EFFECTS OF PRENATAL METHYLMERCURY EXPOSURE ON MOTOR COORDINATION, ACTIVITY LEVELS AND MITOCHONDRIAL MEMBRANE POTENTIAL IN ADULT MICE

A Senior Honors Thesis

by

JESSICA MARIE MACKEY

Submitted to the Office of Honors Programs
& Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements of the

UNIVERSITY UNDERGRADUATE
RESEARCH FELLOWS

April 2006

Major: Biomedical Science

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Approved as to style and content by:

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Louise C. Abbott   Edward A. Funkhouser
(Fellows Advisor)   (Executive Director)

April 2006

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ABSTRACT

Effects of Prenatal Methylmercury Exposure on Motor Coordination, Activity Levels and

Mitochondrial Membrane Potential in Adult Mice (April 2006)

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Methylmercury has cytotoxic effects on animals and humans and a major target

organ for methylmercury is the central nervous system (CNS). Methylmercury

(MeHg) is a developmental neurotoxicant and the prenatal CNS appears to be even more

sensitive to MeHg than the mature CNS. Previous research has shown that high doses of

MeHg have severe cellular and behavioral effects. More recently, concern has been

raised with respect to possible deleterious effects of chronic low doses of MeHg exposure

to the developing CNS. The objective of this study was to determine if chronic low dose

MeHg exposure would result in deficits in cognition and coordination and a decrease

in mitochondrial membrane potential in cerebellar granule cells. Pregnant mice were

divided into control and treatment groups. Developing fetuses were exposed to chronic

low doses of MeHg through feeding food containing MeHg to pregnant C57Bl/6J female

mice at a daily dose of 0.03 µg/Kg of body weight. Footprint analysis, rota-rod, vertical

pole, open field and Morris water maze were used to test for changes in coordination,

activity levels, spatial learning and memory. Differences were observed between control and MeHg groups in rota-rod, footprint analysis, open field and Morris water maze. The results follow below. Controls stayed on the rota-rod twice as long as treated mice on the last day of trials. Left and right foot angles were decreased in treated mice. Treated females displayed less rearing movement in the first five minutes of open field compared to both treated males and the controls. In the Morris water maze, treated mice took longer than controls to find the platform on days 1-4. The probe trial results indicated that treated mice swam significantly less than the controls.

Cerebellar granule cells were isolated using dissociation media containing DNAse, and mitochondrial membrane potential was measured on the acutely isolated cells using tetramethylrhodamine methylester fluorescent dye. Images were captured with a fluorescence microscope. The data indicated no significant differences in fluorescence between control and MeHg mice.

These conclusions indicate prenatal *in vivo* methylmercury exposure in mice may not significantly impair mitochondrial membrane potential; however, other abnormal neuronal functions lead to the subtle changes in motor and cognitive behavior described in this research. As a consequence, other cellular mechanisms must be delineated to understand how prenatal methylmercury exposure causes CNS pathogenesis.

ACKNOWLEDGMENTS

Many people have assisted me through the course of this project and it would have been impossible to do without them. I am very thankful to them all.

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I would also like to thank Dr. Bhupinder Bawa for his time and expertise in cell isolations and microscope analyses.

Lastly, I would like to thank my parents, Gary and Natalie Mackey, for their unconditional love and support. At times I have been difficult to deal with but you have made it easier for me to stay positive and keep going. Thank you for your almost nightly phone calls and for caring. I love you.

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INTRODUCTION

Methylmercury is the most common form of organic mercury found as a ubiquitous environmental contaminant all over the world (Siciliano et al., 2003; Eisler, 2004). Currently, the major source for methylmercury exposure to humans comes from consumption of contaminated foods, primarily fish such as swordfish and tuna. Methylmercury has cytotoxic effects on animals and humans and a major target organ for methylmercury is the central nervous system (CNS). Methylmercury also is recognized as a developmental neurotoxicant and the prenatal CNS appears to be even more sensitive to methylmercury than the mature CNS (Clarkson, 2002).   Many studies have shown severe deleterious effects due to high doses of methylmercury, but more recently concern has been raised with respect to possible deleterious effects of chronic low dose of methylmercury exposure to the developing nervous system. The current recommended upper limit of exposure to methylmercury in humans is 0.1µg/kg body weight/day (Clarkson, 2002). The Environmental Protection Agency, Food and Drug Administration and National Academy of Science/National Research Council recently have all issued warnings to pregnant women and women considering becoming pregnant to avoid eating high-risk fish such as tuna, which are sources of significant human methylmercury exposure.

Today, chronic low doses of methylmercury are the most common form of human exposure; however, little is known about the molecular and cellular effects of prenatal exposure in environmentally relevant doses. Several studies have given insight into the mechanisms underlying prenatal brain damage at classical high dose exposures.

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This thesis follows the style and format of the Journal of Neuroscience.

The findings indicated neuronal cell division and migration, the most basic processes in brain development, were affected. Normally ordered parallel arrays of neuronal cells in the cortex were also disrupted, indicating disturbances in the developmental growth of the brain. Published evidence also indicates that prenatal high dose methylmercury exposure in children results in adverse outcomes (Cordier et al., 2002). It has been shown that motor and eye-hand coordination are decreased in some populations of children exposed to mercury compounds (Grandjean et al., 1998). Ataxia, which correlates with cerebellar dysfunction, is typically one of the prominent human clinical signs of methylmercury poisoning (Limke & Atchison, 2002) and animal models, when exposed to methylmercury also show CNS signs including ataxia. Continuing research into the mechanisms of methylmercury toxicity has shown that postnatal *in vivo* methylmercury exposure in mice impairs mitochondrial membrane potential, resulting in abnormal neuronal function and death and leads to adverse motor and cognitive outcomes.

Reports of methylmercury exposure in human populations and in experimental studies in non-human primates indicate that overt toxicity signs may be delayed for weeks or even months after exposure (silent or latent phase) (21st International Neurotoxicology Conference, February 10-14, 2004). Mercury is sequestered in brain tissue and it can continue to cause cellular damage and death as it remains in the cells of the CNS. The toxic effects of sequestered methylmercury have been linked to a disruption of a variety of normal biochemical processes and cellular constituents in neurons, including mitochondrial function. The purpose of this study was to analyze adult mice two months after exposure to prenatal, chronic low doses of methylmercury.

It was hypothesized that prenatal methylmercury exposure decreases mitochondrial membrane potential and thus motor coordination and activity levels in adult mice.

Analysis of motor coordination and activity revealed several subtle changes in behavior. Methylmercury appeared to effect no cellular changes in regards to mitochondrial membrane potential. Therefore, methylmercury did not compromise cell health through impaired mitochondrial function and the behavioral changes seen are the result of other cell mechanisms affected by methylmercury.

This project is important because it explores the effects of chronic, low dose prenatal MeHg exposure at the cellular and behavioral level. Greater understanding of how prenatal exposure to methylmercury causes neuronal damage and death is needed. This research has enabled us to better understand potential mechanisms through which methylmercury causes CNS pathogenesis and further methylmercury research can be directed towards delineating other cellular mechanisms that affect the motor and cognitive outcomes described in this research. With this knowledge, strategies could be developed to reduce the burden of morbidity and dysfunction caused by methylmercury toxicity. This study will be beneficial to developing future therapies or possibly even eventually lead to the prevention or reduction of deleterious effects of methylmercury toxicity in humans.

The following section reviews the literature for methylmercury as relevant to this study and presents several case studies in humans.

LITERATURE REVIEW

<u>History</u>

For ages, countries such as China, Egypt, Greece and Rome have employed the use of mercury in medicine and industry. Hippocrates used it as a drug in Greece around 400 B.C. Mercurous chloride was used as a treatment for syphilis in the 16th century (Sigerest, 1996) and various forms of elemental and inorganic mercury were used in cathartic, antihelminthic and diuretic preparations to name a few, up until the 20th century (Sanfeliu et al., 2003).

Mercury has also acted as a toxin or poison: 2000 years ago in Pliny the Elder's *Naturae Historiarium Libri,* he documented mercury (II) sulfide poisoning in Spanish miners (Rackman, 1952).

Mercury poisoning has long been a historical occupational hazard; the term "mad hatter" stems from hatters who used mercury compounds to shape and finish felt hats. Oftentimes, they worked in poorly ventilated areas and breathed in the mercury compounds. Sadly, they suffered from debilitating personality changes and "hatter's shakes," a term coined to describe symptoms of mercury poisoning to include tremors, loss of coordination and memory, and slurred speech (Quinion, 2001).

<u>The Mercury Cycle</u>

Atmospheric deposition is the main source of mercury in the environment; however, other anthropogenic sources such as coal-burning power plants and metal processing plants, medical wastes and mining also contribute. Natural sources of mercury can come from geological deposits, volcanoes and volatilization from the ocean. Mercury can circulate in its gaseous form for years, traveling through the atmosphere and

becoming widely distributed. It is then photochemically oxidized to inorganic mercury where it combines with water vapors and comes back to the surface as rain (Pierce, 2005). Once in surface water, mercury enters a complex cycle and can be methylated by sulfate-reducing bacteria through metabolic processes (Mercury, 2002), turned into other forms, or vaporized and re-entered into the atmosphere.

Methylmercury can also enter the food chain (Mercury in the Environment, 2000). Fish can ingest the methylated bacteria or waste from the bacteria can be excreted into the water and absorbed by plankton which fish then eat. Fish that eat other contaminated fish also have compounded levels of mercury in their flesh. Human consumption of these fish can lead to methylmercury deposition in all body tissues, including the brain. Understanding the methylation of mercury is important because it is much more toxic than inorganic mercury and it takes much longer for the body to eliminate methylmercury (Pierce, 2005). Thus, accumulation of methylmercury in body tissues is compounded over time. Previous reports of methylmercury exposure in human populations and in experimental studies in non-human primates indicate that these overt toxicity signs may be delayed for weeks or even months after exposure (silent or latent phase) (21st International Neurotoxicology Conference, February 10-14, 2004). Mercury is sequestered in brain tissue and it can continue to cause cellular damage and death as it remains in the cells of the CNS.

Two forms of mercury can cross the blood brain barrier and accumulate in neuronal cells: mercury vapor and organic mercury (methylmercury, for example). Inorganic mercury such as mercuric chloride cannot. Rather, mercuric chloride is distributed to other organs in the body and not the central nervous system. It is gradually

metabolized by the liver and excreted. Once in the body, Amin-Zaki's (1974) results indicated that methylmercury passes readily from mother to fetus and neonatal blood mercury levels are maintained through ingestion of mercury in mothers' milk. Methylmercury crosses the placental barrier and enters the endothelial cells of the blood–brain barrier as a complex with L-cysteine. Levels in the fetal brain are about 5–7 times that in maternal blood and the brain-to-blood ratios in adult humans and other primates are approximately in the same range.

Methylmercury builds up in growing scalp hair and is proportional to concentrations in the target tissue, the brain (Cernichiari et al., 1995). Consequently, measurements of MeHg in hair samples are reflective of MeHg concentration's in the body. Although methylmercury is the predominant form of mercury during exposure, it is slowly metabolized to an inorganic, insoluble mercury form in the brain. Methylmercury is oxidized into an ionic form, which makes it more difficult to re-cross the blood brain barrier and leave the central nervous system. The complete mechanism of organic mercury transformation into inorganic mercury is unclear in the brain; however, it is known that the inorganic mercury form accumulates over time and resides for long periods in the central nervous system (Clarkson, 2002).

<u>Symptoms of High Dose Methylmercury Exposure in Adults</u>

In adults, high doses of mercury vapor cause bizarre behavior such as extremes of shyness or aggression, gingivitis and tremors (Clarkson, 1997). Tremors may also be accompanied by neuropsychological effects ranging from emotional instability at high doses to subtle performance deficits at lower doses. Most adults encounter high doses of mercury vapor in occupational settings; however, if children are exposed at high levels, a syndrome known as acrodynia, characterized by erythema of the extremities, chest and

nose, polyneuritis (inflammation of several nerves marked by paralysis, pain and muscle wasting) and gastrointestinal disorders (Davidson et al., 2004). In the same respect, methylmercury exposure in high doses has an extensive impact on the CNS. Sensory disturbances followed by visual field constriction, ataxia, cognitive decline, and death can occur in adults. Neuropathology indicates that the occipital cortex and cerebellum are most affected. In prenatal high dose MeHg exposure, first reported in Japan and Iraq, diffuse CNS damage with disruption of cellular migration occurred (Choi, 1989).

Observations of Classical High Dose Exposure

Organic mercury poisoning (methylmercury, for example) became important in the mid-20th century when several outbreaks occurred. Contaminated food became a problem (the Iraq outbreak) because organic mercury forms were used as anti-fungicides for seed and crop preservation (Sanfeliu et al., 2003). Industrial (the Minamata outbreak) and environmental exposures remained problems. Today, methylmercury is the most common and toxic form of organic mercury found as a ubiquitous environmental contaminant all over the world (Siciliano et al., 2003; Eisler, 2004). Currently, the major source for methylmercury exposure to humans comes from consumption of contaminated foods, primarily older and larger carnivorous fish at the top of the food chain such as tuna, swordfish, shark and king mackerel and those fish that are bottom dwellers.

