NEUROBEHAVIORAL CONSEQUENCES OF AGING AND CHRONIC

METHYLMERCURY EXPOSURE: INTERACTIONS

WITH DIETARY SELENIUM

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NEUROBEHAVIORAL CONSEQUENCES OF AGING AND CHRONIC METHYLMERCURY EXPOSURE: INTERACTIONS WITH DIETARY SELENIUM

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A Dissertation

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Doctor of Philosophy

Auburn, Alabama
August 9th 2008

NEUROBEHAVIORAL CONSEQUENCES OF AGING AND CHRONIC METHYLMERCURY EXPOSURE: INTERACTIONS WITH DIETARY SELENIUM

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John Charles Heath was born in Westcliff, Essex, England on August 8th 1949. He graduated from Parkstone Sea Training School in 1963. He attended Southend College of Technology and Riversdale Technical College graduating in Mechanical engineering in 1968. In 1977 he emigrated from England to the USA and pursued a career in engineering. Returning to School in 1996 he graduated from Utah State University summa cum laude with a Bachelor of Science degree in Psychology in 2001. He entered Graduate School at Auburn University's Department of Psychology, in August, 2001 and received his Master's degree in August of 2005.

DISSERTATION ABSTRACT

NEUROBEHAVIORAL CONSEQUENCES OF AGING AND CHRONIC METHYLMERCURY EXPOSURE: INTERACTIONS WITH DIETARY SELENIUM.

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Doctor of Philosophy, August 9, 2008
(M.S., Auburn University August, 2005)
(B.S., Utah State University, 2001)

75 Typed Pages

Directed by M. Christopher Newland

Methylmercury (MeHg), although a well-described toxicant to the adult and developing nervous system, is inadequately understood in adult onset, low-level chronic exposure. Among the known effects are somatosensory deficits and weakness in the extremities. Selenium (Se), an essential nutrient, might allay chronic neurotoxic effects of MeHg. To examine this, sixty-day-old female Long Evans rats were fed a diet containing 0.06 or 0.6 ppm of Se as sodium selenite; both diets providing adequate Se. After 100 days, methylmercuric chloride was introduced into their drinking water in concentrations of 0.0, 0.5, 5.0, or 15.0ppm. Both exposures continued daily for 16 months. Approximately every two months, forelimb grip strength, tactile sensitivity in the tail, and overnight running distance were tested. Flexion and hindlimb cross (clasping reflex) were also examined on a regular basis. Age-related changes were noted in control groups

on grip strength, tail sensitivity, and running. MeHg-related effects were observed only in the 5.0 and 15.0 ppm exposure groups. The severity and latency to effects were influenced by MeHg dose and dietary selenium. In unexposed animals running increased with age, the increase being larger in the high-Se animals. The 5.0 ppm exposure groups showed a smaller increase in running, and Se influenced this effect. Running during young adulthood was a good predictor of running in older rats for unexposed animals. Age-related correlations were also noted on other measures. MeHg exposure generally weakened or eliminated these correlations, even in the 0.5 ppm exposure group for which no other signs appeared. Flexion always appeared before hindlimb cross, and forelimb grip strength together with tail sensitivity were both delayed by selenium in the higher methylmercury groups. Dietary selenium afforded a degree of protection against MeHg's effects, largely by delaying their onset.

ACKNOWLEDGEMENTS

I would like to thank Dr. M. C. Newland for his patience, kindness, and guidance. Without his support this manuscript would not have been completed. I would also like to thank my committee members Dr. Chris Correia, Dr. Jeff Katz, and Dr. Elaina Freida, for their help and support. Special thanks are given to my wife Yuk Fun whose patience and understanding made the production of this document that much easier.

Style manual or journal used: Publication Manual of the American Psychological Association (5th Ed.)

Computer software used: Microsoft Word 2007, Microsoft Excel 2007, SigmaPlot 10.0, Systat 11, and Dr paper 5.

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CHAPTER 1

LITERATURE REVIEW

Introduction

Mercury (Hg) is a naturally occurring metallic element that is ubiquitous throughout the environment. Mercury is present in several inorganic forms including metallic (Hg^0), mercurous (Hg_2^{2+}), and mercuric (Hg^{2+}) valence states (Daintith, 1996). The dominant form of mercury in the atmosphere is ionic mercury (mercuric) (Hg^{2+}), which can be transformed into several organic forms of which Methylmercury (MeHg) is one (National Research Council, 2000).

Methylmercury (MeHg) is known to be a neurotoxicant i.e., a substance that disrupts the function of the nervous system by interacting with it directly or by interacting with supporting cells in the nervous system. Methylmercury is also known to be a developmental neurotoxicant, an agent that causes malformation of an embryo or a fetus, by being passed to the fetus from the mother via the placenta (Takeuchi, Morikawa, Matsumoto, & Shiraishi, 1962).

Selenium (Se) is an essential nutrient that is required for a number of different roles in both the nervous system and in different organs.. Its function has been shown to be important as a growth factor, as well as having powerful antioxidant and anticancer properties (Raymond & Ralston, 2004). It also supports, amongst other functions, normal thyroid hormone homeostasis, immunity and fertility (Raymond & Ralston, 2004) .

Selenium has a particularly high affinity for MeHg i.e., the two substances join together very easily and form a strong chemical bond, and as such Se may act as a protective mechanism against MeHg onslaught. Conversely, it may be possible that the observed effects of MeHg are associated with Se deficiency because MeHg binds so closely to Se, making less available for Se related biochemical processes that take place within an organism (Raymond & Ralston, 2004).

Methylmercury

The first modern large-scale contamination by MeHg was in Minamata Japan in 1956, although it was not until 1957 that MeHg was suspected as the toxic agent. In 1959 it was reported that what had come to be known as Minamata Disease was most probably caused by MeHg poisoning. A similar outbreak took place in Niigata Japan. Both outbreaks were cases of chronic exposure of MeHg caused by the dumping of industrial waste into public waterways (Clarkson, 1989).

In a survey of 2383 victims of Minamata Disease 1540 (64.6 %) suffered sensory disturbances which the authors of this survey described as the stocking and glove type. This was a loss of sensory function in the lower section of the arms and legs from a region just above the elbow or knee to the digital extremities. Among other signs and symptoms reported were incoordination (33.6%), muscular weakness, (35.3%), and tremors (27.7%) (Harada, 1997). Initial Signs and symptoms of MeHg intoxication were non specific, such as parethesia. After a latent period of weeks or months sensory disturbances such as constriction of the visual field, deafness, and motor aberrations similar to ataxia of the gait appeared (Clarkson, 1989).

A study on potential health effects of low-level MeHg exposure, in a village on a tributary of the Amazon, reported, on 91 adults whose hair mercury levels were lower than 50µg/g. Manual dexterity, adjusted for age, decreased significantly with hair mercury levels, and there was a tendency for muscular fatigue to increase and muscular strength to decrease in women. Hair mercury levels were also significantly higher for subjects who presented disorganized movements on an alternating movement task (Lebel et al., 1998).

A population in Iraq, in the early 1970's, experienced acute exposure caused by seed grain that had been treated with a fungicide that contained MeHg. People who used the grain to make bread were exposed to high levels of the toxicant. Exposure took place from November through December 1971 with the appearance of neurological signs after approximately one month. The order of appearance was parethesia, ataxia, and dysarthria. Exposure lasted from 43 to 58 days with the mean latent period from the end of contamination to the onset of signs ranging from 16 to 32 days, and concentration levels ranging from 500 to 4000ng/ml. Mercury hair concentrations revealed that the minimum level that correlated with specific signs were approximately 80µg Hg/g for parethesia and 130µg Hg/g for ataxia (Bakir, 1973).

From the preceding reports it can be established that MeHg ingestion is instrumental in the appearance of signs such as parethesia, ataxia, and dysarthria, together with loss of sensory function on the extremities of the body.

Neurological Effects of Methylmercury

Methylmercury is known to cross the blood brain barrier in order to enter the central nervous system (CNS) (Clarkson, 1995). It forms a complex in plasma with the amino acid cysteine, which is similar to the neutral amino acid methionine. The methionine molecule has the structure, $CH_3 - S - CH_2 - CH_2 - CH_{\,|\,NH_3^+}^{\,|\,-\,COO^-}$ while the methylmercury cysteine complex has the structure. $CH_3 - Hg - S - CH_2 - CH_{\,|\,NH_3^+}^{\,|\,-\,COO^-}$ This similarity allows MeHg to cross the blood brain barrier disguised as an amino acid (Aschner & Aschner 1990).

Postmortem examination of 16 victims of Minamata Disease showed varying degrees of gross damage to neurological areas including the calcarine fissure, and the pre and post central cerebral cortex. Microscopically there was cellular degeneration with gliosis i.e., activation of glial cells, which is an acute response to trauma and other forms of injury that lead to CNS damage (Worobey, Tepper, & Kanarek, 2006), in the granular cells of the cerebellum. There was also involvement to a lesser degree of other cortical areas, the hypothalamus, midbrain and basal ganglia (Kurland, Faro, & Sielder, 1960). In adults destruction of neuronal cells has been found in the calcarine fissure in the occipital lobe, the cerebellum and the precentral gyrus (Eto, Tokunaga, Nagashima & Takeuchi, 2002).

A pathological study of Minamata disease patients revealed a diffuse loss of granule cells in the cerebellum, while the Purkinje cells were, in general, spared. In severe cases, both cell types entirely disappeared (Takeuchi et al., 1962). A review of research on Minamata disease made the following observations. Pathological changes were recognized in the nervous system, especially in the cerebral cortices and peripheral

sensory nerves. Neurons in the cerebral cortex showed swelling, or acute shrinkage with an increase in Eosinophils (white blood cells active in allergic diseases, parasitic infections, and other disorders), and occasionally severe ischemic changes with neuronophagia (destruction of neurons by phagocytic cells) (Stedman, 1995).

When comparing the reported physical signs of MeHg ingestion with the reports of neurological damage a correlation is observed between the impairment reported to motor abilities, and loss of sensation with the areas of the CNS that have reported damage. The four areas of damage frequently (though not exclusively) reported, are the calcarine fissure, precentral gyrus, postcentral gyrus, and the cerebellum. Of these the latter three areas are all implicated in motor control and somatosensory function (Naito, 2004).

The precentral gyrus is the origin of many of the neurons in the pyramidal system, which is a major pathway from the brain to motorneurons (Rosenzweig, Breedlove & Watson 2005). Damage in this area can result in partial paralysis on the side of the body opposite to the lesion. The effect can be greatest in distal muscles such as the hands or feet. In contrast, the post-central cortex is the primary cortical receiving area for somatosensory sensation. Lesions in this area tend to produce sensory deficits (Naito, 2004).

