats as well as delayed physical maturation (Lubics et al., 2005). These results indicate that the CN environment ameliorated the effects of HI injury on physical maturation and weight gain. It is possible that this normalization of growth trajectory in the CN group was induced through the hypothesized increased frequency of maternal CTB with the presence of a nest box. Kuhn, Paul, and Schanberg (1990) found that separation from the dam in the first two weeks of life causes an immediate and dramatic reduction in the secretion of growth hormone. Furthermore, this study found that maternal CTB was necessary to promote secretion of growth hormone at healthy levels in rat pups at PND 10. This evidence further supports the notion that the CN environment promotes maternal CTB, and that through this mechanism, the CN environment counteracts some of the detrimental effects of HI injury on development.

The second aim of the study was to examine the relationship between nesting environment and neurological sequelae of HI injury. We expected that pups reared in the closed nest environment would demonstrate less functional impairment on tests of neurobehavioral development than pups reared in standard housing. In the early pre-weaning period, tests of neurodevelopment showed that pups reared in CN exhibited the ear twitch reflex in the left ear and auditory startle reflex significantly earlier in development than pups reared in SH. These tests were specifically developed and tested in a rodent model of neonatal HI by Lubics et al. (2005) by comparing the performance on a range of neurodevelopmental tests between normal control rat pups and pups with

HI using the Levine model of RCC ligation followed by hypoxia. In comparison with the findings reported by Lubics et al. (2005), CN pups displayed the development of the auditory startle reflex at the same time in development as non-injured rats, and ear twitch reflex earlier than non-injured rats (see Table 4.). We hypothesize that the effects of nesting environment were exerted through its effects on maternal CTB. Studies have shown that the offspring of dams that display high levels of CTB show greater neural proliferation and synaptic density in the hippocampus, and pre-frontal cortex (Bredy, Grant, Champagne, & Meaney, 2003) (Liu, et al., 1997) and the absence of maternal CTB has been shown to decrease neural proliferation (Chocyk, Dudys, Przyborowska, Majcher, Mackowiak, & Wedzony, 2011) and accelerate the rate of glial and neuronal apoptosis in white matter tracts (Zhang, et al., 2002). The findings from the current study demonstrate a more normal development of reflexes in CN pups than is typically observed in HI. This demonstrates that greater frequency of CTB may either promote more robust neural connectivity before injury or facilitate recovery by increasing neural proliferation after injury. Given the evidence in the literature discussed above, it is likely that both processes are working in tandem in order to lessen the impact of the injury and promote repair.

The lack of a significant difference between CN and SH on the development of right ear twitch may be explained by facial weakness on the ipsilateral side of the damaged hemisphere that was exhibited immediately after injury in all pups with HI. Most pups regardless of housing condition, exhibited a weakness in the muscles of the

right eye resulting in partial opening of the eyelid. In some cases the right eye remained closed for the majority of the time after initially opening early in development. This weakness may have extended to the right ear as well, inhibiting the development of a twitch reflex in the ear ipsilateral to the injury. Damage this severe may have been irreparable by environmental intervention. There was no difference between the groups on eye twitch, which may have been partially because of the early development of the eye twitch for both groups. Both SH and CN pups developed eye twitch on day 8 on average (8.3 and 8.5 days), which was on the first day of testing.

Table 4.

Reflex development compared to norms established in Lubics et al. 2005.

Measure	**Control** (*Lubics et al., 2005)*	**Standard housing**	**Closed Nest**
Eye opening	14.6 ± 0.2	15.4 ± 0.2	13.5 ± 0.1*
Ear unfolding	13.2 ± 0.2	15.5 ± 0.2	13.2 ± 0.2*
Incisor eruption	9.7 ± 0.2	8.3 ± 0.1	8.4 ± 0.4
Eye twitch reflex	12.4 ± 0.5	8.3 ± 0.8	8.5 ± 0.9
Ear twitch reflex	15.6 ± 0.7	15.0 ± 0.7	13.1 ± 0.5*
Auditory startle	13.5 ± 0.2	14.5 ± 0.3	13.1 ± 0.3*

