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Exploring the Mitigating Effects of Early Somatosensory (Tactile) Stimulation and Acoustic Discrimination Experience in Neonatal Hypoxic-Ischemic Male Rats

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STIMULATION AND ACOUSTIC DISCRIMINATION EXPERIENCE

IN NEONATAL HYPOXIC-ISCHEMIC MALE RATS

A Master's Thesis Presented

by Patricia Taubin

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Abstract

The focus of this study was to determine the effects of early somatosensory (tactile) stimulation and acoustic discrimination experiences in hypoxic-ischemic (HI) male rats on long-term behaviors, learning sensory, and brain weight outcomes. 58 Wistar rats were randomly assigned to one of the three conditions: no stimulation, somatosensory stimulation and auditory stimulation. To observe the effects of the early life stimulation on adult behavioral measures, the following testing was performed: analysis of exploratory behavior, acoustic discrimination, spatial/memory learning, and brain weight. Overall we hypothesized that somatosensory and auditory interventions earlier in life would have beneficial effect on subjects' performance in all the testing. Results suggested that tactile and auditory stimulation in early life did not have any significant beneficial effects on improving spatial learning, auditory processing or exploratory behavior in HI and sham subjects. However, some beneficial effect was found in the spatial memory task but only for the tactile HI and auditory sham group.

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Chapter 1

Introduction to Early Somatosensory Stimulation and Acoustic Discrimination Experience in Neonatal Hypoxic-Ischemic Male Rats

Medical observations in humans and previous research in rodents have demonstrated that at risk full term infants and premature babies are particularly vulnerable to experience oxygen deprivation (hypoxia) and reduced cerebral blood circulation (ischemia). These vascular irregularities affect normal neurological and behavioral development (Chou et al., 2001).

Some of the impairments caused by hypoxia and ischemia (HI) can be seen in animal and humans at cognitive level (exploratory behavior, spatial and non spatial learning, etc. (Alexander, Garbus, Smith, Rosenkrantz & Fitch, 2014; Hill, 2001; McClure, Threlkeld, Rosen, & Fitch, 2006)), at the acoustic level as deficits in the complex auditory process, (McClure et al., 2006), at neurological levels (injury of certain brain cells and areas, (Grafe, 1994; Johnston, Trescher, Ishida, & Nakajima, 2001)), at the motor level (reflexes like grasping and gait, motor coordination, and hypertonia, (Lubic,et al., 2005; Derrick et al., 2004)), and the acquisition and development of language in humans (Martinez et al., 2014). Currently, there are few medical interventions that can prevent these pathologies or ameliorate their cognitive, motor, and neurophysiological effects. For that reason, this current study explored the use of early tactile and auditory stimulation to mitigate the long-term effects of hypoxia and ischemia (HI) in rodents.

Previous studies have demonstrated that the introduction of handling, tactile and other environmental interventions, such as auditory stimulation, early in life have had effects on the structure of the brain (Kolb & Gibb, 2010) and on subsequent cognitive function (Costa, Tamascia, Nogueira, Casarini, & Marcondes, 2012). Some of these effects have included improvement of acoustic discrimination performance (Threlkeld, Hill, Rosen, & Fitch, 2009), learning capability (Chou et al., 2001) memory (Bilbo et al, 2007), reduction of anxiety (Costa et al., 2012; Imanaka et al., 2008), and prevention of cognitive impairments in adult rodents, even those suffering from hypoxic-ischemic induced alterations.

The beneficial effects of handling and tactile stimulation have been shown on animal and human behaviors and brain structure. The terms tactile and handling intervention seem to be used as synonyms to describe any action of touching, rubbing, holding, shaking, or stroking one subject and/or a group of subjects at a time (Anisman, Zaharia, Meaney, & Merali, 1998; Gibb, Gonzalez, Wegenast, & Kolb, 2010; Gilad Rabey, Eliyayev, & Gilad, 2000; Gschanes, Eggenreich, Windisch, & Crailsheim,1998; Imanaka et al. 2008; Jansen & Low, 1996; Lehmann et al., 2001; Muhammad, Hossain, Pellis, & Kolb, 2011; Pham, So¨derstro¨m, Henriksson, & Mohammed, 1997; Richards, Mychasiuk, Kolb, &Gibb, 2012; Rodriguez et al., 2004; Schanberg &Field, 1987). There has been a lack of standardization in the methods utilized for handling or tactile stimulation across studies. Differences observed in a variety of studies have included how subjects are handled, for how many days, and how often each day. Despite these differences, the effects of handling have been observed at the brain, physiological, and

behavioral level across studies. For example, Lehmann and colleagues (2001) focused their study on stress and compared the effects of early handling and maternal separation in rats. They found that early handling promoted superior spatial cognition and reduced corticosterone levels related to the stress response. In addition, Anisman and associates (1998) showed that earlier handling reduced adverse behavioral responses and neuroendocrine reactions to stressors encountered during adulthood. Others have found similar results on stress related behavior and observed that handling can improve long-term cognitive function (Pham et al., 1997). Handling studies have also shown improvements to learning, memory, and anxiety reduction in adolescent rats, (Costa et al., 2012), and even in neonatal rats exposed to infections such as Escherichia coli which typically impairs memory capacity (Bilbo et al., 2007). Schanberg and Field's (1987) review paper on the effects of tactile stimulation on sensory deprivation stress in rats and preterm neonates showed that babies who received tactile and kinesthetic stimulations improved weight, were awake and active longer, and better performed certain orientation, habituation, and motor behaviors.

In relation to acoustic stimulation, research studies have shown that early auditory intervention has led to cognitive improvements in neonatal hypoxic and ischemic rats. Auditory discrimination is frequently impaired after neonatal brain damage in rats and after neuropathology in preterm human infants at risk of brain injuries (Benasich & Tallal, 2002; Threlkeld et al., 2009). Rats subjected to unilateral hypoxia and ischemia at an early age exhibited significant rapid auditory processing deficits (McClure, Threlkeld, Rosen, & Fitch, 2006). An inability to discriminate rapidly changing acoustic signals has

been proposed to interfere with some aspects of language development in humans with abnormal brain development (Threlkeld et al., 2009). Further, Threlkeld and associates (2009) studied the early effects of acoustic discrimination experience in male rats with induced cortical developmental anomalies. They found that prior auditory experience in rats improved acoustic discriminatory performance, mitigating the effects of cortical developmental anomalies. More recently, a study of early musical training in humans showed that participants with more than two years of childhood musical training were able to process and identify speech syllables significantly faster than those without early music training (Kraus et al., 2014a). Furthermore, it was observed by using an Electroencephalogram (EEG) that superior speech discrimination performance was accompanied by faster auditory evoked potential activity (EEG) in the cerebral cortex of participants with two or more years of early musical training as compared to those without the early experience (Kraus et al., 2014a; Kraus et al., 2014b).

