THE EFFECTS OF METHYLMERCURIC CHLORIDE EXPOSURE ON IMMEDIATE EARLY GENE INDUCTION IN THE MURINE BRAIN

by

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ABSTRACT OF THE DISSERTATION

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The present set of experiments was designed to examine the effects of dose, route of administration, and frequency of MeHg exposure on activation of neural stress nuclei, altered behavior in the open field and splenic cytokine production. MeHg exposure is associated with gross neuropathological alterations, behavioral deficits, and suppressed function of the immune system. Results from these experiments show that acute IP and ICV exposure of C57BL6J mice to MeHg results in increased recruitment of stress-associated nuclei, as measured by c-Fos immunoreactivity. Acute IP MeHg administration also causes dose-dependent decreases in exploratory behavior. Peripheral stimulation of the gastrointestinal vagus nerve by acute doses of MeHg could be responsible for stimulation of brainstem nuclei and activation of central stress nuclei, as peripheral exposure to LPS in vagotomized mice results in attenuated c-Fos response in limbic and autonomic regions of the murine brain. Proposed malaise-induced alterations in exploratory behavior may also be linked to vagal activation. In Experiment 2, multiple

exposures to MeHg resulted in activation of fewer neural stress nuclei and attenuation of the dose-dependent effects of MeHg on open field behavior noted in Experiment 1. Repeated MeHg treatment followed by exposure to LPS resulted in a significantly increased c-Fos response, demonstrating that the reduction in c-Fos noted in response to repeated MeHg was not due to altered protein synthesis, but more likely due to habituated effects on neurotransmission. The splenic cytokine response to acute MeHg did not significantly affect proinflammatory cytokine production, but did increase IL-2; an effect that could have resulted from T-cell proliferation. Repeated exposure to MeHg increased splenic TNF- α, IL-6 and IL-10 levels, through unknown mechanisms. However, the spleen, which functions to both monitor the circulatory system for immunological stimuli and is the central site for the reticuloendothelial immune system, could be increasing cytokine production through macrophage-induced T-cell activation. Methylmercury has been shown in this thesis to uniquely activate stress-associated nuclei in the murine brain, inhibit exploratory behavior in the open field and increase splenic cytokine production likely due to its documented effects on both the nervous and immune systems.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
ACTH	adrenocorticotropic hormone
AD	Alzheimer's disease
A.D.M.E.	absorption, distribution, metabolism, excretion
ANOVA	analysis of variance
AP	area postrema
AP-1	activator protein-1
ASD	autism spectrum disorder
ATP	adenosine triphosphate
AVP	arginine-vasopressin
BML	blood mercury level
BSTm	medial bed nucleus of the stria terminalis
BSTs	supracapsular bed nucleus of the stria terminalis
Ca^{2+}	divalent calcium cation
CD	cluster of differentiation
CeA	central amygdaloid nucleus
CNS	central nervous system
CRH	corticotrophin-releasing hormone
DA	dopamine
DAB	diaminobenzidine
Dg	dentate gyrus of the hippocampus
DOPAC	dihydroxyphenylacetic acid

ELISA	enzyme-linked immunosorbent assay
GABA	gamma-aminobutyric acid
GI	gastrointestinal
GSH	glutathione
HC	home cage
Hg^{o}	elemental mercury
Hg^{2+}	divalent mercury cation
HgS	mercuric sulfide
HPA	hypothalamic-pituitary-adrenal
HT	hydroxytryptamine
HVA	homovanillic acid
ICV	intracerebroventricular
IEG	immediate early gene
IL	interleukin
IFN	interferon
IP	intraperitoneal
K^{+}	monovalent potassium cation
Lc	locus coeruleus
LiCl	lithium chloride
LPS	lipopolysaccharide
LS	lateral septum
MAP	mitogen-activated protein
MeHg	monomethylmercury

mg/kg	milligrams per kilogram
MS	multiple sclerosis
MSH	melanocyte stimulating hormone
NE	norepinephrine
$NiSO_4$	nickle sulfate
NK	natural killer
NTS	nucleus tractus solitarius
OF	open field
PVA	anterior paraventricular thalamic nucleus
PVN	paraventricular hypothalamic nucleus
ROS	reactive oxygen species
SMART	spontaneous motor activity recording and tracking
SRB	sulfate reducing bacteria
TCA	tricarboxylic acid cycle
TLR	toll-like receptor
TNF	tumor necrosis factor
TUNEL	terminal uridine deoxynucleotidyl transferase dUTP nick end labeling

“Mad Hatter” by John Tenniel

Alle Ding' sind Gift und nichts ohn' Gift; allein die Dosis macht, dass ein Ding kein Gift ist. "All things are poison and nothing is without poison, only the dose permits something not to be poisonous.” - Philip “Paracelsus” von Hohenheim

General Introduction

Mercury and Human Culture

The importance of mercury in human culture originated with a sense of fascination and mysticism, largely because little was known regarding its physical, chemical, and biomedical properties. Elemental mercury has been found adorning Egyptian tombs dating back to 1500 BCE, and around this time the mercury-containing mineral cinnabar (HgS) was used by Hindu and Tibetan people to dye clothing (Goldwater, 1973). Mercury was believed at one time to bring eternal life and in 210 BCE China's first emperor Qin Shi Huang became insane and died after overdosing on mercury-containing pills in an attempt to obtain immortality. Ancient Romans called elemental mercury hydrargyrum meaning "liquid silver" and from which its chemical symbol, Hg, is derived. Additionally, ancient Romans and Greeks used mercury in cosmetics and ointments. Throughout the Dark and Middle Ages mercury was used by alchemists as a "first matter" that when alloyed with other metals was thought would produce more precious metals such as gold and silver.

In the early half of the 16th century Philip "Paracelsus" von Hohenheim, father of modern toxicology, doctor and alchemist, published *On the Miners' Sickness and Other Diseases of Miners*, which documented the occupational hazards associated with mining and metalworking (von Hohenheim & Waite, 1976). Ironically, von Hohenheim while being the first to document the occupational maladies associated with exposure to heavy metals, as a physician, continued to prescribe mercury as a standard treatment for

syphilis, gout, edema, and as a diuretic, laxative, and topical disinfectant (von Hohenheim & Goodrick-Clarke, 1990; von Hohenheim & Jacobi, 1951).

With the onset of the eighteenth century and the Age of Enlightenment came a better documented understanding of mercury's deleterious effects on human health. Bernardino Ramazzini (b.1633 d.1714), described as the father of modern occupational medicine, addressed the perilous consequences of mercury exposure among Italian metal workers and mirror makers in his book *Diseases of Workers* (Ramazzini, 198 3; Buchanan, 1991). Ramazzini describes the severity of mercury exposure on neurological function when he states "mercury is the most cruel of all poisons dealing death and destruction to miners….demons and ghosts are often found to disturb miners. The diseases to which miners and other workers of that sort are exposed are chiefly dyspnoea, phthisis, apoplexy, paralysis, cachexy, swollen feet, loss of teeth, ulcerated gums, pains in the joints, and palsy" (Ramazzini, 1983).

It is during this period and extending through the 19th century that the phrase "mad as a hatter" came to describe the peculiar behavior noted among workers in the felt hat making industry. However, it wasn't until 1860 that John Addison Freeman M.D. of Orange, NJ published "Mercurial Disease among Hatters" in the *Transactions of the Medical Society of New Jersey*, the first literature to link the use of mercuric nitrate during the "carrotting" process of hat making by which fur was separated from the pelt, to the neurological manifestations prevalent among these workers (Freeman, 1860). Epidemiologically, Freeman had noted that 25% of hat makers in northeastern New Jersey exhibited "ulceration of gums, loosening of teeth, abnormal saliva, tremors of the upper extremities, or a shaking palsy,…the result of inhaling air impregnated with

mercury vapor."; this percentage would later rise and include 80% American felt hat makers in 1934 as noted by the U.S. Public Health Service (Neal & Jones, 1937). One year later in 1861, Adolf Kussmaul, a student of Rudolf Virchow and prominent German physician, similarly reported tooth loss, stomacitis, reddening of the pharynx, tremors, and irritability in mercury-poisoned German mirror makers (Kussmaul, 1861).

During the first half of the 20th century, while the United States and several European countries began to appropriate restrictions on the use of mercury containing compounds, other countries had yet to fully appreciate the threat that mercury posed to human health. Effluent from Chisso Corporation's acetaldehyde production in Minamata, Japan containing a mercury sulfate catalyst was released into Minamata Bay from 1932 to at least 1959. The mercury was biomethylated by bacteria to the more toxic methylmercury, which then bioaccumulated up the food chain. As a result of the bioaccumulated methylmercury, the people consuming fish and shell fish from the bay exhibited a spectrum of neurological deficits that included paresthesia in the extremities, ataxia, paralysis, concentric narrowing of the visual field, blurred vision, blindness, dysarthia (slurred speech), difficulty hearing and convulsions, coma, and death in the most severe cases (Takeuchi, 1968). It was also noted that the severity of clinical symptoms increased markedly among the younger population at the time of exposure, with *in utero* exposure resulting in the most deleterious outcomes.

Not long after the Minamata Bay gained world wide attention, an extensive episode of methylmercury poisoning occurred from February to August of 1972 in Iraq. Exposure resulted from consumption of grain seed treated with a mercury-based fungicide that had been intended to be used for planting, but was instead prepared in

home-made breads. Clinical symptoms for adults and children were similar to those exhibited in the Minamata cases. In all, more than 6530 people were hospitalized, with 459 hospital deaths in response to consumption of the mercury-tainted grain (Bakir et al., 1973).

Recently, a well-documented human exposure to a fatal dose of organic mercury occurred when a chemistry professor at Dartmouth College in Hanover, NH was transdermally exposed to as little as 0.44ml of dimethylmercury, which had permeated through the latex gloves she was wearing. This small volume of mercury, due to its remarkably toxic physical and chemical properties, allowed for an estimated total exposure of 1344mg of mercury (Nierenberg et al., 1998). Onset of clinical symptoms occurred at 6months with rapid progression of neurological dysfunction, eventually resulting in a persistent vegetative state, followed by death 298 days after exposure.

While the history of mercury-containing compounds has been punctuated by mysticism and fascination as well as considerable ignorance, contemporary views of mercury recognize its industrial utility amidst its potential role as a ubiquitous environmental toxicant. Today mercury is used in energy-efficient fluorescent light bulbs, high temperature thermometers, sphygmomanometers, barometers, diffusion pumps, neon signs, home thermostats, production of sodium hydroxide, chlorine, and insecticides, as industrial catalysts, and as antiseptic and antifungal agents.

Mercury Bioavailability and Biomethylation

After its release, the chemical species, distribution and bioavailability of mercury plays a significant role in determining the potential for exposure. Mercury derived from

ocean sediments cycles between the ocean and atmosphere, and with the addition of volcanic and human sources, result in increased atmospheric deposition of elemental mercury onto the terrestrial environment. Further, terrestrial soils are considered the most significant direct sources of mercury and methylmercury to terrestrial bodies of water (Mason et al., 1994; Grigal 2002; Bishop et al., 1991). Within aquatic environments inorganic mercury can then be biomethylated, clearing room for more inorganic mercury, and increasing the amount of methylmercury available to bioaccumulate within the food chain (Andren & Nriagu, 1979).

Once inorganic mercury codfmpounds are released and deposited into the environment they may be biomethylated by bacterial species to produce organic mercury. Factors that promote inorganic mercury methylation within the aquatic environment include anoxia, pH of 5-7, low salinity, high redox potential and a dissolved organic matter concentration of 500-2600μM (St. Louis et al., 1996; Miskimmin et al., 1992). Within both lacustrian and oceanic environments, centimeters below the sediment-water interface, where oxygen levels are lowest, pH is highest (due to acid production from degraded organic matter), and redox species are available, are found the sulfate reducing bacteria (SRB) that are most responsible for converting inorganic to organic mercury (Lambertsson & Nilsson, 2006; Jensen & Jernelov, 1969). Methylation of elemental mercury (Hg^{o}) by SRB, such as *Desulfovibrio desulfuricans*, is first dependent on its oxidation to the Hg^{2+} cation via redox reaction in which sulfate groups act as electron acceptors and carbon as the electron donor (Jensen & Jernelov, 1969; Compeau & Bartha, 1985). While the exact mechanism of mercury biomethylation by SRB is currently not well understood, it likely involves donation of the methyl-group from

microbial coenzyme methylcobalamin coupled with pyruvate metabolism (Ekstrom et al., 2003; Branfireun et al., 1999; Ridley et al., 1977). Recent research suggests that Hg^{2+} may increase its permeability by reacting with a sulfide group to form HgS, where it can more readily enter the bacterial cell through passive diffusion across the cell wall as neutrally charged species (Benoit et al., 1999; Jay et al., 2002). Also, predictions of HgS as the major Hg species in sediment-pore water would imply that a major percentage of mercury within the sediment-water interface is available for biologic uptake and methylation (Fitzgerald et al. 2007).