In Japan during the 1950s, an outbreak of methylmercury poisoning occurred most notably in Minamata, located on the southwestern coast of Kyushu. Its source was traced to industrial discharges of inorganic mercury into Minamata Bay by the Chisso Hiryo Company, a large chemical factory. It took several years to determine that the mercury was being methylated by microorganisms in the water and bioconcentrated in the food

chain (Boening, 2000). Contaminated fish that swam in these areas were caught and eaten by local residents. The residents poisoned by the methylmercury in the fish began to experience what became known as Minamata disease and displayed symptoms similar to Hunter-Russell syndrome, a syndrome that previously described one of the first documented cases of organic mercury poisoning.   Symptoms included paresthesias, dysarthria, sensory deficits and speech impairments, postlabryinthine deafness, cerebellar ataxia and progressive visual field constriction (Takizawa & Kitamura, 2001).

Pregnant women who ate the contaminated fish had mild symptoms of methylmercury intoxication. However, methylmercury crossed the placental barrier and they gave birth to infants that appeared normal but had a history of delayed developmental milestones and displayed severe neurologic abnormalities including cerebral palsy, mental retardation, and seizures. Congenital Minamata disease, as it came to be called, was the first indication that the fetal brain may be much more sensitive to MeHg than the adult brain.

Some 21,000 people filed claims with Japanese officials as victims of Minamata disease. Almost 3000 were certified as having the disease. Close to 600 people died (Takizawa & Kitamura, 2001).

 In 1972 in Iraq, seed grain coated with methylmercury fungicide was used to make bread which resulted in the poisoning of 6530 people and the death of 439 (Bakir et al., 1973). Of the pregnant women who were in a limited developmental study of their children, prenatal methylmercury levels ranged from 1 to 600 ppm in hair samples collected from the mothers. At 30 months of age, the children were examined for neurologic symptoms developmental setbacks.

Adverse motor and mental impairments were seen at doses as low as 10-20 ppm in maternal hair and the results of the study suggested a dose-response curve that was associated with developmental milestones (Cox et al., 1989). In other words, the higher the exposure dose, the greater the postnatal risk of adverse neurobehavioral development. These findings were used for many years as a basis for determining the acceptable daily intake of methylmercury in food sources (WHO, 1990).

Effects of High Dose Methylmercury Exposure in the Prenatal CNS

The Minamata Bay and Iraq occurrences have given insight into the mechanism underlying prenatal brain damage and its resulting behavioral outcomes. Findings from the occurrences indicated that neuronal cell division and migration, the most basic processes in brain development, were affected. The Minamata brain samples indicated widespread damage to all areas of the fetal brain, as opposed to the focal lesions seen in adult tissue. Tissues from Iraq showed that the normally ordered parallel arrays of neuronal cells in the cortex were disrupted, which is an indication of disturbances in the developmental growth of the brain. Purkinje cells that had failed to migrate to the cerebellum were also present (Choi, 1978). Other parameters affected in the developing CNS also include postmigratory CNS cytoarchitecture, various enzyme activities and protein expression (Burbacher et al., 1990).

Outward symptoms of prenatal exposure to high doses of methylmercury produced mental retardation, cerebral palsy-like symptoms, cerebellar ataxia, dysarthria, decreased motor and eye-hand coordination, constriction of visual fields, sensory disturbances and hearing impairment in the exposed children (Watanabe and Satoh, 1996; Grandjean et al., 1998).

Prenatal Low Dose Methylmercury Exposure

Many studies, like those described previously, have shown severe deleterious effects due to high doses of methylmercury, but more recently concern has been raised with respect to the possible deleterious effects of chronic low doses of methylmercury exposure to the developing nervous system. Chronic low doses of methylmercury are the most common form of human exposure today; however, little is known about the molecular and cellular effects of prenatal exposure at environmentally relevant doses. Strong preliminary data show that chronic low dose methylmercury exposure results in consistent mercury accumulation in fetal brain tissue.

Methylmercury is recognized as a developmental neurotoxicant and the prenatal CNS is five to ten times more susceptible to methylmercury than the mature CNS (Clarkson, 2002). Mild CNS deficits, most notably language, attention and memory and to a lesser extent, visuospatial and motor function, in children have been associated with methylmercury exposure at levels that cause little to no signs of toxicity in adults. Thus, the effects of prenatal methylmercury exposure on brain function are widespread, and early impairment is detectable at exposure levels currently considered safe (Grandjean et al., 1997).

Observations of Prenatal Methylmercury Exposure

Two controversial studies done in the 1980's, the Seychelles Island study (Davidson et al., 1998; Myers et al., 2003; Myers et al., 2000) and the Faroe Islands study (Grandjean et al., 1997), examined the effects of prenatal methylmercury exposure. Both islands were ideal to conduct epidemiological studies because those communities consumed large amounts of seafood, the most common form of methylmercury exposure. Exposure levels were similar (mean: 4.0 ppm in the Faroes and 6.0 ppm in Seychelles)

and the children were examined for neurodevelopmental effects at various stages of their postnatal development.

However, the Seychelles and the Faroe Island studies also differed in several important ways. Almost daily fish consumption provided the methylmercury exposure in the Seychelles, while the Faroe Island residents were exposed by occasional pilot whale meat consumption. Pilot whales contain higher levels of mercury than fish and it is possible that a high intermittent dose of methylmercury may affect CNS development differently than daily low-dose exposure. This hypothesis has yet to be tested. While both the Seychelles and Faroe studies tested maternal hair, which reflects methylmercury exposure to the fetus over the course of the entire pregnancy, the Faroe study also used umbilical cord blood. Cord blood only reflects exposure received over the last trimester of pregnancy; earlier exposures (intermittent consumption of whale meat in the Faroe study) would not be indicated. Results from the Faroe hair samples still indicated adverse but weaker developmental outcomes.

The findings from the two studies differed. Expert groups reviewed the Faroe and Seychelles studies on several occasions and addressed the scientific merit of the studies. They concluded that both were methodologically sound and reached scientifically valid conclusions for their respective populations. They also concluded that the different results may be a reflection of the differential influences of biological factors not yet identified (Davidson et al., 2004).

The National Research Council's 2000 report titled "Toxicological Effects of Methylmercury" concluded that prenatal methylmercury exposure in the Faroe Islands caused subtle yet significant changes in neuropsychological development. Grandjean et

al. (1997), noted that memory, attention span, visual spatial perception, and language skills were affected in the first age tested (7 years old). As of 2003, neurobehavioral deficits from the second age tested (14 years old) were not examined.

However, in the most recent updated findings of the Seychelles Island study (Myers et al., 2003), several views have posited that current "safe" levels of methylmercury pose little risk of serious neurodevelopmental impairment to developing embryos. In the Seychelles, 46 endpoints were measured and only one showed adverse prenatal methylmercury effects. In boys at the oldest age tested (107 months), performance on a grooved pegboard using their nonpreferred hand showed deficits. In other words, subtle motor deficits became more pronounced with age. Language function (at 66 months) and hyperactivity (at 107 months) became more pronounced when levels of prenatal methylmercury increased.

Conclusion

Despite thousands of years of history of human exposure and intense research activity, many of methylmercury's toxic mechanisms remain unexplained. Many of the previous studies have observed the effects of moderate to high doses of methylmercury on the developing central nervous system. This review reveals key gaps in our knowledge, gaps that highlight important research needs. Studying chronic, low doses would be helpful in delineating the cellular mechanisms underlying the neurotoxic effects of environmentally relevant doses of methylmercury. Once the mechanisms through which methylmercury exposure occurs is understood, future therapies to counteract exposure and prevent CNS impairment can be developed. By exploring these understudied areas of methylmercury research, we have gained insight into the nature of methylmercury toxicity.

MATERIALS AND METHODS

<u>Animals</u>

Adult male and female C57BL/6J: +/+ wild type mice that were used for breeding were purchased from The Jackson Laboratory (Bar Harbor, MA, USA). The mice were housed at the Texas A&M Laboratory Animal Resources and Research building under a

constant temperature of 21-22° C, a humidity of 45-50% and a 12 hour light/dark cycle.

All procedures were carried out in accordance with the regulations, policies and

guidelines set forth in the Public Health Service Policy for the Care and Use of

Laboratory Animals (PHS Policy, 1996), the United States Department of Agriculture's

(USDA) Animal Welfare Regulations (Animal Welfare Act, AWA, 9CFR, 1985, 1992)

and the Association for the Assessment and Accreditation of Laboratory Animal Care

(AAALAC International). A total of 40 animals were used in this study, 15 male and 4

female treated mice, and 13 male and 8 female control mice. The mice were weaned at

29 days of age. Males and females from the same litter were separated according to

gender and lived with their same gender littermates for the duration of the experiment.

Chemicals and Dosing

Methylmercury was made by dissolving 95% pure methylmercuric chloride

(MMC) from Alfa Aesar (Ward Hill, MA, USA) in sterile deionized water. For addition

to food, the MMC was further diluted in sterile deionized water. Pregnant females were

randomly divided into control and treatment groups and fed a daily meal of 4.0 grams

moistened rodent chow containing vehicle (deionized water) for the first 5 days of

gestation. Control females continued to receive rodent chow moistened with deionized

water for the remainder of the gestational period. Treated females were given the

moistened rodent chow and methylmercury at a daily dose of 0.03 μg/Kg of the body

weight of the pregnant mouse starting on day 6 of gestation through birth.

The pups were weaned at 29 days of age and fed dry rodent chow containing no

methylmercury *ad libitum*. When the pups reached 60 days of age, behavioral tests and

cell isolations were performed. Mice had access to water at all times.

Behavioral Tests

Open Field

A VersaMax open field activity chamber (Accuscan Intstruments, Inc., Columbus, OH) capable of detecting horizontal and vertical movement was used to test for spontaneous activity. Two acrylic chambers, each measuring 42 x 42 cm and 30 cm high, were divided vertically and horizontally by acrylic partitions into four smaller square chambers. Figure 1 shows the activity chamber. Thus, a total of 8 smaller chambers, each measuring 21 x 21 cm comprised the system. The chambers were equipped with x, y and z photo beams to detect movement in all directions. Only two diagonal 21 x 21 cm compartments in each large 42 x 42 cm chamber were used to prevent overlapping of photo beams. A maximum of 4 mice could be tested at one time using both 42" chambers, with each mouse in a separate 21" chamber diagonal to one other mouse. If mice could not be run in pairs, a decoy mouse was placed in the diagonal chamber to standardize conditions. Control and prenatally treated MeHg mice were placed individually in the open field chamber for 30 minutes on days 1 and 2 of behavioral testing. Factors measured over the two consecutive days included distance traveled (in cm), number of rearing movements, and center time (seconds).
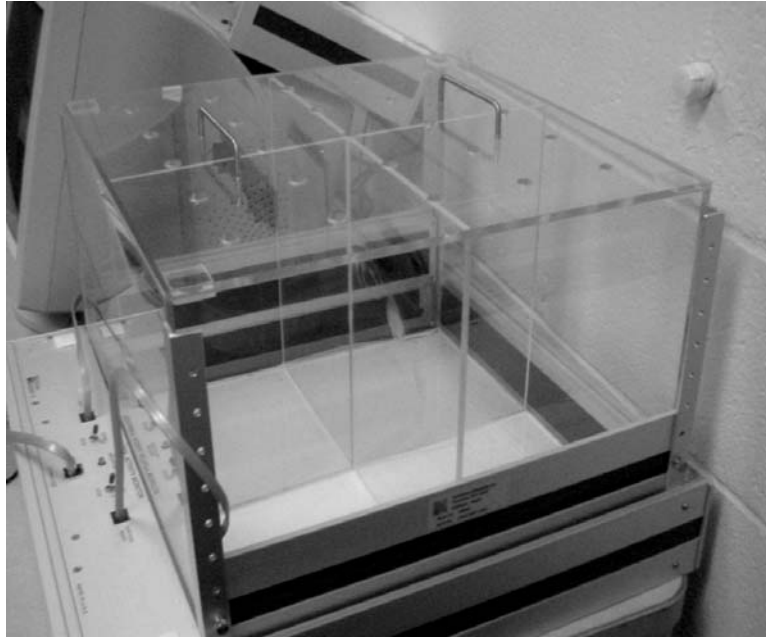
Figure 1. Open Field Activity Chamber

Rota-rod

The motor coordination of control and prenatally treated MeHg mice was quantitatively measured using a standard mouse accelerating rota-rod (model 7650, UGO Basile, Comerio, VA, Italy). The rota-rod was 3 cm in diameter and 30 cm long and was covered in clear plastic tubing to provide a smoother surface for treading. Two circular plastic disks were placed at the ends of the rod and 4 disks equally spaced were placed along the length of the rod. This created 5 compartments so 5 mice could be tested at once. The disks prevented escape and served as a barrier between mice. The rota-rod was anchored 16 cm above a platform of 5 levers, one lever per compartment. See Figure 2.