All these results have suggested that tactile and auditory stimulation in early life improve different mechanisms of brain function that may underlie recovery. Several studies have focused on somatosensory stimulation and have found its beneficial effects on: Brain injury, stress, anxiety, infections, and brain plasticity, in rats (Anisman et al., 1998; Bilbo et al., 2007; Costa et al., 2012; Kolb & Gibb, 2010; Lehman et al., 2001; Pham et al., 1996; Schanberg &Field, 1987). Other studies have demonstrated how early auditory stimulation mitigates the long-term auditory processing deficits induced by hypoxia and ischemia in neonatal male rats, and improves some language capabilities in children (Krauss et al., 2014a; Krauss et al., 2014b; Threlkeld et al., 2009). However, no

studies have yet explored the long-term mitigating effects of both stimulations on cognitive and neurological impairments of hypoxic-ischemic male rats. The purpose of this project was to explore the relative long-term effects of neonatal somatosensory (tactile) stimulation and acoustic discrimination experiences on exploratory behavior (anxiety), acoustic discrimination, and spatial memory-learning in adult rats with neonatal hypoxic-ischemic injury. Brain weight testing was also performed to investigate the possible relative benefits of both or either tactile and auditory stimulation respectively. We expected that these interventions would alleviate the cognitive and sensory impairments caused by injury, with the goal that the outcome of this study would result in information relevant to the development of new therapeutic methods that could help mitigate the effects of vascular irregularities in high-risk term infants and premature babies. Overall we hypothesized that early handling and auditory stimulation would result in better sensory, cognitive, and neurological outcomes in hypoxic-ischemic adult rats than rats with no stimulation. The HI group included all the subjects with induced brain injury, and the sham group or control group comprised all the subjects that did not experience the hypoxia and ischemia insult. We expected that the tactile stimulation subjects (HI and Sham) would perform better in the spatial memory/learning, exploratory behavior tests, and have a greater brain weigh than the non-stimulation groups (HI and sham). We also predicted that rats in the auditory stimulation groups (HI and sham) would show more complex acoustic discrimination ability and have greater brain weight than those in the no stimulation groups (HI and sham).

Chapter 2

Methods

Subjects and Surgical Treatment

Experimental subjects were 58 (27 HI and 31sham) male Wistar rats born to 14 time-mated dams at Rhode Island College. Dams were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed using a 12-h light/dark cycle with food and water available *ad libitum*. On postnatal day one (P1), pups were organized into 8 litters of eight males and two females.

On P7, animals were randomly selected to receive HI injury or control (sham) procedures. Before the surgical procedure male subjects were randomly assigned to one of the treatment groups: hypoxia-ischemia (HI) or sham. Male subjects were studied, given prior evidence of behavioral deficits in male but no female rodents with neonatal brain injury (Hill, Threlkeld, & Fitch, 2011; Peiffer, Rossen, &Fitch, 2004)- findings that parallel higher diagnostic rates of neurodevelopmental disorders (e.g., dyslexia, epilepsy, autism, and intellectual disability) in human males as compared to females (Liederman et al, 2005; Raz et al., 1995; Rutter, Capsi, & Moffitt, 2003)

Prior to surgery, subjects were weighed and anesthetized using 4% isoflurane and maintained with 1-3% during the surgical procedure. The midline of the neck was swabbed with alcohol and betadine. Following a 1 cm midline incision of the neck, the right common carotid artery (RCCA) was located and completely cauterized (inducing

ischemia; McClure et al., 2006) using a surgical cauterizing tool. Following ligation of the RCCA, the pups' skin was sutured using two interrupted Vicryl sutures and labeled with paw ink injections for identification (approximately 10µl ink injections). Sham subjects underwent identical surgical procedures without cauterization of the RCCA. Body temperature was maintained at 37 °C preoperatively, during surgery and during postoperative recovery using isothermal heating pads. After surgery, the pups returned to their dams to be fed for 2–3 h before being placed in an airtight acrylic chamber and exposed to 8% humidified oxygen balanced with 92% nitrogen (Hypoxia) for 120 minutes. Sham subjects were placed in an open-air container for 120 minutes as a control procedure. After all those procedures were completed animals were placed back in their home cages with their mothers.

Prior to any intervention (P8), subjects were randomly assigned to one of three condition groups: tactile or somatosensory stimulation, 22 subjects (hypoxia-ischemia (HI) n=10 and sham n=12), auditory stimulation 23 subjects (HI n=12 and sham n=11) and no stimulation 13 animals (HI n=5 and sham n=8).

SOMATOSENSORY (TACTILE) STIMULATION (SS) n-22		**AUDITORY STIMULATION (AS) n=23**		**NO STIMULATION (NS) n=13**	
HI **SSHI** 10	Sham **SSS** 12	HI **ASHI** 12	Sham **ASS** 11	HI **NSHI** 5	Sham **NSS** 8

All procedures were performed according to the National Institutes of Health guide for the care and use of laboratory animals and reviewed and approved by the Rhode Island College Institutional Animal Care and Use Committee.

Early Intervention and Behavioral Testing

Tactile Stimulation. This procedure was performed to observe whether or not somatosensory stimulation has any beneficial effect in mitigating the cognitive and behavioral impairments caused by the HI insult. The day following surgery, on postnatal day eight (P8), the 22 pups (HI=10 and sham=12) randomly assigned to the tactile group started the somatosensory stimulation process. Animals received seven consecutive days of tactile stimulation twice a day (P8-14), between the hours of 9:00am-11:00am and 1:00 PM-3:00 PM. Pups were divided in 3 groups, all the animals that were in one litter were kept together in that group. To avoid stimulation order effects, experimenters randomly alternated the order in which litters were selected for stimulation. (i.e., on day P8 cage number one received stimulation first, on day two, cage two receives stimulation first, etc.). Each day, the first group of experimental pups were removed from their mothers and moved to an alternate cage, which was placed on an isothermal heating pad to maintain the animals' body temperature. The pups were moved to another room where the stimulation session began. Pups were together and were rubbed with a soft duster in a circular motion for a period of 10 minutes, two times a day (Mychasiuk, et al., 2013, See Figure 1-Met, to observe the somatosensory stimulation). Each group of pups had their

own duster to eliminate cross litter scent confounds. After the first group received the tactile intervention, pups were reunited with their respective dam and the second group started the same process, once the second litter ended the procedure, the experimenters started with the third group. Finally, those rats assigned to the auditory (ASHI and ASS) and the no stimulation (NSS and NSHI) groups were also separated from their mothers to control for any separation effects that could have had an influence on the result of this study. Thus, during the same 7 consecutive days (P8-P15) two times a day and between the hours of 9.00 AM-11.00 AM and 1.00 PM-3.00 PM pups in each litter were removed from their dams and held together for 10 minutes in separate cages in the stimulation room. Their cages were placed on isothermal heating pads to maintain their body temperature. Following the 10 minutes window all the pups returned to their respective dams.

Auditory Stimulation and Testing. To observe the effects of auditory stimulation on complex acoustic discrimination in adult subjects, the modified acoustic startle paradigm was used at two different moments of this study: During juvenile period (P25-29), to elicit the auditory stimulation, and during the adult period (P68-74), to assess the long-term effect of the early auditory stimulation on the rats. The modified acoustic startle paradigm allows detection and measurement of the behavioral reflex response (muscle contraction) that an animal manifests following an unexpected intense auditory stimulus, in this case a loud noise burst, in order to identify that the animal detected the stimulus. The auditory testing/experiences also involved the use of a modified pre-pulse inhibition paradigm In this paradigm, detection of a pre-stimulus presented within 50 ms

prior to a loud (105dB) startle eliciting stimulus (SES) resulted in attenuation of the startle response relative to an uncued trial. This task has been frequently used to assess basic sensory-motor gating (Holly Fitch, McClure, Peiffer, & Threlkeld, 2008).