Abiotic conversion of inorganic mercury to organic mercury by reaction of chemical species is currently being explored as an additional pathway for methylmercury production. Chemical methyl-group donors within aquatic sediments include small and large organic molecules such as acetate (Akagi et al., 1973; Falter, 1999), α-methylated organic acids (Falter, 1999), other methylated metals (Howell et al., 1986; Cerrati et al., 1992) and humic compounds (Nagase et al., 1982, Nagase et al., 1984). Experiments have demonstrated that when mercuric cations (Hg^{2+}) are mixed in solution with the cofactor methylcobalamin, methylmercury is formed in the presence of biologically inactive SRBs or in the absence of SRBs (Postgate, 1984; Bertilsson & Neujahr, 1971; Nobumasa et al., 1971).

Methylmercury Bioaccumulation

While biological and chemical processes are both capable of producing methylmercury, how organic mercury is incorporated and bioaccumulated in aquatic organisms is poorly understood. Mercury deposited into aquatic environments enters the

water column with most of the mercury settling and adhering to bottom sediment (DeSimone et al., 1973). Within the water column and sediment, binding of metals to algal (amino, carboxylic, hydroxo, hydroxy-carboxylic, and thio) and bacterial (phosphoryl) cell-surface functional groups has been demonstrated to be a key first step in metal bioaccumulation within natural bodies of water (de Carvalho et al., 2006; Sigg, 1987; Xue et al., 1988). Consumption of these mercury-containing, simple eukaryotic organisms by more complex organisms is thought to be the main mechanism of methylmercury bioaccumulation within the aquatic food chain. Research has demonstrated that methylmercury bioaccumulates in greater concentration as plankton size increases and the amount of consumed plankton is directly related to total levels of bioaccumulated mercury in fish (Coughlin et al., 1983; Kainz et al., 2006; Cabana & Rasmussen, 1994). It was noted that planktonic bioaccumulation of methylmercury is independent of fatty acid/ lipid content of these microorganisms (Coughlin et al., 1983). Methylmercury has been found in all species of fish and its bioaccumulation continues up the marine food-chain resulting in large predatory fish such as tuna, marlin, sailfish, tilefish, shark and king mackerel having the highest concentrations of methylmercury (US EPA, 2006; Barber & Whalin, 1983; US EPA, 2003). Due to the abundance of methylmercury in fish common in the human diet and its inherent toxicity, the United States Environmental Protection Agency has established fish consumption limits for adults and pregnant women, recommending consumption of only 12-20oz of tuna, salmon, pollock, and catfish per week, while advising not to eat shark, swordfish, king mackerel, or tilefish (US EPA, 2003). Mercury levels in these fish range from ~0.12ppm in tuna to 1.45ppm in tilefish (US FDA, 2006).

Methylmercury: Absorption, Distribution, Metabolism, Excretion (A.D.M.E.)

The toxicity associated with methylmercury exposure is dependent on its physical and chemical properties in addition to the underlying A.D.M.E. mechanisms. One general reason why methylmercury elicits such a powerful toxic response is because very few, if any, animal species have found an effective way for metabolizing and excreting this heavy metal.

Methylmercury's unique physical and chemical properties contribute toward its toxicity. Organomercurials, such as methylmercuric chloride and dimethylmercury have extremely high specific gravities (4.06 and 2.96g/ml, respectively) allowing for small volume exposures (in solution) to transfer large, potentially life-threatening, total doses. From the Dartmouth College exposure scientists were able to determine that a severely toxic dose of 100-200mg of mercury would require absorption of only 0.1ml of dimethylmercury solution (Nierenberg et al., 1998). While lipophilicity, as measured by partition coefficient (with two liquids) or reverse phase high pressure liquid chromatography (RP-HPLC) (between a liquid and a solid), certainly plays a role in the A.D.M.E. of methylmercury, absorption may depend more on the molecular weight of the organomercurial; with lower weights resulting in increased intestinal absorption (Endo et al., 1989).

Absorption: The main route of exposure of methylmercury for humans is the gastrointestinal (GI) tract via consumption of contaminated food, primarily as fish and shellfish (Boening, 2000; WHO, 1976). Experiments have demonstrated that doses of methylmercury salts, whether in solution or bound to fish protein, are essentially completely absorbed by the human GI tract, while inorganic mercury bound to food

protein is only 15% absorbed (Clarkson, 1972[A]; Miettinen, 1973). Further, methylmercury uptake in the GI tract occurs as a complex when it is bound to non-protein sulfhydryl compounds, as found in bile (Urano et al., 1990). Methylmercury excreted into the gut lumen bound to biliary salts can be subsequently reabsorbed, resulting in enterohepatic recycling (Norseth & Clarkson, 1971; Norseth, 1973). The oral LD50 for methylmercuric chloride is 29.92 mg/kg in rats, 57.6 in mice (Sigma Aldrich, MSDS), and estimated to be 200 mg (total dose) in humans (Bakir et al., 1973). Mouse studies have demonstrated that oral or intraperitoneal (IP) exposure to methylmercuric chloride results in similar toxicokinetics and identical biological half-lives (Clarkson, 1972[B]; Nielsen, 1992). Therefore, experimentation involving IP administration of mice appears to be a valid approach for simulating oral exposure to methylmercury.

***Distribution*:** While methylmercury has been shown to diffuse across lipid bilayers *in vitro*, *in vivo* it is thought to enter and leave the central nervous system through neutral amino acid transporters complexed to a sulfhydryl-containing ligand, such as cysteine (Lackowicz & Anderson, 1980; Hughes, 1957; Olendorf, 1970; Aschner & Clarkson, 1988). It is believed that the methylmercury-cysteine complex is able to be actively transported across nearly all cell membranes, including the blood brain barrier, due to its structural resemblance to the amino acid methionine (Clarkson, 1987).

In general, organ level methylmercury concentrations have been shown to depend mainly on tissue thiol levels and less on lipid content (Clarkson, 1997). Research has demonstrated that lipophilic methylmercury compounds only traverse across membranes at very high and non-physiologic pHs, while nearly all other studies have shown methylmercury to be associated with water-soluble peptides, proteins or thiol-containing

amino acids (Gage, 1961; US EPA, 1983). Therefore, it can be said that the lipophilicity of methylmercury only marginally helps its distribution, while its high affinity for thiol groups and organ thiol levels determine organ-specific concentrations (Carty & Malone, 1979).

Organ distribution of methylmercury is much more uniform than inorganic mercury species and thus allows blood, brain and kidney levels to remain within a two- or threefold range of each other (Verity and Sarafian, 2000). For example, after acute exposure to methylmercury, humans and mice show the highest mercury levels in blood, kidney, liver and brain (Nierenberg et al., 1998; Mehra & Kanwar, 1980; Mehra & Choi, 1981; Doi & Kobayashi, 1982; Vandewater et al., 1983; Nielsen & Andersen 1991; Nielsen, 1992).

After oral exposure, methylmercur y enters the bloodstream and is primarily bound to hemoglobin with additional binding to intracellular glutathione (GSH) (Naganuma et al., 1980). Single IP doses of methylmercury given to mice result in blood mercury levels that reach peak levels hours after exposure, maintaining these levels through the first 24h and decreasing exponentially thereafter (Doi & Kobayashi, 1982). Mouse studies have further demonstrated that blood and kidney mercury-levels peak within hours of a single injection, while brain mercury levels peak at 2-3 days (Mehra & Choi, 1980; Doi & Kobayashi, 1982; Nielsen, 1992). Additional work has shown that female mice typically have higher mercury levels in brain, muscle, fat, dermis and hair than males, after single, acute oral and IP doses (Nielsen, 1992).

Intrarenal distributions of different organomercurials are similar to one another with higher mercury concentrations in the renal cortex and cells of the proximal

convoluted tubules (Clarkson, 1972). Lower mercury levels are noted in the distal convoluted tubules and loop of Henle with non-detectable levels in the collecting ducts and glomeruli.

After exposure to a single acute dose, methylmercury levels in the liver are highest on day one (typically lower than levels found in the kidney) and decline thereafter (Mehra & Choi, 1980). Within the liver, mercury is found in hepatocytes and Kupffer cells after acute exposure and has similar kinetic half-times across inbred and outbred mouse species (Doi & Kobayashi, 1982).

Methylmercury toxicity is capable of strongly affecting both the central and peripheral nervous systems. Mercury deposition in the human brain after oral exposure is found in all brain regions with largest concentrations noted in the cerebral cortex, calcarine cortex, and central and transverse gyri of the telencephalon, cerebellum, spinal cord and peripheral nerves (Hunter & Russell, 1954; Takeuchi & Eto, 1975, 1977, 1986; Eto, 2000). Mercury levels in the human brain's visual cortex had an average value of 3.1μg/g after acute dimethylmercury exposure (Nierenberg et al., 1998). Direct intracerebral administration of methylmercury in the mouse resulted in preferential accumulation in the cerebellum when compared to the cerebrum, pons/medulla and olfactory lobes (Fair et al., 1987).

Metabolism and Elimination: Metabolism of methylmercury involves its binding to endogenous proteins for subsequent elimination (Goyer & Clarkson, 2001).

Elimination of mercury in human and several animal models has demonstrated first-order kinetics (Nierenberg et al., 1998; Nielsen & Andersen, 1991; Nielsen, 1992). Approximately ninety percent of acutely and chronically administered methylmercury in

humans is excreted in the feces with the remainder eliminated thorough the urine (Miettinen, 1973).The mean whole-body half-life (retention) of methylmercury in humans after a single, acute exposure ranges from 45 to 75 days (Bakir et al., 1973; Nierenberg et al., 1998; Goyer & Clarkson, 2001).

Human levels of methylmercury excreted through the urine after a toxic dose can reach 250µg per 24 hours (normal range of 1 to 5, toxicity at levels >50) (Nierenberg et al., 1998). Use of heavy metal-chelation therapy involving binding of the methylmercury to a hydrophilic compound can result in a dramatic increase in urinary clearance of total mercury (39,800µg/ 24hours). Heavy metal-chelation therapies utilize chemicals with multiple thiol groups, such as 2,3-dimercapto-1-propanesulfonic acid, and have had mixed results in improving clinical symptoms in response to mercury poisoning (Nierenberg et al., 1998; Kosnett, 1992; Aaseth et al., 1995; Clarkson et al., 1981).

Methylmercury Impact on Human Health – Clinical and Epidemiological Findings

Information gathered in response to the methylmercury poisonings of Minamata Bay, Iraq, and Dartmouth College provided fundamental insight into how methylmercury toxicity is clinically manifested in adult humans. Neurologic impairments such as loss of sensation or tingling in extremities (paresthesia), loss of coordinated gait (ataxia), slurred speech (dysarthia), concentric narrowing of visual field, and loss of hearing dominate adult clinical symptoms and typically occur after a latent period of weeks or months after the first exposure (Takeuchi, 1968; Bakir et al., 1973; Nierenberg et al., 1998). More severe methylmercury poisonings result in blindness, deafness, coma and death. Iraqi blood mercury levels (BMLs) correlated well with severity of clinical symptoms. “Less

severe" neurologic symptoms (paresthesia) were found in half of the patients with a BML of 100-1000ng/ml, more severe visual/ hearing/ speaking aberrations were found in ~50% of people with 1000-3000ng/ml BML, with death and coma occurring in patients with 3000-5000ng/ml levels (Bakir et al., 1973).

Nierenberg et al. reported similar findings in the dimethylmercury poisoning of the Dartmouth College chemist. Observations made 154 days after the initial exposure revealed a BML of 4000µg/L that first resulted in upper-extremity dysmetria, dystaxic handwriting, wide gait, and scanning speech approximately four months after the exposure (Nierenberg et al., 1998). Days later these symptoms quickly evolved into paresthesia, flashes of light in both eyes, background noise in both ears, and progressive deterioration speech, vision, hearing and walking days later. Aggressive chelation therapy began 170 days after exposure decreasing the mercury half-life from 74.6 days to ~29 days 10mg/kg orally every eight hours and increasing urinary output of mercury from 257ug to 39,800ug every twenty-four hours. Despite this therapy, twenty-two days (day 176) after development of first neurologic symptoms the patient became unresponsive to all verbal, visual, and light-touch stimulation, which was then followed by spontaneous yawning, moaning, and limb movements. Neuropsychiatric tests revealed marked deficits in all areas. Despite three months of continued chelation therapy, the patient died 298 days after the initial exposure.