Mice were placed on the rota-rod perpendicular to the long axis of the rod, with their heads facing away from the experimenter. As soon as a mouse was placed on the rod, the experimenter lifted the lever and a timer started. The rota-rod could gradually

accelerate from 4 rpms to 40 rpms over the course of five minutes. When a mouse could no longer keep pace with the rod it fell off, thus depressing the lever and stopping the time. Four trials were run on each of days 3, 4, and 5 of behavioral testing for each mouse with two minutes of resting time in between trials. Each trial was recorded (in seconds), however, the first three trials of each day were training trials and only the amount of time the mouse stayed on the rod on the fourth trial of each day was analyzed.

Figure 2. Rota-rod

Vertical Pole

A PVC pipe wrapped with cloth tape to provide grip was used to assess coordination as illustrated in Figure 3. The pipe, 1 inch in diameter and 60 cm long, was held 50 cm above a soft "egg crate" foam cushion. Each mouse was placed on the pipe at the center when it was in a horizontal position and the experimenter held both ends of the pipe. A timer was started and the experimenter raised one end of the pipe gradually over 10 seconds to a 45 degree angle. Over the next 5 seconds, the experimenter raised the pipe quickly from a 45 degree angle to a 90 degree vertical position. Latency to fall from

0-45 and 45-90 degrees was recorded. The vertical pole test was performed on day 6 of behavioral testing.

Figure 3. Vertical Pole Apparatus

Footprint Analysis

Coordination was assessed using footprint analysis. A strip of paper was placed on the floor of a plastic-lined walkway, 100 cm long, 6 cm high, with 15 cm walls and walled off at one end. The walled off end of the walkway was covered with cardboard to provide a dark area mice would be attracted to. The walkway is shown in Figure 4. Hindpaws were dipped in non-toxic paint and the mice then walked down the strip of paper towards the dark end of the walkway. For each mouse, five or more consecutive strides were averaged. If more than one trial had to be performed on a mouse, all the trials were averaged together. Parameters measured included stride length (in mm), distance between the two hind feet (base stance, in mm), and angle of foot placement (in degrees). The footprint analysis was done on day 6 of behavioral testing immediately following the vertical pole test.
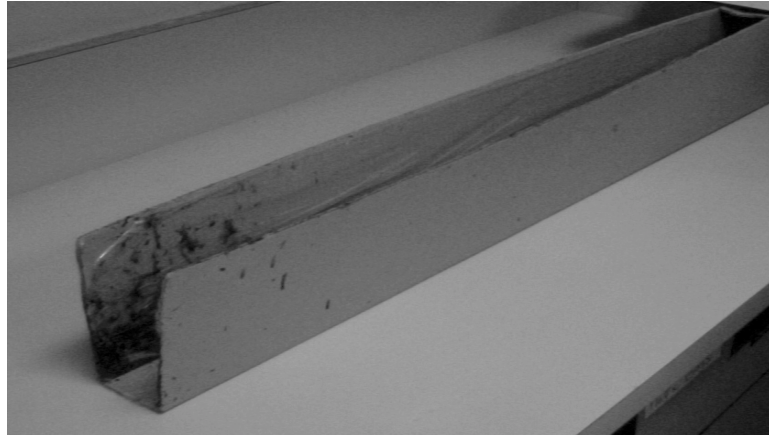
Figure 4. Walkway for Footprint Analysis

Morris Water Maze

The Morris water maze was the most stressful of the behavioral tests in this experiment and so was the last test done to eliminate any confounding effects of stress on the other behavioral tests performed. The water maze assessed learning and memory over the course of five consecutive days-days 7, 8, 9, 10 and 11 of behavior. Figure 5 illustrates the test setup. The test consisted of a circular pool of room temperature water, approximately 100 cm in diameter and filled to a depth of 50 cm. The pool was divided into 4 equal quadrants and labeled N (North), S (South), E (East) and W (west), respectively. In one quadrant, a 5 cm x 5 cm clear platform was submerged 1 cm under the surface of the water so that the mice could not see it. The platform was also far enough away from the edge so that the mice would not find it just by simply circling the pool. A video camera recorder was anchored to the ceiling directly above the circular pool to record the swimming pattern of each mouse. The trajectories and parameters were recorded by a video-tracking system (Smart Program version 2.5, Panlab, Barcelona, Spain). The room light was turned off and two 100W halogen lamps facing two different walls were turned on to indirectly illuminate the surface of the water. Two

experimenters standing around the edge of the pool and objects on the walls of the room

served as visual cues. The mice were to use the visual cues to learn the position of the
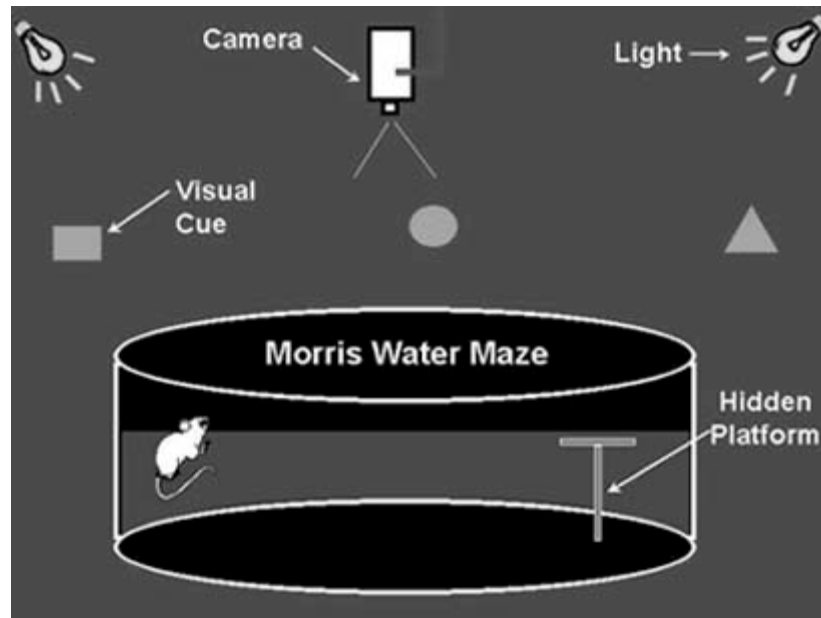
submerged platform.

Figure 5. Schematic Diagram of Morris Water Maze.
www.mcg.edu/Core/Labs/sabc/Morriswatermaze.htm

On days 1-4, each mouse was placed by the tail and facing the pool perimeter into

the water. Four trials were run each day at each of the 4 quadrants labeled N (North), S

(South), E (East) and W (west). The mouse had a maximum time of 60 seconds to reach

the platform. If it reached the platform before the 60 seconds passed, it was removed

from the platform and returned to its cage; if it did not find the platform within the 60

seconds, it was guided by the tail to the platform and allowed to sit for 3 seconds. A one

minute resting time was allotted in between trials. On each of the subsequent trial days,

the original starting direction was moved clockwise. If a mouse performed the Morris

water maze starting at N, then proceeding to E, S and W on day 1, day 2 would start at E,

then S, W, and N. Examples of swim patterns are illustrated in Figure 6. On day 5,

termed the "probe" trial, the submerged platform was removed. Starting at the W (West)

quadrant, each mouse had only one 60 second trial.

The parameters measured in Morris water maze were distance (in cm), time to

reach the platform on days 1-4 (in seconds), and percent of time spent in each quadrant

on

day 5. A summary of behavioral tests conducted can be seen in Table 1.
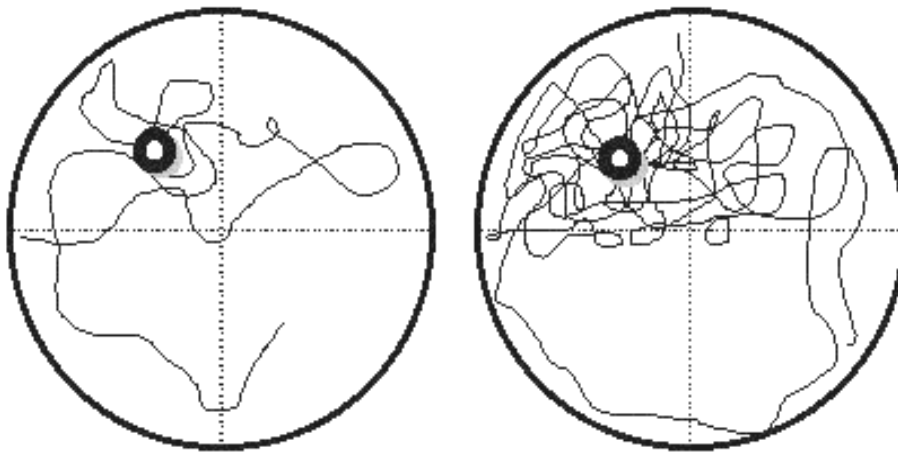

Figure 6. Examples of Swim Patterns. Picture 2 is a probe trial
and the concentrated swim pattern in the platform quadrant
indicates learning has occurred.
www.med.unc.edu/alcohol/cenline/12_3_1.htm

Test	Behavior Assessed
Open Field	activity
Rota-rod	coordination
Vertical Pole	coordination
Footprint Analysis	coordination
Morris Water Maze	learning and memory

Table 1. Summary of Behavioral Tests

Acute Isolation of Cerebellar Granule Cells

Brain Extraction

Mice were placed in an enclosed chamber containing 2-mL isoflurane. The genotype birthdate, sex, and weight of each mouse were recorded. The head and neck were rinsed with 70% ethanol and the anesthetized mouse decapitated with 7-inch curved Mayo scissors. The head was placed on a sterile paper towel and the body in a plastic bag for disposal. Fine-angled micro dissecting scissors were used to cut the skin from the neck to the snout and the skin was pulled back and separated to expose the skull. A #11 sterile surgical blade was used to score the midline and remove the skull flaps. The brain was detached from the rostral side and the cerebellum separated from the rest of the brain. The meninges were then removed from the cerebellum using #55 Dumont forceps. The cerebellum was sliced into 5-6 pieces using a sterile surgical blade and the pieces placed in a sterile 50-mL falcon tube containing 10-mL of filtered minimal essential media (MEM; Sigma, St. Louis, MO).

Dissociating Cerebellar Granule Cells

10-mL of filtered dissociation media (DM) containing protease was added to a 50-mL beaker with a stir bar and placed on a magnetic stirrer. The 10-mL MEM from the 50-mL falcon tube containing the cerebellar pieces was carefully poured into a waste beaker without pouring out any pieces and 2-mL filtered (DM) containing protease was pipetted into the tube. The 2-mL tube containing the cerebellar pieces was swirled to unsettle the pieces at the bottom of the tube; all pieces and the 2-mL DM were poured into the 50-mL beaker containing 10-mL DM on the stirrer. The cerebellar pieces and DM mixture was stirred for 10 minutes at low speed (#3-4 or 60 rpm). The speed was

adjusted accordingly depending on the type of stir plate used and by visual judgment. If the media began to turn cloudy, the speed was reduced.

While the mixture was stirring, 10 15-mL falcon tubes were placed in two rows on ice, 5 in each row. 5-mL of filtered MEM was pipetted into each tube. One aliquot of DNAse I was then removed from a -20° C freezer and thawed. After the cerebellar pieces and DM mixture had been stirred for 10 minutes, it was removed from the stir plate and placed at a 45° angle for 2 minutes to allow the nondissociated tissue to collect at the bottom of the beaker. A 10-mL Sigma coated serological glass pipette was then used to aspirate up to 10-mL of dissociated cells in the clear portion of the fluid. The fluid was then divided equally into the first 15-mL falcon tube containing MEM in each row of the ice bucket (extraction 1).

10-mL of DM was added again to the 50-mL beaker. The thawed aliquot (75-80 μL) of DNAse I was also added to the beaker using a micro-pipetter and allowed to stir on the stir plate for 10 minutes. In the meantime, a 30 μL working solution of tetramethyl rhodamine methyl ester (TMRM) with a concentration of 30 μM was prepared. See Table 2 for the preparation of TMRM. TMRM accumulates in mitochondria in proportion to mitochondrial membrane potential and is light sensitive. Thus, it was loaded into an aliquot tube wrapped in foil. The room lights were also turned off. The foil wrapped aliquot tube containing the TMRM solution was placed in the ice bucket with the 10 15-mL falcon tubes. After the 10 minutes passed, the beaker sat at a 45° angle for 2 minutes and the clear fluid was aspirated and divided equally into the second pair of 15-ml falcon tubes containing MEM(extraction 2).

Ingredient	Concentration	Amount
DMSO (dimethyl sulfoxide)	1 mM	66.6 µL
TMRM (tetramethyl rhodamine methyl ester)	1 mM	2 µL

Table 2. Preparation of TMRM Solution

10-mL of DM was added again to the 50-mL beaker and stirred for 10 minutes. The speed of the stir plate was gradually reduced with each extraction to prevent the cerebellum pieces from tumbling violently into each other and causing excessive cloudiness. However, the speed was still fast enough to allow cells to be dissociated from the cerebellar sections; the pieces were still able to circle in the beaker smoothly and were not stagnating in the corners. After the 10 minutes passed, the beaker sat at a 45° angle for 2 minutes and the clear fluid was aspirated and divided equally into the third pair of 15-ml falcon tubes containing MEM (extraction 3).