During the juvenile period, rats in the auditory stimulation groups (ASS and ASHI) received acoustic discrimination test-experience starting on day P25 and lasting 5 consecutive days (P25-P29). During the auditory stimulation process, subjects were placed on a load cell platform (Med Associates, Georgia, VT, USA), which transduced each subject's ballistic motor response to the SES in mV. Signals were acquired and passed through a linear load cell amplifier (PHM- 250-60U) into a Biopac MP150WS acquisition system (Biopac Systems, Santa Barbra, CA) connected to a computer, which recorded the subject's movement and acoustic startle response (ASR) as a mV signal. The maximum peak value defining the ASR for each trial was extracted by algorithm from the 200 ms following the onset of the SES. This ASR represents a dependent variable. Auditory stimuli were generated using a Dell PC with custom programmed software and a Tucker Davis Technologies (RX6) real time processor. Stimulus files were played through a Niles SI-1260 amplifier (Niles Audio Corporation, Miami, FL) connected to 4 Cambridge Sound Works speakers (MC110), with sound levels calibrated by sound-level meter. Each pair of platforms had one speaker centered and mounted 50cm above. Attenuated response scores (ATT) were calculated from the peak ASR using the formula ([mean cued response/mean uncued response] X 100). In this formula, absolute response scores (as measured by load-cell displacement for each subject's startle response) for cued and uncued trials were expressed as a ratio and multiplied by 100; thus ATT scores

represent a percentage. ATT scores were analyzed as a second dependent variable for all tasks. Scores at or near 100 reflect no difference (no detection) between cued and uncued trials, while lower ATT scores represent more significant detection. ATT scores higher than 100 reflect sensitization or exceedingly greater motor response to the elicited SES.

To avoid possible confound variables at the end of each auditory testing (P-25-30), rats in the non-auditory stimulation groups (SS and NS) were transported in their cages to the testing room. The animals stayed there for the same length of time that each experiment took that day (45 minutes for Single Tone and Oddball tests respectively, and 90 minutes for Silent Gap test).

Normal single tone (NST). Pre-pulse inhibition or normal single tone startle paradigms are commonly used to assess sensory-motor gating. In the current paradigm the single tone task was used as a method of acoustic stimulation in the juvenile period and to assess basic auditory acuity and pre-pulse inhibition prior to the evaluation of more complex temporal processing (e.g., Silent gap, Oddball and FM sweep discrimination) analogous to auditory temporal tasks used to test language learning impaired populations in the adult period. The ASS and ASHI groups received NST as the initial (1 day) auditory experience on P25 and again on the first day of testing in adulthood (P68), to assess baseline PPI in the juvenile period and experience effects in adulthood.

The normal single tone test (NST) session comprised 103 trials (cued or uncued), presented in a pseudo-random order. Uncued trials consisted of a silent background

followed by the 105 dB, 50 ms SES. In cued trials a 75 dB, 7 ms, 2300 Hz tone was presented 50 ms prior to the SES. Trials were variable in duration (16–24 s, 20 s on average). Somatosensory and No Stimulation HI and sham animals, (SS, NS) were not exposed to any auditory experience or stimulation during the juvenile period.

Silent Gap Procedure. A silent gap (SG) procedure (similar to single tone) was utilized to assess simple auditory temporal processing (a commonly used tool for this purpose in human populations and rodent models). Juvenile subjects (P26-27) experienced a total of two consecutive days on silent gap detection tasks, and adult subjects (P69-70) were tested also for two days in this procedure. A long gap duration (SG-100) version of the SG detection task was presented for one day, followed by one day of a short gap version (SG-10). In the juvenile procedure each session, regardless of gap duration, included 300 trials, while in the adult testing each session included 200 trials, each consisting of the presentation of variable duration silent gaps (Long SG (0, 2, 5, 10, 20, 30, 40, 50, 75, or 100 ms); Short SG (0, 2, 3, 4, 5, 6, 7, 8, 9, 10 ms)) embedded in continuous 75 dB broadband white noise. Each gap was presented 50 ms prior to a 105 dB burst of white noise. The uncued trials used a ‘‘gap’’ of 0 ms. The cue-burst interval for each task was maintained at 50 ms [59]. SS and NS HI and sham animals were not exposed to the silent gap task at any time during the juvenile period.

Oddball Procedure. Oddball sessions comprised of 103 trials, and a total of two sessions (one per day over two days) were administered to ASS and ASHI in the juvenile period (P28-29) and again over the adult periods (P71-72) where the animals were tested.

This procedure involves the repeated presentation of a background 75 dB, high-low tone sequence (2300-1100 Hz, respectively) separated by a within-stimulus inter-stimulus interval (ISI) of variable duration (275, 225 ms; one interval used per session). Each sequence was separated by a between sequence ISI, which is always 200 ms greater than the inter-stimulus interval to maintain perceptual contiguity of the tone-pair. In uncued trials, the last tone sequence was followed by 50 ms of silence, then by the 105 dB/50 ms SES. In cued trials, a reversal of the tone sequence occurred (low-high, 1100–2300 Hz) followed by 50 ms of silence, and then the SES. Again, if stimuli were discriminated (high-low tone pair from low-high), and the stimulus change was detected subjects would show inhibition of the startle response to the SES. The No Stimulation (NSHI-NSS) and Tactile stimulation (SSHI-SSS) groups did not experience any auditory discrimination task during the juvenile period. See Figure 2-Met to observe an example of the cued and uncued Oddball trial. (See Figure 2-Met)

Adult Testing

During the adult period (P60+), rats in all groups were evaluated to observe the effects of handling and auditory stimulation on exploratory behavior, spatial learning-memory, and auditory processing..

Elevated Plus-Maze (Exploratory behavior-Anxiety Measures). The Elevated Plus Maze is an apparatus developed to observe and assess rodents' exploratory behavior in enclosed areas or in the edges of a confining space, as well as their aversion of moving in open areas.

The apparatus used for the elevated plus maze test was made of plastic material and had the shape of a (+) sign, (See Figure 3-Met- to observe an example of the Elevated Plus Maze). This apparatus was comprised of two open arms (50 cm long) across from each other and perpendicular to two closed arms (50 cm long) with a center platform (10 cm). The open arms possessed a very small edge to prevent animal falls; the closed arms had a high wall (30 cm tall). The entire maze was elevated 50 cm from the floor and placed in a square empty area surrounded by low thin metal walls to protect the animal from escaping. The color of the platform and the walls of the maze were off white.

Before the experiment started on (P66), animals were located in the experiment room, where they were transferred to individual opaque cages. They stayed in their cages for at least five minutes prior to the test to acclimate. Behavioral testing was performed between 9 AM and 6 PM. Before testing, equipment and lights were checked to keep condition uniform during the test session. Animals were tested following the order their cages were placed in the vivarium. During the experiment each rat was placed in the center area of the maze with its head facing directly toward an open arm. Rats were allowed to move freely around the maze for 5 minutes; during that period, animal's movements were recorded using a video camera located above the maze. A remote device connected to a computer controlled the camera. The time spent (duration) in the open arms, close arms, and the center area of the maze, were recorded and calculated by the EthoVision XT video tracking program. To control olfactory cues all arms and the center area were cleaned with isopropyl alcohol after each trial (Komada, et al., 2008). After the

experiment, animals were transferred to their original cages and moved back to the animal house.

Adult Acoustic Discrimination. To evaluate the long-term effects of juvenile auditory experience and possible interactions with somatosensory stimulation, adult subjects (P68-74) from each testing group received auditory testing using the same tasks as described for juvenile AS subjects with the addition of a novel FM sweep detection procedure.