The cerebellum, located caudal to the pons, is instrumental in the coordination of movement. The cerebellar cortex (the outer layers of the cerebellum) is dominated by Purkinje cells, which synapse deep within the cerebellar nuclei with granule cells. Purkinje cells produce only inhibitory postsynaptic potentials, thus, the cortical areas of the cerebellum guide movement by inhibiting neurons. Inputs to the cerebella cortex

come from sensory sources e.g., muscle and joint receptors as well as vestibular, somatosensory, visual and auditory systems. Because the cerebellum is active in many aspects of movement, damage to this area of the brain can result in many abnormalities of behavior, including (but not limited to) disturbances of balance, ataxia in the legs, and difficulties of motor coordination (Takeuchi et al., 1962).

Somatosensory function is mediated by sensory fibers whose cell bodies are in the dorsal root ganglia adjacent to the spinal cord. With adult-onset exposures, MeHg accumulates readily in this structure (Somjen et al., 1973, Chang, 1987) where it induces pathology and apoptosis (Chang, 1987; Wilke, 2003).

The Effects of Methylmercury Ingestion in Rats

Rats have been used in many studies on the effects of methylmercury, for examples see (Day, Reed, & Newland, 2004; Kakita et al., 2000; Roegge et al., 2004; Sakamoto et al., 2002; Sakamoto et al., 1998; Su, Wakabayashi, Kakita, Ikuta, & Hitoshi, 1998; Wakabayashi et al, 1995., Kinoshita, Ohnishi, Kohshi, & Yokota, 1999). Many of these studies document the physiological signs and neurological consequences of MeHg ingestion.

A study by Sakamoto et al., (2002) evaluated the changes in Hg concentration in the brains of rat offspring, and its effects on the developing rat brain, based on consecutive and moderate doses throughout gestation and lactation. Fourteen female Wistar rats, separated in to two groups, (4 controls and 10 experimental) were fed 16 grams per day of either chow diet (control) or a diet containing 5ppm of MeHg. This translates to an intake of 80µg/day of Hg. Although the weights of subjects were not

reported for this study, based on average weights for female Wistar rats of approximately 212gms at 7 weeks old, (Harlan Laboratories, 2007), the average daily intake of Hg would be 0.38 mg/kg/day (380 µg/kg/day). After eight weeks on this diet the females were mated. Both groups continued on their respective diets through gestation and lactation of the pups. After postnatal day 30 the pups were placed on the MeHg diet for approximately two months while control pups were placed on the chow diet. At the age of five weeks the offspring were given a motor coordination test in which the time the rats could stay on an 8 cm diameter rod rotating at 10 rpm was recorded. The rats exposed to the MeHg diet stayed on the rods for a significantly lower percentage of time than the animals on the control diet (12.5% MeHg diet, 87.5% control). Histological examination revealed abnormal development in the cerebella cortex resulting from the atypical location of granule and Purkinje cells (Sakamoto et al., 2002).

In a previous study by the same author (Sakamoto et al., 1998), the administration of MeHg was based on body weight and not the concentration in the food. Wistar rat pups were administered 5 mg/kg/day of MeHg in a distilled water and condensed milk solution. This translates to 5000µg/kg/day, thus a l00mg rat would receive 500µg of MeHg. Administration continued for more than 30 days until the rats died. The animals were compared with a control group that did not receive MeHg. At day 18 the experimental group started to gain weight at a slower rate than the control group and at day 25 they began to lose weight. Neurological signs were observed starting at day 30 in the form of the retraction of the hind legs when suspended by their tail, paralysis of hind legs, piloerection (erection of the hair of the skin) and unsteadiness of gait. By day 32 severe contractions of the hind legs and tail erection started to appear. Animals in the

experimental group started to die on day 32 and the most resistant rat (author's definition) survived to day 40. A histological examination showed neuronal degeneration in the cerebral neocortex, neostriatum, red nucleus, brainstem, cerebellum and spinal sensory ganglia (Sakamoto et al., 1998). These two studies demonstrated not only that neurological damage occurred at a high dose of MeHg which was fatal after a maximum of 40 days (Sakamoto et al., 1998), but that neurological signs were seen, and similar (though less severe) neurological damage was observed at lower non fatal doses of MeHg (Sakamoto et al., 2002).

In a longitudinal study in which the neurological effects of MeHg on rats placed on a diet either high or low in docosahexaenoic acid (DHA) was studied, effects on several sensory and strength factors were observed (Day et al., 2004). Sixty-six of 72 female Long Evans rats that were purchased at the age of 80 days were tested on motor and strength tasks over a period of 18 months after being bred and weaning their pups. Additionally, 49 offspring of these females were also tested on various motor and strength tasks.

The breeding females were initially assigned to either a diet high or low in DHA at the age of 12 weeks. At 15 weeks they were further assigned to one of three MeHg groups in which they were exposed to 0.0, 0.5 or 5.0 ppm methylmercury in their drinking water. These doses translate approximately to a MeHg intake of 0.0, 40, or 400μg/kg/day. Three weeks after the beginning of exposure these females were bred with non-exposed males, and except during weaning of the pups, (so pups were not directly exposed to MeHg through drinking water) chronic exposure of MeHg continued for the remainder of their lives. Offspring were only exposed to MeHg gestationally, however,

they were fed the same diet as their dams and remained in MeHg exposure groups according to exposure received gestationally (Day et al., 2004).

Testing for the F0 generation (dams) included forelimb grip test, running wheel activity, gait, hindlimb cross and flexion observations. Hindlimb cross was defined by the position of the hindlimbs (crossed: impaired, or uncrossed: not impaired) when suspended from their tail, flexion was the closing of the hindlimb digits similar to a clenched fist. Testing of the F1 generation (offspring) included forelimb grip strength and running wheel activity.

For forelimb grip strength the F0 generation, who were subjected to a lifetime chronic exposure, showed a main effect for MeHg exposure at 11, 14 and 18 months of age, but not at 7 months of age. Post hoc analysis confirmed only the highest MeHg exposure group produced a significant reduction in hindlimb strength. For the F1 generation, who were exposed only gestationally, forelimb strength was unaffected by MeHg exposure.

Mercury decreased running wheel performance in a dose-related manner beginning at 11months and continuing for 30 months, for the F0 generation. Performance was severely impaired by the 400µg/kg/day MeHg dose. Hindlimb cross and gait deficiencies was also seen in a dose related manner with only the chronic high MeHg (F0) animals showing these signs at 11 months and 18 months. Gait deficiencies and hindlimb cross were highly correlated (> 0.9) at all ages. Observed signs included ataxia, impaired coordination, excessively splayed or adducted hind limbs, and dragging of hind limbs. (Day et al., 2004).

Although the onset of flexion was reported as roughly coincident with hindlimb crossing by Day et al (2004), Kinoshita et al., (1999) showed in photographs that rats subcutaneously injected with 10 mg mercury/kg body weight) daily for 7 days had developed flexion by the ninth day after onset of exposure and hindlimb cross by the eleventh day of exposure. Although not reported as such, this would suggest that flexion occurs slightly before hindlimb cross.

The studies reported above indicate that the neurological signs seen in rats after ingestion of MeHg show a similar pattern as seen in human victims. This pattern is also seen in the neurological damage observed by histological studies of rats brains subjected to MeHg insult. Two of the studies (Day et al., 2004; Sakamoto et al., 2002) reported deficiencies in motor tasks i.e., running wheel activity (Day et al., 2004), rotating rod (Sakamoto et al., 2002), after chronic ingestion of MeHg through food contaminated with MeHg (Sakamoto et al., 2002), or MeHg added to the drinking water (Day et al., 2004). Two of the studies (Day et al., 2004; Sakamoto et al., 1998) reported impairment of mobility in the form of hindlimb restriction, deformation, or paralysis. Histological reports in two of the studies (Sakamoto et al., 2002; Sakamoto et al., 1998) revealed neurological damage in the cerebellar cortex, cerebral neocortex, cerebellum, and spinal sensory ganglia, all areas known to effect motor coordination and use of limbs.

Selenium

Selenium is a trace mineral that is essential to human biology and human health. Deficiency of Se has been implicated in viral infection, reproduction (male and female), mood disorders, cardiovascular disease, and cancer (Rayman, 2000). As selenocysteine

(the 21st amino acid), Se is a component of approximately 35 selenoproteins, some of which have important enzymic functions although, the roles of many are not yet fully understood (Sunde, 1997).

Selenium enters the food chain through plants, which take it from the soil. Se deficiency throughout the world has been correlated with areas that have poor soil Se content. Some effects of severe Se deficiency are Keshan disease (a potentially fatal form of cardiomyopathy) and Kashin-Beck disease, a deforming form of arthritis. Immune deficiency has also been associated with Se deficiency possibly because immune cells in the liver spleen and lymph nodes need considerable amounts of Se, however, Se diet supplementation has also shown to increase activated T-cells (Rayman, 2000). The deficiency of Se has also been associated with harmless viruses becoming virulent, such as in the case of the cocksaxie virus in which it is thought that Se deficiency alters the genome of this virus. This is considered a co-factor in the cardiomyopathy of Keshan disease (Beck, Esworthy, Ho, & Chu, 1998).

In reproductive studies selenium deficiency has been linked to deficits in reproduction in both males and females. In a study in which four generations of male rats were fed a low selenium diet the findings indicate that testicular morphology and functions were affected by severe selenium deficiency. Selenium was also found to be necessary for testosterone biosynthesis and the formation and normal development of spermatozoa (Behne, Weiler, & Kyriakopoulos, 1996). Barrington and colleagues found significantly lower serum selenium in women who had had either first-trimester or recurrent miscarriages. They suggest that early pregnancy loss may be linked to reduced antioxidant protection of biological membranes and DNA by low concentrations of the

selenium dependent Glutathione peroxidase. A subsequent study found lower selenium levels in non-pregnant women who had experienced recurrent miscarriages, than in controls (Barrington, Taylor, Smith, & Bowen Simpkins, 1997).

Selenium in the Brain

Because it is virtually impossible to deplete Se in certain tissues such as the thyroid, pituitary, and especially the brain (Behne, Pfeifer, Rothlein & Kyriakopoulos 2000), it appears that Se in the form of selenoproteins, may be particularly important to these tissues (Raymond & Ralston, 2004). In studies in which animals were subjected to extreme Se deficiency over six generations the brain still retained 60% of the Se found in control animal while there was a decrease to less than 1% of Se in liver, skeletal muscle, and blood. Follow up studies showed the brain concentration of Se in rats was maintained through 16 generations of selenium-deficient diet (Behne, Pfeifer, Rothlein, & Kyriakopoulos, 2000).

Selenoprotein P knockout mice had their brain selenium levels reduced to 43% of normal when being fed a diet containing less than 0.1 ppm selenium, the lowest brain Se concentration achieved at that time. The mice developed poor motor coordination, lost weight, and fertility in males was reduced drastically. When fed a diet containing 2ppm Se, the Se concentration in their brain and motor coordination were restored to normal (Hill et al., 2003). It would appear from these studies that the regulation of Se in the brain is of prime importance and is therefore tightly regulated with the brain having priority over other organs.