10-mL of DM was added again to the 50-mL beaker and stirred for 10 minutes. After the 10 minutes passed, the beaker sat at a 45° angle for 2 minutes and the clear fluid was aspirated and divided equally into the fourth pair of 15-ml falcon tubes containing MEM (extraction 4).

10-mL of DM was added again to the 50-mL beaker and stirred for 10 minutes. After the 10 minutes passed, the beaker sat at a 45° angle for 2 minutes and the clear fluid was aspirated and divided equally into the fifth pair of 15-ml falcon tubes containing MEM (extraction 5).

Five total extractions were performed because after that point, usually only fibrous tissue remained in the 50-mL beaker. The dissociated cells and medium from the 15-mL falcon tubes were then pooled into two 50-mL falcon tubes, one 50-mL tube for five 15-mL tubes.

Pelleting Cerebellar Granule Cells

The two 50-mL falcon tubes were then centrifuged for 10 minutes in a 4° C swinging bucket rotor to pellet the suspended cells. The centrifuge brake was set at high and the speed was set at five so a speed of 1700 rpm could be achieved. After the tubes were centrifuged, the media was poured into a waste beaker and the pelleted cells remained stuck to the bottom of the tubes. The pelleted cells at the bottom of the empty 50-mL falcon tubes were resuspended in 3-mL of MEM per tube. Gentle pipetting with a Sigma coated serological glass pipette helped to resuspend the cells.

Mitochondrial Membrane Potential Measurement

Both falcon tubes containing 3-mL each of the pooled cell suspension were poured into a Petri dish containing two coverslips. The day prior to cell isolation, coverslips were prepared. Two coverslips were used per mouse, one served as an extra. The coverslips soaked in glycine for 10 minutes and were then transferred into poly-D-lysine overnight. On the day of cell isolation, the coverslips were removed from the poly-D-lysine and were dried under a UV light for 30 minutes. The dried coverslips were then transferred to an autoclaved Petri dish. Thus, a total of 6-mL of pooled cell suspension was poured into the Petri dish over the two coverslips. The Petri dish was incubated at 37° C with 5% CO_2 for 25 minutes. The foil wrapped TMRM was allowed to thaw during the incubation time. After 25 minutes, the Petri dish was removed from

the incubator and the room lights turned off. The 30 μL, 30 μM working solution of

TMRM was then added to the 6-mL of media in the Petri dish to give a 150nM final

concentration. The Petri dish was swirled to evenly distribute the dye and placed back in

the incubator at the same temperature for 15 minutes. PFM imaging media was also

placed in the incubator to allow it to reach the same temperature as the coverslips in the

Petri dish.

During the second incubation, an Olympus IX70 inverted fluorescence

microscope (Olympus America Inc., Melville, NY) connected to Simple PCI software

(Compix Inc., Sewickley, PA) was set up. The hardware and software specifications are

listed in Tables 1.3 and 1.4.

Hardware	
Filter	TRIDC
Objective	40x oil immersion
Condenser	No. 4
Polarizer	Out
Analyzer	In

Table 3. Microscope Specifications

Software	
Exposure	D FREM RED=0.06 DIC=0.04
Contrast Enhance Grain	D FREM RED=1 DIC=1
Device Setup/Capture Resolution	672 x 512
Two Color Image	16 Bit at 2 Binning

Table 4. Simple PCI Specifications

The rest of the procedure was performed in a minimal amount of light due to the light sensitive TMRM dye. The cerebellar granule cells infused with dye were removed from the incubator after 15 minutes. A coverslip was removed from the dish, inverted and loaded onto a custom made stage cassette. The cassette was then flipped over so the cells on the coverslip were exposed at the bottom of the cassette well. The cassette well was loaded with 1-mL of PFM imaging media and the cassette placed on the microscope stage. The microscope was focused and cerebellar granule cell pictures were taken by pressing the capture 1 button. All components of the pictures were saved if any granule cells had fluorescing mitochondria. At least 30 cells per mouse were saved and the images analyzed with SimplePCI software. The fluorescent intensity of each cell was measured if it fell within the threshold region minimum of 11,000 or maximum of 30,000.

Statistical Analysis

The data was analyzed using a two way ANOVA at $\alpha=0.05$, using Microsoft Office Excel 2003. Significant differences among treatment and control groups were interpreted by the Bonferroni correction post hoc test at $\alpha=0.05$. A Chi-Square test was performed to analyze the vertical pole test data.

RESULTS AND DISCUSSION

<u>Open Field</u>

The open field is a test of activity. Only activity during the second day of open field was analyzed. Three measures of activity were assessed in this test: distance traveled (in cm), number of rearing movements, and time spent in the center of the chamber (in seconds). For each measure of activity the first 5 minutes, second 5 minutes, and total 30 minutes were examined. Looking at the first 5 minutes is important in assessing animal behavior because it measures an animal's activity when it is first introduced to a new and novel situation. Factors such as fear and unwillingness to explore a new environment are a few factors that could decrease activity in the first five minutes. A five minute open field session is a sufficient preliminary assessment of motor activity and for the evaluation of gross abnormalities in locomotion (Crawley, 1999).

Rearing Movement

In the 1st five minutes of activity, treated females tended to have less rearing movement than both the treated males and the controls. Anxiety or fear to explore a novel situation could be due to the effects of estrus and the stage of the estrous cycle the treated females were in. MeHg treatment could also have affected females more than males when initially exploring a new environment. During the 2nd five minutes, treated females began to explore the activity chamber more and the number of rearing movements they displayed was not significantly different from the treated males. However, as a whole, the treated group had significantly fewer rearing movements than the control group. During the second five minutes, mice are still in a relatively new situation and MeHg seems to affect new exploration. By the end of the 30 minute session

when the activity chamber was no longer a new environment, treated mice seemed to overcome their anxiety and the number of rearings did not differ significantly from the controls. Figures 7, 8 and 9 show the number of rearing movements during the 1st five, 2nd five, and total 30 minutes, respectively.
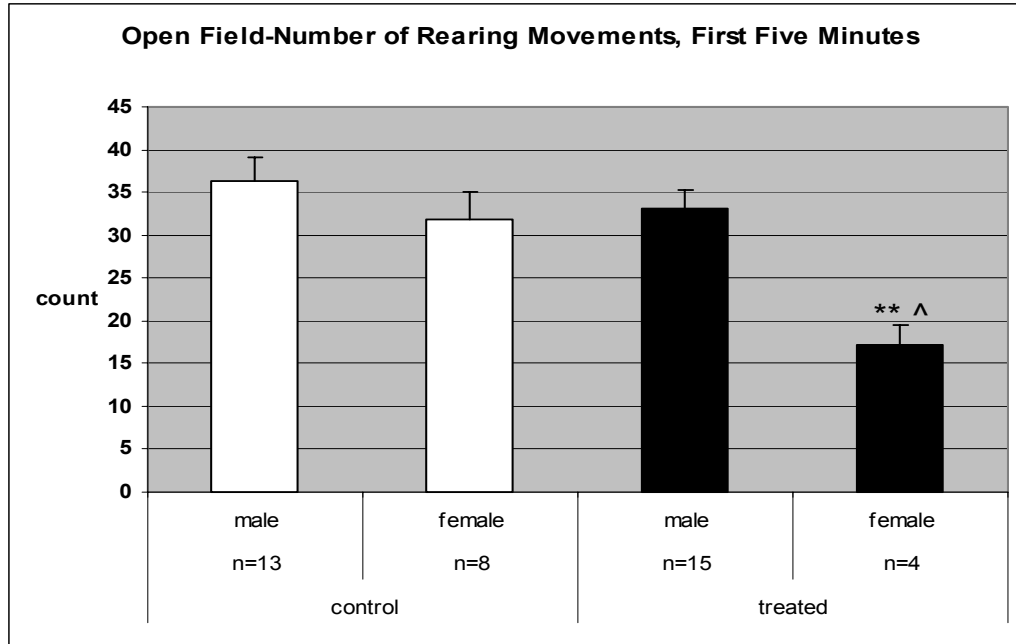

Figure 7. Rearing Movement in the First Five Minutes of Open Field. Graph shows average number of rearing movements in control males (36.38; n=13), control females (31.88;n=8), prenatally treated MeHg males (33.07; n=15) and prenatally treated MeHg females (17.25; n=4). Single-factor and two-way ANOVA indicated no significant differences between gender in the control group or between treated males and the control group. Treated females differed significantly (p=0.002) from treated males (**) and also differed (p=0.001) from the control group (^). α was 0.05.
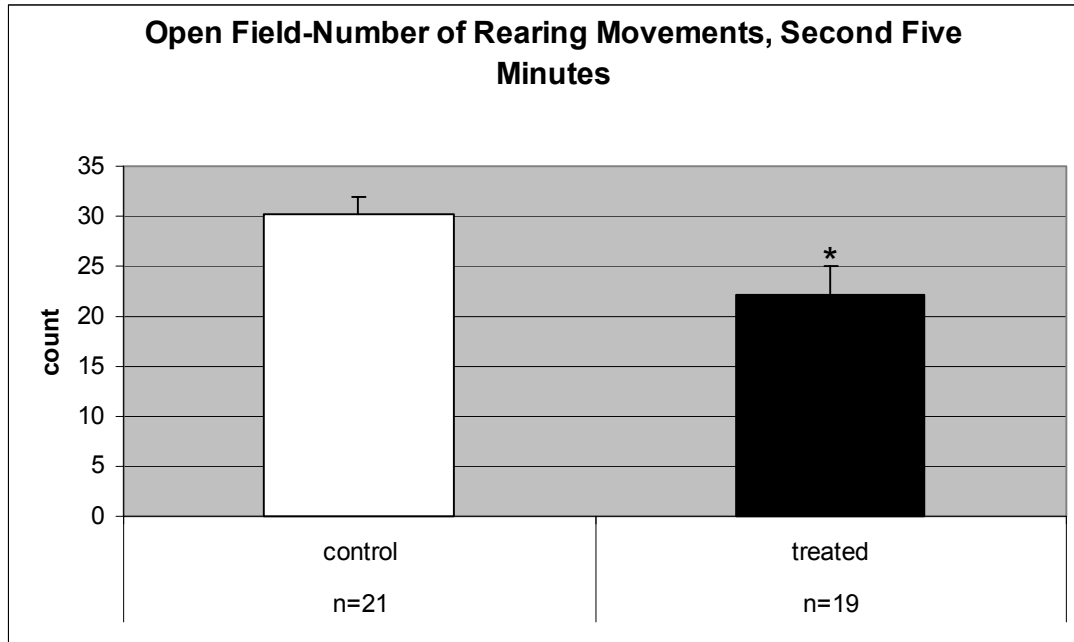
Figure 8. Rearing Movement in the Second Five Minutes of Open Field. Graph shows average number of rearing movements in the control group (30.19; n=21) and treated group (22.16; n=19). Single-factor ANOVA indicated no significant differences between gender in both the control and treated group. The treated mice differed significantly (p=0.01) from the controls (*). α was 0.05.
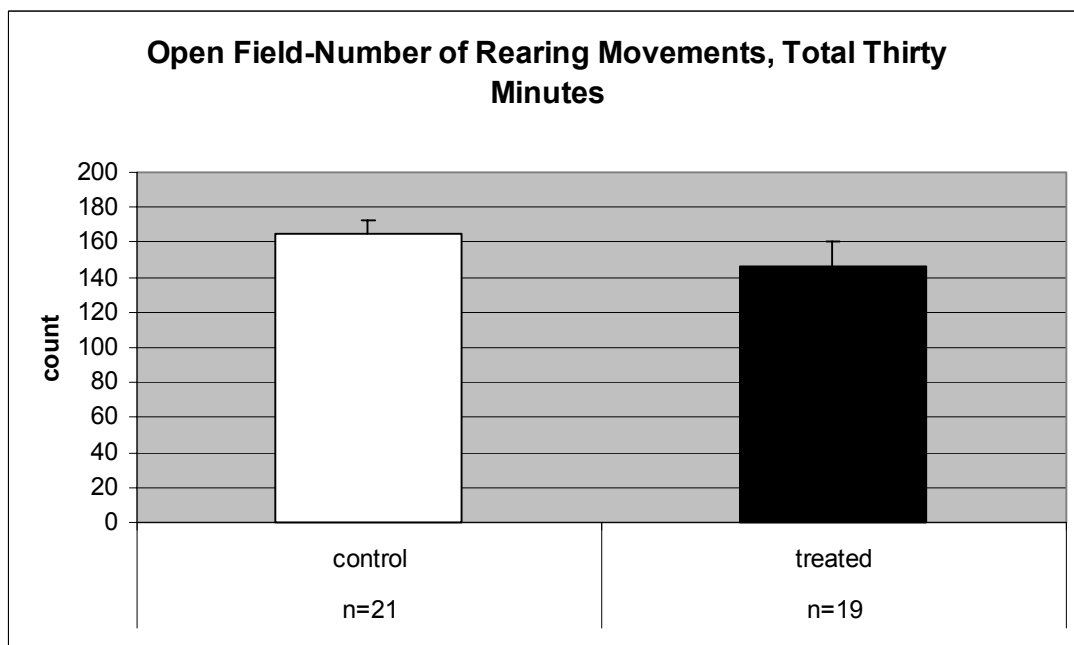

Figure 9. Rearing Movement During Total 30 Minutes. Graph shows average number of rearing movements in the control group (164.86; n=21) and treated group (146.63; n=19).