FM Sweep Procedure. A novel FM sweep discrimination task was used to assess complex auditory temporal processing capabilities in adult subjects. The FM sweep discrimination task provided increasing processing demand beyond that of more basic silent gap detection and oddball tasks and shares similarities to frequency shifts seen in human phonemic sweeps (Fitch et al., 2008a,b; Tallal, 2004). FM sweep sessions consisted of 102 trials, and a total of two sessions (one per day across 2 days). Conversely, both were presented with the FM sweep battery for the first time in adulthood, following oddball testing (4 days of testing). This procedure involves the repeated presentation of a background 75 dB, downward FM sweeps (2300–1900 Hz) separated by a within- stimulus inter-stimulus interval (ISI) of variable duration (225, 175ms; one interval used per session). Each sequence was separated by a between sequence ISI, which was always 200 ms greater than the sweep duration. On uncued trials, the last FM sweep was followed by 50 ms of silence, followed by the 105 dB, 50 ms SES. On cued trials, an upward FM sweep (the reversal of the standard sweep, 1900–

2300 Hz), was followed by 50 ms of silence and then the SES. As with the other tasks detection, FM was measured by comparing cued and uncued response to the SES and ATT. Scores were calculated for between group comparisons of relative detection thresholds. See Figure 4-Met. to observe an example of cued and uncued FM Sweep trial.

Morris Water Maze (Spatial Memory-Learning Measure*).* This is a task typically used to assess the spatial learning in rodents. During this procedure, subjects used specific visual cues outside the maze (Doors, shelves, painted shapes on the wall) to locate a submerged escape platform while navigating in a circular pool. In this study the spatial learning performance is evaluated by analyzing the total distance that animals traveled over five days of trials. At age P76-80, rats from all groups were exposed to a spatial learning assessment using the Morris Water Maze apparatus**. (**See figure 5-Met to see an image of the Morris Water Maze). Testing was conducted in a round 122 cm diameter tub filled with water (temp 22 °C) with a 20.3 cm diameter submerged (invisible) platform, consistently placed in the southeast (SE) quadrant, two cm below the water surface. Fixed, extra-maze cues were abundant (wall images, computer, sink, door, table), while precaution was taken to eliminate intra-maze cues (tub was painted black so the transparent submerged platform blends into a consistent background). A camera located above the tub tracked and recorded the animal's movements. To avoid experimenter bias, two experimenters observed animals' movements and verified the time animal entered to the tub and reached platform. Experimenters also assisted the animals when they needed help in reaching the platform once their time was completed, or trying to exit the tub. On each of five testing days, subjects underwent four trials, with

each trial starting from a different randomly selected compass point (North, South, East, West). On day one, trial one, each subject was placed on the platform for 10 s, removed from the platform and then released from one of the starting locations. Each trial had a maximum time of 45s. Subjects unable to reach the platform within this time window were guided to the target and allowed to remain for 5 s. Latency and distance traveled to reach the platform for each trial was recorded as dependent variables. On day five of testing, following the fourth trial for each subject, the platform was removed and subjects were released from the quadrant previously adjacent to the platform. Within this probe trial, distance traveled and time spent in each quadrant were recorded to assess memory retention for the previous platform location. Following the 45-second trial subjects were removed from the water maze. During all testing sessions subjects were kept warm by placement of isothermal heating pads under the holding cages (Bromley et al., 2011). Animals were tested in a random order each day.

Morris Water Maze- Probe Trial. This is a task used to assess spatial memory in rodents. On day five of testing, following the fourth trial for each subject, the submerged escape platform was removed from the pool, and subjects were released from the quadrant previously adjacent to the platform. This procedure allowed observing and testing subject's preference to locate the platform using their previous spatial learning experience. Within this probe trial distance traveled in each quadrant were recorded to assess memory retention for the previous platform location. Following the 45-second trial subjects were removed from the water maze. During all testing sessions subjects were

kept warm by placement of isothermal heating pads under the holding cages (Bromley et al., 2011)

Brain weight. Rats were weighed and euthanized on P81and 82 following adult behavioral testing with an overdose of Pentobarbital (Sleepaway, 100mg/kg) and they were perfused using .9% PBS and 10% formalin. The brains were removed and weighed. Transcardial perfusion represented the end point for all adult experimental subjects in which tissue collection was required.

Chapter 3

Statistical Analysis and Results

Statistical Analysis

Repeated measures ANOVAs were performed to assess main effects of treatment and condition when multiple days of testing or repeated testing conditions were presented (e.g., Morris water maze, Silent Gap and Oddball tasks). MANOVAs were used to assess main effects of treatment and condition for between subjects' tasks. A One-Way ANOVAs was used to assess the results of exploratory behavior generated during the Elevated Plus Maze (EPM) experiment (open arms, closed arms, and center of maze). ARepeated Measure ANOVA was used to analyze the outcome of the Morris Water Maze across the five days of testing. A One-Way ANOVA was used to analyze the Morris Water Maze probe trial. Tukey's test was used for post hoc analysis, and t-tests were used for planned comparisons when warranted. SPSS with a criterion of alpha 0.05 was used in the analysis of all variables.

Results

Exploratory Behavior: Elevated Plus Maze

An overall three (condition; tactile, auditory, and no stimulation) by two (treatment; HI and sham), by three (duration; center, open arm, and close arm) Multivariate Analysis of Variance (MANOVA) revealed a main effect of treatment in the open arm zone with HI subjects spending more time in the open arm than sham animals, $F(1,52)= 4.627$, p=

0.036; partial $n^2 = 0.082$ (See Fig.6 EPM). Post-hoc analyses of treatment across conditions using Tukey's HSD revealed no significant difference on the time each condition (tactile, auditory, and no stimulation) spent in the open arm. One way ANOVAs were used to analyze differences in the time subjects spent in the open arm between the following groups: Tactile HI and sham, auditory HI and sham, and no stimulation HI and sham, to assess the treatment effect in each condition, no significant results were found in any of the comparisons (See Fig.6 EPM).

Auditory Testing

Oddball. A two (treatment; HI and sham) by three (condition; tactile, auditory, no stimulation) by two (Interstimulus interval (ISI); 275 and 225) Repeated Measure Analysis of variance revealed that animals across groups performed similarly on the Oddball acoustic discrimination task. The statistical results indicated no significant effects of condition, treatment, or interactions.

FM Sweep. Similar to the Oddball auditory test, a two (treatment; HI and sham) by three (condition; tactile, auditory, no stimulation) by two (ISI; 225 and 175) Repeated Measure Analysis of variance showed no statistically significant condition or treatment effects or interactions. The results indicated that animals across groups performed similarly on the FM Sweep acoustic discrimination task.

Spatial Learning: Morris Water Maze (MWM)

A two (treatment; HI and sham) by three (condition, tactile, auditory, and no stimulation) by five (day; 1,2,3,4,5) Repeated Measures Analysis of variance was used to compare the total distance that each animal traveled each day to reach the platform. Results showed a significant effect of day, F(4,208)= 51.382, p= 0.01, $\eta^2 = 0.497$, indicating a significant decrease in the total distance traveled by all subjects over five days, suggesting that animals were able to spatially learn the platform location (Fig. 7 MWM).

Results also revealed a significant treatment effect, F(1,52)= 8.23, p<0.01, $\eta^2 = 0.137$ indicating that HI subjects traveled longer distance across of the five days of testing to reach the platform as compared to shams. The increase in distance traveled by HI animals as compared to shams, was indicative of spatial learning impairment (See Fig. 8 MWM). In contrast, no significant effect of condition was observed on distance traveled to reach the platform indicating early tactile and auditory interventions did not influence the acquisition phase of the Morris Water Maze task.

Probe Trial-MWM. An overall three (condition; tactile, auditory, and no stimulation) by two (treatment; HI and sham), by one (total distance traveled in northeast, northwest, southeast, southwest quadrants, and platform zone) Multivariate Analysis of Variance (MANOVA) revealed no significant effects of condition or treatment, but a statistically significant treatment by condition interaction was observed in the zone formerly occupied by the platform in the MWM (platform zone), a F(2,52)= 4.817, p=

0.012; $n^2 = 0.156$ respectively. This result indicated that HI and sham subjects performed differently depending on the early intervention condition

Results from multiple comparisons revealed a statistically significant difference between the tactile HI and tactile sham group, t (20)= 2.274, p= 0.034 (two tailed), indicating that tactile HI traveled more distance in the platform zone than the tactile sham.