Selenium and Methylmercury Interactions

As discussed above, not only has mercury has been shown to cross the blood brain barrier (Clarkson, 1995) but it also has a high affinity for Se (Raymond & Ralston, 2004). Affinity is a pharmacological concept that describes the degree of attractiveness between two substances. It is the product of the mutual attraction of the molecular structures of the substances in question (Jeffries, 1999). The selenium in selenocysteine, and mercury has an affinity constant of $\sim 10^{-22}$. During each cycle of selenocysteine synthesis the free selenides that form have a high affinity constant for mercury of 10^{-45} (Raymond & Ralston, 2004).

Because of high affinity between Se and MeHg the adverse effects of MeHg ingestion may be caused by one of two factors. If the MeHg binds to an excessive amount of selenium then adverse effects may be caused by a deficiency of Se in the brain. However this may only show a transitory effect as demonstrated in the Hill study (Hill et al., 2003) where restoration of a diet containing 2 ppm Se restored Se brain concentrations and motor coordination. If the amount of MeHg that crosses the blood brain barrier is too great to be bound up by available Se then not only may the effects of Se deficiency be apparent but there may be neurological damage caused by MeHg that has not bound to available Se.

Studies on the effects of pre and postnatal exposure to MeHg via fish consumption in Seychelles Island children showed an association with beneficial effects and only one detrimental effect which was performance on the grooved pegboard test with the non-dominant hand (a test of motor speed and coordination) (Davidson, Myers, Weiss, Shamlaye, & Cox, 2006). However, studies in the Faroe Islands, another large

seafood consuming population, reported mercury-related neuropsychological dysfunctions in the areas of language, attention, and memory, and to a less extent in visuospatial and motor functions (Grandjean et al., 1997). Thus two large studies with seafood eating populations show results that appear to contradict each other.

One reason for the apparent anomaly in the results of these two studies may be the source of MeHg exposure. The seafood diet consumed by participants in the Faroe Islands study included whale whereas the Seychelles Islanders' diet did not. The mature pilot whale, which was consumed by Faeroes Island study participants, had very high total mercury levels, and mercury concentration increased with body size. Selenium levels also increased with body size with significant correlation coefficients being found between the total mercury and selenium in the liver and kidneys, where Se was found in molar excess relative to mercury. However, the opposite was found in muscle tissue where mercury was found in excess of Se (Julshamn, Andersen, Ringdal, & Morkore, 1987). In contrast, selenium and MeHg levels in fish, which was a main constituent of Seychelles study participants, both rise with age thus as MeHg concentration increase so does the Se concentration (Lourdes, Cuvin-Aralar, & Furness, 1991). It has been reported there is no known instance of mercury exceeding Se concentrations in fish (Rayman, 2000). For these reasons, it could be speculated that detrimental effects found in the Faeroes study and not in the Seychelles may be related to the greater availability of Se from fish consumption as opposed to whale consumption. And that the increase incidence of Se in fish is a protective factor in the Seychelles diet that is not available in the Faeroes diet.

The protective effects of Se on MeHg ingestion is supported by a study that reported the effect of freeze-dried swordfish on methyl mercury toxicity in rats. Subjects were separated into two experimental groups consisting of different diets in which either casein or swordfish were mixed with other nutritional ingredients. Both groups were sub-divided in to diets that were either supplemented with or without MeHg at concentrations of 20 and 40ppm.

Both the casein and the swordfish groups that were supplemented with 20ppm MeHg gained weight at a slower and equal rate than the rats that did not receive MeHg. At the ninth week of the study the rats that received casein and 20ppm MeHg started to lose weight while the group fed swordfish and 20ppm MeHg continued to gain weight (though at a slower rate) (Freidman, Eaton, & Carter, 1978). Fatality data given in this study indicates that there was a slower rate of mortality in both the 20 and 40 ppm MeHg/swordfish diet compared with the equivalent MeHg/casein diets. The 40ppm MeHg/swordfish diet group survived 15% longer than the casein group, and the 20ppm casein group recording one death in the ninth week and the 20ppm swordfish group recording no deaths. Neurological signs, which were reported as "crossed rear legs" show a considerable difference between groups. The 40ppm groups (both casein and swordfish) showed similar rates of acquisition of rear leg crossing starting at the third week. The author reports the severity of response was greater in the casein group, however he does not report how that was determined. In the 20ppm groups there was a considerable difference in rear leg crossing with only one animal showing signs by week 10 in the swordfish group while 50% of the animals showed signs, in the same period, in the casein

group. The MeHg and Se analysis of the swordfish diet was report as 1.05ppm (+/- 0.41) for Hg and 2.18ppm (+/- 1.19) Se (Freidman et al., 1978).

There are several problems with this study that should be noted. No definition of rear leg crossing was reported, thus, the severity of the condition is not known. With the exception of the swordfish Hg and Se content analysis no statistical analysis was reported on the data and in the reporting of the graphical data it appears the author has mislabeled graphs 2 and 3. This is surmised from the reported data in the text. Furthermore the dependent variable axis were not labeled, however it is assumed that the scale on mortality (0-100) represents the percentage of subjects that died, and the scale for neurological signs (0-1) represents subjects showing signs.

Summary

From evidence suggested by the studies presented above the following may be surmised. The neurological effects of MeHg ingestion have similarities in humans (Bakir, 1973; Clarkson, 1989; Harada, 1997; Lebel et al., 1998) and rats (Day et al., 2004; Roegge et al., 2004; Sakamoto et al., 2002; Sakamoto et al., 1998). In all cases, both human and animal, motor deficiencies were seen as a result of the ingestion of MeHg. Furthermore, neurological damage observed was in equivalent areas in human and rats brains e.g., pre and post central cerebral cortex (Worobey et al., 2006), the granule cells of the cerebellum (Kurland et al., 1960), diffuse loss of granule cells (Takeuchi et al., 1962) in humans and cerebella cortex resulting from the atypical location of granule and Purkinje cells (Sakamoto et al., 2002), neuronal degeneration in the cerebral neocortex, cerebellum and spinal sensory ganglia (Sakamoto et al., 1998) in rats.

The time course for the onset of the signs of MeHg intoxication have been hard to assess in human populations, however the unfortunate accidental consumption of mercury contaminated seed grain in Iraq allowed data to be collected on the onset of these signs. Signs began to appear within approximately one month in the order of parethesia, ataxia, and dysarthria. Exposure in Iraq lasted from 43 to 58 days and blood concentrations were 500ng/ml to 4000ng/ml. The mean latent period from the end of contamination to the onset of signs ranged from 16 to 32 days. Also, the lowest mercury hair levels that correlated with specific signs were approximately 80 µg Hg/g for parethesia and 130 µg Hg/g for ataxia (Bakir, 1973).

Data for the effects on rats are more specific. Wistar rat pups administered 5mg/kg/day of MeHg had a slower weight gain at day 18 and started losing weight at day 25. Hindlimb cross was observed at day 30 with paralysis of hind legs, piloerection and unsteadiness of gait by day 32 together with the first deaths on the same day (Sakamoto et al., 1998). Day et al., studied rats exposed chronically to MeHg in drinking water at concentrations of 40 and 400 µg/kg/day. There was a significance difference in the proportion of animals in the high exposure group with hindlimb cross after 11, 14, 18,and 30 months and analysis of running wheel performance revealed a main effect of MeHg at 11, 12 and 30 months. Additionally, there was significant difference in the high exposure group, over low exposure and controls, in mean grip strength at 11, 14, and 18 months (Day et al., 2004). Finally, swordfish diet, with a reported content of approximately twice the concentration of Se than Hg appeared to give a protective effect to rats administered 20ppm MeHg in their diet on rear leg crossing and mortality, and at a concentration of

40ppm in their diet appeared to have a delaying effect on the onset of the reported effects (Freidman et al., 1978).

Selenium, an essential trace mineral that has been shown to be important to the brain has a high affinity with mercury (Raymond & Ralston, 2004). (Ralston, Blackwell & Raymond, 2007) (Ralston, Blackwell & Raymond, 2007)

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CHAPTER 2

NEUROBEHAVIORAL CONSEQUENCES OF AGING AND CHRONIC METHYLMERCURY EXPOSURE: INTERACTIONS WITH DIETARY SELENIUM

Abstract

Methylmercury (MeHg) is a well-characterized developmental neurotoxicant but the effects of chronic, low-level, adult-onset exposures are poorly understood. Among the known effects of MeHg are somatosensory deficits and weakness. Selenium (Se), an essential nutrient, may mitigate MeHg's chronic neurotoxicity. Sixty-day-old, female Long Evans were fed a diet containing 0.06 or 0.6 ppm of Se as sodium selenite; both diets provided adequate Se. After 100 days, the subjects consumed methylmercuric chloride in their drinking water in concentrations of 0.0, 0.5, 5.0, or 15.0ppm. These exposures continued for 16 months. Approximately every two months the subjects were tested in running wheels overnight. Forelimb grip, flexion , and hindlimb cross ("clasping reflex") were also examined on a regular basis. Age-related changes were noted in control groups on running, tail sensitivity, and grip strength. MeHg-related effects were noted only in the 5 and 15 ppm exposure groups. The severity and latency to effects were influenced by MeHg dose and dietary selenium. Flexion always appeared before hindlimb cross. In unexposed animals, running increased with age, and the

increase was larger in the high-Se animals. The 5 ppm exposure groups showed less of an increase in running, and Se influenced this effect. Running during young adulthood was a good predictor of running when older, for unexposed animals and age-related correlations were also noted on other measures. MeHg exposure generally weakened or eliminated these correlations, even in the 0.5 ppm exposure group for which no other signs appeared. Dietary selenium afforded a degree of protection against MeHg's effects, largely by delaying their onset.

Introduction

Methylmercury (MeHg) is a well known developmental neurotoxicant (Murata, Grandjean & Dakeishi, 2007; Newland, Donlin, Paletz & Banna, 2006; Trasande, Schechter, Haynes & Landrigan, 2006; Newland, Paletz, and Reed, under review), however studies of chronic, low level adult exposure are sparse. Selenium (Se), an essential nutrient, has numerous physiological roles including antioxidant and anticancer properties, as well as supporting normal thyroid hormone homeostasis, immunity and fertility (Alexander, 2007; De Lorgeril & Salen, 2006; Raymond & Ralston, 2004). Its potential importance to neural function is evident in observations that brain selenium concentrations are aggressively defended, even during severe deficiency (Behne, Pfeifer, Rothlein, & Kyriakopoulos, 2000). Selenium has a high affinity for MeHg, binding it into an insoluble complex that reduces the bioavailabilty of mercury. Thus, Se may protect against MeHg's neurotoxicity but it has also been noted that this may also result in a deficiency of bioavailable selenium (Hill et al., 2003; Ralston, Blackwell & Raymond, 2007), even if joint exposures result in an increase in total brain selenium (Newland et al., 2006).