Two-way ANOVA indicated no significant differences between gender or treatment group in the number of rearing movements seen. α was 0.05.

Center Time

Time spent in the center of an area is also an indicator of fear. It is thought that if an animal spends its time in the middle of an area, it is unwilling to move outward beyond what it sees and knows. Others believe that if an animal spends its time in the periphery of an area, it is also frightened. It is thought that the animal may be looking for a way out when it actively spends its time there. Figure 10 shows the amount of time spent in the center of the open field chamber for all measurements of time. There were no gender differences within control and treated groups. There were no differences in the amount of time spent in the center between control and treated groups, indicating that both treatment groups were aware of the bounds of the chamber and could see the surrounding area. Therefore, both groups were willing to explore without fear.
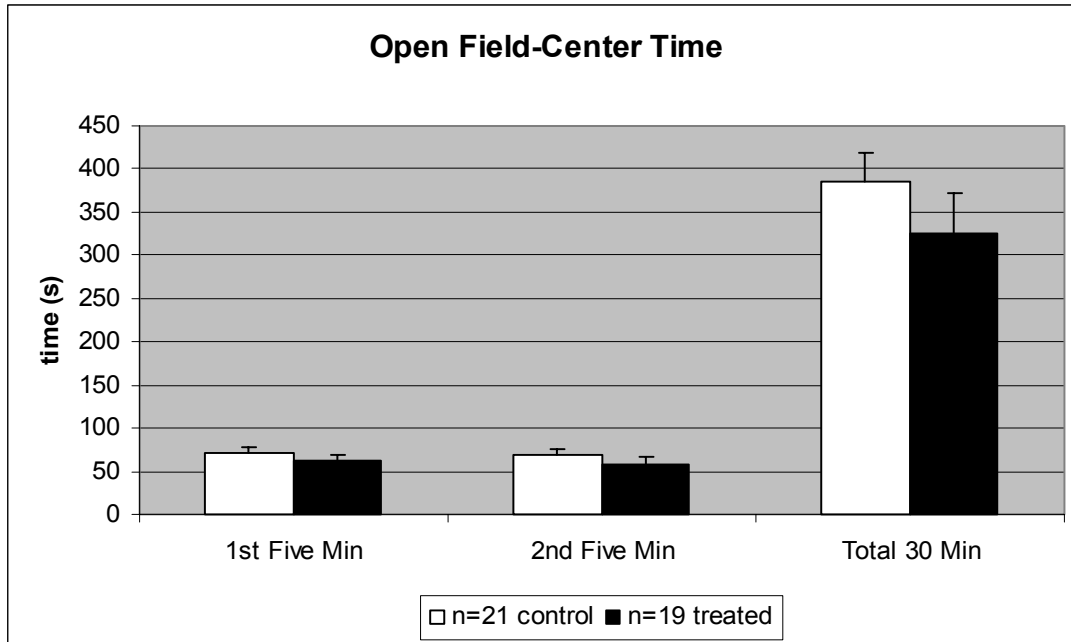
Figure 10. Center Time During 1st Five, 2nd Five, and Total 30 Minutes. Graph shows 1st five minute control (71.40; n=21) and treated (62.71; n=19), 2nd five minute control (69.47; n=21) and treated (57.14; n=19) and total 30 minute control (384.40; n=21) and treated (324.52; n=19). Two-way ANOVA indicated no significant differences between gender or treatment and time spent in the center. α was 0.05.

Distance in the Open Field

In the 1st five minutes of activity, treated females differed from treated males, just as in the 1st five minutes of rearing movements. Thus, treated females were indeed less active and less willing to explore in a novel situation. During the 2nd five minutes treated females became more active and did not differ from the treated males. However, as a whole the treated group was less active than the controls, also like the 2nd five minutes of rearing movements. Accordingly, methylmercury seems to decrease activity and thus, exploration, in new environments. Because the treated mice did not spend more time in the center than control mice, as would be expected if they were afraid and unwilling to explore, it can be concluded that they remained relatively inactive in the periphery of the chamber for the first 10 minutes. The treated mice were not active

around the corners of the activity chamber so they were not looking for a way out; rather they were unwilling to examine their surroundings initially. Total distance in the last 30 minutes did not differ between treatment groups. This indicates that the treated mice gradually became more active as the chamber became less novel. Figures 11, 12 and 13 show distance traveled during the 1st five, 2nd five, and total 30 minutes, respectively.
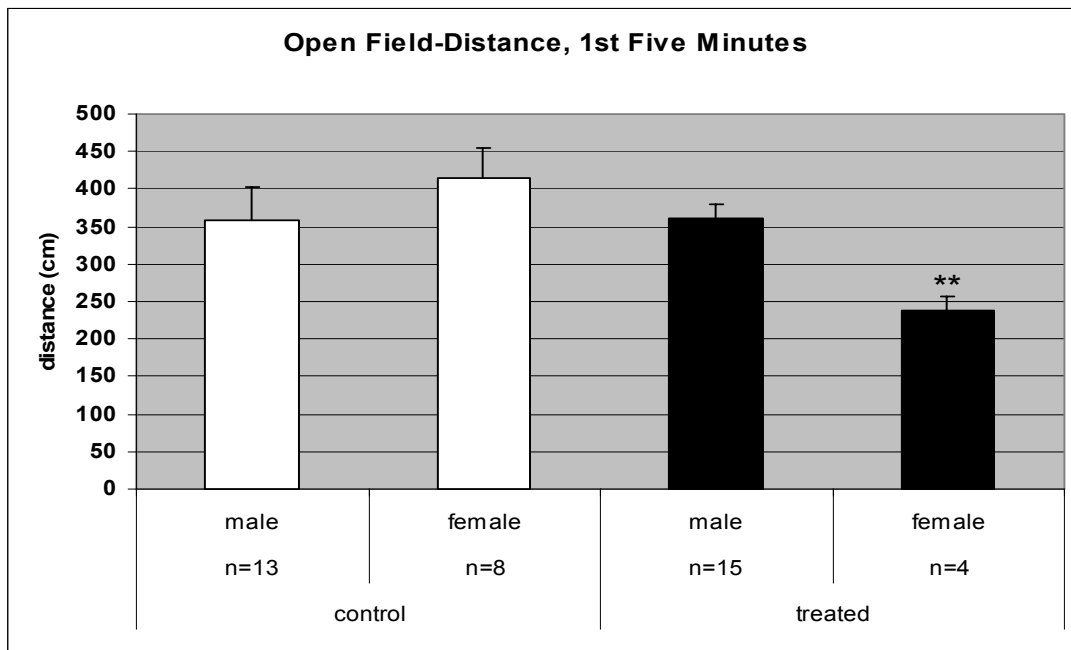
Open Field-Distance, 1st Five Minutes

male	female	male	female
n=13	n=8	n=15	n=4
control		treated	

Figure 11. Distance in the First Five Minutes of Open Field. Graph shows average distance in control males (358.46; n=13), control females (414.13;n=8), prenatally treated MeHg males (359.8; n=15) and prenatally treated MeHg females (239; n=4). Single-factor and two-way ANOVA indicated no significant differences between gender in the control group or between treated males and the control group. Treated females differed significantly (p=0.01) from treated males (**). α was 0.05.
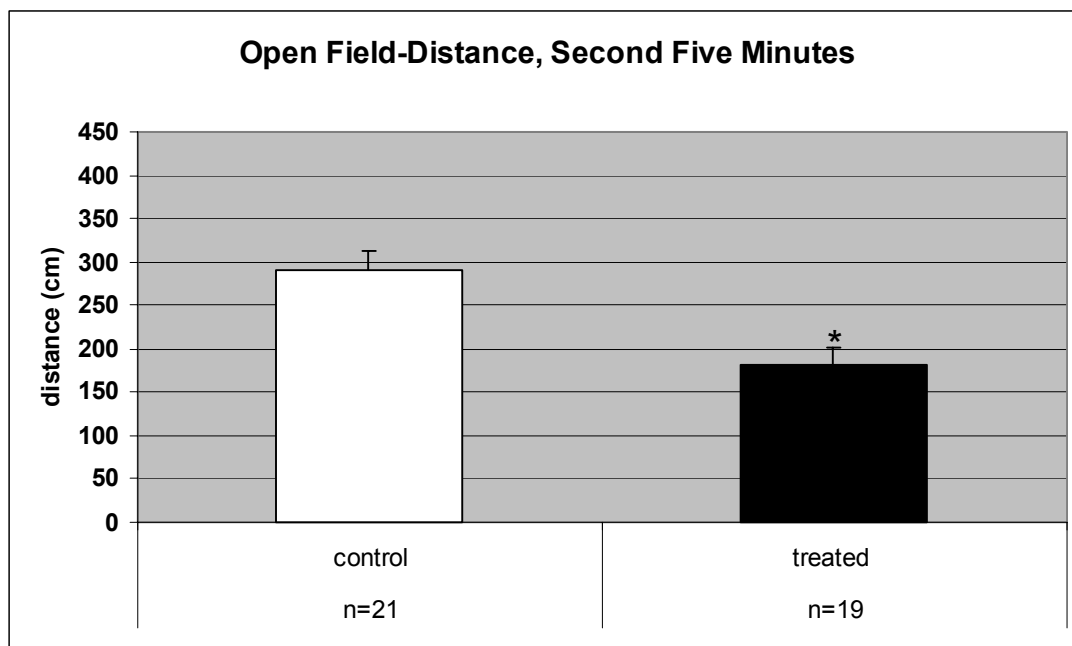
Figure 12. Distance in the Second Five Minutes of Open Field. Graph shows average distance in the control group (290.24; n=21) and treated group (182.53; n=19). Single-factor ANOVA indicated no significant differences between gender in both the control and treated group. The treated mice differed significantly (p=0.0006) from the controls (*). α was 0.05.
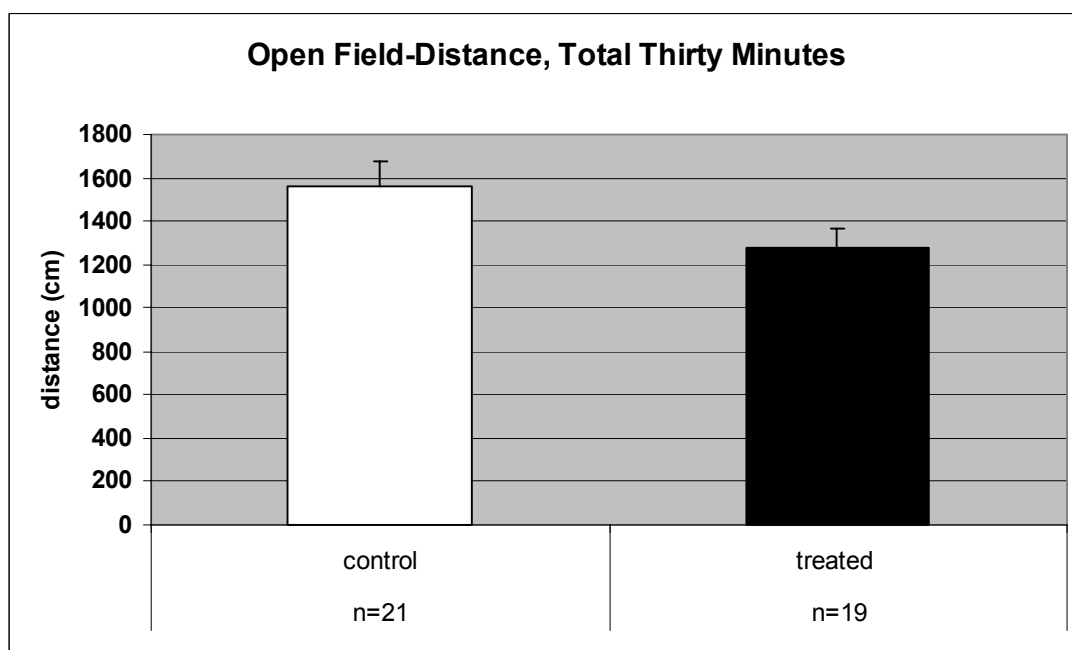

Figure 13. Distance During Total 30 Minutes. Graph shows average distance in the control group (1563.76; n=21) and treated group (1273.74; n=19). Two-way ANOVA

indicated no significant differences between gender or treatment and distance traveled. α was 0.05.