A significant result was also found by comparing the auditory HI and sham group in the platform zone, t (21)= 2.128, p= 0.045 (two tailed), indicating that the auditory sham group traveled more distances in the platform zone that the auditory HI subjects. No significant result was found in the comparison between no stimulation HI and no stimulation sham, indicating that both groups traveled similar distances in the platform zone. (See Fig 9, Probe Trial-MWM).

Brain Analysis

Brain Weight. Result for brain weight using a two (treatment; Hi and sham) by three (condition; tactile, auditory, and no stimulation) Univariate ANOVA, revealed significant effect of condition, F(2,52)= 4.509, p= 0.016; $\eta^2 = 0.148$ and effect of treatment , F(1,52)= 28.554, p= 0.01; $\eta^2 = 0.354$. The results indicated that tactile and auditory HI subjects presented less brain weight than the tactile and auditory sham animals. No significant result was observed between the no stimulation HI and control sham groups (See Fig. 10.Brain Weight). Post-hoc analysis showed that neonatal HI

injury resulted in more significant reduction of brain weight in the tactile subjects as compare to the no stimulation group ($p<0.05$). No significant brain weight differences were observed between the tactile and the auditory groups.

RESULTS SUMMARY		
EXPERIMENT	**PURPOSE**	**STATISTICAL RESULT**
Elevated Plus Maze	**Assess exploratory behavior**	. Significant effect of treatment in the open arm zone, $F(1,52)= 4.627$, $p= 0.036$; $n^2 = 0.082$ *
Auditory Oddball 275 – 225	**Assess complex auditory processing**	. No significant effects of condition, treatment, or interactions
Auditory FM Sweep 225 – 175	**Assess complex auditory processing**	. No significant effects of condition, treatment, or interactions
Morris Water Maze	**Assess spatial learning**	. Significant effect of day, $F(4,208)= 51.382$, $p= 0.01$, $\eta^2 = 0.497$* . Significant treatment effect, $F(1,52)= 8.23$, $p<0.01$, $\eta^2 = 0.137$ *
Probe-Trial Morris Water Maze	**Assess spatial learning and memory**	. Significant treatment by condition interaction (platform zone), $F(2,52)= 4.817$, $p= 0.012$; $n^2 = 0.156$ * . Significant difference between the tactile HI and tactile sham group (platform zone), $t(20)= 2.274$, $p= 0.034$ (two tailed)* . Significant difference between the auditory HI and sham group (platform zone), $t(21)= 2.128$, $p= 0.045$ (two tailed)*
Brain Weight	**Assess brain volume and severity of injury**	**. S**ignificant effect of condition, $F(2,52)= 4.509$, $p= 0.016$; $\eta^2 = 0.148$ * . Significant effect of treatment, $F(1,52)= 28.554$, $p= 0.01$; $\eta^2 = 0.354$*

Chapter 4

Discussion and Conclusion

Medical observations in humans and previous research in rodents have demonstrated that high-risk term infants and premature babies are susceptible to experience oxygen deprivation (hypoxia, H) and reduced cerebral blood circulation (ischemia, I). These vascular irregularities lead to abnormal neurological and behavioral development (Chou et al., 2001; Threlkeld et al., 2014) that cannot be easily alleviated by the used of hypothermia, one of the most common current medical interventions. Looking for new approaches to ameliorate the effects of these pathologies led us to explore the effects of tactile and auditory stimulation in this type of brain injury.

Historically, previous studies in animals and humans have demonstrated that the introduction of tactile and auditory stimulation early in life has had different effects on the structure of the brain (Kolb et al., 2010) and on subsequent behaviors and cognitive functions (Costa et al., 2012). Some of these beneficial effects have been seen in animals and humans in the improvement of spatial learning capability (Chou et al., 2001; Pham et al., 1997) spatial memory performance (Bilbo et al, 2007), exploratory behavior (Costa et al., 2012), brain plasticity (Kolb & Gibb, 2010), reduction of anxiety and stress (Costa et al., 2012; Imanaka et al., 2008; Pham et al., 1997), reduction of certain hormones related to stress, and increase of proteins related to development (Anisman et al., 1998 Lehmann et al., 2001; Pham et al., 1997). Other beneficial effects were also observed with the use of auditory stimulation, such as improvements of acoustic discrimination performance and mitigating effects of cortical developmental anomalies (Threlkeld et al., 2009). Even

studies on hypoxic and ischemic rodents have shown improvement in spatial learning (Chou et al., 2001) and acoustic discrimination performance (Threlkeld et al., 2009. In humans, for example, the use of handling has also shown that preterm neonates who received tactile and kinesthetic stimulation improved weight, were awake and active more time, and better performed certain behaviors. In relation to acoustic stimulation, more recently, studies in humans have shown that early auditory intervention has led to improvements in acoustic discrimination performance and speech comprehension in patients with stroke (Ilvone et al. 2003), identification of speech syllables in children with musical training (Kraus et al., 2014), vocabulary in children with congenital hearing loss (Vohr et al, 2010), and language improvement in preterm infants (Caskey, Stephens, Trucker, & Vohr, 2015). Over all these results have suggested that tactile and auditory stimulation in early life improve different mechanisms of brain synapsis that underlie functional recovery.

Thus, the primary purpose of this study was to explore the relative long-term effects of neonatal somatosensory (tactile) stimulation and acoustic discrimination experiences early in development (P8-14 at tactile intervention, P25-29 at auditory intervention) on adult behavioral performance in male rats that experienced neonatal HI injury. In particular, this study explored whether or not tactile and auditory early stimulation could improve the learning, exploratory and auditory impairments produced by neonatal HI injury, and/or provide any beneficial neurobehavioral and anatomical outcome or sparing of brain weight in treated subjects. A series of experiments were used to observe and compare the effect of the stimulation on exploratory behavior, acoustic discrimination,

and spatial memory-learning performances of HI and sham control subjects, as well as their brain weight. Many studies mentioned above have already shown the beneficial effects of somatosensory (tactile) and auditory stimulations on brain injury, stress, anxiety, infections, and brain plasticity, long-term auditory processing deficits, and improving some language capabilities in children. However, information about the long-term mitigating effects of both stimulations on cognitive, behavioral, and neurological impairments of the HI male rats is not currently available.

The Elevated Plus Maze allowed us to observe and assess whether or not the tactile or auditory stimulation had any long-term beneficial effects on exploratory behavior in rats, in particular those with HI injury, in enclosed areas or in the edges of a confining space, as well as their aversion of moving in open areas. In this experiment we found no significant effect of condition (tactile or auditory stimulation). However HI animals spent significantly more time exploring the open arm zones in comparison with the other groups. Further analysis was done to investigate differences among HI groups (tactile, auditory, and no stimulation), but results did no show any significant effects of stimulation on exploration on the plus maze arms. Previous studies on the effect of HI on rodents showed that the injury and the severity of the brain injury induced by the HI insult affects subjects' performance in the Elevated Plus Maze model. Similar to our findings, Wan Fan et al. (2005) in their research on the effects of HI on neurological dysfunction observed that HI animals not only had more entries to the open arms, but also spent more time there as well. Further, Pesold and Treit (1992), in their study on brain injury of the septum (septal nuclei) in rodents, found that lesions in that area of the brain

led animals to increase the time spent in the open arms as well as increased the number of entries to the open arms of the elevated plus maze. Since the EPM test assesses animal anxiety to explore the open arms, they concluded that HI injury led to a reduction of anxiolytic behavior in HI subjects. The HI injury may have led to damage of brain regions important for anxiety fostering the exploratory behavior in the open zones in the present study. Further research will be needed to confirm the exact anatomical structures affected by the injury.