Autopsy findings revealed many consistencies between the Minamata and Dartmouth methylmercury poisoning episodes. Acute exposure to methylmercury results in neuronal necrosis, perivascular edema and demylination, swelling of cortical neurons, cytoplasmic eosinophilia, and ischemic aberrations within the human telencephalon

(Takeuchi & Eto, 1975; 1977; 1986; Eto, 2000). Cerebellar alterations in response to methylmercury toxicity involve death of granular and glial cells deep within the cerebellar hemisphere, with little early effect on neighboring Purkinje cells. Also, in patients with acute onset of mercury poisoning followed by long-term survival, secondary degeneration was noted of the pyramidal tracts, internal sagittal stratum and the central portion of the cerebral white matter (Takeuchi & Eto, 1975; 1977; 1986; Eto, 2000). The spinal cord showed secondary Wallerian degeneration of the pyramidal tracts in patients with similar acute onset and long survival. Within the periphery, sensory nerves of the spinal ganglion have been found to be more sensitive to mercury toxicity than motor neurons, although both are affected in response to more severe mercury exposure (Takeuchi & Eto, 1975; 1977; 1986; Eto, 2000). Fibroblastic scar tissue and an increased number of Schwann cells and fibroblasts further suggest incomplete regeneration of these peripheral nerves (Eto, 2000).

Similarly, autopsy of the Dartmouth College chemist noted cortical thinning of the cerebral hemispheres, gliosis of the visual cortex, calcarine fissure and superior temporal gyri (Nierenberg et al., 1998). The cerebellum displayed diffuse atrophy characterized by extensive granule cell, and basket cell loss and gliosis. Broad neuronal atrophy and gliosis were noted in the primary visual and auditory cortices with milder neuronal loss and gliosis within the sensory and motor cortices. The four month lag from time of exposure to dimethylmercury to onset of neurological symptoms is at least partially due to its *in vivo* conversion to monomethylmercury, as toxicity studies have demonstrated that dimethylmercury toxicity is mediated by monomethylmercury metabolites in mice (Ostland, 1969). Conversion of dimethylmercury to the

monomethylated species is a slow process and has been demonstrated by gut flora methyltransferases (Rowland, 1988), and thus may contribute to the prolonged or delayed onset of clinical symptoms.

Experimental Findings – Mechanisms of Methylmercury Neurotoxicity

While the clinical and neuropathological manifestations attest to the effects of methylmercury on the nervous system, experimental research has helped to illuminate the mechanisms underlying its neurotoxic properties. In general, the neurotoxicity associated with methylmercury exposure likely involves several biochemical/ molecular sites of action, with the relevance of each site to neurotoxicity depending on the specifics of the dose, manner of exposure, and underlying metabolic characteristics of the species involved. The combinatorial nature of methylmercury-induced neurotoxicity, as addressed in detail below, would then discount the notion that any single mechanism is responsible for the neurotoxic effects elicited by exposure to methylmercury.

Disruption of protein synthesis is believed to be one of the earliest responses of the nervous system to methylmercury insult (Verity et al., 1977). Reduced protein synthesis in the rat brain after exposure to methylmercury occurs concomitantly with morphological changes in neuronal rough endoplasmic reticulum of the CNS (Miyakawa & Deshimaru, 1969; Chang & Hartmann, 1972) and PNS (Cavanagh & Chen, 1971). Additional studies have shown that inhibition of protein synthesis likely precede the structural alterations noted in cerebellar neuron cell membranes (Brown & Yoshida, 1965). Further, this reduction in protein synthesis occurs independently of RNA synthesis, mitochondrial function and intracellular Na^+/ K^+ levels in cerebral neuron cell

cultures (Sarafian et al., 1984). These results also suggest that methylmercury inhibition of protein synthesis likely occurs by direct interaction with protein synthetic machinery (Miura & Imura, 1987). Cheung and Verity (1985) were able to demonstrate that both *in vivo* and *in vitro* methylmercury exposure directly inhibited the activity of phenylalanyl-tRNA synthetase with subsequent reductions in aminoacylation of tRNA in a manner that was not dependent on initiation, elongation, or ribosomal function. Also, lower cellular ATP/ ADP ratios resulting in defective translation has also been demonstrated (Kuznetzov et al., 1987).

Neurotoxicity induced by methylmercury exposure can also result from intracellular effects on mitochondria and reactive oxygen species (ROS) balance. Methylmercury exposure resulting in reduction of brain mitochondrial respiration rates could occur by increases in potassium (K^+) ion permeability across the inner mitochondrial membrane, potentially via decreasing succinate dehydrogenase levels and in doing so inhibiting the tricarboxylic acid cycle (TCA) (Yoshino et al., 1966; Verity et al., 1975). Methylmercury also causes ATP-dependent and independent reduction in Ca^{2+} uptake and release from preloaded mitochondria, which could result from MeHg affecting the Ca^{2+} uniporter of the inner mitochondrial membrane (Levesque & Atchison, 1991). Additionally, methylmercury has been shown to induce lipidperoxidation and ROS production in regions that are preferentially sensitive to MeHg induced neurotoxicity, with ROS production partially attenuated through use of antioxidants (Sarafian & Verity, 1991; Chang et al., 1978). However, only glutathione (GSH) and the iron-chelator deferoxamine have been shown to reverse cell loss due to oxidative stress following methylmercury exposure (LeBel et al., 1992). Free radical scavenging

compounds such as mannitol, catalase and superoxide dismutase are ineffective at reversing this neurotoxicity (Atchison & Hare, 1994).

Exposure to methylmercury is also capable of strongly affecting mitotic function. *In vitro* studies have demonstrated that methylmercury insult increases the incidence of mitotically-arrested and multinucleated cells (Umeda et al., 1969; Rodier et al., 1984). Electron microscopic analysis of methylmercury exposed mouse glioma cells showed reduced proliferation, increases in mitotic indexes and absence of microtubules as mitotic spindle fibers (Miura et al. 1978). Studies indicate that cell mitosis inhibition occurs through effects on tubulin polymerization, as a result of both inhibition of tubulin polymerization (Imura et al., 1980) and depolymerization of assembled microtubules (Miura et al., 1998). Inhibition of microtubule polymerization and depolymerization of microtubules are both dependent on the ability of methylmercury to bind to tubulin sulfhydryl groups (Kuriyama & Sakai, 1974 and Vogel et al., 1985, respectively). Microtubules appear to be one of the most susceptible intracellular components affected by methylmercury exposure (Miura & Imura, 1987) and disruption of these microtubules is at the root of the effects on cell growth and proliferation. Specifically, inhibition of microtubule reassembly is noted in the mitotic spindle (Miura et al., 1978), while disassembly of microtubes is more associated with metaphase arrest (Sager, 1988) following methylmercury insult. Additionally, effects on microtubules could be leading to faulty neuronal migration and inhibition of axonal elongation, both of which have been noted *in vitro* following methylmercury exposure (Philbert et al., 2000).

Methylmercury has also been demonstrated to enhance DNA transcription and RNA translation *in vitro* of ovalbumin in chicken oviduct cells by partial denaturization

of the mRNA translation complex (Payvar & Schimke, 1979). Supporting the role of methylmercury as a transcriptional modifier, Frenkel and Ducote (1987) showed *in vitro* increases in RNA levels in HeLa cell nuclei and in rat neuroblastoma cells, an effect that was dependent on RNA polymerase II.

The mechanisms of methylmercury-induced neurotoxicity can include effects on synaptic transmission, membrane depolarization and effects on membrane ion channels, which irreversibly affect cell function. Methylmercury (MeHg) has been shown to decrease nerve-evoked release of acetylcholine (ACh) at the neuromuscular junction by first transiently increasing, then blocking postsynaptic end-plate potential (Juang, 1976; Atchison & Narahashi, 1982). The mechanism inhibiting transmitter release by methylmercury is currently unknown. Methylmercury also causes spontaneous release of several central neurotransmitters from brain homogenates (Bondy et al., 1979). Further it is not clear if decreases in neurotransmitter content result from MeHg-induced neurotransmitter release or inhibition of reuptake (Atchison & Hare, 1994). Enzymatic activity associated with synthesis and degradation of neurotransmitters is inhibited by chronic methylmercury exposure (Tsuzuki 1981; Omata et al., 1982). Further, reuptake of divalent calcium ions (Ca^{2+}) into synaptosomes is inhibited in a noncompetitive, irreversible manner by methylmercury (Atchison et al., 1986).

In addition to its effects on synaptic transmission, methylmercury can also directly affect axonal membrane properties and thus conduction of nerve impulses (Shrivastav et al., 1976; Pennock & Goldman, 1972). Increases in intracellular Ca^{2+} could be mediating the methylmercury- induced increases in neurotransmitter release, as reducing the amount of extracellular Ca^{2+} available for uptake reduces neurotransmitter

release (Atchison, 1986). In summary, alterations in Ca^{2+} homeostasis may underlie neurotransmitter disturbances, which could help to explain the neurological symptoms that accompany methylmercury exposure.

Experimental Findings – Methylmercury Effects on Behavior

While the cellular and sub-cellular neurotoxic effects of methylmercury have been demonstrated, how these effects underlie changes in behavior should be addressed. Significant neurobehavioral effects of methylmercury in humans were observed in Minamata Bay, Iraq, and Dartmouth College and included weakness, fatigue, inability to concentrate, with progressive neuropsychiatric deficits in the more severe cases (Takeuchi, 1968; Bakir et al., 1973; Nierenberg et al., 1998). Methylmercury exposure also leads to similar affects on motor function, emotion and arousal, and attention in animal models, as reviewed below.

Methylmercury Effects on Motor Function

Human exposure to large doses of methylmercury can result in marked affects on motor function, often manifested as loss of coordinated gait (ataxia) (Takeuchi, 1968; Bakir et al., 1973). In rodent models, the Rotarod test is a commonly used method for sensitively addressing sensorimotor function; such that animals with impaired coordination fall off the apparatus more quickly than non-impaired animals (Dunhan & Miya, 1957). Deficits in voluntary motor movement are often linked to dysfunction within the primary motor cortex and/or the cerebellum; the later of which is preferentially sensitive to methylmercury-induced neurotoxicity (Franco et al., 2006). Motor

coordination and balance in young mice (post-gestational day 34), administered a single oral dose of 5mg/kg, demonstrate reductions in falling latency on the Rotarod and increased number of falls in the vertical pole test (Bellum et al., 2007). Additionally, mice repeatedly exposed to methylmercury in drinking water over a period of 17 days display marked decreases in falling latency when compared to controls and animals treated with MeHg and the metal chelator 2,3-dimercapto-1-propanesulfonic acid (Carvalho et al., 2007). Thus both single and repeated dosing regimens of methylmercury are capable of affecting motor function in the mouse. Dare et al. (2003) were also able to show that while low-level methylmercury exposure (0.5mg/kg/day) in the rat from day 7 of pregnancy to day 7 of lactation does not significantly affect Rotarod performance on day 20, it does increase locomotion in the open field in response to a low dose of apomorphine, a finding that correlated with a reduction in dopamine receptor binding.

Methylmercury Effects on Exploratory Behavior

Human exposure to methylmercury often leads to observable effects on emotional well-being, frequently characterized by increased feelings of agitation (Likosky et al., 1970) and depression (Takeuchi, 1968). As noted above, methylmercury exposure also induces neuromuscular abnormalities, affecting coordination of gait (Takeuchi, 1968; Bakir et al., 1973). Analysis of open field behavior/ locomotor activity is commonly used in rodent models as a measure of neurological dysfunction, emotion and arousal (Janssen et al., 1960; Crawley, 1983; Weiss et al., 1999). Using this open field model of exploratory behavior, adult mice exposed to methylmercury IP have demonstrated lower frequency and duration of hind limb rearings (Salvaterra et al., 1973; Morganti et al.,

1976). Repeated exposure to methylmercury resulted in a reduction in open field locomotor activity as measured by number of line crossings (Carvalho et al., 2007). Additionally, total dose of mercury and brain mercury levels were both found to be inversely proportional to the number of rearings within an open field design (Morganti et al., 1976). Alterations in cortical and cerebellar glycolytic metabolite levels were also correlated with changes in ambulatory and rearing behavior in adult mice exposed to methylmercury (Salvaterra et al., 1973).