Chronic, adult-onset exposure to high levels of MeHg produces sensory-motor dysfunction including paresthesias, incoordination, muscular weakness, and ataxia. (Clarkson, 1989; Harada, 1997; Lebel et al., 1998). Postmortem examination of 16 victims of Minamata disease showed varying degrees of pathology and gliosis in the pre- and post-central gyrus and in granular cells of the cerebellum (Kurland, Faro, & Sielder, 1960). In adults, destruction of neuronal cells has been found in the calcarine fissure in

the occipital lobe, the cerebellum and the precentral gyrus (Eto, Tokunaga, Nagashima & Takeuchi, 2002).

Signs and symptoms of MeHg ingestion are notable for the latent period between the onset of exposure and the appearance of signs (Weiss, Clarkson & Simon, 2002) This latent period can be weeks to months, or even years (Kinjo, Higashi, Nakano, Sakamoto & Sakai, 1993). In a large scale, accidental, acute exposure to MeHg in Iraq in the early 1970's parethesia, ataxia, and dysarthria, first appeared approximately one month after exposure (Bakir et al., 1973). Once present, these signs are irreversible, having been described in Minamata victims' decades after exposure ended (Ninomiya et al., 2005; Takaoka, Fujino, Sekikawa & Miyaoka, 2004). This characteristic of chronic MeHg exposure has been reproduced in rodent models using high (Sakamoto et al., 2002; Sakamoto et al., 1998) and more moderate (Day et al., 2004) exposure levels. Characteristic signs include retraction of the hind legs, paralysis of hind legs, piloerection and unsteadiness of gait, which were associated with damage to the cerebral neocortex, neostriatum, red nucleus, brainstem, cerebellum and spinal sensory ganglia (Sakamoto et al., 1998). However, there are no good data on the relationship between chronic low level MeHg ingestion, concentrations of MeHg, time course for the onset of neurological signs, and any beneficial effects Se may have.

It has long been known that selenium confers protection against MeHg's neurotoxicity but these observations have been largely qualitative. The roles of MeHg and Se dose on the degree of protection or latency to the onset of signs have not been investigated until very recently (Friedman, Eaton & Carter, 1978; Iwata, Okamoto & Ohsawa, 1973; Ralston, Blackwell & Raymond, 2007). The potential for protection by

Se against MeHg-induced neurological signs and mortality is evident in a lower rate of mortality in rats fed a MeHg/swordfish diet containing Se compared with rats fed MeHg/casein diets not containing Se (Freidman et al., 1978). Neurological signs reported as "crossed rear legs" also showed a substantial difference between groups.

The present study was designed to examine quantitatively the latency to, and magnitude of, sensorimotor deficits associated with chronic, low-level exposure. The protection conferred by dietary selenium was also examined. Adult rats were provided a diet containing 0.06 or 0.6 ppm (nominally) in selenium content. These are on the low and high end of what is considered appropriate for laboratory rats (National Research Council, 1995). They were also exposed chronically to MeHg in their drinking water in concentrations of 0.0, 0.5, 5.0 or 15.0 ppm. A Se concentration of 0.06ppm Se is the lowest that can be obtained with a casein-based diet (Newland et.al., 1999), while 0.6ppm is at the high end of an adequate non-toxic level (Reeves, Nielsen & Fahey Jr, 1993) The MeHg exposure (with the exception of the 15ppm group) was selected to span a range of exposures that might be characterized as low to moderate (Burbacher, Rodier & Weiss, 1990), however even the high exposure level of 15 ppm was lower than that used in many studies of adult-onset studies (Kinoshita, Ohnishi, Kohshi & Yokota, 1999; Sakamoto et al., 1998).

These exposure constituted a 2 (diet) X 4 (MeHg) design that permitted the detection of main effects of each treatment, protection, as well as natural aging. Dependent measures were selected to test sensorimotor function and to examine signs characteristic of chronic MeHg exposure. These include spontaneous running wheel activity in overnight sessions, forelimb grip strength, tactile sensitivity, hindlimb flexion

and hindlimb cross (sometimes called clasping reflex). Testing was conducted over a 16 month period to track the onset and severity of MeHg's neurotoxicity and the degree of protection by Se.

Methods

Subjects

One hundred thirty one female Long-Evans rats, approximately 60 days of age, were purchased from Harlan Laboratories (Indianapolis, IN). The subjects were housed in polycarbonate shoebox-cages with open wire tops, (2 animals per cage) containing aspen bedding. A Flexan© diagonal barrier separated cage mates, always in the same experimental group. The colony was maintained on a 12-hour light-dark cycle (lights on at 06:00 am) in a temperature and humidity controlled, AAALAC accredited facility. Each subject was implanted subcutaneously with an electronic chip (BioMedic Data Systems Inc, Seaford, DE) containing a unique identification number. Seven days after entering the colony the subjects were assigned to their diets groups and fed ad libitum until body weight reached approximately 240 – 250gms, at which time feed was restricted to maintain this weight. When subjects reached approximately 12 months of age, the animals' body weight was increased to 250 – 260gms. Two selenium diets and four MeHg exposures were produced in a 2 (diet) X 4 (MeHg) full factorial design. There were 16-17 animals in each of the eight experimental groups.

Diet Exposure

Diets, purchased from Research Diets (New Brunswick, NJ), were based on the AIN 93 semi purified formulation but adjusted for selenium content. Seven days after entering the colony subjects were randomly assigned to one of two isocaloric diet groups. The "low" and "high" selenium diets contained 0.06 and 0.6 ppm (nominally) respectively. These were the same casein-based semipurified diets used in Newland, Reed, LeBlanc, & Donlin, (2006), in which selenium concentration ranged from 0.05 to 0.1 ppm in the low Se diet, and from 0.6 and 0.9 ppm in the high Se diet. Selenium intake is estimated to be 2.4µg/kg/day and 24.0µg/kg/day for the low and high diets respectively. Dietary mercury in these diets has been reported below the level of detection of 50 ppb (Newland et al., 2006).

Methylmercury Exposure

MeHg exposure to 0, 0.5, 5, or 15 ppm of Hg as methylmercuric chloride in drinking water began 108 days after arrival in the colony and 100 days after the onset of the diet exposure. The first three concentrations (0, 0.5, 5.0 ppm of Hg as MeHg) have previously been reported as concentrations that produce approximately 0, 40, and 400 µg/kg/day of mercury intake (Newland et al., 2006) and the 15 ppm group likely consumed 1200 µg/kg/day. Subjects from each diet group were randomly assigned to one of the four MeHg groups.

Apparatus and Procedure

Animals were evaluated on a number of markers of neurobehavioral function. The procedures are described below and the timing of the procedures can be seen in Figure 2.1.

Running wheels. Eight running wheels were used for testing. The wheels were 35.5 cm in diameter and 14 cm wide. The floor was constructed of wire mesh to allow the subjects to grip the surface when running. A 21.6 x 11.4 x 11.4 cm chamber containing a water bowl was always accessible. A limit switch recorded each complete revolution. The apparatus sat over a large tray filled with ground corncob bedding. Subjects were divided into groups of eight, counterbalanced across experimental groups, and then randomly assigned to a running wheel. Subjects received their daily ration of food approximately 2-3 hours before the session began. Each running wheel was tested prior to a session. Subjects were placed in the wheels between 3.00pm and 4.00pm on the afternoon they were being tested and allowed free access to the running wheel for 16.5 hours, of which 12 were during their dark cycle, after which they returned to their home cages. It took 17 days to test all 133 rats. The pre-exposure baseline test commenced 56 days before MeHg administration began. The second test sessions commenced 28 days after MeHg administration began and then occurred approximately every two months for 16 months. A total of six tests were conducted (Fig 2.1).

Forelimb grip. A grid approximately 7.7 cm deep by 11.4 cm wide was constructed of #8 steel wire (0.33 cm diameter) consisting of 1.9 cm squares such that it was three squares deep and six squares wide. The grid was attached to an Imada (Northbrook, IL) digital force gauge (model DPS-4) by a small threaded rod so it

projected down at approximately 450 when the gauge was clamped to a standard height table. The gauge was set to record peak force applied during a trial.

An assistant obtained the rat from the colony so as to keep the tester blind to treatment. The rat was held by the tail and torso and allowed to grip the top bar of the grid with its forelimbs. The rat was pulled away in a rapid motion parallel to the floor. The mean of three trials was used for analysis. The same experimenter performed all trials for all test sessions. The first baseline session was conducted 28 days before MeHg administration began. Thereafter, testing was conducted approximately every 10 weeks for 16 months. A total of seven sessions were completed (Fig 2.1).

Tail sensitivity. The same Imada digital force gauge was assembled with a 90^0 chisel point attachment on a 5.7 cm extension. An assistant obtained a subject so as to keep the experimenter blind to the experimental group. The rat was placed on a laboratory cart and gently wrapped in a towel to occlude its vision and exposed its tail. While holding the subject gently but firmly the chisel point was placed on the subjects' tail approximately 5cm from the tip with the axis of the chisel point at 90^0 to the axis of the tail. Pressure was applied to the tail with the force gauge over a period of approximately 1 second. When the subject flinched, flicked its tail, or squeaked, the force gauge was removed. The mean of three readings was used for analysis. If the rat did not react then a force of 2 kg was recorded. Testing followed the same timeline as the forelimb grip test. A total of 7 sessions were conducted (Fig 2.1).

Flexion and hindlimb cross. Tests for flexion (FLX) and hindlimb cross (HLC) were conducted once a week starting 56 days after the start of MeHg administration and continued for the duration of the experiment. The tester, who was blind to the treatment

group, held the subject perpendicular to the floor by the base of its tail for approximately 3 sec and observed the position of the hindlimbs and the manner in which the subject held its paws. For flexion, a zero (normal) was recorded if the hindlimb digits were splayed with toes pointed away from the body. A one (flexion) was recorded if all five hindlimb digits of either or both feet curved inward, as in the form of a clenched fist. Hindlimb cross was recorded with a score of zero (normal) if both hind limbs were splayed outward. A score of one (partial cross) was recorded if the hindlimbs were adducted toward the midline, but not crossed or if one limb crossed the midline. A score of two (full cross) was recorded if both hindlimbs crossed the midline. Flexion and HLC were assessed at the same time.

Within variable correlations. In order to determine whether final performance was related to previous performances in the same measure, within-variable correlations were performed in which the results of the last session were correlated with the results for the previous sessions from the same variable. The results are reported in table 2.2.

Mortality and criteria for euthanasia. Eight subjects died of natural causes unrelated to exposure to MeHg, mostly due to trichobezoars in the gastric tract. Four subjects died of an accidental overdose to pentobarbital or ketamine/xylazene. These injections were given as an anesthesia while biopsy samples were taken from the hind limbs approximately a year into the experiment. All were in the 5.0 ppm exposure group so their death could have been due to sensitivity to these drugs."

All other deaths were due to euthanasia administered when an animal displayed rapid loss of weight, loss of hind-limb function such that it was becoming difficult to eat, or were found in a moribund state.

Data Analysis

Stratified Kaplan-Meier estimates followed by a log-rank test were conducted for the dichotomous measures, mortality, flexion and hindlimb cross. For the analysis of mortality, the independent variable for mortality was age in weeks and subjects that died of causes unrelated to MeHg exposure were right censored. For FLX and HLC analyses, the independent variable was duration of MeHg ingestion until the sign was observed and a subject was right censored if it died before the sign was first detected.