The use of acoustic discrimination tasks such as Oddball and FM Sweep allowed us to observe and evaluate the long-term effects of the early auditory stimulation on complex acoustic discrimination in adult subjects. The aim of the tasks was to determine the ability that the animals had to detect certain sounds by measuring their startle responses to cued and uncued elicited noises. The results of these tasks revealed no significant condition or treatment effects or interactions indicating that subjects across all groups, (HI and shams), performed similarly on both acoustic discrimination tasks. A previous study with P7 HI injured subjects using Silent Gap 0-100 task (Alexander et al., 2014) observed that HI animals showed a long-term deficit in rapid auditory processing as juveniles and as adults in comparison with the P3 HI injured group and the sham group. However, our study showed no significant detrimental difference in the responses to the acoustic discrimination tasks between HI rats as compared with the sham group. Contrary to this result, Threlkeld, Hill, Rosen and Fitch (2009) studied the effect of early auditory intervention on rats with developmental cortical injury (microgyria). They found that early auditory experience significantly improved auditory performance in adult

subjects. This variation in outcomes between Threlkeld et al. (2009) research and this current study may be influenced by differences in the methodology (more days of auditory stimulation in the juvenile period, 17 vs. 5 days) and the type of brain injury (mycrogyria vs. HI). Factors such as the number of days tested and the age at which early auditory testing began differed between the current study and those previously reported. These differences in methods could explain the lack of effects seen in the present study. Since the difference in methodology and results have been observed in other auditory studies, more research replicating the same methodology should be done to explore whether or not these difference between the HI and the control sham group are attributable to that factor. In addition, future studies should be implemented to systematically test the effects of age at testing and intervention duration.

To assess the long-term effects of tactile stimulation on spatial learning we used the Morris Water Maze. During this procedure, subjects used specific visual cues outside the maze to locate a submerged escape platform while navigating in a circular pool. In this study we evaluated spatial learning performance by measuring the total distance that animals traveled over four trials each day for five consecutive days. Our finding showed no significant effects of condition in the acquisition phase of the MWM task (first five days of testing). Further, results showed a significant decrease in the distance all animals traveled to reach the platform over the five days. This reduction of distance traveled was indicative of a significant day effect, i.e. each day subjects traveled less distance to reach the platform. This result suggested that all subjects were able to spatially learn the platform location, even those with HI injury. A significant treatment effect was found,

showing that HI animals traveled a longer distance to reach the platform as compared to the sham group regardless of early sensory stimulation. Previous studies support this finding as well, since they have revealed that overall HI animals present neurobehavioral and motor impairments caused by the HI injury, but still were able to spatially learn the task (Alexander et al., 2014 & Wan Fan et al., 2005). Other studies exploring the effect of caffeine treatment and its implication on spatial memory using MWM showed that HI animals, not exposed to the caffeine treatment, showed a significant deficit in the spatial learning performance in comparison to the sham group, (Alexander, Smith, Rosenkrantz, & Fitch, 2013). Contrary to what we expected, early tactile intervention did not show a beneficial effect on spatial learning performance in HI and sham animals. This discrepancy in results across those studies that used the MWM to evaluate spatial learning in rodents with brain injury may be influenced for different factors, such as, the age at which animals received the brain injury (e.g. P1, P3, P7), the measures used to evaluate spatial learning performance, (e.g. distance traveled, latency, frequency, or duration of each animal in the quadrants and platform areas), and the daily number of trials used in the MWM task. For that reason, it would be important to take into consideration this discrepancy in the design of future studies to better evaluate and compare animals' spatial learning across studies. Further studies would also be needed to explore more deeply the absence of the condition effect (tactile and auditory stimulation) in the spatial learning process, and if the method used to implement the stimulation were the more appropriate for this type of study or this type of the injury (e.g. the use of more ecological sounds for auditory stimulus).

The spatial memory- probe trial of the Morris Water Maze (MWM) is a task used to assess the spatial memory for the previously learned MWM platform location in rodents. On day five following the fourth trial for each subject, the submerged escape platform was removed from the pool. The purpose of this task was to observe whether or not subjects were efficiently able to locate the absent platform zone by traveling longer distances in that area. This task also was selected to explore if the early tactile intervention had any beneficial effect in the subject performance during this experiment. Within this probe trial distance traveled and time spent in each quadrant were recorded to assess memory retention for the previous platform location. Results from the probe trial at the MWM in the current study showed a condition (tactile, auditory, and not stimulation) and treatment (HI and sham) interaction that indicated that HI and sham animals performed differently depending on their early intervention condition. Tactile and auditory early stimulation influenced performance in subjects' spatial memory. Tactile HI and auditory sham traveled more distance in the zone previously occupied by the platform indicating that they spatially remembered the location of the platform that allowed them to escape from the water. Future anatomical analyses of brain regions important for spatial memory may help explain why there was an interaction between stimulation conditions (tactile and auditory) and treatment.

To assess the effect of tactile and auditory stimulation on possible brain plasticity, once the animals were euthanized, the brains were removed from the animals and weighted. By observing the results we found out that overall HI animals had more brain damage as compared with the sham control group. Brain weight results revealed a

significant effect of condition, indicating that tactile and auditory HI animals presented less brain weights than the other groups. We also observed the severity of the HI insult in subjects through treatment blind visual observation. Although preliminary observation in this study revealed that animal visible injury varied from moderate to severe across subjects, tactile and auditory HI subjects appeared to have more severe visible brain injury and less brain weight than the other subjects. The study of Alexander et al. (2014) explored the behavioral and neurological difference in animals that experience HI insult at P3 and P7. Overall, they observed that rats injured at P7 presented more behavioral deficit and more damage in the certain structures of the brain compared with the P3 and control group. Future work will seek to quantify injury severity in an effort to shed light on the interaction seen between groups on the spatial memory task. Taking into account the brain weight and injury results in this current study, it is interesting to observe and difficult to understand how HI and sham subjects still performed similarly in many of the assessment tasks we used. Because this current study could not determine clearly which factors affected its results, future research needs to explore in detail other aspects that could have influenced animals' performance across the current battery of tasks, and also deeply explore the relation between the time when HI injury is induced and the possible effectiveness of the stimulation. It would also be important to determine what areas of the hypoxic and ischemic brain are more affected depending on the level of brain damage (moderate, mild, and severe) and what type of behavioral and cognitive deficit are associated with it. This information may lead us to look for other methods or other interventions that can be more effective to mitigate the impartments of HI.

Conclusion

In this study we replicated previous investigations showing that the effect of HI injury in P7 rats leads to spatial learning deficits and changes in the exploratory behavior on the elevated plus maze. Results in this study obtained from the probe trial MWM suggested that treatment and condition interaction may be related to beneficial effects of tactile and auditory experience, given that HI animals with tactile stimulation and sham subjects with early acoustic intervention spent more time in the formerly learned water maze platform zone as compared to the other groups. Another important result is related to HI brain injury. HI animals in the tactile group performed better in the probe trial than the other subjects and they were able to performed similarly to other animals in some of the tasks used for this study. The lack of a significant difference in performance of HI animals as compared with the sham group may be related to the effects of auditory experiences in the adult period or the constant tactile manipulation of the animals across the entire study. Since HI animals present particular characteristics because of their brain injury, future studies should take into consideration specific factors and conditions that are unique to that particular injury in this type of animals (e.g. the severity of brain damage caused by the HI insult at different ages, which areas of the brain are more affected, what particular behaviors can be expected based on the affected area of the brain, etc.). This information may help to understand better what approaches or stimulations should be more beneficial and what type of effect can be expected.