Methylmercury Effects on Attention

In addition to exploratory and motor impairments, the effects on attention following human exposure to methylmercury have been reported. In human studies, mercury hair levels following consumption of methylmercury-containing fish were shown to be directly correlated with a decrease in concentration in performance assessment tests (Yokoo et al., 2003). Also, cohort studies examining neurobehavioral function in 7-year-old Faroe Island children and later at 14 years in the same children revealed deficits in finger tapping speed, reaction time in continued performance tasks and cued naming tests following prenatal methylmercury exposure; an effect that was statistically independent of potential confounders, such as polychlorinated biphenyl exposure (Debes et al., 2006). Blood-mercury levels in children have also been positively correlated with an increased risk of attention-deficit hyperactivity disorder and the accompanying affects on attention (Cheuk & Wong, 2006). Presently there is a paucity of research examining how methylmercury affects attentive behavior in the mouse. Use of a novel object within an open field has been used as a non-specific stimulus to assess

changes in behavior between treatment and control groups (Cheal, 1980). More recently the novel object/ open field paradigm has been used to address measured changes in rodent development of attention behaviors (Cheal, 1987) and attentional deficits following brain damage (Hayne et al., 1992). In this way behavior in the open field was used experimentally within this thesis to assess effects on motor function, exploratory behavior and attention/ arousal following exposure to methylmercury.

The IP dose of methylmercury required to elicit strong behavioral responses in adult mice has been shown from previous acute and repeated studies to be in the low (1-10) mg methylmercury /kg body weight range (Bellum et al., 2007; Morganti et al., 1976; Salvaterra et al., 1973). Given this body of research this thesis research utilized similar mg/kg doses.

The Stress Response and Neurologic Disease

The physiological basis of stress was first elucidated by the American physiologist W.B. Cannon and then later by pathologist/ endocrinologist Hans Selye. Cannon first used the term "homeostasis" to describe "the coordinated physiologic processes which maintain most of the steady states of the organism" (Cannon, 1939). In his now classic book, The Wisdom of the Body, Cannon was able to demonstrate the role of the sympathetic nervous system played in the homeostatic response to hunger, thirst, and perturbations in body temperature, blood pH and sugar levels. Cannon also noted that release of sympathetic adrenal hormones (epinephrine, norepinephrine) following stressful stimuli was associated with both a negative emotional state and disequilibrium of the homeostatic systems noted above (Dougall & Baum, 2001). While still in medical

school Selye astutely noted that “patients suffering from the most diverse diseases exhibit strikingly similar signs and symptoms” leading him to examine “the problem of a stereotyped response to any exacting task” (reviewed in Selye, 1975). Over the course of a lifetime Selye shed a great deal of light on the neuroendocrine response to stress in terms of his “general adaptation syndrome” (GAS). Selye noted that the GAS was a non-specific response to stress, characterized by activation of the hypothalamic-pituitary-adrenal (HPA) axis, production of “syntoxic” (pro-inflammatory) and “catatoxic” (anti-inflammatory) mediators, adrenocortical hypertrophy, atrophy of the spleen, thymus and lymph nodes, and stomach ulcers; all as a non-specific response to this “stress syndrome” (Selye, 1936, 1975). After a lifetime of scientific observation, Selye would ultimately define stress as “the nonspecific response of the organism to any demand made upon it” (Selye, 1971).

While Cannon and Selye’s work addressed the importance of both the nervous and endocrine responses in producing a stress-associated arousal state, other scientists have focused on different physiological and psychological parameters when defining the stress response. In the 1970s JW Mason argued that stressors were capable of affecting normal function of nearly all bodily systems, endocrine responses were stressor specific and that the main function of the stress response was to maintain elevated levels of circulating glucose (Mason, 1971). Around this time Richard Lazarus was developing the “cognitive-mediational theory” of emotion whereby he stated that individuals cognitively assessed the stressor at hand, which then would determine the level of emotion/ arousal (Lazarus, 1966). Lazarus’ idea that cognition initiates the stress response was later disputed by scientists, including Stephen Hobfoll whose “conservation of resources”

stress theory proposed that individuals will respond to real or perceived stress in a manner that minimizes the amount of lost resources (Hobfoll, 1989). More recently Pfaff et al. (2007) have highlighted the overlap between the "generalized arousal" theory and stress. Pfaff et al (2007) define a stressful stimulus as one that "causes release of corticotrophin releasing hormone to a level that raises the release rate of ACTH", noting human and animals that have "higher CNS arousal (i) shows greater responsiveness to stimuli in all sensory modalities; (ii) emits more voluntary motor activity; and (iii) is more reactive emotionally." This paper then states that the relationship between the physiology of stress and arousal are mutually inclusive, where stress always includes some level of arousal but an individual that is aroused may not necessarily be stressed (Pfaff et al., 2007). Additionally, it is noted that both stress and arousal similarly "involve large domains of adequate stimuli characterized by their non-specificity" (Pfaff et al., 2007).

After examining how each of these theories attempts to best define stress, the idea that adaptation and adjustment occur in response to external changes is consistent throughout. Additionally, the mechanisms by which homeostasis is maintained and non-specific activation of the underlying biological framework support these responses are both considered measures of stress (Dougall & Baum, 2001). It is this notion of non-specific activation that will be used when addressing the concept of stress within the context of this thesis.

The human immune system is evolutionarily designed to allow for adaptive responses to physical, chemical and psychological stimuli that result in changes in homeostasis. In recent years, a distinction has been made between stressors that engage

neural circuitry by way of information processing (hence, processive stressors) and systemic stressors, which impact the brain as a function of internal physiologic changes (Herman & Cullinan, 1997). Processive stressors would include exposure to a novel environment, forced swim test or restraint, while examples of systemic stressors would be injection of hypertonic saline, insulin, bacterial antigens or metals such as lead or mercury.

Regulation of glucocorticoid production by the paraventricular nucleus of the hypothalamus (PVN) is crucial in maintaining a controlled response to stress. After stimulation by a stressor, neurons of the PVN secrete a mixture of releasing hormones, including corticotropin-releasing hormone (CRH) and arginine-vasopressin (AVP) into the pituitary portal circulation (Whitnall et al., 1993; Herman & Cullinan, 1997). Activation of the anterior pituitary (adenohypophysis) by CRH results in secretion of corticotrophin (ACTH), which then causes release of glucocorticoids (cortisol in humans; corticosterone in rodents) from the cortex of the adrenal gland. Response to acute stressors induces rapid production of immediate early gene (IEG) expression in catecholaminergic neurons of the brainstem (Cullinan et al., 1995; Sawchenko PE, 1996), suggesting that activation of these cells, as measured by induction of the IEG c-Fos, is involved in the stress responses of the HPA axis (Herman & Cullinan, 1997). Limbic stress nuclei including the PVN (Plotsky et al., 1989), amygdala (Dunn & Whitener, 1986), bed nucleus of the stria terminalis (BST) (Dunn, 1987), and locus coeruleus (Lc) (Smith et al., 1991) are thought to have an HPA-activating role in response to stress, while the hippocampus is implicated as a region functioning to modulate HPA axis activity (Jacobson & Sapolsky, 1991). Several PVN-projecting stress-associated nuclei

have the ability to inhibit HPA axis activation, which include the BST, hypothalamus and preoptic area (Herman and Cullinan 1997).

Improper regulation of stress and its effects on the HPA axis are implicated in the pathogenesis of psychological disorders (post-traumatic stress disorder, anxiety, depression) (Kathol et al., 1989; Charney et al., 1993; McEwen et al., 2004), systemic diseases (colitis, asthma, hypertension) (McEwen & Stellar, 1993) and neurodegenerative diseases such as Alzheimer's disease (Landfield & Eldridge, 1991). This may be due to excessive stimulation of corticotopin-releasing hormone neurons in the PVN (Raadsheer et al., 1994).

Activation of the HPA axis also leads to production of soluble inflammatory mediators, such as cytokines. Cytokines are produced mainly by cells of the immune system (neutrophils, activated monocytic macrophages, lymphocytes, NK cells, Kupffer cells) and also by glial cells of the CNS (microglia, astrocytes) as part of the innate immune response (Rivest, 2001). The immunologic function of a cytokine is typically thought of as pro-inflammatory, anti-inflammatory, or a combination of the two. Cytokines with primarily pro-inflammatory effects include tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and interferon (INF)-γ, while the main anti-inflammatory cytokines would include IL-2, IL-10, and transforming growth factor (TGF)-β. Induction of exogenous cytokines by exposure to the Gram-negative endotoxin lipopolysaccharide (LPS) strongly activates the HPA axis, as well as increasing inflammatory cytokine levels in the brain (Rivest, 2001). Overall, both pro- and anti-inflammatory cytokines are thought to play a key role in the pathogenesis of several neurological diseases including

multiple sclerosis, Alzheimer's disease, AIDS dementia and cerebral ischemia (Correale & Villa, 2004).

Proinflammatory cytokines function to increase the activity of the immune system in response to changes in homeostasis. *TNF-α* is mainly produced by activated macrophages (Miller et al., 1993), and functions to stimulate neutrophil and monocyte recruitment to the site of infection and to activate phagocyte responses. Immune cell production of small amounts of TNF-α has been shown to alter autonomic and endocrine function, to induce fever and HPA axis activation/ CRH release (Cohen, 2002). Large amounts of circulating TNF-α, as produced during Gram-negative bacterial sepsis, can induce a dramatic drop in blood pressure and shock (Rivest 2001). TNF-α is also capable of inducing cell death by the MAPk/JNK and Fas-associated death domain (FADD) pathways (Laflamme & Rivest, 1999). *Interleukin-1* is produced primarily by activated macrophages, monocytes, and dendritic cells. IL-1 functions to alert the hypothalamus of potentially threatening peripheral immunologic stimuli, can induce the production of TNF-α and IL-6 (Steinman, 2004), and facilitates localization of immune cells to a site of injury by up-regulating endothelial cell adhesion molecule expression. Interleukin-1 also plays an important role in modulating immune function during systemic and central inflammatory events. Produced by activated macrophages and T-cells in response to pathogenic foreign molecules, the pleiotropic function of *interleukin-6* includes mediation of fever, lymphocyte differentiation, induction of IL-2 production in T cells and hematopoiesis (Taga & Kishimoto, 1997; Hirano, 1998).

After an acute-phase immunologic stimulation, anti-inflammatory cytokines work to attenuate the resulting endogenous immune response and at times induce a more pro-

inflammatory state. The release of *interleukin-2* by activated T-cells and its subsequent binding to the IL-2 receptor stimulates growth and differentiation of cytotoxic T-cells and NK cells (Steinman, 2004). IL-2 is also utilized in the development of memory T- cells after a challenge to the immune system and facilitates endogenous production of B-cell antibody (immunoglobulin). *Interleukin-10* is expressed in monocytes, mast cells, and activated T- and B-cells and when released from cytotoxic T-cells inhibits NK cell activity. Further, IL-10 is capable of inhibiting antigen presentation and synthesis of the proinflammatory actions of TNF-α, INF-γ, and IL-2 (Steinman, 2004).

It evident from the foregoing that the physiological response to stress is a dynamic process of complex interactions between hormones, neurotransmitters, cytokines and immediate early genes. Given the detrimental effect that methylmercury has on neurological function and that increases in cytokines levels and over activation of stress-associated brain nuclei have both been associated with several neurological diseases, it would then be of interest to examine how methylmercury affects activation of neural stress-circuitry, behavior and cytokine production. Measurement of immediate early gene levels and the levels of their protein products are common methods for examining activation of neuroanatomical nuclei following physiological (Morgan et al., 1987; Herrera and Robertson, 1996) or behavioral (Matsuda et al., 1996 and Miczek et al., 1999) stressors. Analysis of open field behavior has been used extensively as a model for measurement of motor function (reviewed in Walsh & Cummins, 1976), exploratory behavior (Janssen et al., 1960; Crawley, 1985) and in attention/ arousal paradigms (Cheal, 1987; Hayne et al., 1992). Also, alterations in measures such as number and duration of hind limb rearings within the open field have been noted in adult mice

exposed to methylmercury (Salvaterra et al., 1973; Morganti et al., 1976). The experimental focus of this thesis was to characterize the open field behavior and the immediate early gene response of stress-implicated brain regions following methylmercury exposure.

Rationale for Thesis Research

Methylmercury is a well-documented and potent neurotoxicant that is of immediate relevance to human health. Biogeochemical accumulation of mercury compounds in our bodies of water and the persistence of methylmercury in seafood warrants better understanding of how these compounds are affecting neurologic function. Similarly, activation of stress-associated brain nuclei of the limbic system and the downstream production of inflammatory cytokines has been implicated in the etiology of many neurodegenerative and neuropsychiatric diseases.