* $p<0.05$

To further examine the relation between CTB and neurological sequelae of HI, CN and SH housing groups were tested on locomotor activity as well as motor coordination and strength. Animals in CN exhibited significantly more vertical counts during the 5 minutes of free ambulation in the locomotor activity chamber than SH animals. Vertical exploration, or rearing, is understood as an exploratory behavior, indicating curiosity and less inhibition related to anxiety around novelty (Roy & Chapillon, 2004). Since rearing behavior is a sign of lower anxiety response to novelty, it may be an indicator of HPA-axis response to stress, with more rearing activity and exploration being related to less reactivity in the HPA-axis. Research has shown that HPA-axis reactivity may be dampened in animals that experience more CTB early in development (Nunez, Ferre, Escorihuela, Tobena, & Fernandez-Teruel, 1997). This apparent difference in HPA axis reactivity between the two housing groups may have also been responsible for the faster reaction times of the SH animals in Negative Geotaxis. While faster performance on Negative Geotaxis may indicate more intact reflex development to an extent, it can also reflect an exaggerated reactivity and hyperactive ambulation. The higher frequency of rearing behavior and less reactivity in Negative Geotaxis observed in the CN group indicates that the nest box may have facilitated more CTB, as Hypothesis 1 stated, and that this probable change in HPA-axis reactivity occurred despite HI injury occurring on day 7 of life. This is an important piece of

information because it points to CTB as the mechanism of the CN intervention and demonstrates that it is capable of creating functional neurological changes despite experiencing a significant insult early in development. Chou et al. (2001) found a similar effect in an Open Field test, with HI rats handled during early development exhibiting more exploratory behavior and less fear avoidance than non-handled rats with HI. This effect was also linked to CTB, which was found to be more frequent with handled pups. In the current study, there were no significant differences found between housing groups on the amount of ambulation or distance travelled by the pups in the locomotor activity chamber. McAuliffe, Miles, and Vorhees (2006) found that rats with hippocampal damage in severe HI injury showed excessive ambulation compared to less severely injured rats. While this was not seen in LMA, the faster climbing speeds of SH animals in Negative Geotaxis may be an early indicator of excessive ambulation. Negative Geotaxis is tested over a shorter period of time (30 sec) than LMA (5 min) and therefore may have been more sensitive to such differences. A larger sample size would allow for groups to be striated by injury severity, which would illuminate possible differences in ambulation.

In the rope suspension task, which is a test of motor coordination and strength, animals in the CN condition exhibited greater strength in the affected forelimb in contralateral rope suspension than animals in SH. In HI injury, somatic effects are observed on the side of the body that is opposite to the injury (contralateral). In the model used for this study, the right common carotid artery was severed; therefore, most motor effects would be expected to occur on the left side of the body. In contralateral rope

suspension, the forelimb on the ipsilateral side, which should not be directly affected by the injury, is restrained with tape so that the animals cannot compensate for any weakness on the affected side. In bilateral rope suspension, animals can adapt and compensate for one-sided weakness by relying primarily on the strength of the unaffected forelimb. The task is timed with a maximum of 30 seconds, which animals can adapt to easily with bilateral suspension as they mature. This may explain why animals in SH and CN did not differ on fall-off latencies for bilateral rope suspension, while CN animals exhibited superior performance in contralateral suspension. This finding is consistent with the study by Lubics et al. (2005), which found no difference between HI animals and uninjured animals in the bilateral rope suspension, but found that HI animals performed significantly worse on contralateral suspension. The finding that CN animals showed better performance in contralateral rope suspension points to a functional improvement in motor strength with less one-sided weakness in animals reared in the CN environment when compared to a SH environment. Interestingly, results from both bilateral and contralateral rope suspension revealed a diversion between the performance of the two housing groups later in development, with the performance of animals in the SH group reaching a plateau or declining, while animals in CN continued to improve. This difference was most pronounced in the results of contralateral suspension, which showed CN animals continuing to improve five weeks after the injury while SH animals plateaued two weeks after injury. This supports the theory that the metabolic changes stimulated by the CN environment through the proposed mechanism of CTB involve the stimulation of growth factors and stabilization of GCs; and that trophic support better

enables neural recovery for neuromuscular tasks. This effect could be exerted through increased neurogenesis in the motor cortex and other cortical areas. In future tissue analysis, an examination of the motor cortex in both groups would shed more insight into the mechanism behind this functional improvement. This could have important implications for the clinical applications of this model, as motor deficits and cerebral palsy frequently result from HI injury.