Future studies can also increase the number of subjects to better compare any possible difference in performance or response between the groups. Additional research

should also take into consideration the effects of any stimulation in male and female animals, since previous studies have shown that female rats react differently to treatments and interventions as compared to male rats. Previous research has demonstrated abundant significant results that showed the beneficial effect of somatosensory and auditory stimulation in HI and animals with no injury. However, we found those beneficial effects only on the spatial memory task in tactile HI and auditory sham subjects.

Finally, the lack of standard methods across studies (e.g., numbers of days for stimulation or testing, way the stimulation is implemented, duration in the hypoxia chamber, age when the HI insult is performed, different measurements used to evaluate the performance of the animals, etc.) could be some of the factors that led to finding differences in results among all the investigations. Another suggestion for future studies would be the use of more ecological interventions or stimulations in the neonatal and juvenile period, which could replicate a natural interaction and sounds between dams and pups.

Chapter 5

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Chapter 6

Figures

Figure 1- Met. Somatosensory Stimulation

Figure 1.Met. Somatosensory Stimulation

Figure 2 Met. Example of uncued and cued Oddball trial

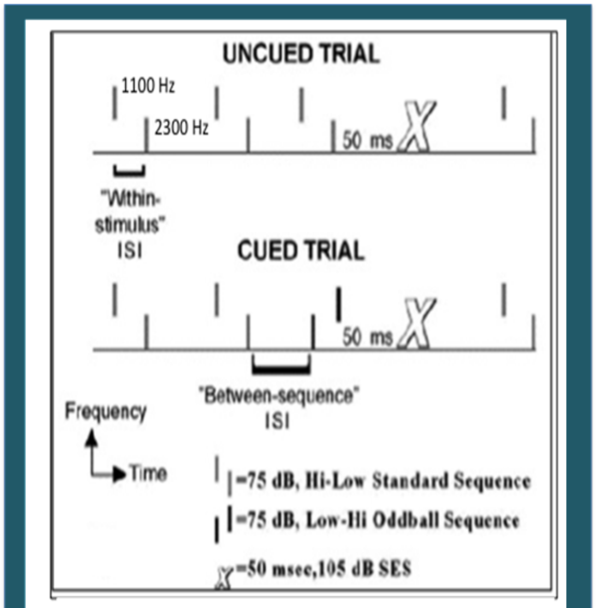

Figure 2- Met. Example of uncued –cued Oddball trial

Figure 3 Met. Image of Elevated Plus Maze

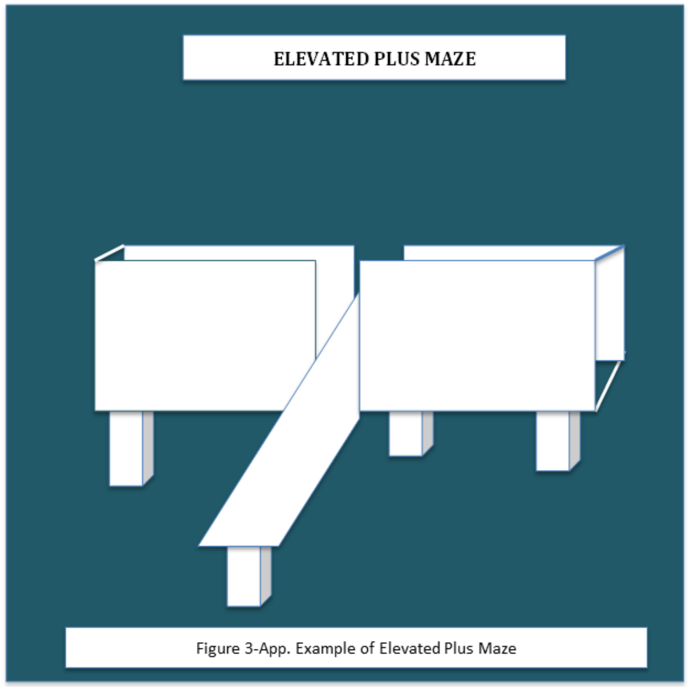

Figure 3-App. Example of Elevated Plus Maze

Figure 4-Met. Example of cued and uncued FM Sweep trial

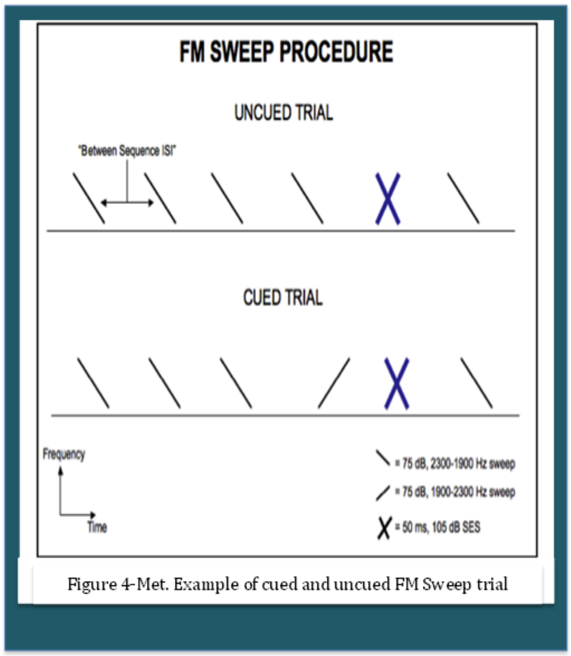

Figure 4-Met. Example of cued and uncued FM Sweep trial

Figure 5-Met. Image of a Morris Water Maze

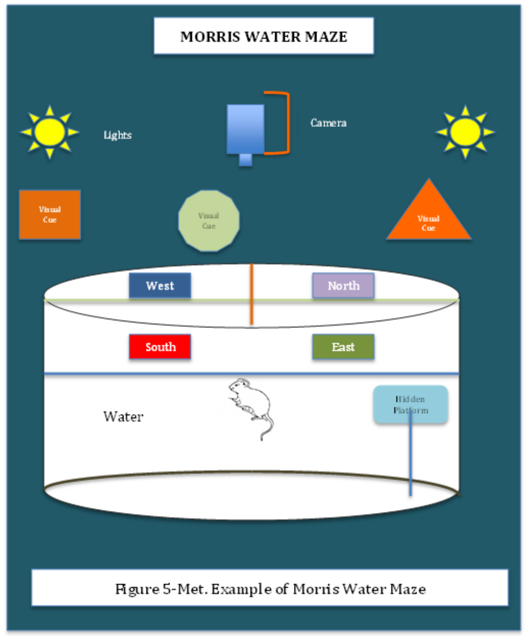

Figure 5-Met. Example of Morris Water Maze

Figure 6. EPM- Results of Elevated Plus Maze

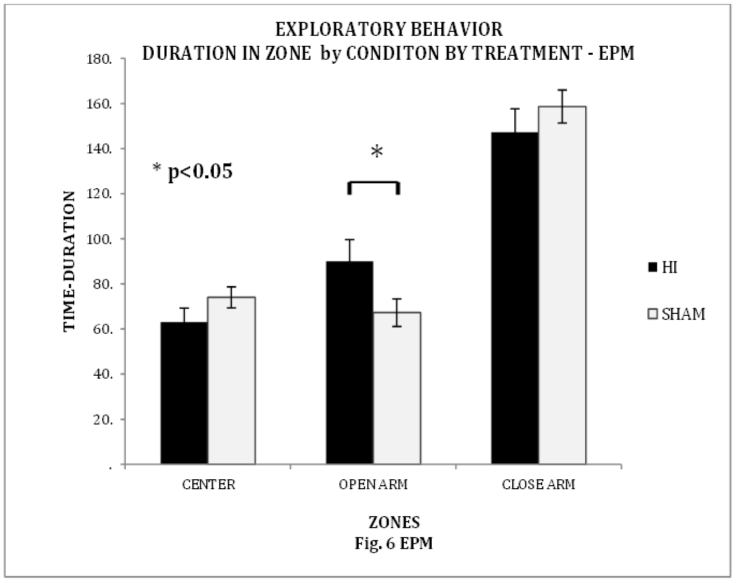

Fig.6 EPM. Comparison of HI and sham subjects in the time they spent in all zones. HI animals spent more time traveling in the open arm that shams.