There is little question that exposure to environmental toxicants, such as methylmercury, disrupts homeostasis and may be viewed as a systemic stressor. This may impact the brain and recruit similar neural pathways as other systemic stressors. Given that over stimulation of central neural stress circuits has been associated with several neurological diseases (AD, PTSD, depression, anxiety) and the well-established ability of methylmercury to induce severe neurological dysfunction, it would be of interest to determine if methylmercury is capable of activating these same neural circuits and if this activation occurs along-side functional changes in behavior. Additionally, the persistence of methylmercury in fish species common to human consumption, would lead one to wonder if acute or repeated exposure to methylmercury is capable of consistently

activating neural circuitry and if so could this be contributing to the etiology or severity of these neurological diseases? However, no information is presently available on the extent to which acute or repeated methylmercury exposure engages recognized neuronal pathways involved in the stress response. Methylmercury exposure has also been associated with decreases in open field exploratory behavior, which is most often measured in terms of ambulatory activity (Dare et al., 2003) and/ or time spent within particular zones of the open field (Ivinskis, 1970). In the present thesis, this question was addressed using either an acute peripheral (intraperitoneal-IP), acute central (intracerebroventricular-ICV) dose of methylmercury or repeated IP exposure to lower doses of methylmercury. In addition, the degree to which acute and repeated IP exposures to methylmercury has c-fos induction in addition the interaction with a psychogenic stressor was also determined. That is will exposure to both methylmercury and open field psychological stress affect c-Fos levels in manner that is based on the interaction of these two experimental conditions, but which is significantly different from either condition alone. Finally, the impact of repeated MeHg exposure on the subsequent activation of c-fos by lipopolysaccharide was examined to determine if pretreatment with methylmercury was capable of sensitizing or desensitizing the response to lipopolysaccharide, a strong immunological stimulus and c-fos activator. If previous exposure to methylmercury were to heighten activity of neural stress nuclei to subsequent stressor, this would further expand the potential for methylmercury to deleteriously affect neurological diseases associated with over active stress circuitry.

SPECIFIC AIMS

Specific Aim One: **To determine if acute intraperitoneal and intracerebroventricular exposure to methylmercuric chloride (2-8 mg/kg) induces production of the immediate early gene protein product c-Fos within stress-associated brain nuclei and effects on open field behavior in the mouse. Also, differences in c-Fos production should be examined when comparing intraperitoneal and intracerebroventricular routes of administration.**

Methylmercury is a toxicant that inhibits mitotic function (Miura et al., 1978; Rodier et al., 1984), protein synthesis (Verity et al., 1977), ATP production (Yoshino et al., 1966), proper neurotransmitter system function (Bondy et al., 1979; Atchison & Narahashi, 1982) and hence affects the fundamental ability of an organism to maintain homeostatis. Due to the multifactorial nature of methylmercury toxicity, one could hypothesize that methylmercury exposure will act as a physiological stimulus, activating stress nuclei and increasing c-Fos positive cell counts in the murine brain.

The route of administration for methylmercury will also be examined. The most common route of exposure for methylmercury in humans is mainly through the gut by consumption of aquatic animals (US EPA, 2006). Experimentally, intraperitoneal and oral exposure in the mouse results in very similar toxicokinetic profiles for methylmercury accumulation in the brain (Nielsen, 1992). However, communication of information from the periphery to the central nervous system, resulting in activation of neural stress nuclei, has been demonstrated (Cohen, 2002). Therefore to determine if and

to what extent peripheral processes may play in activation of central stress nuclei, methylmercury should be administered both peripherally (intraperitoneal) and centrally (intracerebroventricular). Indeed production of c-Fos protein in response to the physiological stressor lipopolysaccharide has been demonstrated following both peripheral and central routes of exposure (Wan et al., 1993). Therefore, one could certainly postulate that methylmercury given centrally or peripherally will activate and induce c-Fos production within stress-associated nuclei of the brain.

Given that methylmercury exposure within the 1-10 mg/kg range has demonstrated decreases in motor function (Bellum et al., 2007) and hind limb splay (Salvaterra et al., 1973) in mice, with attention deficits in humans (Yokoo et al., 2003; Debes et al., 2006), it would be plausible to conclude that effects of methylmercury on total distance traveled and time spent exploring will likely also be inhibited.

Specific Aim Two: To examine how repeated intraperitoneal exposure (2-4 mg/kg) to methylmercuric chloride engages neural stress nuclei, as measured by induction of c-Fos, and affects behavior in the open field.

Repeated exposure to methylmercury has been demonstrated to result in severe neurological dysfunction in both humans (Takeuchi, 1968; Bakir et al., 1973; Likosky et al., 1970) and rodents (Berglund et al., 1971; Mehra & Choi, 1981). In humans, this neurological dysfunction is characterized by an increase in the frequency of patients experiencing paresthesia, ataxia, coma and/ or death (Bakir et al., 1973). In mice, repeated exposure to methylmercury has been demonstrated to result in more severe neurodegeneration in several stress-associated brain nuclei (thalamus, hypothalamus, and

cortex) (Berthoud et al., 1976). Given that repeated administration of methylmercury elicits more severe effects on the central nervous system than acute/ lower-level exposures, it remains a homeostatic threat, and as such will likely continue to activate brain stress nuclei, induce production of c-Fos protein within these areas and inhibit exploratory behavior.

Repeated administration of methylmercury has also been demonstrated to reduce the frequency and duration (Morganti et al., 1976) of hind limb rearings in the open field; with duration inversely proportionate to brain mercury levels (Morganti et al., 1976). Repeated exposure to methylmercury, within the constraints of this thesis, would then be hypothesized to again inhibit locomotory behavior within the open field.

Specific Aim Three: To determine how repeated pre-treatment with methylmercury (2-4 mg/kg) affects the ability of neural stress nuclei to become active following exposure to the established physiological stressor and c-Fos inducer, lipopolysaccharide.

Over the course of an individual's life it is not uncommon to experience several different stressors at one particular time or one after the other. However, while repeated exposure to methylmercury has been used experimentally to examine how neurological dysfunction in humans may be occurring (Takeuchi, 1968; Bakir et al., 1973; Eto, 2000), there is very little understanding as to how pre-exposure to methylmercury may affect the ability of these neural stress nuclei to respond to a subsequent and different physiological stressor.

Given the ability of methylmercury to inhibit protein synthesis, it is possible that repeated exposure to methylmercury could inhibit the ability of these stress-associated nuclei to produce c-Fos. Treatment with lipopolysaccharide, a well characterized immunologic stimulus (Cohen, 2002) and c-Fos inducer (Lenczowski et al., 1998), and LPS administration following pre-treatment with methylmercury would allow one to determine if methylmercury is capable of inhibiting c-Fos protein production within stress nuclei of the brain. Within this scenario it would be plausible to hypothesize that pretreatment with methylmercury would limit the c-Fos response to a subsequent exposure of lipopolysaccharide.

Specific Aim Four: To examine the pro- and anti-inflammatory cytokine response of the spleen following acute and repeated intraperitoneal methylmercury exposure.

Most of this thesis focuses on the central c-Fos response to peripheral injection of methylmercury. However, methylmercury can influence peripheral cytokine levels and peripheral administration of these same cytokines can induce c-Fos production in the brain (Tolchard et al., 1996; Rivest et al., 1992; Konsman et al., 2000). In this way methylmercury administration could activate these stress-associated brain nuclei through peripheral cytokine induction. The final study was conducted to measure pro- and anti-inflammatory cytokine production in the spleen following acute and repeated intraperitoneal methylmercury exposure.

Experiment 1

Effect of Acute Methylmercury Exposure on Activation of Neural Stress Circuitry and Open Field Exploratory Behavior

2.1 - Introduction

The deleterious effects elicited by exposure to methylmercury on the mammalian nervous system are pronounced and wide-ranging. Histopathological assessments from human methylmercury studies show cortical thinning, neuronal necrosis, gliosis of the visual cortex, calcarine fissure and superior temporal gyri, perivascular edema, demylination, Wallerian degeneration of spinal chord pyramidal tracts, and peripheral fibroblastic and Schwann cell hypertrophy (Takeuchi & Eto, 1975; 1977; 1986; Eto, 2000; Nierenberg et al., 1998). The subcellular effects of methylmercury include inhibition of protein synthesis (Yosino et al., 1966; Verity et al., 1977), attenuation of energy production through mitochondrial dysfunction (Verity et al., 1975), and disassembly of mitotic spindles and induction of mitotic arrest (Miura et al., 1978). Further effects on neurophysiological function consist of perturbations in Ca^{2+} homeostasis and neuronal death (Limke et al., 2003), alterations in mitochondrial membrane potential (Insug et al., 1997), disruption of ROS balance by increases in pro-oxidative species (Usuki et al., 2001), and impairment of glutamate uptake (Aschner, 1996).

Methylmercury has also been demonstrated to significantly influence neurobehavioral function. Cases of human exposure to methylmercury frequently involve changes in behavior and neurological function that include paresthesia, dysarthia,

spontaneous yawning, moaning, and limb movements and the inability to concentrate (Takeuchi, 1968; Bakir et al., 1973; Nierenberg et al., 1998). Behavioral effects in adult humans after exposure to methylmercury are characterized by impaired locomotory function/ uncoordinated gait (Takeuchi, 1968; Bakir et al., 1973), feelings of agitation (Likosky et al., 1970), depression (Takeuchi et al., 1968) and decreased ability to concentrate (Yokoo et al., 2003). Progressive and dramatic neuropsychiatric deficits are noted in the more severe cases (Nierenberg et al., 1998). In response to low-level methylmercury exposures in children, effects involved deficits in language, attention, and memory (Grandjean et al., 1997). In animal studies, open field behavior of adult mice given methylmercury displays a reduction in number and duration of hind limb rearings within the open field (Morganti et al., 1975; Salvaterra et al., 1973). Further, prenatal exposure to methylmercury has been demonstrated to limit open field exploratory movement, impair working memory (T maze) and motor function (hind limb splay) (Kim et al., 2000; Dore et al., 2001; Goulet et al., 2003). As noted previously, Salvaterra et al. (1973) demonstrated that changes in open field ambulatory and rearing behavior were correlated with alterations in cortical and cerebellar glycolytic metabolite levels, demonstrating that effects of methylmercury on subcellular processes could be underlying alterations in exploratory behavior.

In recent years, activation of neural stress circuitry and subsequent activation of the hypothalamic-pituitary-adrenal (HPA) axis has been implicated in the etiology and progression neurodegenerative conditions such as Alzheimer's disease (Landfield & Eldridge, 1991) and the neuropsychiatric conditions, post-traumatic stress disorder, anxiety, depression (Kathol et al., 1989; Charney et al., 1993; McEwen et al., 2004).

Also, increased production of inflammatory cytokines is also believed to contribute to neurologic dysfunction (Correale & Villa, 2004).

The most commonly used tool for neuroanatomical mapping of the stress response is expression of immediate early genes (IEG), and in particular, detection of the *c-fos* gene and its protein product c-Fos (Morgan et al., 1987; Herrera and Robertson, 1996). Of particular importance is that neuronal c-Fos production is elevated by social stressors and fearful and/or novel stimuli (Matsuda et al., 1996 and Miczek et al., 1999), which then allows for determination of important regions of stressor-related information processing in response to the stressor. Additionally, open field exposure has been demonstrated to act as a psychogenic stimulus capable of activating the brain's stress circuitry and increasing production of c-Fos protein (Herman et al., 1998; Rossi-George et al., 2005; Badowska-Szalewska et al., 2006; Coryell et al., 2007). Following upregulation, a rapid drop in c-Fos protein levels occurs through both protein degradation and post-translational modifications, thus contributing to its transient activation profile (Salvat et al., 1998; Bossis et al., 2005).

Exposure to methylmercury has been demonstrated to elicit a range of effects on the nervous system, all of which are capable of disrupting homeostasis. However, there is a paucity of research addressing the extent to which methylmercury is capable of activating the brain's stress circuitry. Determination of methylmercury's ability to activate underlying neural stress circuitry is of immediate relevance in that its potential as a physiological stressor may affect the ability of neural stress circuits to function properly. As stated previously, dysfunction of these brain regions is implicated in neurodegenerative (Landfield & Eldridge, 1991) and systemic (McEwen & Stellar, 1993)

diseases as well as neuropsychiatric disorders (Kathol et al., 1989; Charney et al., 1993; McEwen et al., 2004). Given that the open field can be utilized to measure locomotory/ exploratory behavior and attention/ arousal, each of which are affected by exposure to methylmercury in humans, it was therefore believed that open field analysis would provide this thesis with a relevant behavioral measure following methylmercury treatment. It was also noted in the introduction that several experiments have demonstrated alterations in behavior in the open field following exposure to slightly different methylmercury doses and/ or dosing regimes than proposed for use in this thesis (Salvaterra et al., 1973; Morganti et al., 1976; Kim et al., 2000; Dore et al., 2001; Goulet et al., 2003). Salome et al. (2004) were able to show that rats bred to exhibit high-anxiety related behaviors had greater c-Fos induction in the hypothalamus, which was correlated with higher plasma ACTH levels and shorter distances traveled within the open field when. Currently there is little information available regarding how time spent exploring distinct inner and peripheral zones and locomotor measurement of total distances traveled is affected by an acute dose of methylmercury. This experiment will therefore examine the extent to which a single acute dose of methylmercury activates the brain's stress circuitry and affect behavior in the open field.