Repeated measures ANOVAs with time of testing as the repeated measure and experimental group as between-subjects measures were conducted for continuous measures: running speed, forelimb grip, and tail sensitivity. Because of the high early mortality rate in the 15.0ppm MeHg group, data were analyzed twice. First, a 4(MeHg) x 2(Se) x 3 (time of testing) factorial design with duration of exposure at the time of each testing session treated as a repeated measure, was used for running and grip strength and a 4(MeHg) x 2(Se) x 4 (time of testing) factorial design was used for tail sensitivity. The data were then re-analyzed without the 15.0ppm MeHg, but with all testing sessions (6 running wheel, 7 forelimb grip strength and 7 tail sensitivity). A log transform was used for forelimb grip and tail sensitivity to stabilize variability across groups. The hypotheses interest were 1) a main effect of exposure duration/age, showing age-related differences, 2) MeHg X time interactions, to show the cumulative effects of MeHg, 3) Se X time interactions to show the effects of dietary selenium, and 4) MeHg X Se X time interactions, to show protection by dietary Se of MeHg's chronic neurotoxicity. Therefore, only the results of repeated measures analyses (main effects of duration and

interactions of duration with exposure) are emphasized. Between-group main effects of MeHg and Se are also documented.

For running, grip strength, and pressure sensitivity, the stability of a subject's performance across test sessions was examined using correlational analyses. Performance on the last session of each variable was correlated with that of previous sessions from the same variable. Correlations across endpoints were also conducted between sessions in which subjects were of comparable age to determine the degree to which these measures provided independent information about neurotoxicity. The 15 ppm-exposed groups were not included in these correlational analyses.

Results

Mortality

Of the original 131 subjects 84 survived to the end of the experiment. Fourteen died of non-MeHg related causes. During the course of the experiment, 36 subjects were euthanized because of advanced MeHg neurotoxicity. Of these, 34 represented the whole population of the 15.0 MeHg group, and 2 came from the low Se 5.0 MeHg group.

The 15.0 MeHg subjects had lower survival rates than the other MeHg exposure groups (log rank test $\chi^2_{(3, 129)} = 195$ $p < .001$). Of this group the high Se subjects survived longer than the low Se subjects ($\chi^2_{(1, 34)} = 19.5$ $p < .001$). Further analysis indicated the 5.0ppm High Se group survived significantly longer than the MeHg equivalent low Se group ($\chi^2_{(1, 33)} = 3.91$ $p = .048$). Mean survival times for the Low Se, 15 MeHg, High Se 15 MeHg, and low Se 5 MeHg exposure groups were 36.6, 52.0, and 89.1 weeks respectively (see figure 2.2).

Flexion and Hindlimb Cross

Table 2.1 presents the mean latency from the onset of MeHg exposure to the appearance of FLX and partial and full HLC. With the exception of one subject in the high Se 0.5 MeHg group, all cases of FLX and HLC only appeared in the two higher MeHg groups. Within these groups FLX always preceded HLC. The high selenium diet delayed the appearance of FLX by approximately 5 weeks in both the 5.0 and 15 ppm exposure groups.

For animals exposed to 5.0 MeHg, FLX appeared about 12 weeks and 20.5 weeks before partial HLC, for the low- and high-Se groups, respectively. For the 15 MeHg group, latency from FLX to partial HLC was less than a week for both diet groups. In all cases, as MeHg exposure levels increased, the period between the appearance of FLX and HLC decreased. Although the high Se groups took longer to show signs of flexion than the low Se groups, full HLC appeared at approximately the same time for both diet groups. Figure 2.3 displays the Kaplan – Meirer survival curves for FLX for the 5.0 and 15.0 MeHg groups separated by diet. The top chart indicates the low Se 5.0 MeHg group had a shorter latency to flexion than the high Se group (39.5, 57.9 weeks) respectively, ($\chi^2_{(1, 28)} = 4.84$ p =.028). In the 15.0ppm MeHg groups (bottom chart) the low Se group had a shorter latency to flexion than the high Se group (9.5, 14.3 weeks), respectively ($\chi^2_{(1, 32)} = 19.7$ p < .001).

The low Se 5.0 MeHg group showed signs of partial and full HLC earlier than the high Se group but the difference due to diet was significant ($\chi^2_{(1, 32)} = 5.18$ $p = .023$) for partial HLC but not for full HLC ($\chi^2_{(1, 32)} = 2.50$ $p = .113$) (see figure 2.4). Similarly, the

low Se 15.0 MeHg group displayed partial and full HLC before the high Se diet subjects. In this exposure group, the low Se was always significantly earlier than the high Se subjects (partial HLC, $\chi^2_{(1, 32)} = 12.1$ $p = .001$), (full HLC, $\chi^2_{(1, 32)} = 15.4$ $p < .001$).

Running

The average distance run during baseline was 1.93 km and was similar for all exposure groups. Figure 2.5 displays the distance run as a percent of the pre-exposure baseline. Repeated measures analysis of all groups over the first 3 sessions revealed a main effect of exposure duration ($F_{(2, 228)} = 17.0$, $p < 0.001$), and an interaction between exposure duration, and MeHg ($F_{(6, 228)} = 16.7$, $p < .001$). Between-groups analyses revealed a main effect of MeHg ($F_{(3, 114)} = 4.7$, $p = .004$).

When the data were analyzed using all sessions (with the 15.0 MeHg group omitted), repeated measures analysis revealed a main effect of exposure duration ($F_{(5, 405)} = 19.2$, $p <.001$). An interaction was found between exposure duration and MeHg ($F_{(10, 405)} = 3.25$, $p < 0.001$). Control animals increased running while exposed animals showed no increase or a decrease, except for the 15.0 MeHg group. An interaction was found between exposure duration and Se ($F_{(5, 405)} = 4.2$, $p = 0.001$). The high Se subjects increased their running over the course of the study while the low Se tended to stay steady or decline. By the last session all high Se groups were running more than the low Se groups. No between-groups main effect of MeHg or Se (in the absence of duration as a factor) was found.

Forelimb Grip

Forelimb grip during baseline sessions was 377gms and did not differ across exposure groups. Figure 2.6 shows forelimb grip as a percent of the baseline session. The first three sessions were analyzed separately, so as to include the 15 MeHg subjects. The data were log-transformed prior to statistical analyses for these sessions. Repeated measures analyses revealed a main effect of exposure duration ($F_{(2, 226)} = 61.1$, $p < 0.001$), and an interaction between exposure duration and MeHg ($F_{(6, 226)} = 15.8$, $p < .001$). There was no interaction of exposure duration and Se ($F_{(2, 226)} = .292$, $p = .747$), but there was an interaction among exposure duration, MeHg and Se ($F_{(6, 226)} = 3.1$, $p = .006$). There was a between-groups main effect of MeHg ($F_{(3, 113)} = 8.89$, $p < .001$, an interaction between MeHg and Se ($F_{(3, 113)} = 2.68$, $p = 0.051$), but no main effect of Se ($F_{(1, 11)} = .006$, $p = 0.974$).

Repeated measures analysis of all the sessions (with the 15.0ppm MeHg group omitted) revealed an effect of exposure duration ($F_{(6, 492)} = 34.4$, $p <. 001$), an interaction between exposure duration and MeHg ($F_{(12, 492)} = 4.12$, $p = 0.001$) and an interaction among exposure duration, MeHg, and Se ($F_{(12, 492)} = 3.51$, $p < 0.001$). No interaction between exposure duration and Se ($F_{(6, 492)} = 2.051$, $p = .058$) was found. Forelimb grip strength declined by about 20% with age in control subjects, and MeHg accelerated this decline, especially in the low Se, 5 ppm MeHg exposure group. Between-groups analyses found no overall main effect of MeHg ($F_{(2, 82)} = 1.08$, $p = .344$) or Se ($F_{(1, 82)} = 3.31$, $p = .073$) and no interaction between them ($F_{(2, 82)} = 1.94$, $p = .150$).
Post-hoc tests for a Se effect in the 5.0 MeHg group revealed that after 23, 31 and 42 weeks of exposure, there was a significant effect of diet on grip strength of the diet

groups; 23 wks ($F_{(1, 31)} = 4.31$, $p = .046$), 31 wks ($F_{(1, 31)} = 6.68$, $p = .015$), and 41.5 wks ($F_{(1, 31)} = 7.67$, $p = .009$).

Tail Sensitivity

Tail sensitivity during baseline sessions was 450gms and did not differ across exposure groups. Figure 2.7 shows tail sensitivity tests as a percent of the baseline session. Repeated measures analysis of the first 4 sessions, which include the 15 MeHg subjects, revealed a main effect of exposure duration ($F_{(3, 312)} = 74.8$, $p < 0.001$), an interaction between exposure duration and MeHg ($F_{(9, 312)} = 14.3$, $p < .001$), no interaction between exposure duration and Se ($F_{(3, 312)} = 1.7$, $p = 0.168$), but an interaction among exposure duration, MeHg and Se ($F_{(9, 312)} = 2.35$, $p = 0.014$). There was a between-groups main effect of MeHg ($F_{(3, 104)} = 7.2$, $p < .001$), but no effect of Se ($F_{(1, 104)} = 2.95$, $p = .089$), and no interaction between MeHg and Se ($F_{(3, 104)} = .778$, $p = .509$).

Repeated measures analysis of all the sessions (with the 15.0 MeHg data omitted) revealed an effect of exposure duration ($F_{(6, 462)} = 75.5$, $p < .001$), an interaction between sessions and MeHg ($F_{(12, 462)} = 4.03$, $p <.001$), no interaction between sessions and Se diets ($F_{(6, 462)} = 1.11$, $p = .356$), but an interaction among exposure duration, MeHg exposure levels and Se diet ($F_{(12, 462)} = 2.11$, $p = 0.016$). The pressure threshold increased by approximately 50% for control animals as they aged. Mercury accelerated this increase, especially in the low selenium group. Between-groups analyses revealed a main effect of MeHg ($F_{(2, 77)} = 5.63$, $p = .005$), no effect of Se diet ($F_{(1, 77)} = 1.49$. $p = .226$), and no interaction between MeHg and Se ($F_{(2, 77)} = 1.65$, $p = 0.198$).

Correlations

For unexposed animals in both selenium groups, running during the last session at 75 weeks of age was highly correlated with running at 32 weeks of age (session 2) and in the high Se group it was correlated with all sessions, indicating that this measure was quite stable and that running during young adulthood was a good predictor of running when older. The low Se 5.0 MeHg groups showed significant correlations from 32 weeks of age while the high Se group showed a similar effect from 44 weeks of age (session 3). The low Se group had higher correlations. Grip strength and tail sensitivity were weakly, and unsignificantly correlated, with that seen during the first sessions. There were sporadic relationships between later sessions and the last session for the exposure groups and the correlations were weaker. The exception is the low Se 5.0 MeHg in grip, which had moderately high significant correlations from 38 weeks of age, (session 3). The high Se group in tail sensitivity also had moderately high significant correlations from 35 weeks (session 3).