Another measure of functional differences between the housing conditions that was used to test Hypothesis 2a is a test of cognitive ability. Cognitive deficits are observed across the spectrum of severity in HI injury (Ikeda, et al., 2001). The hippocampus is particularly vulnerable to this inflammatory white matter injury and is a hallmark of HI in animal models and clinical presentations (Rodrigues, et al., 2004). It is for this reason that studies using animal models of HI frequently employ the use of Morris Water Maze, which is a test of short and long-term memory as well as adaptive cognitive functioning. To find the platform, animals need to be able to flexibly employ cognitive abilities to search for the platform from all drop-off locations. Animals then need to remember where the platform is in relation to cues, in order to find it more quickly in successive trials, which is indicative of short-term memory functioning. This is repeated on successive days with 20 to 24 hour gaps between testing, and the extent to which animals are able to store and use information in long-term memory is seen in the speed with which they are able to reach the platform on the first trial of each day.

Animals reared in the CN condition exhibited faster escape times than those in the SH condition across all trials and days in both visible platform training and invisible platform testing, demonstrating more robust learning acquisition and working memory profiles. Closed nest animals also demonstrated significantly better long-term memory retention, by finding the platform significantly faster on the first trial of each testing day after the initial introductory trial. This finding shows that CN animals were better able to remember the platform's location across testing days, over a period of 20-24 hours between each testing day, and to use their memory to guide their navigation and reach the platform quickly. SH animals did not demonstrate this capacity to the extent that was seen with CN animals.

We hypothesize that the differences observed between animals reared in the standard housing environment and the closed nest environment are due to differences maternal CTB, with dams expressing more CTB in a CN environment. Offspring of dams who exhibit high frequencies of CTB have been found to have greater hippocampal synaptogenesis and less apoptosis than animals reared by dams exhibiting low frequency CTB (Liu, et al., 1997; Weaver, et al., 2004). Maternal care has been found to have a significant effect on hippocampal development and to have a direct effect on spatial learning and memory, with offspring of high-CTB dams demonstrating better memory functioning (Bredy, Grant, Champagne, & Meaney, 2003). These data may indicate that more frequent and consistent CTB, as induced by CN, buffers the developing brain in such a way that results in less functional memory deficits than those typically seen with

HI. This notion is supported by the finding of much less structural damage in the hippocampal tissue of animals reared in the CN condition, compared to SH animals that showed total loss of ipsilateral hippocampal tissue after HI. In short, the intervention of CN and the resultant increased CTB seemed to rescue the oxidatively-sensitive hippocampal neurons from HI injury compared to the standard housing group that showed increased stress and HPA axis activation. Since this loss must occur over time after HI as well as with the acute injury, we postulate that CTB stimulates on-going processes that may act to stabilize the HPA axis, possibly via glucocorticoid receptor expression, neurotransmitter modulation, and growth factor support for injured cells.

The functional advantage in memory and preservation of hippocampal tissue seen in CN animals may be partially due to an increased sensitivity to circulating GCs in the hippocampus from increased activation of genes that promote proteins necessary for glucocorticoid receptors (GR) in the hippocampus. A greater number of GR promoter genes have been found to be active in offspring of dams that displayed higher frequency of CTB (Weaver et al., 2004). These receptor sites are critical for hippocampal functioning and regulating apoptosis. One possible explanation for the improvement on water maze performance in CN animals, is an increase in specific gene activation for the formation of GRs in the hippocampus, like those seen in offspring of high- CTB dams, which resulted in a phenotype that was better able to withstand hippocampal insult and more capable of re-populating hippocampal tissue with GRs after injury.

These results echo the findings of a study by Chou et al. (2011), which found that rats with neonatal HI handled for 2 hours a day from PND 8 to PND 14 demonstrated shorter latencies to finding the platform in MWM than non-handled rats. This effect is most likely due to the increase in CTB that was found in handled pups. These differences in functional learning and memory were only found in animals with moderate hippocampal damage and were not observed in those with mild or severe damage, indicating that the beneficial impact of handling on HI injury only extended to those with moderate injury. The findings from the present study indicate that the presence of a stable environmental manipulation in the form of a nest box, improved functional working memory and long-term memory. However, based on morphometric comparisons between the two housing groups, this improvement may have been partly driven by the neuroprotective effects of the CN environment. This neuroprotection resulted in less severe injury in CN animals than SH animals on average. Since more CN animals may have been in the category of moderate damage, they may have been more capable of benefitting from the environmental manipulation. This difference in neuroprotection between housing groups, which was not found by Chou et al. (2011) may be driven by earlier timing of intervention (E10). Intervening before parturition, may have provided neuroprotection through an environmental and behavioral form of preconditioning. A study by Davis et al. (2011) found that whisker stimulation 10 minutes before occlusion of the Middle Cerebral Artery improved infarct volumes, whereas stimulation delayed to 3 hours post-injury did not. This supports the notion that sensory stimulation holds the potential for neuroprotection via preconditioning. A preconditioning effect of the nest-