2.2 - Materials and Methods

2.21 - Animals

Six-week-old C57BL/6J male mice (Jackson Labs, Bar Harbor, ME) were housed (4/cage) on a 12h light/12h dark cycle (lights on 0700h), with *ad libitum* access to food

and water. Upon arrival mice were acclimated for at least one week prior to experimentation. Six week old mice were chosen on the basis of previous research that found this age to show sufficient sexual and neurological maturity for studying the effects of methylmercury exposure in mice (Doi and Kobayashi, 1982; Yasutake et al., 1990; Nielsen, 1992; Adachi et al., 1994).

Mice were utilized specifically for this *in vivo* study because, unlike rats, they have kinetic A.D.M.E. (Absorption, Distribution, Metabolism, and Excretion) profiles that are more similar to that of humans (Berglund et al., 1971; Nielsen & Andersen 1991; Nielsen 1992). The C57BL/6 strain was specifically chosen as they are often used in methylmercury studies and have been shown to be adequately responsive to lower to mid-level methylmercury doses (up to 8mg/kg) (Mehra & Choi, 1980; Doi & Kobayahsi et al., 1982; Nielsen & Andersen, 1991; Yasutake et al., 1991; Adachi et al., 1994; Yee and Choi, 1994). All procedures were approved by the Rutgers Institutional Animal Care and Use Committee.

2.2.2 - Acute Intraperitoneal Methylmercury Experimental Procedure

Mice were administered 2, 4 or 8mg/kg methylmercuric chloride (MeHg) (Sigma, St. Louis, MO) or 0.9% saline in a volume of 0.2 ml by intraparitoneal (IP) injection and subsequently exposed to either an open field (with and without a novel object) or allowed to remain in the home cage (HC). Therefore, there were four main groups: (i) MeHg/Home Cage, (ii) MeHg/Open Field, (iii) Saline/Home Cage, (iv) Saline/Open Field. Exposure to the open field (OF) occurred 1 hr after injection and lasted 15 minutes as described below (section 2.3). For all mice exposed to the open field, the first 10

minutes were without a novel object, while for the last 5 minutes a novel object was present. The total time in the open field was 15 minutes. Following exposure to the OF, mice were returned to the home cage for 45 minutes, at the end of which (2 hours after the injection with methylmercury) they were anesthetized for perfusion (see section 2.4).

Methylmercury was given IP after noting that previous research has demonstrated that IP and oral exposure results in similar toxicokinetic and biological half-lives in the mouse and exposure through injection facilitated administration and was more reproducible across experiments (Nielsen, 1992). The 8mg/kg "high" dose of MeHg was used in this experiment because it was well below the mouse LD_{50} of 57mg/kg (Sigma Aldrich, MSDS), yet high enough to elicit significant neurobehavioral and neurophysiological responses (Salvaterra et al., 1973; Morganti et al., 1975; Annau & Cuomo, 1988; Dore et al., 2001; Cheng et al., 2006). The lower doses (2 and 4 mg/kg MeHg) and saline controls were included in order to establish a dose-response relationship. Mice were sacrificed 2 hours after the injection because during the first hours after exposure c-Fos levels are at maximal levels (Cheng et al., 2006; Akasaka et al., 2006) and behavioral responses to methylmercury are most affected (Salvaterra et al., 1973). All mice were injected 6 hours into the 12 hour light cycle and mice were never injected on days that cages were cleaned.

2.2.3 - Acute Intraperitoneal Methylmercury Open Field/Novel Object Test

The open field/novel object test was conducted as previously described by Kawashima & Kusnecov (2002). The open field apparatus was a 63x57x28 cm (LxWxH) Plexiglas box with opaque walls and floor. A smooth white curtain enclosed the area and

an overhead video camera connected to a VHS tape player recorded all animal movements. Animals were allowed to freely explore the field for 10 minutes, at the end of which a novel object (metal cylinder) was placed in the center of the field for the remaining 5 minutes of the test. Therefore, total exposure time to the open field was 15 minutes.

Spontaneous Motor Activity Recording and Tracking (SMART) software (San Diego Instruments, San Diego, CA) was used as a video-tracking system to monitor location of the animal within the open field. For the purpose of analysis by SMART, the open field was divided into two zones (outer perimeter and inner zones) for the initial 10-minute period and divided into three zones (outer, inner, and centered novel object zones) during the 5-minute novel object period. The outer/peripheral zone was a 9.5cm wide area bordering the outer sidewall; the inner zone was the remainder of the central zone during the initial 10 minute period. During the period of exposure to the novel object the outer zone remained the same, while for purposes of analysis within the inner zone a third central zone was introduced. This central zone was larger than the novel object itself was introduced to register movements involving the area immediately surrounding the novel object. Percent time spent in each zone was measured for the 10 and 5-minute time periods as was the total distance traveled within each time period.

Use of the open field to observe subtle changes in exploratory behavior first began with psychopharmacological studies involving antibiotics (Janssen et al., 1960) and since has been used effectively when characterizing behavioral changes in number and duration of hind limb rearings and ambulation/ locomotor activity in response to

methylmercury exposure (Spyker et al., 1972; Salvaterra et al., 1973; Morganti et al., 1976; Kobayashi et al., 1981; Dore et al., 2001; Goulet et al., 2003).

2.2.4 – Acute Intracerebroventricular Methylmercury Experimental Procedure

All materials and methods were the same as presented in the acute methylmercury study except where noted below. For further detail regarding materials and methodology not described below please refer to Section 2.2.1

The procedure for intracerebroventricular (ICV) administration of MeHg was conducted according to methods previously described (Kanteta and Kusnecov, 2005). Briefly, three days prior to experimentation mice underwent stereotaxic surgery to place an indwelling cannula into the right lateral ventricle. Mice were anesthetized with ~0.20-0.25ml of ketamine/ xylazine (80/12 mg/ml) from Sigma (St. Louis, MO), placed into the stereotaxic apparatus (David Kopf Instruments, Tujunga, CA), and fitted with a 26-gauge stainless steel guide cannula (Plastics One, Roanoke, VA) unilaterally implanted in the right lateral ventricle using the following coordinates: 0.25mm anterior to bregma, 1.0mm lateral from midsagittal suture, and 2.5mm ventral. A dummy cannula was then inserted and the entire assembly secured with cyanoacrlyate glue (Plastics One, Roanoke, VA). Mice also received 50ug (in 0.1ml of saline) dose of gentamicin sulfate (ICN Biomedicals, Aurora, OH) to prevent potential infection. One- and two-days after surgery mice underwent sham infusion as habituation and on the third day mice were administered saline, 19ng MeHg, or 0.5ng LPS ICV with a Harvard Apparatus infusion pump (Holliston, MA). Sham infusions consisted of picking up the mice, removing the dummy cannula, inserting the real cannula with the pump off, placing the animal back in

their home cage for 1 minute, picking the animal back up, removing the real cannula, reinserting the dummy cannula and placing the animal back in the home cage. All injection volumes were 2.5µl. Sham mice received ICV surgery but were not fitted with an indwelling cannula. Two hours after infusion, animals were perfused as described below in Section 2.25. Accurate cannula placement was validated for each animal after tissue was mounted on slides. Animals failing to show proper placement were excluded from analysis. All animals were naïve to ICV treatments.

The ICV MeHg dose for this experiment was determined to be 19ng, which is equivalent to the amount that would be present in the brain after an IP dose of 8mg/kg followed by intracardial perfusion, as determined by Cheng *et al* (2006). The total mercury concentration in the brain following the 5mg/kg injection was measured as 472ng MeHg/ kg brain tissue, which was determined to be 756ng/kg following an 8mg/kg MeHg exposure. For a 25 gram (0.025kg) mouse with an average brain weight of 0.43g the brain mercury level would then be ~18.9ng, giving us the 19ng ICV dose used in this experiment. Previous intracerebral injections of methylmercury to mice were three-fold higher, in the low microgram range (Fair et al., 1987) and after noting that Cheng et al (2006) had demonstrated that brain mercury levels in the nanogram range was predictive of neurotoxicity, the total dose used for the ICV portion of this experiment was 19ng.

Intracerebral exposure to methylmercury in rodents has demonstrated the histopathological and neurological abnormalities that are consistent with those described in human cases of methylmercury poisoning (Gallagher et al., 1982; Fair et al., 1987),

making inferences from this mouse model useful when translating these potential mechanisms to humans.

2.2.5 - Tissue Preparation and c-Fos Immunohistochemistry

Animals were anesthetized IP with 0.4ml of 7.5mg/ml of pentobarbital (Sigma Aldrich, St. Louis, MO) and then transcardially perfused with saline and 4% paraformaldehyde solutions. Brains were post-fixed in paraformaldehyde for 7 days and in a 30% sucrose solution for at least 7 days. Brains were sectioned at 30μm on a Leica SM 2000R freezing microtome and stored in cryoprotectant until assayed. Immunohistochemical detection of c-Fos was conducted using a free-floating assay as described previously (Rossi-George et al., 2005). Briefly, cryopreserved sections were washed with potassium phosphate buffered saline (KPBS), incubated with rabbit anti-mouse, anti-Fos primary antibody (1:15,000) (CalBiochem, La Jolla, CA) for 2h at room temperature, and then at 4°C for 48h. After the primary incubation, sections were washed and incubated in a secondary anti-rabbit antibody (1:500) for 1.5h. Tissue was then washed and incubated for 1h with the VectaStain ABC streptavidin horseradish peroxidase reagent (Vector Labs, Burlingame, CA), and subsequently reacted with $NiSO_4$-diaminobenzidine (DAB) substrate solution (Sigma Chemical, St. Louis, MO) for 3-5 minutes. The reaction was stopped with a 0.175M sodium acetate solution. Sections were then mounted onto Fisher Superfrost slides, allowed to dry overnight, counterstained in neutral red for 4.5 minutes and coverslipped.

2.2.6 - Image Analysis and Cell Quantification

Regions analyzed for Fos-positive cells included the anterior paraventricular nucleus of the thalamus (PVA), posterior paraventricular nucleus of the thalamus (PV), paraventricular nucleus of the hypothalamus (PVN), central amygdaloid nucleus (CeA), dentate gyrus (Dg), locus coeruleus (LC), bed nucleus of the stria terminalis (medial and supracapsular), and lateral septum (LS). During the stress response, specific neuroanatomical regions function to direct adaptive physiological and psychological responses to the particular stressor. Many of the brain regions that respond to stressful stimuli are located in the forebrain, and include thalamic, hypothalamic, amygdaloid, and septo-hippocampal nuclei (Herrera and Robertson, 1996). It is for this reason that the aforementioned brain regions were chosen for analysis. Images were taken on a Nikon Eclipse E400 microscope with the 10x objective. Fos-positive cells were counted with NIH Scion Image software. The quantification of c-Fos positive cells was conducted on coded slides thereby rendering the experimenter blind to all treatments. Additionally, all brains were coded after post-fixing and prior to sinking in sucrose by the lab technician, such that slides were labeled according to their coded brain descriptor. The code was broken after the raw data was placed into a spread sheet and confirmed with Dr. Kusnecov.

Cell counts used for data analysis for a given nuclei were arrived at by counting the number of c-Fos positive cells twice present on each section, across the entire structure, and then taking the average count per mounted section; thus the number used for statistical analysis was the average number of c-Fos positive cells per section of tissue. It should be noted that the c-Fos positive cell counts reported in this thesis

represent the average number of c-Fos positive cells for a given stress-associated nucleus and not the total number of immunoreactive cells per nucleus, as previously reported by Rossi-George et al., 2005. Additionally, the number of sections was near-equivalent across animals, thus ensuring that statistical differences were not due to examination of a particular nucleus at different positions along the anterior-posterior axis.

2.2.7 - Data Analysis

For behavioral tests, two-way ANOVAs were conducted, with repeated measures incorporated where comparisons of behavior before and after novel object exposure were assessed. For the c-Fos experiments, data were subjected to ANOVAs consistent with the factorial nature of the experimental design. Hence, a two-way ANOVA was conducted for the acute MeHg study involving open field exposure or LPS challenge. Further, for the repeated and intracerebroventricular MeHg experiments, one-way ANOVAs were conducted. In follow up analyses, post-hoc comparisons were restricted to comparisons between groups according to the significance of main treatment effects. For this purpose, the Fisher's LSD test was used. Finally, where *a priori* expectations were present based on previously published literature on the effects of open field on c-Fos immunoreactivity in the brain (e.g., Rossi-George et al, 2005), a Student's t-tests was conducted. In all statistical tests, differences were considered significant at $p < 0.05$.