Correlation among variables. Correlations were calculated between the sessions across variables when subjects were of comparable age to determine whether the deficits tended to co-occur. Only the 5 ppm exposure group is shown because they showed deficits whose appearance was spread out over a long time. Many correlations were significantly different from zero (Table 2.3). Overall, these dependent measures showed changes in concert. Correlations between running and forelimb grip were found in the low Se subjects from age 29 weeks (running wheels). Significant correlations between running wheel and tail sensitivity were found for the low Se subjects starting at 39 weeks of age. The low Se subjects had significant correlation between forelimb grip strength

and tail sensitivity from 40 weeks, while the high Se animals had significant correlations from 52 weeks.

Discussion

Chronic exposure to MeHg produced sensorimotor deficits in a dose- and time-dependent fashion. For animals consuming 15 ppm of mercury (as MeHg) the signs began to appear after about ten weeks of exposure and nearly all animals showed signs within 20 weeks. The onset was slower and less universal in rats consuming 5 ppm and no neurological signs appeared in lower exposure group or in controls. Selenium generally delayed and sometimes prevented neurotoxicity as expressed in these measures but the pattern of selenium-mercury interactions effects depended upon the daily mercury dose and the dependent variable examined. The selenium concentrations of 0.06 (low) and 0.6ppm (high) in the diets, together with the MeHg concentrations of, 0, 0.5, and 5 ppm were the same as those reported in (Newland, Reed, LeBlanc & Donlin, 2006). In that experiment it was reported that even though brain molar selenium content was elevated in the high-selenium, high-mercury condition, (but not in the low Se 5.0 MeHg group) it still did not bind up all the mercury present. It was suggested that homeostatic systems may draw from a pool of excess selenium to replace unavailable, mercury-bound selenium and this implies that the target of regulation is biologically active selenium, such as that incorporated into selenoproteins (Newland et al; 2006). This would explain why the 5.0 MeHg subjects on the high Se diet still showed MeHg signs even though, by implication from Newland et al; (2006), there was an excess of selenium present in the brain.

The study was conducted until the rats were nearly two years of age so we were able to track the time course of natural aging and the role of selenium and MeHg in this process. Overall, unexposed animals showed greater running but diminished somatosensory function as they aged. For controls, performance during aging was correlated with that as young adults, but MeHg exposure diminished this correlation, even at the lower exposure level of 0.5 ppm that did not produce overt signs.

Running Wheels

Spontaneous running showed an age-related increase in both 0-MeHg exposure groups. All animals on the high Se diet, except those in the 15 ppm group, increased running as they aged and unexposed animals showed a greater increase than those on the low selenium diet. At the last session, the distance run by the high Se group was approximately double their baseline levels and for the low Se animals there was a 33 percent increase. It is not clear at present how high dietary selenium increased running, but it can be noted that exercise promotes the release of reactive oxygen species and thiol groups. Supplementation with Vitamin E and Se (Reddy, Kumar, Prasad & Reddanna, 1998) or dietary Se (Soares, Folmer and Rocha (2003) attenuates this release and enhances exercise.

Selenium attenuated MeHg's effect on running in the 5 and 15 ppm exposure groups. Animals in the 5 ppm exposure group and high dietary Se showed only a modest increase in running and this began to appear at a later age than seen with the lower exposure groups. Animals in the 5 ppm exposure group but low Se showed no increase in running over levels at young adulthood. Animals exposed to 15 ppm showed only

declines in running. Day et al., (2004) using 0, 0.5, and 5 ppm Hg examined running-wheel exposure using a MeHg exposure regimen similar to the current one, but with manipulations of dietary n-3 polyunsaturated fatty acids rather than selenium. In that study, no effect of diet and no interaction between diet and MeHg exposure were found. The 5 ppm, but not the 0.5 ppm, exposure group showed a reduction in running at 12 and 30 months of age, after about 8 and 26 months of MeHg exposure, respectively. There was also an age-related decrease in running at 30 months of age, older than the animals in the present report.

Forelimb Grip

Forelimb grip strength declined for all animals as they aged. Both low and high selenium control groups had similar grip strength throughout the study so selenium did not influence this age-related effect, but it did influence MeHg's effect. Grip strength in the 5 ppm/high Se group did not differ from low-selenium controls throughout the study but the 5 ppm/low Se group decline after approximately16 weeks of MeHg exposure and eventually reached a lower level. The15ppm group showed a rapid decline beginning at about 10 weeks of exposure.

The decline in grip strength in the MeHg groups is consistent with the findings in a study that showed a decline in forelimb grip strength in mice exposed to mercury vapor (Stankovic, 2006). Day et al., (2004), reported that 5.0ppm MeHg weakened forelimb grip in exposed animals at 11 months of age, after seven months of exposure, while in the present study an effect was seen in the low-Se at 16 weeks of exposure. A study undertaken in two villages in Brazil, where gold mining has increased the levels of MeHg

exposure, showed women had a tendency towards reduced grip strength with increased total hair Hg content (Lebel et al., 1996). This data also lends itself to the results found in this study showing MeHg had an effect on grip but that Se ameliorated the effects to some extent.

Tail Sensitivity

All animals showed a gradual decline in tail sensitivity as they aged. This decline was not influenced by dietary selenium but it was accelerated by MeHg exposure, especially in those on the low Se diet as indicated by a Se X diet X age interaction. Animals in the 5.0 MeHg group showed a diminished sensitivity by 16 weeks of exposure and this continued through the remainder of the study. The highest MeHg exposure group showed rapid decline in sensitivity after 10 weeks of exposure with the low Se group requiring almost double the pressure on their tails before they reacted compared to the high Se group. This differential had disappeared by 16 weeks of exposure.

Somatosensory function is mediated by sensory fibers whose cell bodies are in the dorsal root ganglia adjacent to the spinal cord. With adult-onset exposures, MeHg accumulates readily in this structure (Somjen et al., 1973, Chang, 1987) where it induces pathology and apoptosis (Chang, 1987; Wilke, 2003). Selenium can protect against MeHg-induced pathology in the dorsal root ganglia after exposure to a single injection of 2 mg/kg (Chang, 1987) but with chronic exposure to 20 ppm of mercury in drinking water (higher than our highest exposure group) no such protection by Se was noted. MeHg's impairment of somatosensory function may be mediated by this accumulation in

the dorsal root ganglia, and the degree to which selenium confers protection is likely to be dose-related. That is, protection is more likely to appear at lower MeHg doses. A role for the cerebral cortex cannot be ruled out at present, as this region also accumulates MeHg with adult-onset exposure (Ekino, Susa, Ninomiya, Imamura & Kitamura, 2007) and its response to somatosensory stimulation can be affected by systemic inorganic mercury administration (Pecze, Papp & Nagymajtényi, 2004).

Flexion and Hindlimb Cross

Flexion of peripheral joints like wrists and ankles are characteristic signs of adult-onset MeHg exposure. Hind-limb cross (also known as "clasping reflex") has also been noted consistently (Day et al., 2004; Kakita et al., 2000; Kinoshita et al. 1999; Sakamoto et al., 1998), but the exact sequence and time that each sign appears is not well documented. In the present study, flexion preceded partial hind limb cross by about 12 weeks in the 5.0ppm exposure group. Dietary Se delayed the onset of flexion by approximately 5 weeks. After flexion appeared, Se delayed the onset of Partial HLC by nearly 14 weeks in the 5.0 MeHg group and 4 weeks in the 15.0 ppm group. With regard to the progression from partial to full HLC, the high Se group took nearly 3 times longer to exhibit full HLC in the 5.0 MeHg group and twice as long in the 15 MeHg group. Thus, dietary Se delayed the onset of flexion and HLC, and the extent of this delay is smaller at the higher MeHg exposure.

Mortality

Mortality from non-mercury causes was similar for all groups and resembled the control function shown in fig 2.2. Methylmercury increased mortality in a dose-related fashion with all the 15.0 MeHg animals dead by one year of age. Most mortality was by euthanasia due to MeHg neurotoxicity, so in this case the mortality is another indicator of MeHg neurotoxicity. By the end of the study the animals in the Low Se, 5.0 group MeHg animals had approximately 50% survival rate while the 0.5 MeHg animals had better than a 90% rate. The selenium diet increased survival of both the 15.0 and the 5.0 MeHg groups, with mean survival rates in the high selenium group being nearly double that of the low selenium group. A previous study reported that 1 ppm of dietary Se did not mitigate the effects of chronic exposure to1.25mg/kg MeHg /day, (Beyrouty & Chan, 2006). The daily dose is similar to that used in the present study but differences in the route of administration may by important. They administered a daily bolus by gavage while the in the present study exposure was via drinking water. However, in our study Se administration was considerably longer, beginning 100 days before MeHg administration, as compared to 28 days, and continuing throughout the study.

Correlation

In this long-term study, an unexposed animal's performance on several of the tests when it was young was a good predictor of its performance when it was older. Wheel-running showed the highest correlations. The relationship between an animal in session 2 (32 weeks of age) had a correlation of 0.9 with its performance at session 6 (75 weeks of age). This means that animals that ran a long distance when young also ran a

long distance when older. Methylmercury exposure degraded this correlation. For the MeHg-exposed rats, performance in youth was a poorer predictor of performance during aging. Interestingly, this degradation also occurred in the 0.5 ppm exposure groups, for which no other MeHg effect had been noted.

A more muted pattern occurred for forelimb grip strength. The relationship between testing in session 1 and the last session was weaker than that seen with running. This relationship was weakened further by MeHg exposure. consistency seen in the case of the low 5.0 MeHg and this was because they had consistently low grip strength from the third session forward. The high Se 5.0 MeHg animals also showed consistent significant correlations from the third session but these were considerably lower than the low Se subjects.

The tail sensitivity data showed little systematic temporal correlations except in the high Se 5.0 MeHg group toward the end of the study. About half-way through the study the animals that did poorly in this exposure group continued to do poorly with continued MeHg exposure.

Correlations among the different neurological tests were conducted to determine the degree to which the tests used provided independent measures of neurotoxicity. If these tests were highly correlated within an animal then that would indicate that running, grip strength, and pressure sensitivity were different measures of the same or highly similar effects. The correlations among these were modest to high ranging from a magnitude of 0.51 to 0.85. Thus, performance on one test accounted for 25% to 70% of the variabity on another test. The correlations between grip strength and pressure sensitivity, were the highest, suggesting perhaps similar mechanisms. It seems plausible

that somatosensory deficits could have contributed to an early release of the grip strength apparatus, for example. Running and grip strength was correlated approximately the same as grip and tail, with the other measures showing more moderate correlations. It is interesting that these animals evidently continued to run despite experiencing other sensorimotor deficits. The p values are raw and that applying a bonferonni adjustment for multiple comparisons reduced the number of significant differences, but not the overall pattern.