Figure 7.MWM- Results of Morris Water Maze

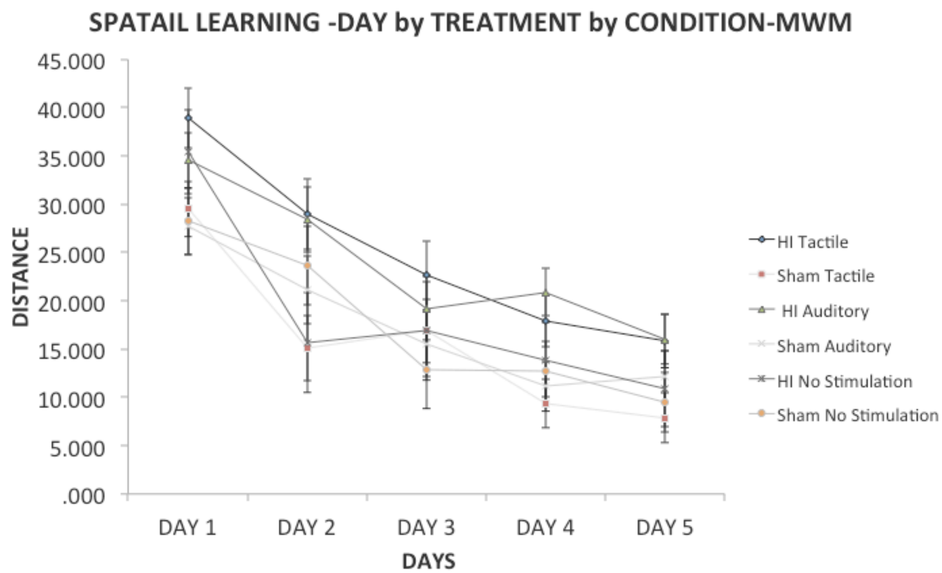

Fig. 1 MWM

Fig.7 MWM. Comparison showed in effect of day, indication that subjects decreased the total daily distance traveled to reach the platform across days.

Figure 8. MWM- Results of Morris Water Maze

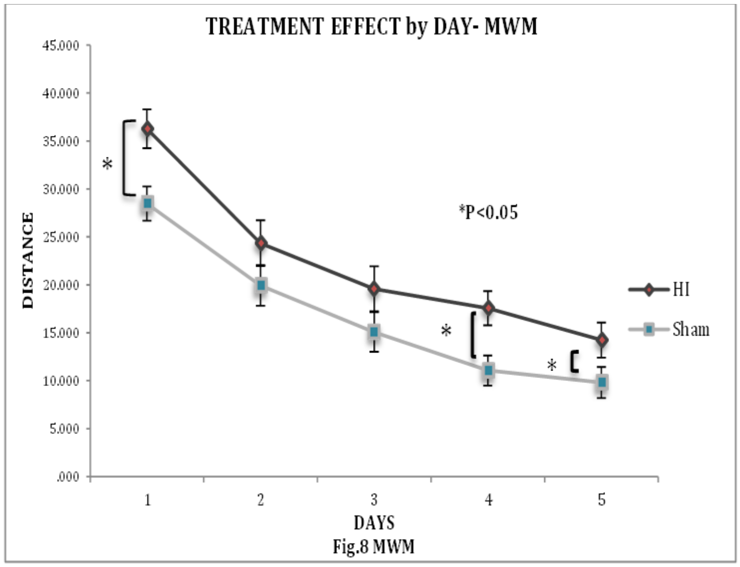

Fig.8 MWM. Comparison showed that the HI subjects overall traveled more distance across days than the sham group, indicating a significant spatial learning impairment for HI animals.

Fig.9 Probe Trial-MWM.

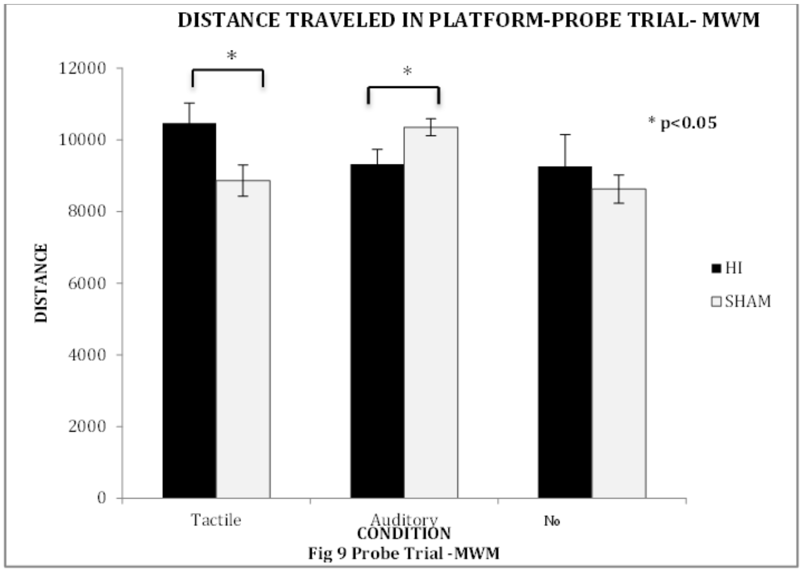

Fig.9 Probe Trial-MWM. Comparison showed that the tactile HI and auditory sham subjects traveled more distance in the platform zone than tactile sham and auditory sham groups respectively. No significant difference in the distance traveling in the platform zone was found between the no stimulation HI and sham animals.

Figure 10. Brain Weight- Results of Brain Weight

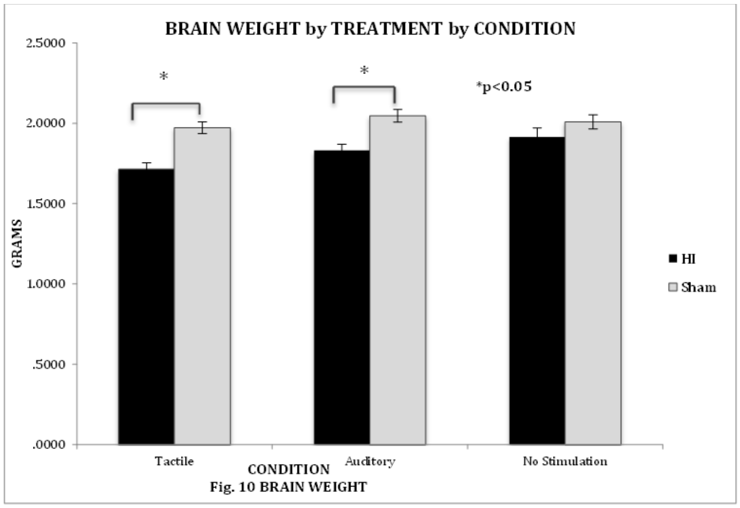

Fig. 10 Brain Weight. Comparison of tactile Hi and sham and auditory HI and its control sham revealed significant difference in brain weight. No significant results were found between no stimulation HI and its control sham group.