2.3 - Experimental Results

2.3.1 – Acute Intraparitoneal MeHg: Open Field Behavioral Testing

Analysis of the open field/novel object behavioral data in response to acute 2, 4, 8 mg/kg doses of MeHg or saline treatment revealed several significant findings as detailed below and shown in Figures 1-6.

Outer Zone

Analysis of the percentage of time spent in the outer zone, with and without the novel object, revealed that irrespective of acute treatment the time spent in the outer zone increased significantly after the novel object was introduced ($F_{(3,17)}$=12.29, $p<0.001$) (Figures 1 and 2, respectively). Acute open field data without the novel object showed a dose-dependent increase in time spent in the outer zone following MeHg treatment ($F_{(3,17)}$=12.81, $p<0.001$), with the 4 and 8 mg/kg groups showing the longest duration of time in the outer zone when compared to saline treatments ($p<0.001$ and $p<0.0001$, respectively). Provocation with a psychogenic stressor (novel object), resulted in acute saline and 2 mg/kg animals increasing the time spent in the outer zone, and the 4 and 8mg/kg MeHg being significantly different from saline ($p=0.03$ and $p=0.04$).

Inner and Novel Object Zones

Exploration of the inner zone after acute treatments was influenced by the presence of the novel object, as expected from previous findings (Kawashima & Kusnecov, 2002). In the absence of the novel object, mice spent a significantly greater percentage of time in the inner zone ($F_{(3,17)}$=14.06, $p<0.0001$) (Figure 3). There was a

reciprocal reduction in the percentage of time spent in the inner zone as a function dose of MeHg treatment, with the most significant reduction occurring for the acute 4 and 8mg/kg MeHg doses. Introduction of the novel object produced a significant attenuation of inner zone exploration for all acute groups, with the 4 ($p=0.03$) and 8mg/kg MeHg ($p=0.04$) doses producing the greatest reductions (Figure 4). Analysis of the percentage of time spent in the novel object zone revealed no significant statistical differences between treatment groups.

Total Distance Traveled

Distance traveled in the open field without the novel object present exhibited a dose dependent decrease in total distance traveled following acute MeHg treatment ($F_{(3,17)}=23.35$, $p<0.0001$), with the same dose dependent decrease in distance traveled noted in the presence of the novel object ($F_{(3,17)}=7.75$, $p=0.0018$) (Figures 5 and 6, respectively). Without the novel object present, only comparisons of acute saline-2mg/kg MeHg ($p=0.055$) treatments were found to not be significant. In the presence of the novel object there was less overall statistical significance between acute groups, as saline-2mg/kg MeHg ($p=0.273$) and 4-8mg/kg ($p=0.883$) MeHg were not found to be significantly different.

2.3.2 - Acute Intraperitoneal MeHg: c-Fos Immunohistochemistry

Stressor-dependent changes in neuronal c-Fos immunoreactivity were noted within stress-associated brain regions. Analysis of selected brain regions was guided by previous evidence that exposure to psychogenic stressors activates brain nuclei located in

the hypothalamus, thalamus, hippocampus, amygdala, and lateral septum (Senba and Ueyama, 1997). In addition, attention was directed to the locus coeruleus, a critical region responsive to stress, and implicated in anxiety-like behaviors (Carrasco and Van de Kar, 2003).

Paraventricular thalamic nucleus

In the thalamus, the paraventricular nucleus (PVA/PV) showed marked increases in the number of c-Fos positive immunoreactive cells after acute MeHg exposure in response to both open field ($F_{(1,34)}$=20.07, $p<0.0001$) and treatment with MeHg ($F_{(3,34)}$=3.98, $p<0.05$) when compared to the home cage and saline controls (Figure 7). The most statistically significant differences were between acutely treated saline mice and the 2 and 8mg/kg MeHg treatments (both $p<0.01$). Interestingly, there was a trend toward increased c-Fos cell counts when 4mg/kg was compared to 8mg/kg MeHg ($p=0.07$). There was no significant interaction between open field exposure and MeHg treatment.

Paraventricular hypothalamic nucleus

Increased numbers of c-Fos positive cells were noted in the paraventricular hypothalamic nucleus (PVN) following acute MeHg treatment ($F_{(3,34)}$=7.11, $p<0.001$). Significant differences were noted between saline-HC and the 2, 4, and 8mg/kg MeHg-HC treated mice ($p<0.001$, $p<0.01$ and $p<0.01$, respectively). The number of c-Fos positive cells also increased in response to exposure to greater concentrations of MeHg (Figure 8). In the open field treatments, the 4mg/kg MeHg had significantly greater c-

Fos immunoreactivity when compared to saline controls ($p<0.05$). No MeHg-open field interaction effects were noted.

Based on data previously published from our lab noting that open field stress produces statistically significant increases in the number of c-Fos positive cells within the PVN (Rossi-George et al, 2005), an *a priori* Student's T-test was conducted comparing saline-HC to saline-OF mice ($t_{(18)}=2.21$, $p<0.05$), confirming that open field exposure increased the number of c-Fos immunoreactive cells in the PVN.

Central amygdaloid nucleus

The central nucleus of the amygdala (CeA) showed increased numbers of c-Fos immunoreactive cells in response to all doses of acutely administered MeHg ($F_{(7,34)}=10.24$, $p<0.0001$) (Figure 9). Specifically, acute treatment with MeHg resulted in increased c-Fos counts in 2, 4, 8mg/kg MeHg-HC animals (all $p<0.001$) when compared to the saline-HC control. Also open field groups given 2 or 8mg/kg MeHg had a greater c-Fos response when compared to saline (both $p<0.05$). There were no significant differences in c-Fos positive cell counts noted in response to open field stimulation or for potential open field-acute MeHg interaction effects.

Dentate gyrus of the hippocampus

The dentate gyrus of the hippocampus (Dg) expressed significant increases in the number of c-Fos positive cells with respect to both open field ($F_{(3,34)}=25.32$, $p<0.0001$) and acute MeHg ($F_{(1,34)}=3.16$, $p<0.05$) exposure (Figure 10). Saline, 2, and 4mg/kg acute MeHg-OF groups had higher c-Fos immunoreactivity when compared to all home cage

treatments (all $p<0.05$). No open field-MeHg interaction effects were found to be significant.

Locus coeruleus

The response of the locus coeruleus (Lc) to acute MeHg exposure resulted in significantly increased numbers of c-Fos positive cells after acute MeHg treatment (general trend, $F_{(3,34)}=11.62$, $p<0.001$). Specifically, home cage mice given acute dosed of 2mg/kg MeHg ($p<0.01$), 4, and 8mg/kg MeHg (both $p<0.0001$) had significantly higher c-Fos cell counts than saline controls (Figure 11). Within the open field exposed groups, a greater number of c-Fos cells was associated with acute 4 and 8mg/kg MeHg (both $p<0.05$) when compared to saline-OF. The number of c-Fos positive cells was not significantly affected by open field stress or interaction effects with MeHg and the open field.

Lateral septum

Fos positive cell counts in the lateral septum (LS) were increased significantly following exposure to the open field ($F_{(1,34)}=30.51$, $p<0.0001$) and acute MeHg exposure ($F_{(3,34)}=6.45$, $p<0.01$) (Figure 12). In response to open field stimulation, saline, 2 and 4mg/kg MeHg-O.F. treatments had greater c-Fos positive cell counts when compared to respective home cage treatment. Methylmercury exposure led the 4 and 8mg/kg MeHg-O.F. groups to have statistically different c-Fos responses when compared to the saline-O.F. control (both $p<0.05$). Additionally, there was a trend toward OF-MeHg interaction was of borderline significance ($p=0.0568$).

Bed nucleus of the stria terminalis

Counts for c-Fos positive cells in the bed nucleus were divided into medial (BSTm) and supracapsular (BSTs) areas. The BSTm response to the open field and acute MeHg dosing resulted in c-Fos counts that varied significantly with respect to saline and/or home cage controls (Open Field main effect: $F_{(1,34)}=6.35$, $p<0.05$; MeHg main effect $F_{(3,34)}=8.10$, $p<0.001$) (Figure 13). All acute doses of MeHg increased cFos counts relative to saline treated mice ($p<0.0001$). There were no interaction effects between open field and MeHg exposures for the BSTm. In the BSTs exposure to the open field stressor yielded increased c-Fos positive cell counts when compared to home cage controls ($F_{(1,24)}=22.34$, $p<0.0001$) (Figure 14). However, neither MeHg treatment nor combined open field-MeHg treatment significantly altered c-Fos immunoreactivity in the BSTs.

2.3.3 - Acute Intracerebroventricular MeHg: c-Fos Immunohistochemistry

The previous experiment tested the neural effects of systemic MeHg, which may have influenced c-Fos activation in the brain via mechanisms that are independent of direct CNS effects of the toxicant. To determine, whether central administration of MeHg produces a pattern of c-Fos activation similar to systemic MeHg, the present experiment involved direct ICV administration of MeHg followed by perfusion and immunohistochemistry as in the previous experiment. Fair et al (1987) initially demonstrated that ICV dosed MeHg in mice resulted in similar end point neurological impairments, such as ataxia, tremor and paralysis, as noted in humans following oral exposure to methylmercury (Takeuchi, 1968; Vandewater et al., 1983; Nierenberg et al.,

1998). Recently, Cheng et al (2006) demonstrated that a peripheral IP dose of 8mg/kg MeHg would result in a brain-MeHg level of 19ng/ brain, leading to increases in brain c-Fos protein levels that were capable of predicting oxidative-stress mediated neurotoxicity. Furthermore, the present experiment involved infusion of LPS at a total dose of 0.5ng. This dose was selected because was considerably lower than doses demonstrated to mimic systemic infection (2µg) (Cunningham et al., 2005). The decision to use LPS was to provide a positive measure for c-Fos activation, as has previously been shown in published literature (Wan et al., 1993).

The results of this experiment showed that infusion of MeHg led to a significant increase of c-Fos positive cell number and activation of several stress-associated brain nuclei while LPS infusion was typically less active. Analysis of selected brain regions was consistent with the acute IP MeHg study noted in Methods section 2.2.

Activation of stress associated pathways after ICV administration of MeHg and LPS gave results that varied with region when compared to saline and sham controls. Paraventricular thalamic and hypothalamic nuclei showed a strong c-Fos activation response to MeHg treatment ($F_{(3,17)}=12.81$, $p<0.01$), while no effect was noted in response to the 0.5ng LPS dose (Figures 15 and 16, respectively). Responses of the central amygdala and dentate gyrus to MeHg and LPS treatments were not significant when compared to saline controls (Figures 17 and 18). Similar to both the acute and repeated IP MeHg studies, the locus coeruleus responded very strongly to MeHg when administered ICV after comparison with LPS and control groups ($p<0.01$) (Figure 19). Response of the Lc to ICV LPS was similar to that of saline. The lateral septum had significantly higher c-Fos activity in response to MeHg when compared to either LPS or

saline ($p<0.01$ and $p<0.05$, respectively), which themselves were not significantly different from one another (Figure 20). The medial bed nucleus of the stria terminalis (BSTm) was activated after administration of MeHg when compared with vehicle control ($p<0.05$) with no effect in response to LPS (Figure 21). The supracapsular BST did not respond significantly to ICV MeHg or LPS administration (Figure 22). It should also be reported that sham animals had very low levels of c-Fos.

2.4 - Discussion

This study demonstrated that centrally and peripherally administered acute doses of methylmercuric chloride (MeHg) and exposure to a psychogenic stressor, open-field (OF), differentially affected c-Fos protein production across a number of murine limbic brain regions commonly associated with mediating stress responses in the brain. While activation of these stress-associated brain regions by open field exposure is not inconsistent with previously published reports (Herman et al., 1998; Rossi-George et al., 2005; Badowska-Szalewska et al., 2006; Coryell et al., 2007), that exposure of mice to MeHg similarly recruits these very same brain regions has not previously been demonstrated.

Acute IP administration of MeHg impacted open field exploratory behavior in a dramatic way. Behavioral testing showed that acute treatments across several MeHg doses were capable of affecting the exploratory response of mice in the open field. The open field test consisted of two phases. In the first phase, animals were introduced to an empty open field for the purpose of observing their exploratory behavior within the outer (perimeter) and inner regions of the field. This is the traditional test of open field

behavior, and in this manner is thought to provide an index of emotion and/or arousal (Crawley, 1985; Weiss et al., 1999). In the second phase, a novel object was introduced into the open field, a procedure that is designed to provoke an orienting response, which may be interpreted as providing a stimulus-specific measure of arousal and/or defensive behavior, and typically induces a change in ongoing exploratory behavior. Indeed, as shown in the results, saline treated mice displayed a reduction in ongoing locomotor behavior upon introduction of the novel object, with spatial location being confined more to the outer zone of the open field.