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FIGURE CAPTIONS

Figure 2.1 Timeline showing onset of diet and MeHg exposure and the testing schedule. The text "Diet" indicates when the diet started. The text "MeHg" indicates when MeHg exposure started. White shapes represent the start of a trial; black shapes represent the end of a trial. Where there is only a white shape the trial was completed in one day.

Figure 2.2 Kaplan – Meirer survival curves showing the proportion of animals surviving as a function of age. A * indicates a significant difference from unexposed controls (α = 0.05) from control. # indicates a significant difference between groups exposed to the same MeHg concentration but different diets (α = 0.05) MeHg exposure began at 24 weeks of age.

Figure 2.3 Kaplan – Meirer survival curves showing the proportion of animals not showing flexion. The graphs represent flexion in the low and high Se diet groups for (top) 5.0ppm MeHg group and (bottom) 15.0ppm MeHg group. A # indicates there was a significant difference in the curves (α = 0.05). Note the difference in scales on the X-axis.

Figure 2.4 Kaplan – Meirer survival curves showing the proportion of animals' not showing partial (top) or full (bottom) full HLC. A # indicates a significant effect of diet in the curves (α = 0.05) for animals in the MeHg group

Figure 2.5 Distance run as a percentage of the pre-exposure baseline. Error bars = SEM. Average distance run during the pre-exposure baseline was 1.93 km in a 14.5 hour session.

Figure 2.6 Forelimb Grip as a percentage of the baseline session before MeHg exposure began. Error bars = SEM. Average grip strength was 377 gms during the pre-exposure baseline. A * indicates a significant difference (α = 0.05) for high and low Se diets in same MeHg group for a particular session.

Figure 2.7 Tail sensitivity as a percentage of the baseline session before MeHg exposure commenced. Error bars = SEM. Average sensitivity was 450.7 gm. During the pre-exposure baseline.

Figure 2.1

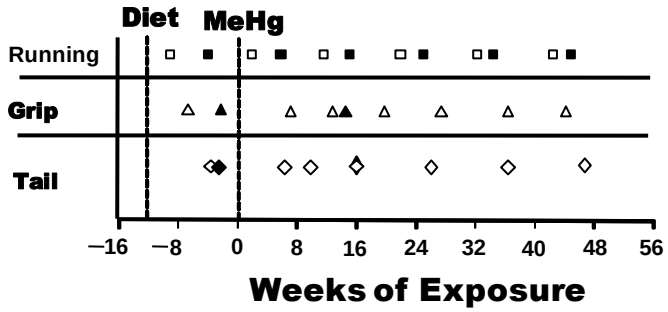
Diet
MeHg
Running
Grip
Tail
−16
−8
0
8
16
24
32
40
48
56
Weeks of Exposure

Figure 2.2

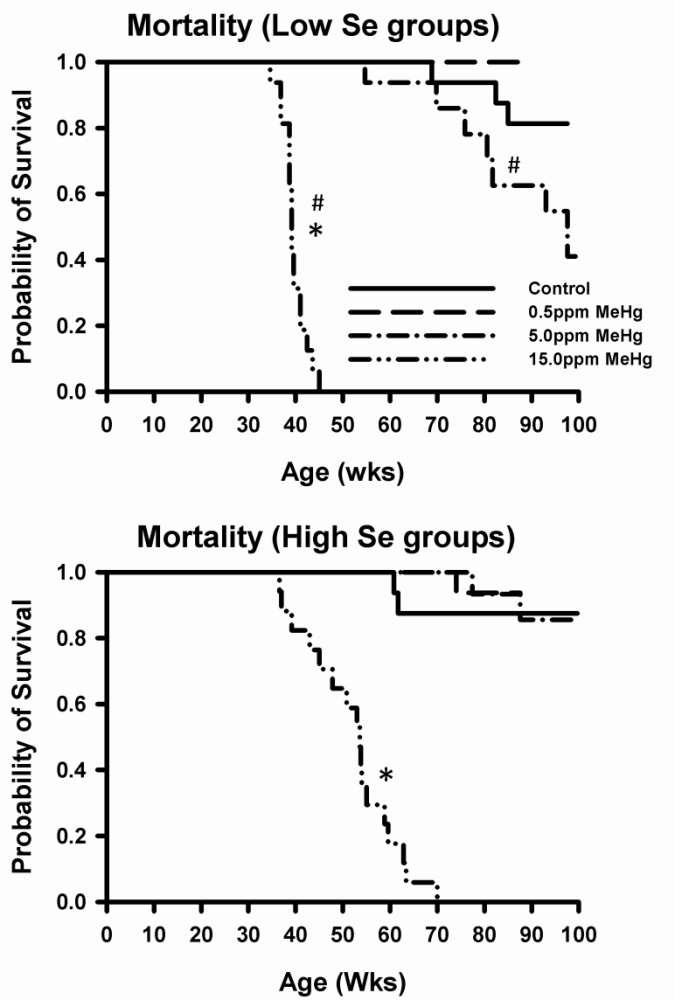
Mortality (Low Se groups)
Probability of Survival
1.0
0.8
0.6
0.4
0.2
0.0
0 10 20 30 40 50 60 70 80 90 100
Age (wks)
#
*
#
Control
0.5ppm MeHg
5.0ppm MeHg
15.0ppm MeHg
Mortality (High Se groups)
Probability of Survival
1.0
0.8
0.6
0.4
0.2
0.0
0 10 20 30 40 50 60 70 80 90 100
Age (Wks)
*

Figure 2.3

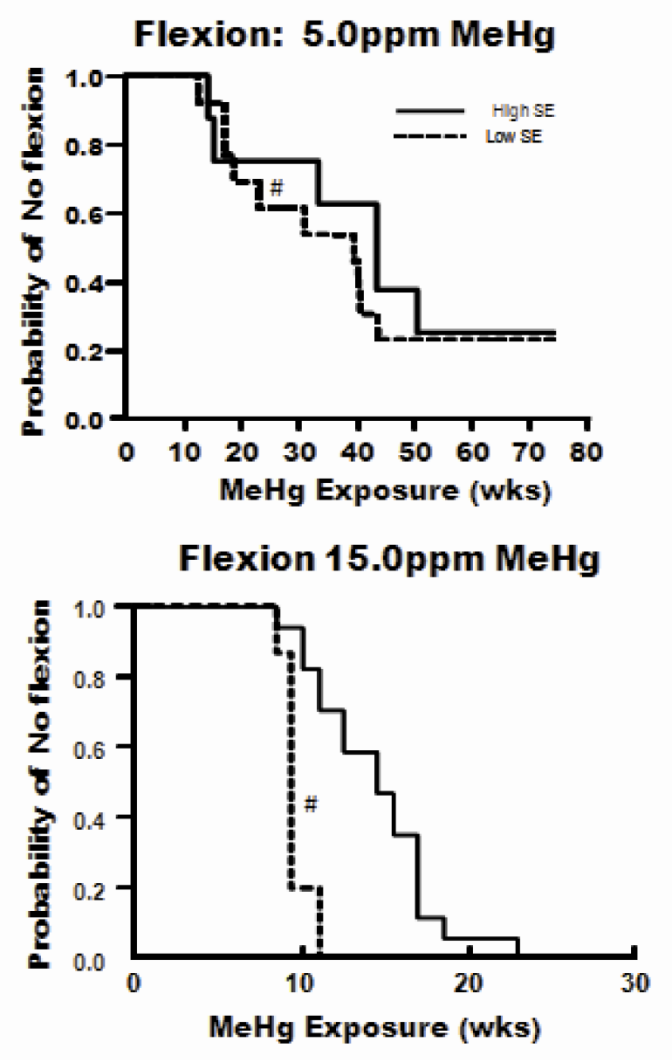
Flexion: 5.0ppm MeHg
Probability of No flexion
1.0
0.8
0.6
0.4
0.2
0.0
High SE
Low SE
#
0 10 20 30 40 50 60 70 80
MeHg Exposure (wks)
Flexion 15.0ppm MeHg
Probability of No flexion
1.0
0.8
0.6
0.4
0.2
0.0
#
0 10 20 30
MeHg Exposure (wks)

Figure 2.4

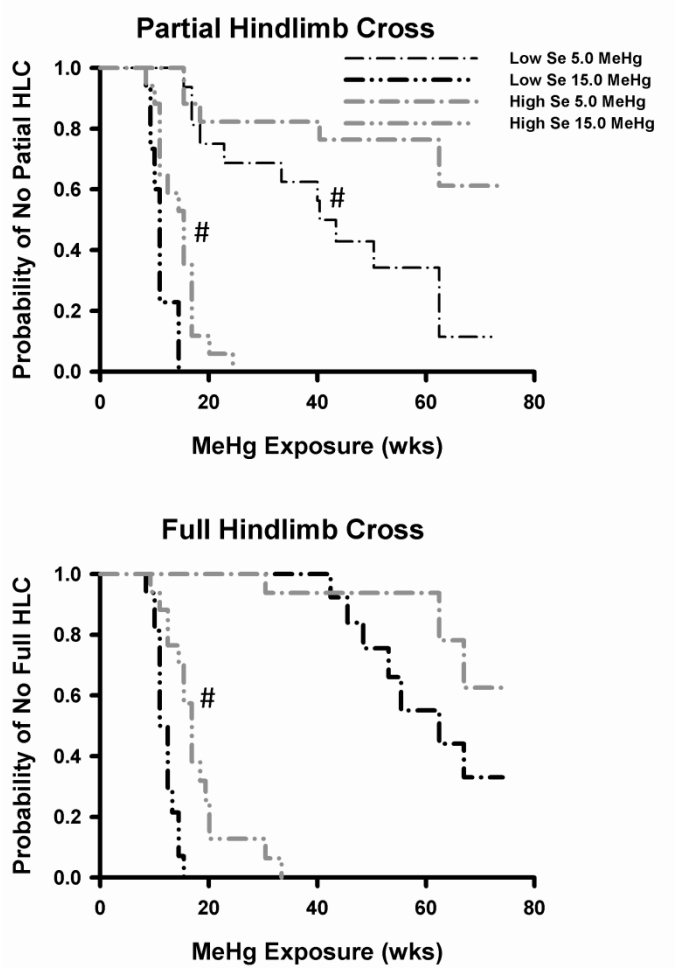
Partial Hindlimb Cross
Low Se 5.0 MeHg
Low Se 15.0 MeHg
High Se 5.0 MeHg
High Se 15.0 MeHg
Probability of No Patial HLC
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
MeHg Exposure (wks)
#
#
Full Hindlimb Cross
Probability of No Full HLC
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
MeHg Exposure (wks)
#