Rota-rod

The rota-rod is a test of coordination. Normal mice learn to keep pace with the rota-rod as it speeds up and can delay falling usually for 3 minutes or more (Homanics et al., 1999), while mice with motor impairments do not learn as well and fall off sooner than normal mice. Figure 14 shows the results of the rota-rod on all 3 days of testing. Treated mice displayed no differences in the amount of time spent on the rota-rod on days 1 and 2. On day 3, treated mice were significantly different from controls in the time in took for them to fall off. This could be because the coordination of the control mice between days 2 and 3 improved much more than the treated mice during that same time. Thus, the time the controls stayed on was much longer. The control mice learned to stay on the rota-rod and had better coordination as the testing days progressed. The treated mice also learned to keep pace; however, they did not learn as well. Methylmercury seems to affect learning and coordination over the course of an extended training period and is illustrated by the decreased amount of time (compared to control mice) spent by the treated mice on the rota-rod.
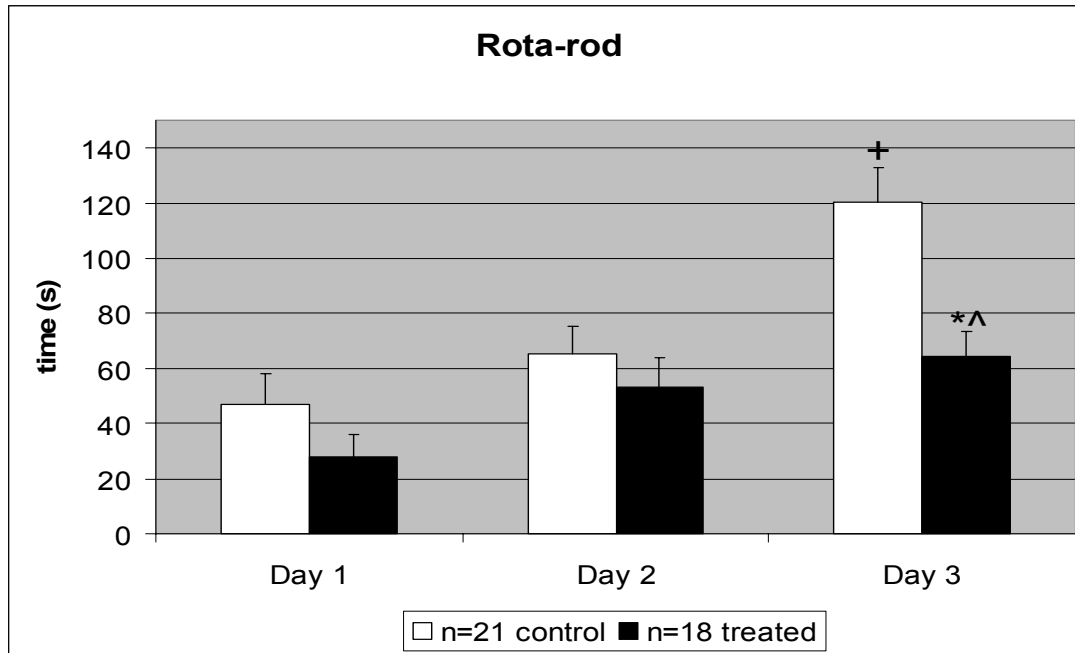
Figure 14. Rota-rod on all 3 Days. Graph shows average time spent on the rod on day 1 in control (46.76; n=21) and treated mice (27.94; n=19), on day 2 in control (65.24; n=21) and treated mice (53.22; n=19) and on day 3 in control (120.24; n=21) and treated mice (64.39; n=19). Two-way ANOVA indicated no significant differences between gender or treatment and coordination on days 1 and 2. Single-factor ANOVA indicated a significant difference (p=0.001) between control and treated mice on day 3 (*). A Bonferroni post hoc test indicated learning occurred (p=0.0003) on days 1 through 3 in control mice (+). Treated mice (p=0.02) also learned (^). α was 0.05.

<u>Vertical Pole</u>

The vertical pole is also a test of coordination. Motor impaired mice are expected to have problems staying on a surface as the incline progressively increases. This is manifested as the mice lose their grip and fall off. Table 5 summarizes the results. A chi-square test indicated no differences in gender or treatment (control or treated) and if they fell off between 0 and 45 degrees or 45 and 90 degrees. Methylmercury treated mice appeared to have no motor deficits in coordination compared to control mice while in a vertical, upright position.

Treatment	Did Not Fall	Fell 0-45	Fell 45-90	Total
Control				
Male	12	0	1	13
Female	7	0	1	8
MeHg				
Male	10	2	3	15
Female	4	0	0	4
Total				40

Table 5. Vertical Pole Results

Footprint Analysis

The footprint analysis test was the last test done to measure coordination. Control mice had a larger foot angle than the treated mice. This could indicate that control mice aren't as stressed during the test and walked normally down the strip of paper. Treated mice were more agitated and tended to jump and move faster down the walkway towards the dark end. As a result, foot placement may not be normal and a decreased foot angle could indicate faster and unnatural movement. The distance between feet, or base stance was not affected by MeHg as both control and treated mice had very similar stance positions. Treated males did display differences in stride length when compared to both the treated females and the control group; the treated males had longer strides. Thus, they could have been more anxious and so moved faster resulting in longer strides down the walkway.

Figures 15, 16 and 17 show the results from foot angle, base stance and stride length measurements.
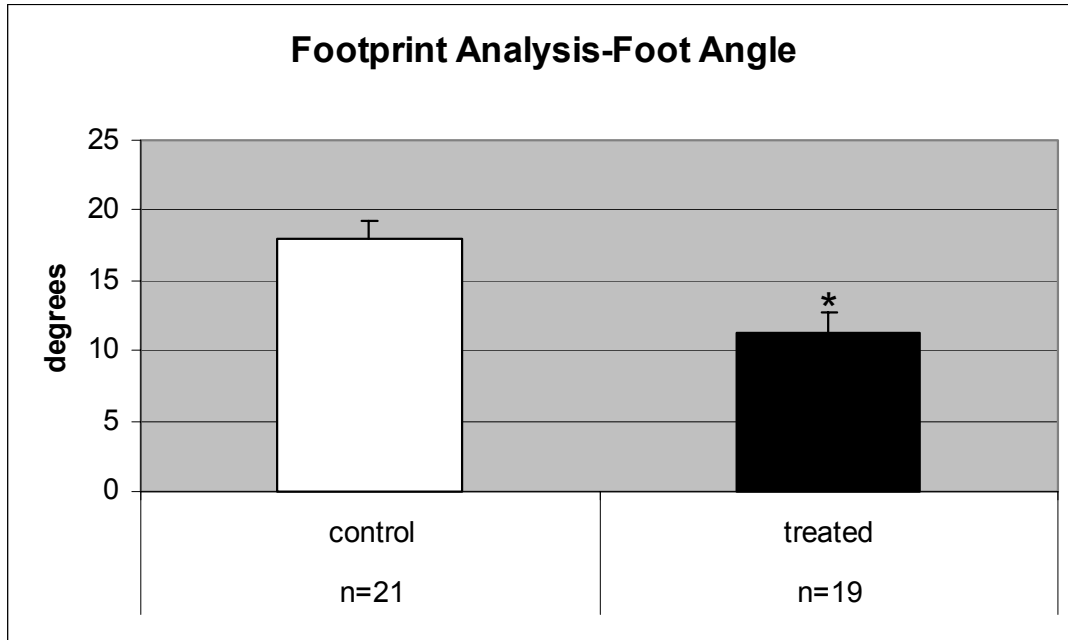

Figure 15. Foot Angles. Graph shows combined right and left foot angles in control (18.07; n=21) and treated mice (11.32; n=19). Single-factor ANOVA indicated no significant differences between gender and foot angle in both control and treated mice. A significant difference in foot angle (p=0.0003) was observed between control and treated groups (*). α was 0.05.
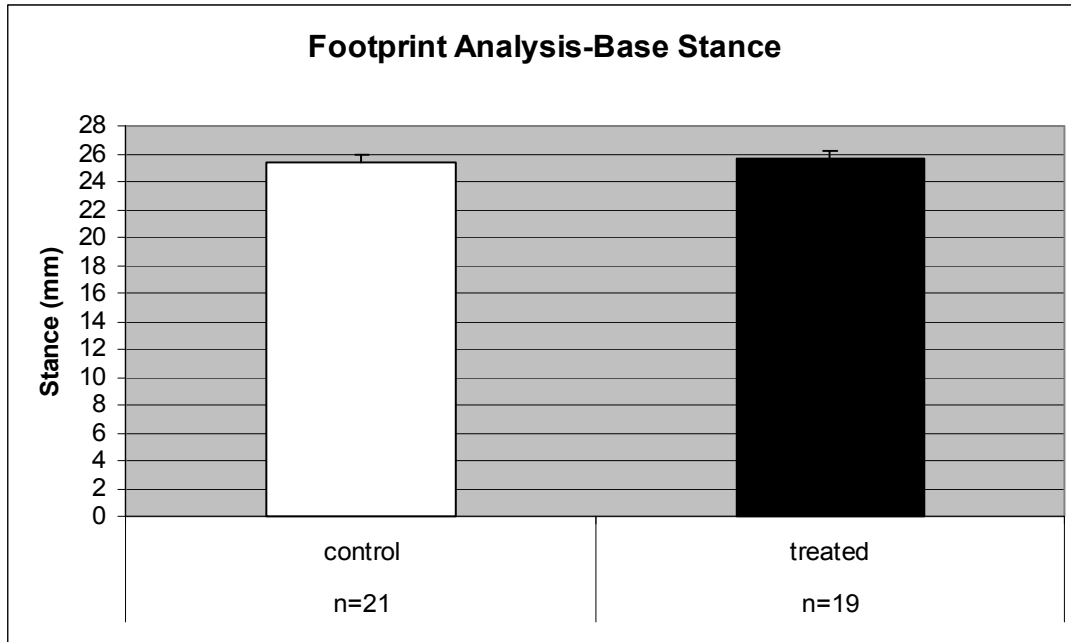
Figure 16. Base Stance. Graph shows base stance in control (25.33; n=21) and treated mice (25.64; n=19). Two-way ANOVA indicated no significant differences between gender or treatment and base stance. α was 0.05.
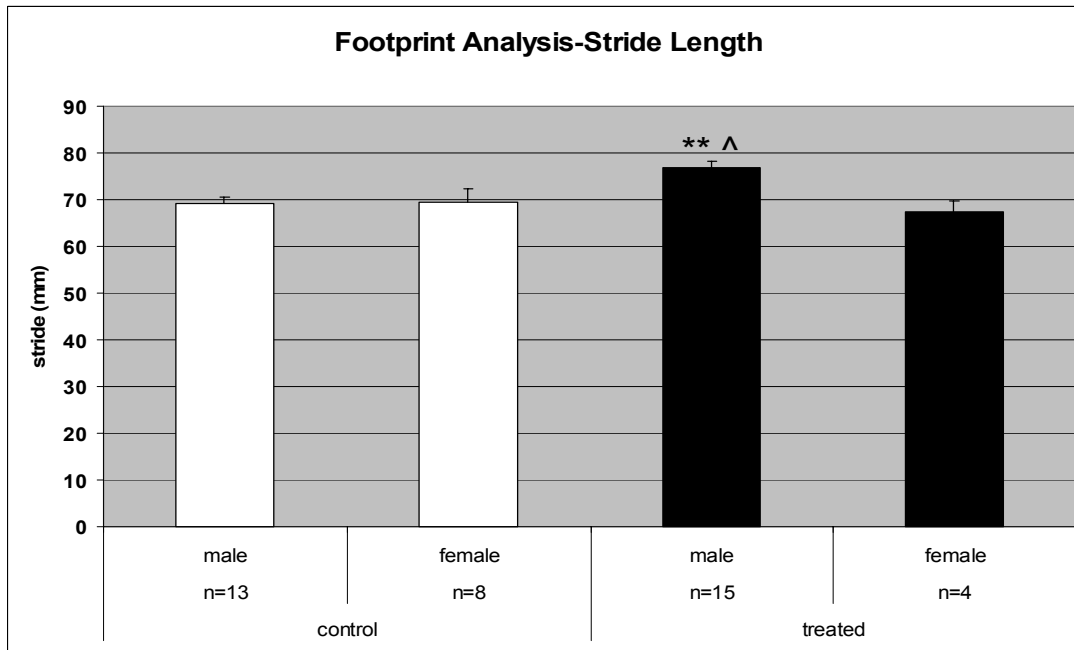

Figure 17. Stride Length. Graph shows combined left and right stride lengths in control males (69.11; n=13), control females (69.53; n=8), treated males (76.85; n=15) and treated females (67.32; n=4). Single-factor ANOVA indicated no significant differences between gender and stride length in controls. There was a significant

difference (p=0.002) between treated males and females (**). There was also a significant difference (p=0.0002) between treated males and the control group in stride length (^). α was 0.05.