box could have lead to a more robust phenotype that was better able to withstand the impact of the injury and more capable of repair following injury than an intervention which was implemented after injury on PND 8 as the one in Chou (2011). Many of the studies demonstrating the beneficial effects of maternal care taking behavior on the development and functioning of the hippocampus begin in the first week of life. Weaver et al. (2004) found that the first week of life was a critical period in development for altering genetic expression in hippocampal tissue, which produced the proteins necessary for GRs. This may be responsible, in part, for the beneficial effects of the CN environment on preservation of hippocampal structure and function observed in this study. In a future study, it would be beneficial to run a Western Blot analysis to quantify mRNA GR expression in hippocampal tissue prior to and after insult in order to determine if HPA-axis is differentially regulated by CN before HI insult or afterwards or both.

One limitation to consider in these findings is the lack of control for motor coordination and strength in the latency to reach the platform due to the inability to measure swimming speed and distance traveled. Results of the rope suspension task demonstrated that CN animals were stronger and exhibited better motor coordination than SH animals. It is possible that this difference in motor ability contributed to the shorter latencies seen in the CN group and partially reflects superior swimming ability rather than only cognitive ability. For the next phase of this study, swimming speed and

distance traveled will be quantified using a motion tracking system in order to account for the contribution of motor ability.

The morphometric analyses and gross pathology partially support the hypothesis that animals in the CN condition, compared to those in the SH condition, would show evidence of less cell death, as quantified by disparities in hemispheric volume, in the ipsilateral hemisphere of carotid artery ligation in a sex-dependent manner, with males exhibiting more damage than females. There were differences in these measures between housing groups and these differences were sex-dependent. However, the finding that males benefitted more from the closed nest condition in overall brain pathology was unexpected. Clinical research and studies using animal models of HI, which have observed sex differences, have shown that males tend to have more severe damage after this type of insult than females (Zhu, 2006; Hill, 2012). A study by Rodrigues et al. (2004) found that male rats that were exposed to tactile stimulation early in development had less severe injury after HI and demonstrated better preservation of hippocampal tissue. However this study did not include female animals, so any sex differences were unknown. Still, these findings, in combination with the overall brain pathology of animals in the current study, may point to a sex-specific neuroprotective mechanism in maternal CTB that is slightly preferential towards males. It will be important to conduct studies with larger sample sizes in order to tease out the sex differences in behavioral data, since the brain pathology indicates a strong interaction between sex and nesting environment on HI injury.

The measurements of cortical areas and hippocampal areas demonstrated a protection of hippocampal tissue and cortical tissue for both males and females in the CN condition. The extent of the HI injury observed in SH animals reveals the potential severity of damage this model can inflict. The striking differences between the pathology of animals in the two groups demonstrate the potential of nesting environment to affect animals' response to insult from HI. As this research continues to progress, more in-depth analysis of brain tissue would shed more light on the extent of this effect and its potential mechanisms.

This study demonstrated that a behavioral intervention of improving the nesting environment can impact the course of neurodevelopment and recovery in neonatal HI, and that decreasing stress in the nesting environment buffers the impact of HI on important learning and memory areas and facilitate repair and recovery in motor, behavior, and learning abilities. The standard housing leaves dams visually exposed to neighboring animals with transparent Plexiglas walls and partial overhead covering, providing a model of antenatal and postnatal stress. This unnatural nesting environment decreases care-taking behavior, and has profound effects on the offspring's resilience to HI injury (Ivy, Brunson, Sandman, & Baram, 2008). This study uses a novel design of providing a nest box, which has not previously been tested in animal research, to create a more relaxed environment and decrease stress in mother and offspring. The novelty of this treatment design, however, was grounded by a highly standardized injury model and testing battery, which has been shown to be reliable over years of research. The Rice-

Vannucci injury model has been used in many studies using animal models of neonatal HI (Northington, 2006). In addition, the proposed effect of the nest-box on maternal CTB is consistent with the results of the study, which demonstrated advantages in areas that have been reported to be related to increased frequency of CTB in the literature, such as physical development, auditory processing, learning and memory, exploratory behavior, and integrity of hippocampal tissue. This provides support for the hypothesis that a closed-nest environment encourages a higher frequency of maternal care-taking behavior. The novelty of this intervention however, does pose a limitation to the study until it is replicated and studied further. In subsequent studies, the reliability and validity of the nest-box as a mechanism of inducing maternal CTB could be improved by quantifying maternal HPA axis status before parturition, maternal CTB with and without a nest-box over the pre-weaning period, and neonatal HPA axis status after injury between CN and SH conditions. It would also be beneficial to assess whether CTB is affected by a pup's injury status.