Figure 2.5

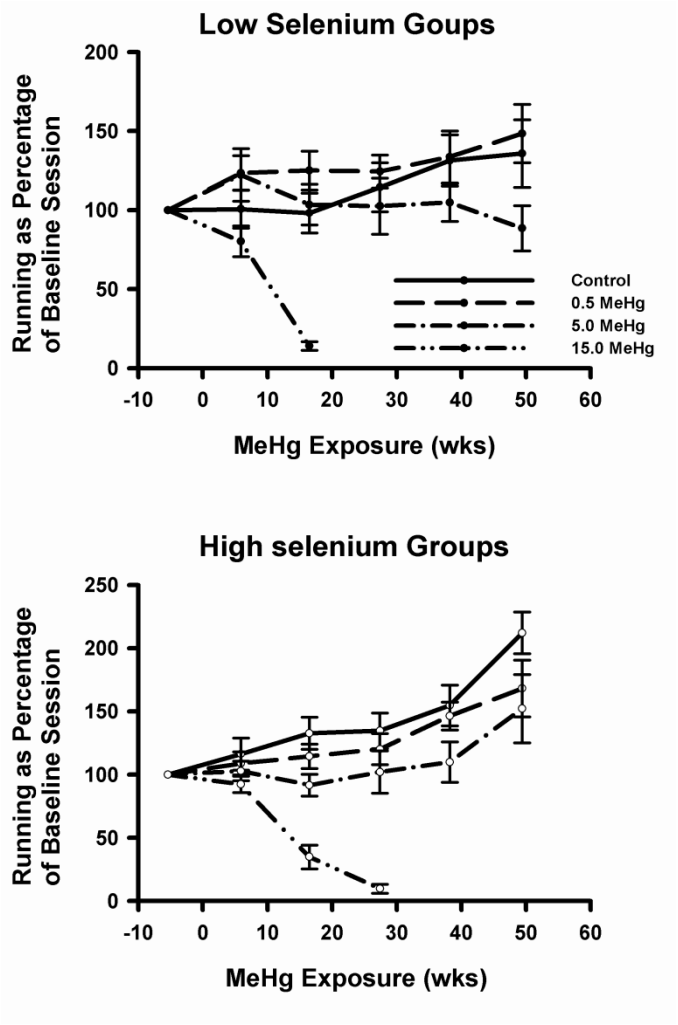
Low Selenium Goups
Running as Percentage of Baseline Session
200
150
100
50
0
-10
0
10
20
30
40
50
60
MeHg Exposure (wks)
Control
0.5 MeHg
5.0 MeHg
15.0 MeHg
High selenium Groups
Running as Percentage of Baseline Session
250
200
150
100
50
0
-10
0
10
20
30
40
50
60
MeHg Exposure (wks)

Figure 2.6

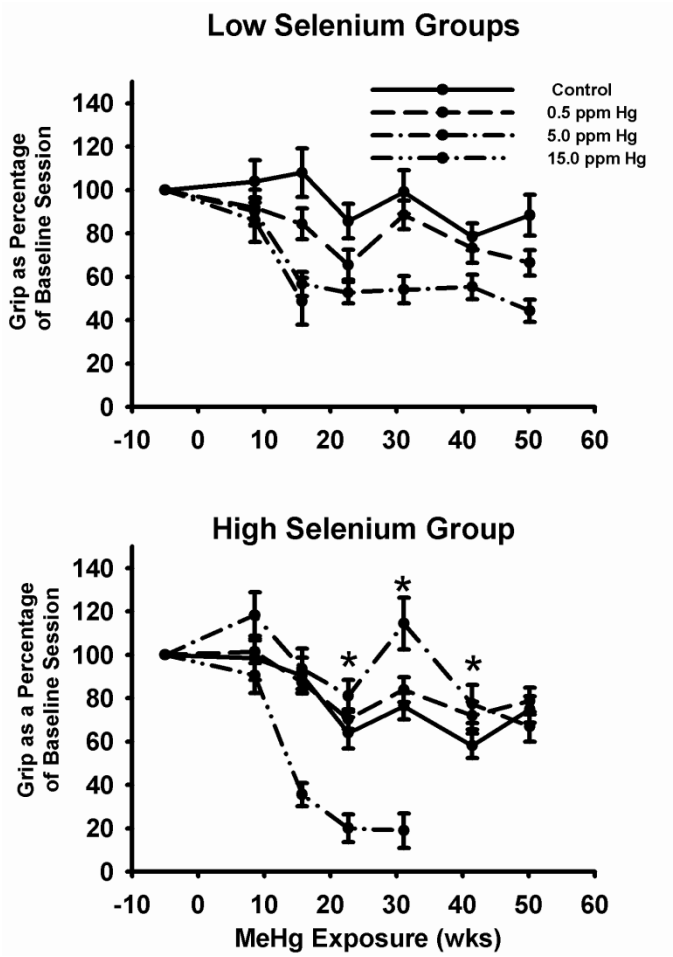
Low Selenium Groups
Control
0.5 ppm Hg
5.0 ppm Hg
15.0 ppm Hg
Grip as Percentage of Baseline Session
140
120
100
80
60
40
20
0
-10
0
10
20
30
40
50
60
High Selenium Group
Grip as a Percentage of Baseline Session
*
*
*
MeHg Exposure (wks)

Figure 2.7

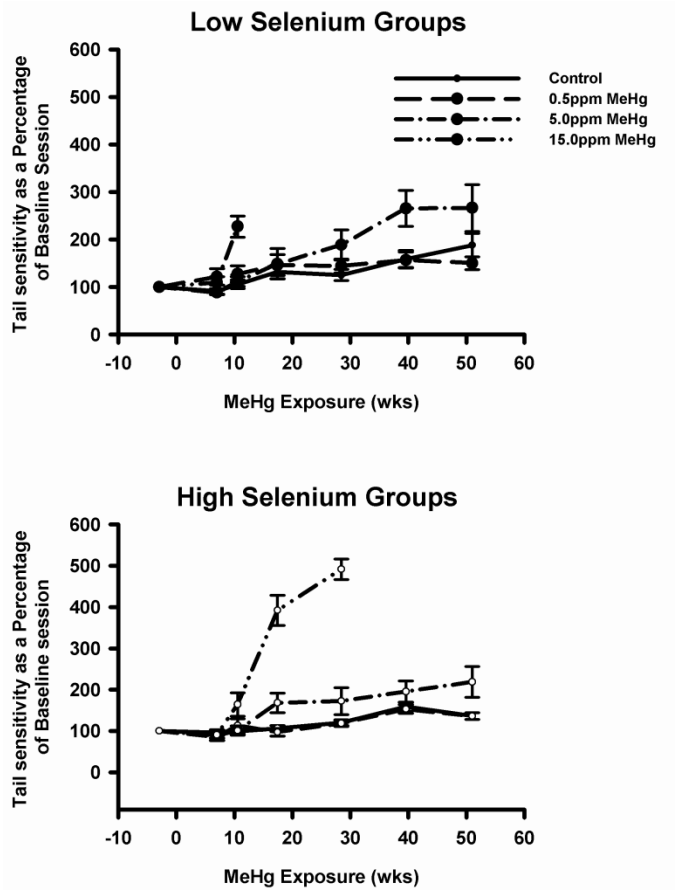
Low Selenium Groups
Tail sensitivity as a Percentage of Baseline Session
600
500
400
300
200
100
0
-10
0
10
20
30
40
50
60
MeHg Exposure (wks)
Control
0.5ppm MeHg
5.0ppm MeHg
15.0ppm MeHg
High Selenium Groups
Tail sensitivity as a Percentage of Baseline session
600
500
400
300
200
100
0
-10
0
10
20
30
40
50
60
MeHg Exposure (wks)

Table 2. 1. Mean weeks to flexion and hindlimb cross

Dietary Selenium (ppm)	MeHg (ppm Hg)	Mean weeks to FLX	Mean weeks to partial HLC	Mean weeks full HLC	Mean weeks from partial to full HLC	Mean weeks from FLX to full HLC
0.06	0.0	~	~	~	~	~
	0.5	~	~	~	~	~
	5.0	28.1	40.0	53.3	13.3	25.2
	15.0	9.5	10.4	11.8	1.4	2.3
0.6	0.0	~	~	~	~	~
	0.5	50.4*	~	~	~	~
	5.0	33.4	53.9	58.8	11	25.4
	15.0	14.3	14.4	17.3	2.9	3

*Only one subject

Table 2. 2. The Pearson correlation coefficients (r) and the significance between the last session of a variable and previous sessions of the same variable separated by experimental groups. * indicates significant to α = .05. ** indicates significant to α= .01. *** indicates significant to α = .001. There is no correlation for session 6 with the running wheels because only six sessions were run due to time constraints

.				Pearson Correlations (r)					
	Dietary Se (ppm)	Subjects in last session	MeHg (ppm Hg)	Session 1	Session 2	Session 3	Session 4	Session 5	Session 6
Running wheel	0.06	15	0.0	0.50	0.90***	0.96***	0.95***	0.92***	`
		15	0.5	0.61	0.50	0.39	0.75**	0.87***	`
		11	5.0	0.46	0.62*	0.94***	0.85**	0.90***	`
	0.6	15	0.0	0.62*	0.89***	0.90***	0.95***	0.93***	`
		16	0.5	0.37	0.66*	0.51*	0.72**	0.76**	`
		15	5.0	0.45	0.44	0.68*	0.69*	0.72*	`
Forelimb Grip	0.06	15	0.0	0.29	0.51	0.60*	0.76***	0.64**	0.51*
		16	0.5	0.23	0.42	0.13	0.31	0.46	0.45
		11	5.0	0.51	0.19	0.77*	0.79*	0.85**	0.86**
	0.6	16	0.0	0.25	0.59*	0.58*	0.45	0.54*	0.78***
		16	0.5	0.01	0.12	0.20	0.42	0.50	0.66**
		15	5.0	-0.03	-0.23	0.56*	0.65**	0.62*	0.59*
Tail Sensitivity	0.06	15	0.0	-0.36	-0.55*	0.26	-0.20	0.53*	-0.05
		15	0.5	0.01	0.10	-0.21	0.03	-0.28	0.11
		11	5.0	-0.16	-0.35	0.67*	0.48	0.49	0.46
	0.6	15	0.0	0.41	0.02	0.05	0.01	-0.02	0.53*
		15	0.5	0.06	0.61*	0.49	0.17	0.48	0.16
		15	5.0	-0.18	0.32	0.56*	0.73**	0.76**	0.84**

Table 2. 3. The Pearson correlation coefficients (r) and the significance between variables when subjects were of comparable age

	Age at testing (wks)		Correlations	
Variables	Run	Grip	Low Se, 5 ppm	High Se, 5 ppm.
Run and Grip	29	32	0.51*	
	40	39	0.63**	0.63**
	52	54	0.72**	
	62	64	0.85***	0.56*
	73	72	0.73**	
Run and Tail	Run	Tail		
	40	39	-0.64**	
	52	52	-0.52*	-0.53*
	62	63	-0.65**	-0.56*
	73	75	-0.74**	
Grip and Tail.	Grip	Tail		
	40	41	-0.49*	
	52	52	-0.78***	-0.81***
	62	63	-0.72**	-0.61*
	73	75	-0.81**	

*indicates significant to $\alpha = .05$ ** indicates significant to $\alpha = .01$
*** indicates significant $\alpha < .001$