Morris Water Maze Analysis

The Morris water maze was used to assess learning and memory. The test required mice to use visual cues in a room to learn the position of a submerged escape platform in a pool of water. The mice would swim 4 trials a day for a maximum of 60 seconds from 4 different quadrants, Q1, Q2, Q3 and Q4 labeled with the cardinal compass directions: N, S, E and W. Quadrant 5 was the platform itself. It was observed that some mice would traverse the periphery of the pool very quickly, looking for a way out. Other mice would just float, neither looking for a way out or for the platform.

Days 1-4

The submerged platform was placed in the pool on trial days 1-4. On all 4 days, treated mice swam a greater distance than the controls. This is because it took the treated mice longer to find the platform. However, significant differences were only observed on days 1 and 2. Thus, MeHg appears to impair learning so that it takes treated mice longer to find and use the platform as a source of refuge when initially learning a new task using visual cues. The treated mice swam more because they did not use the visual cues, two experimenters standing in the room, to guide them to the platform in a shorter amount of time. The results of distance on days 1-4 are shown in Figure 18.
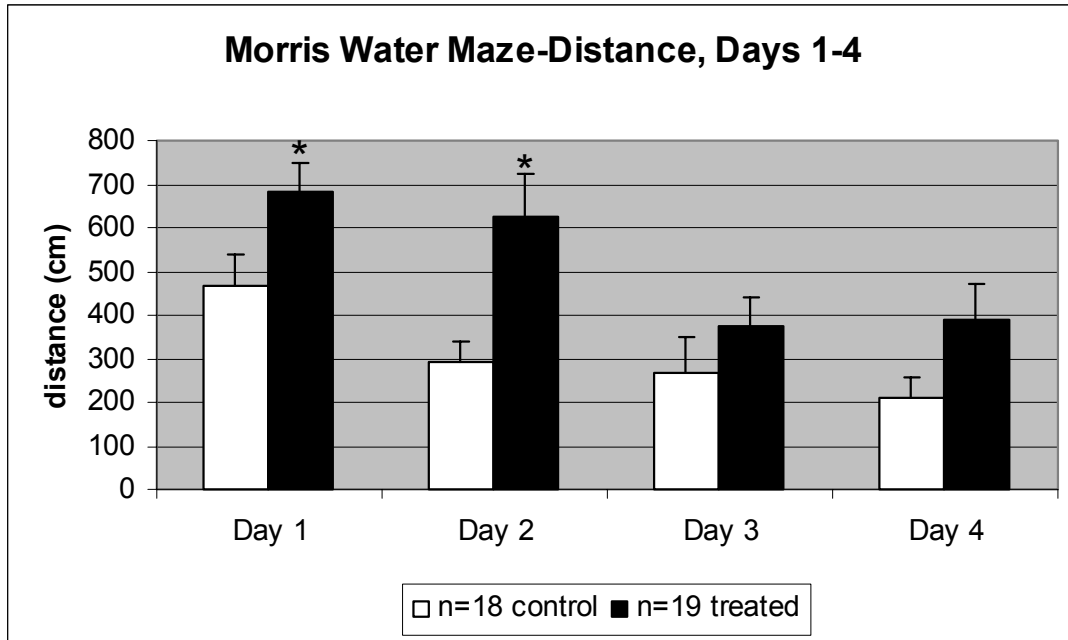
Figure 18. Distance Traveled on Days 1-4. Graph shows day 1 control (465.06; n=18) and treated mice (682; n=19), day 2 control (294.24; n=18) and treated mice (627.56; n=19), day 3 control (267.19; n=18) and treated mice (375.51; n=19) and day 4 control (207.93; n=18) and treated mice (389.26; n=19). Single-factor ANOVA indicated no significant differences between gender and distance traveled in both control and treatment groups. There were significant differences (p=0.03 and p=0.004, respectively) between treated and control mice on days 1 and 2 (*). α was 0.05.

The time it took to reach the submerged platform on days 1-4 was also measured. If a mouse swam a greater distance, it follows that it took him longer to find the platform. If the mouse swam a shorter distance, he was able to find the platform more quickly. Treated mice took longer on all days (1-4) to reach the platform. Except for day 2, where treated males took longer than treated females to reach the platform, there was no difference between gender and platform time in control or treated mice. There were significant differences between control and treated mice on days 1, 3 and 4; treated mice took longer to reach the platform. On day 2, treated males took significantly longer than

controls to reach the platform. There was no difference between treated females and

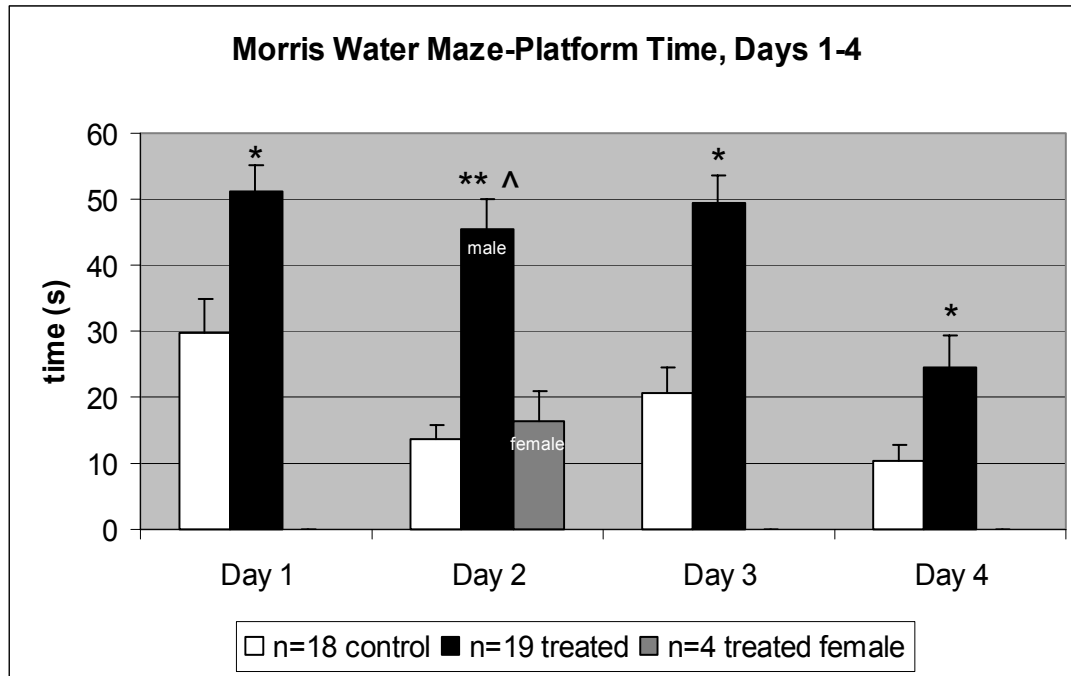controls on day 2. Figure 19 summarizes the results found above.

Figure 19. Time Taken to Reach Platform on Days 1-4. Graph shows day 1 control (29.83; n=18) and treated mice (51.12; n=19), day 2 control (13.5; n=18), treated males (45.32; n=15) and treated females (16.45; n=4), day 3 control (20.54; n=18) and treated mice (49.52; n=19) and day 4 control (10.37; n=18) and treated mice (24.51; n=19). Single-factor ANOVA indicated no significant differences between gender and platform time on days 1, 3 and 4 in the control and treated mice. There was a significant difference (p=0.01) between treated males and females on day 2 (**) and between treated males and the controls (p=0.000001) on day 2 (^). There were also significant differences (p=0.002, p=0.00001 and p=0.01, respectively) between treated and control mice on days 1, 3 and 4 (*). α was 0.05.

Probe Trial

On the last day of the Morris water maze test (day 5), termed the "probe" trial,

the submerged platform was removed. It was assumed that over the course of training,

mice would learn the position of the platform using the visual cues. Thus, they should

have learned what quadrant the platform was in and should theoretically spend more time

in that quadrant looking for it. Figure 20 shows that control mice covered significantly more distance than the treated mice. This could indicate that the controls knew a source of refuge existed and when they could not find it, they kept swimming to look for it. The treated mice covered less distance either because they did not sufficiently learn where the platform was and so swam at a normal pace or knew there was a platform but could not find it and gave up their search for it, knowing they would be removed from the pool after a certain amount of time.
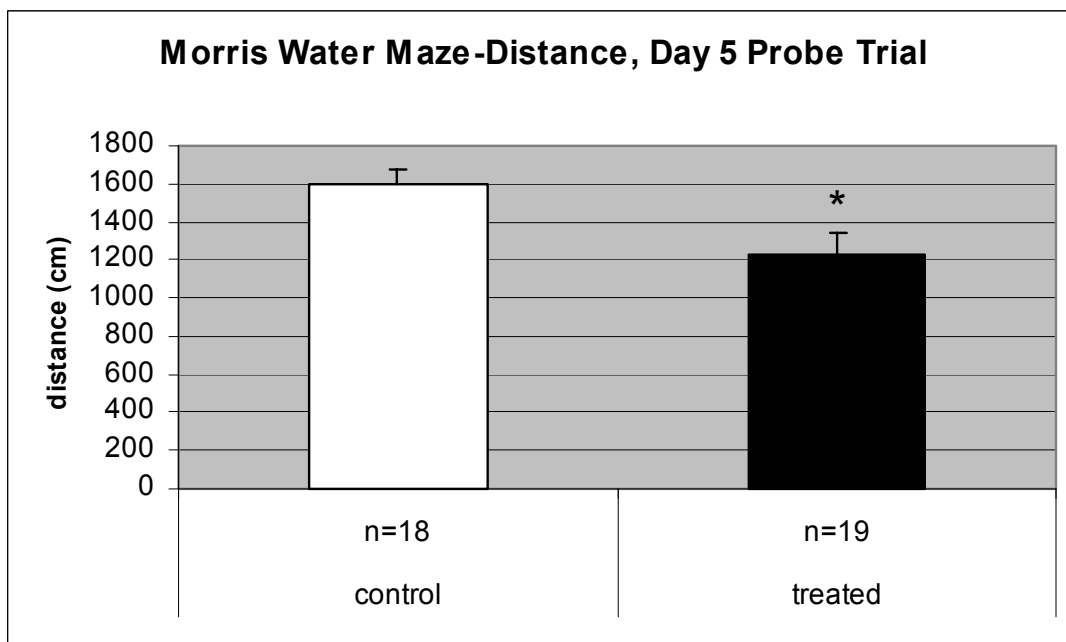
Figure 20. Distance Traveled in the Probe Trial. Graph shows control (1592.03; n=18) and treated mice (1228.82; n=19). Single-factor ANOVA indicated no significant differences between gender and distance in both the control and treated mice. A significant difference ($p=0.01$) was noted between control and treated groups. α was 0.05.

The last Morris water maze parameter measured in the probe trial was the percent of time a mouse spent in each quadrant. Quadrant 5 was the platform itself and was contained in quadrant 4. Q4+Q5 indicates the area where the platform should have been.

Control mice spent slightly more time in Q4+Q5 compared to the treated mice; however, there was no significant difference. This indicates that both treatment groups could not find the platform there and so did not spend a significant amount of time in that area looking for it. This was also seen in quadrants 1 and 3; there were no significant differences in the amount of time that control and treated mice spent in these areas. However, control females significantly more time than control males in Q2. It is interesting to note that the experimenter who served as one visual cue stood by the perimeter of the pool in this quadrant. The control females may have learned to stay in Q2 because they learned that the experimenter would pick them up from that area. There was no significance between control females and treated mice in Q2. Figure 21 shows the percent time spent in each quadrant on the probe trial.
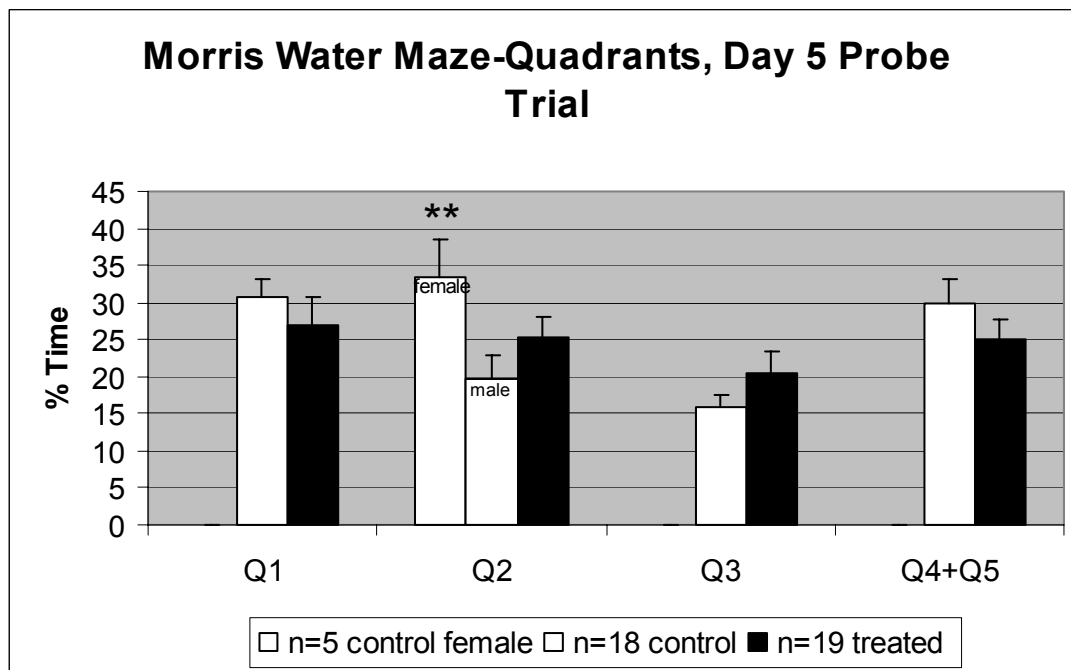

Figure 21. Time Spent in Quadrants on Probe Trial. Graph shows Q1 control (30.77; n=18) and treated mice (26.99; n=19), Q2 control female (33.44; n=5), control male (19.74; n=13) and treated mice (25.25; n=19), Q3 control (15.91; n=18) and treated mice (20.58; n=19) and Q4+Q5 control (29.88; n=18) and treated mice (25.06; n=19). Single-

factor ANOVA indicated no significant differences between gender and % time in Q1, Q3 and Q4 in the control and treated mice. There was a significant difference (p=0.03) between control females and males on day 2 (**). There were no significant differences between treated and control mice in any quadrant. α was 0.05.