Another limitation to this study is the small sample size. This study was designed as a pilot for a larger study that is currently underway. In this study, there were 3 litters, which allowed for only one CN litter. While these results are promising, it is not possible to generalize these findings until several more litters are tested using the same protocol. A larger sample size would also allow for comparisons between males and females in the different housing conditions. As the results from the brain pathology demonstrate, the closed nest environment seems to affect the course of HI injury differently for males and

females. With a larger sample size, it would be possible to determine whether these differences in brain tissue translate to functional sex dimorphism in behavioral tests.

The size of the litters used in the study may be another confounding factor. In larger litters, there is less opportunity for individual pup-directed care-taking behavior, therefore the distribution of litter sizes between the two housing groups could affect the proposed mechanism of the CN intervention. The litters used for this study consisted of two SH litters with six pups in one litter and seven pups in the other; and one CN litter with fourteen pups. When these litters were born, they were equivalent in size (15), however in the SH litters, four to five in each group expired before the injury on P7, and one to two died in hypoxia or within the first two days after injury. One pup in the SH group was too small for surgery on P7 and one pup in the CN group was too large, resulting in the expulsion of both pups from the study. These factors resulted in a large variance in litter size between the two housing conditions. However, if this difference in litter size were to affect the data, it would most likely result in a reduced benefit of the CN environment. If litter sizes are standardized in future studies, it will most likely increase the effect of the nest-box by increasing the frequency of individual pup-directed CTB.

The strength of this study could be further improved upon by the addition of sham-injured pups inter-mixed with HI injured pups in each of the housing conditions. Due to the high intensity and lengthy duration of the study measures, the decision was made to only use HI injured animals in order to reduce the number of animals necessary

to determine whether the CN affected HI injury. Including sham-injured animals, which are exposed to the same surgical anesthetic and rearing environment as HI animals, would allow for a more thorough investigation of the potential mechanisms of the effects of the CN environment on neurodevelopmental and behavioral outcomes.

Additional in-depth tissue analysis would provide a wealth of information about the effects of the CN environment on HI injury and the mechanisms behind it. Much of the brain tissue from the animals used in this study has been collected and stored in order to allow for Western-blot analyses and immunocytochemistry (ICC) in the future. A Western-blot analysis would allow for the quantification of GR promoter mRNA in hippocampal tissue, which would help elucidate the role of GRs and GCs on the functional memory improvement observed in CN animals. Immunocytochemistry could be used to determine the cell population, as a marker of cell death resulting from injury, in important brain regions such as the hippocampus, motor cortex, sensory cortex, basal ganglia, and watershed areas. It could clarify key factors in the resilience and neuroprotection observed in CN animals by quantifying differences in levels of BDNF (brain-derived neurotrophin, implicated in neuroplasticity), Caspase (an enzyme marker of apoptosis), GCs, glutamate receptors and other neurochemical markers that may play a role. These analyses would add important information about how the nesting environment exerts effects on animals with HI, which would facilitate the translation of these findings to clinical populations.

The intent of this study was to determine the effects of early environment on the neurological sequalae of neonatal hypoxic-ischemic injury by studying it in a controlled setting in which the environment could be readily manipulated and the impact could be thoroughly assessed. This research provides a foundation for further investigation into this paradigm and the mechanisms for the effects it exerts. The generalizability of these findings to human caregivers and infants needs to be explored through clinical research in order to bridge this research into the clinical population. These preliminary findings suggest that early interventions that facilitate less maternal stress and greater caregiver-infant interaction have the potential to buffer the impact of HI injury and facilitate functional improvement. As specific pharmaceutical interventions in this population carry significant risk due to the vulnerability and immaturity of the neonates, a behavioral intervention has significant appeal. This is a promising area that has never been addressed clinically, which can supplement standard-of-care medical and therapeutic interventions to offer benefit, without a threat of interfering with standard treatment.

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