Mitochondrial Membrane Potential

MeHg is neurotoxic because of its lipophilicity. Thus, it can cross the blood brain barrier and enter cerebellar granule cells and other cells in the brain. Primary signs of cerebellar dysfunction include ataxia and other motor and cognitive impairments. These motor effects were studied by performing the behavioral tests previously mentioned. Mitochondrial membrane potential is an important indicator of cellular health. High doses of MeHg have been shown to activate the mitochondrial apoptosis pathway. A decrease in MMP could lead to mitochondrial apoptosis. Mitochondria provide cells with ATP, a critical energy molecule, and are essential to proper cell functions. When mitochondria die, the cell loses its source of energy and normal cell mechanisms are altered. If enough mitochondria undergo apoptosis, the cell dies. Cell death and altered cell functions lead to behavioral changes in activity, learning, memory and coordination. This study examined chronic, low dose prenatal MeHg exposure and its effects on MMP. TMRM was used as an indicator dye for MMP because it is a lipophilic cation that accumulates in mitochondria in proportion to MMP. In normal cerebellar granule cells TMRM can cross the lipid bilayer and accumulate in mitochondria. Normal MMP is around -120mV and the TMRM cation is attracted to the negative interior of the mitochondria. Figure 22 shows images of normal cerebellar granule cells loaded with TMRM dye. If MeHg does in fact decrease MMP and the interior becomes less negative, TMRM cannot enter the mitochondria and the MeHg affected cell will have less

fluorescent intensity than a normal cell. Mitochondrial membrane potential was not

affected by prenatal MeHg exposure and there were no significant differences between

control and treated groups. Figure 23 displays the results.
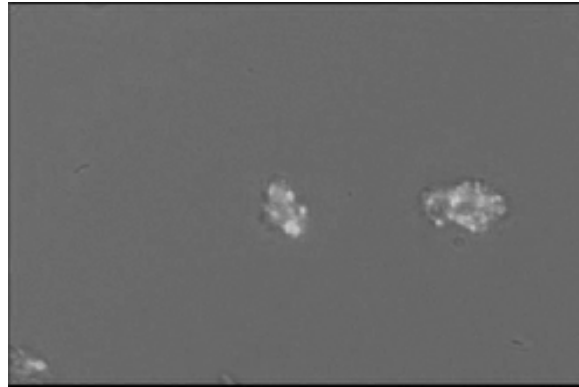
Figure 22. Fluorescent Images of Cerebellar Granule Cells. Clusters of mitochondria (white) loaded with TMRM fluorescent dye that accumulates in proportion to MMP are shown.

Figure 23. Mitochondrial Fluorescence in Proportion to Membrane Potential. Single-factor ANOVA indicated no significant differences between gender and MMP. No significant differences were observed between control (17678.54; n=5) and treated mice (18067.51; n=5) and MMP. α was 0.05.

RECOMMENDATIONS FOR FUTURE RESEARCH

Future studies directed towards studying prenatal methylmercury exposure and its relation to mitochondrial membrane potential should increase the sample size to ensure size was not a limi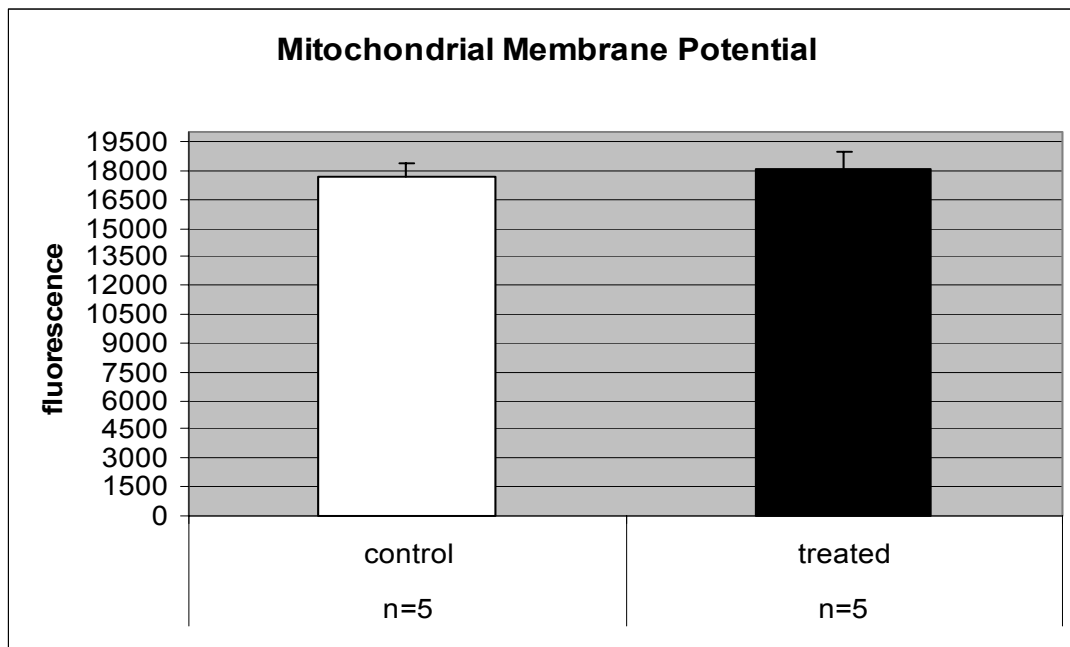ting factor. More treated females should be tested or equal numbers of males and females should be used if possible to reduce the chances of gender differences based on different sample sizes between males and females. Vaginal washes on females should be performed and the cells counted to determine the stage of the estrous cycle because the varying stages of the cycle may have an effect on behavior.

In the open field test, time spent in the periphery of the chamber should be analyzed because this study found that treated mice did not spend significantly more time in the center than controls. This would help us to better understand how much activity occurs away from the center of the chamber in a new situation. It would also enable us to recognize if hiding in the periphery is indeed a sign of fear.

It was noted in the Morris water maze results that control and treated mice did not differ in the time they spent in each quadrant over the course of the 60 second probe trial. If the controls did indeed learn better than the treated mice, they could have lost interest in finding the platform over 60 seconds because it took too much time. Therefore, the first 15 seconds of the probe trial should also be looked at to see if control mice concentrate their focus (spend more time) in the platform quadrant when they are first put in the pool. A curtain with visual cues painted on it should also be used in place of human visual cues. As mentioned earlier, control females spent a significant amount of time in the quadrant where the human experimenter was. They could have associated the visual cue with an escape, since they knew they would eventually be picked up. A

curtain with visuals painted on it would eliminate the visual cue being used as both an aid in finding the platform and as an escape (being removed from the pool).

Continuing prenatal methylmercury research should also be directed towards delineating other cellular mechanisms that affect the motor and cognitive outcomes described in this research.

SUMMARY AND CONCLUSIONS

Summary

There were no differences between control and treated mice in base stance, center time in the open field, vertical pole, percent time spent in each quadrant in the Morris water maze probe trial and mitochondrial membrane potential fluorescence.

In the footprint analyses, treated mice exhibited a decrease in foot angle. In the treated males, stride length was slightly increased compared to both the treated females and the control group. There appears to be a gender difference in this study.

The open field tests also appear to have gender differences. Treated females explored the chamber (i.e.-distance traveled) less than treated males in the first five minutes of the 30 minute test. The treated females also had decreased rearing movement in the first five minutes compared to both the treated males and controls. Analyzing the first five minutes of activity is important because it assesses an animal's initial response to a novel situation. Thus, treated females seem to display less willingness to explore in a new situation.

During the second five minutes, the chamber can still be considered a new environment. Treated mice also reared less and covered less distance in the 2nd five minutes. After this point in time, the treated mice began to explore the open field more and by the end of the test, their rearing movement and total distance traveled did not differ from the controls.

In the rota-rod test, the treated mice differed from the controls on the third (last) day of activity. The rota-rod assesses learning and coordination, and while both groups

displayed a positive learning curve over the course of three days, the treated mice didn't learn to keep pace with the accelerating rod as well as the controls.

On the first two days of Morris water maze, treated mice traveled further. This indicates a slower initial learning time in using visual cues to find the platform. On all four days, it took treated mice significantly longer to find the platform. It is interesting to note that on day 2 it took the treated males longer than both the treated females and control mice to reach the platform. In the probe trial, control mice covered more distance than the treated mice. This is in contrast to the controls' performance on days 1-4 where they covered less distance because they found the platform faster. The control mice swam further because they knew a platform existed and were trying to look for it. This can explain why there was no difference in the time control and treated mice spent in each quadrant because once the controls could not find the platform situated in its normal quadrant, they swam to other quadrants attempting to locate it. However, control females did spend significantly more time in quadrant 2 on the probe trial. The experimenter stood at the perimeter of quadrant 2 and the control females could have stayed in Q2 longer waiting to be picked up. There is also a gender difference in this test.

Conclusions

In this study, methylmercury appeared to effect no cellular changes in regards to mitochondrial membrane potential. Thus, a decrease in mitochondrial membrane potential and consequently impaired mitochondrial function was not responsible for the subtle changes in behavior noted. Some other cellular mechanisms affected by methylmercury are responsible for changes in behavior. Learning and memory (the Morris water maze test) seemed to be more affected by prenatal MeHg exposure than

coordination and activity (the open field, rota-rod, vertical pole, and footprint analysis tests) because there were greater differences between treatment groups in the water maze test than in the activity and coordination tests. Gender also seems to affect the extent of methylmercury effects in the central nervous system as manifested in several behavioral analyses.

Understanding the cellular mechanisms and the behavioral outcomes of methylmercury will help reduce the morbidity and dysfunction associated with methylmercury exposure by creating avenues of prevention and treatment.

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EDUCATION

Texas A&M University   Bachelor of Science Degree
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RESEARCH

Texas A&M University Undergraduate Research Fellows Program Class of 2006
- Thesis: *Effects of Prenatal Methylmercury Exposure on Motor Coordination, Activity Levels and Mitochondrial Membrane Potential in Adult Mice*
- Collaborate with faculty advisor in researching prenatal methylmercury toxicity in adult mice.
- Participant in Student Research Week 2006 with awards for The Lone Star Graduate Diversity Colloquium Choice Award and The International Education Week Choice Award

Texas A&M University Abbott Laboratory, College Station, TX
- Student researcher from January 2004 to present.
- Assist with lab work on Prozac research project in mice
- Skills: behavioral analyses, perfusing, brain extraction and cutting

PUBLICATIONS

Society for Neuroscience 35th annual meeting, Washington, D.C.
- S.E. Wills, J.M. Mackey, M.A. Hughes, L.C. Abbott
  Hippocampal cell proliferation is reduced in adult homozygous leaner mice compared to age-matched wild type mice. Soc. For Neurosci. Abstract # 431.1, Washington D.C., November 11-16, 2005.

MEDICAL EXPERIENCE

St. Joseph Regional Health Center, Bryan, TX
- Volunteer in spring semester 2006; Emergency Room.
- Responsible for taking and recording patient vitals to include blood pressure, heart rate and temperature, setting up and performing the EKG test on patients and removing IV's
- Trained in proper wheelchair procedure, basic department functions, customer service, and stocking medical supplies and specimen trays

Brazos Valley Rehabilitation Center, Bryan, TX
- Volunteered for four semesters in 2004 and 2005; Physical Therapy Department.
- Typed dictations, filed and prepared chart packs, assisted with general housekeeping duties
- Participated in physical interaction with patients during sessions, observed procedures and consultations, recorded measurements

The Med, College Station, TX
- Volunteered for one semester in 2003; Emergency Room.
- Performed housekeeping tasks, observed daily operations

HONORS AND AWARDS
- **Sigma Xi Scientific Research Society Associate Member (2006)**
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