In general, acute treatment with MeHg altered the initial exploratory response upon introduction to the open field, and subsequently, also differentiated mice from saline controls once the novel object was introduced. That is, relative to saline treated controls, IP MeHg treatment increased the percentage time spent in the peripheral zone (during the first 10 min of the open field test without the novel object) and decreased the percentage of time spent in exploring the inner zone when the novel object was introduced. This effect was dose-dependent, with animals treated with the highest dose (8 mg/kg) showing the greatest reduction in exploratory behavior.

It is difficult to determine from these observations whether the treatment with MeHg retarded movement as a function of illness or malaise. However, given that administration of methylmercury to rodents results in immediate appetite suppression (Berthoud et al., 1976; Magos, 1982), it is possible that a sickness-associated behavior may be affecting exploratory movement in the open field. Also, since acutely treated MeHg animals reacted to the presentation of the novel object by reducing entry into the inner zone where the object was placed, it is likely that in spite of any potential MeHg

induced toxicity, attention to environmental modifications was not impaired. At the very least, behavioral testing in the open field confirmed that at least for the two highest doses (4 and 8 mg/kg), acute administration of MeHg strongly influenced behavior in the open field. Of interest is the observation (see below) that the lowest acute dose (2 mg/Kg) was not generally observed to be strongly disruptive of exploratory behavior, but nevertheless induced increased c-Fos protein production in the brain. In attempting to correlate c-Fos expression, across the different groups, with open field behavior it is unclear if there is a connection, particularly after noting that the acute IP 2 mg/kg groups have as high c-Fos positive cell counts as the 4 and 8 mg/kg groups in many of the analyzed nuclei. However, when looking at the open field data there is a strong dose response noted. Thus, the data may be indicating that while the dose of methylmercury was strongly indicative of performance in the open field, c-Fos immunoreactivity was not. Thus, activation of these neural stress circuits and the corresponding increase in c-Fos a may be acting prior to or in conjunction with other homeostatic responses to produce the altered open field behavior. For example, given that loss of appetite occurs rapidly after exposure to methylmercury (Magos, 1982), perhaps gastrointestinal distress and concomitant stimulation of the brain stem-projecting vagus nerve could be resulting in the c-Fos activation and inhibited exploratory behavior in the open field. Indeed it is well established that the vagus nerve plays a major role in the anorexic response to physiological stressors (reviewed by Konsman & Dantzer, 2001), as vagotomy has been shown to inhibit such anorexic responses (Rezek & Novin, 1977) and restore attenuated exploratory behavior in the open field following cholecystokinin exposure (Itoh & Katsuura, 1986).

Analysis of c-Fos positive cell counts in response to administration of MeHg revealed varying intensities of c-Fos activation within stress-associated nuclei of the mouse brain. The paraventricular thalamic nucleus (PVA/PV) is responsible for processing afferent sensory input to the cortex, and in turn mediating influence on both motor and cognitive functions (Spencer et al., 2004). An increase in PVA/PV neuronal activity was observed in response to both IP and ICV acute MeHg treatment and following stimulation by the open field after acute IP exposure. Similarly, the PVN, which regulates autonomic and behavioral responses to various psychogenic and systemic stressors (Feldman et al., 1995), also showed substantial c-Fos immunoreactivity in response to both IP and ICV acute doses of MeHg and open field. Further, appetite suppression with spermine (a cholesterol metabolite) is characterized by increases in c-Fos immunoreactivity in the PVN when given IP or ICV (Ahima et al., 2002). Finally, it is possible that the two major efferent indices of PVN activation, activation of the HPA axis (as alluded to in this thesis by activation of the PVN) and sympathetic nervous system, could be contributing to the behavioral and neuroanatomical IEG effects noted following MeHg treatment.

The central nucleus of the amygdala (CeA) is known to influence the activity of the hypothalamus following input from cortical areas responding to environmental information (Koob & Heinrichs, 1999; Coryell et al., 2007). In general, it has been well demonstrated that changes in neuroendocrine and autonomic functions, such as heart rate, respiration rate, and blood pressure, following exposure to fearful and/or stressful stimuli are mediated by neurons in the CeA (Shekhar et al., 2003). Increased activity in the CeA was noted in response to acute IP MeHg treatment but was not appreciably influenced by

open field exposure or ICV administration of MeHg. While open field exposure per se likely involves induction of a fear-like state due to the unfamiliar circumstances, the lack of a significant c-Fos increase may be due to insufficient intensity of this psychogenic stressor. Indeed, reliable increases in c-Fos levels occur in the CeA in response to a variety of psychological stressors (Pacak et al., 1995; Senba and Ueyama, 1997; Martinez et al., 2002; Coryell et al., 2007). The lack of c-Fos response noted in the CeA in response to psychogenic stress may result from the overwhelming nature MeHg-induced alterations in exploratory behavior demonstrated in this study. However, saline controls exposed to the open field did not have appreciably higher c-Fos positive cell counts, suggesting that within the parameters of this experiment open field exposure was not sufficient to lead to CeA activation. Nonetheless, CeA activation by MeHg suggests engagement of neural substrates necessary for adaptive physiological and behavioral responses, and is consistent with the significant reduction in exploratory behavior observed in mice acutely treated with MeHg.

Exposure of hippocampal neurons to methylmercury has been demonstrated to induce permanent changes in learning and memory with specific sensitivity in neurons of the dentate gyrus (Annau and Cuomo, 1988; Kakita et al., 2000; Andersson et al., 1997). In the current study, it was noted that the dentate gyrus of the hippocampus (Dg), showed a significant increase in the number of c-Fos positive cells in response to open field exposure after both acute IP MeHg treatment. The Dg also had a significantly greater c-Fos response to acute IP administration of MeHg alone, while the response of this nucleus to centrally administered (ICV) MeHg was not found to be significant. In response to stressful stimuli, the Dg takes efferent neuronal excitatory signals from the

limbic system (entorhinal cortex), processes, and relays them to the fornix, which is consistent with data from this study showing that acute MeHg engages the brain's hippocampal stress circuitry.

Robust increases in c-Fos immunoreactive cells within the locus coeruleus were also observed in response to acute IP MeHg exposure, open field stimulation after acute IP exposure and following ICV administration of MeHg. The Lc is a hindbrain region rich in forward-projecting noradrenergic neurons innervating much of the forebrain limbic brain regions (Jones, 1991). Moreover, the Lc has long been considered an important regulator of arousal and anxiety (Ziegler et al., 1999; Berridge and Waterhouse, 2003). Similarly, forebrain regions that receive noradrenergic input, such as the bed nucleus of the stria terminalis (BST) and lateral septum (LS) (Mulders et al., 1997; Forray & Gysling, 2004), known to be critical during the response to stress and anxiety (Morilak et al., 2005), were also activated by the open field and MeHg exposure. Given that the BST, Lc and LS have all been shown to be critical to anxiety-like behavior, this suggests that toxic doses of MeHg generate neural responses that likely induce an anxiogenic state. The reduced exploratory behavior in the open field and novel object test supports this conclusion, although how much of the immobility is due to impaired locomotor function as a result of somatic discomfort and/ or malaise is not known. However, IP exposure to MeHg may be exerting its effects on behavior and activation of stress-associated brain nuclei by stimulation of centrally-projecting peripheral nerves such as the vagus. Indeed vagotomy followed by peripheral injection of appetite suppressing orexin hormones (leptin), attenuates appetite suppression in rodents (Sachot et al., 2007). While not addressed in this thesis, one could speculate that

stimulation of the vagus nerve by IP MeHg may be contributing to the noted effects on open field behavior and activation of central regions, such as the PVN, consistent with the aforementioned appetite-suppression/ c-Fos studies. Also, given that chronic pretreatment with antidepressants results in an attenuated PVN c-Fos response and (HPA axis) blood-ACTH response to LPS (Castanon et al., 2003), it would be interesting to examine the c-Fos response to doses of methylmercury used in these experiments following pretreatment with antidepressants. The similarity of c-Fos activation patterns noted following both IP and ICV MeHg exposure may be due to similar homeostatic responses, perhaps mediated by the locus coeruleus and area postrema, which in both cases could be activated in response to circulating methylmercury (Arvidson, 1992).

When the magnitudes of acute IP and ICV c-Fos responses were compared across brain stress nuclei it was found that the PVN ($F_{(1,10)}$=15.42, $p<0.01$), CeA ($F_{(1,10)}$=6.32, $p<0.05$) and BSTm ($F_{(1,10)}$=5.62, $p<0.05$) had higher numbers of c-Fos cell counts in response to IP MeHg, while the BSTs ($F_{(1,10)}$=8.14, $p<0.05$) had higher c-Fos positive cell counts in response to ICV MeHg. A potential reason for this discrepancy between magnitude of c-Fos response and IP or ICV route of administration may be due to proximity to the injection site. Methylmercury delivered directly into the lateral ventricles (ICV) is likely to affect brain nuclei proximate to the injection site, such as the BSTs. The reason for higher c-Fos positive cell counts in the PVN, CeA, and BSTm following IP MeHg exposure, when compared with ICV, could be due to activation of centrally-projecting peripheral nerves such as the vagus through the brainstem to the PVN and onto the CeA and BSTm, although strong activation of the Lc would have been expected. To

test this notion it would also be of interest to see if these magnitude profiles varied in vagotomized mice.

Data from this set of experiments clearly supports the idea that a peripherally or centrally administered acute dose of methylmercury is capable of activating neural stress circuitry in the mouse. The centrally administered LPS dose of 0.5ng may have been too small to elicit significant production of c-Fos, as Wan et al (1993) have demonstrated that ICV doses of 10ng produce a significant increase in c-Fos levels. However, given that c-Fos response to MeHg was robust, further attempts to establish a LPS positive control for c-Fos activity were not deemed as crucial to this experiment. Acute IP MeHg was also found to result in a dose-dependent decrease in exploratory behavior within the open field. Acute exposure to MeHg has been demonstrated to increase neurotransmitter release, stress hormone production, and inflammatory cytokines (Hrdina et al., 1976; Faro et al., 1997; Ortega et al., 1997) and stress circuit activation (e.g., demonstrated in this experiment). However, larger levels of exposure to methylmercury, whether as similar doses given repeatedly or in larger doses given one time, have resulted in tolerance-like effects on stress-hormone and pro-inflammatory cytokine levels, and inhibit lymphocyte proliferation in response to a subsequent immunologic stressor (Ortega et al., 1997; Hirokawa & Hayashi, 1980). This would lead one to believe that for smaller and chronically administered, biologically relevant exposures the tolerance effects likely do not occur but instead may induce a low-grade stimulation of the immune system (Stejskal et al., 1996), resulting in a sensitized immune response to subsequent immunological challenges.

Acute MeHg data from this experiment demonstrate activation of stress-associated neural circuitry. Also, previous experimental data has shown the stress-associated tolerance to repeated MeHg (Ortega et al., 1997; Hirokawa & Hayashi, 1980). Therefore, an additional experiment to follow this set of experiments could generally hypothesize that repeated administration of MeHg may result in activation of a specific stress-associated parameter (c-Fos), while potentially expressing a tolerance effect within a different measure of stress (open field behavior).

Experiment 2

Effects of Repeated Methylmercury Exposure on Central Stress Circuit Activation and Exploratory Behavior

3.1 - Introduction

Experiment 1 was able to demonstrate that a peripherally administered (intraperitoneal-IP) dose of methylmercury (MeHg) is capable of significantly activating neural stress circuitry and attenuating open field exploratory behavior in the mouse. Additionally, intracerebroventricular (ICV) administration of methylmercury was similarly shown to produce stress-circuit activation as reflected by c-Fos immunostaining. Given that the foregoing experiments demonstrated the acute effects of MeHg on recruitment of murine stress circuitry and exploratory behavior, it was important to consider whether such effects persisted after repeated administration of methylmercury.

While a single acute exposure to methylmercury has been shown to result in marked neurophysiological and neurobehavioral aberrations as shown in Experiment 1 and other laboratories (eg., Nierenberg et al., 1998), the vast majority of documented human mercury poisonings have occurred after weeks to months of repeated exposure (Takeuchi, 1968; Likosky et al., 1970; Bakir et al., 1973). Increased blood mercury levels following repeated exposures to methylmercury have been demonstrated to correlate positively with the severity of neurological symptomology in humans (Bakir et al., 1973) and mice (Berthoud et al., 1976; Morganti et al., 1976; Goulet et al., 2003). Data gathered from the Iraqi poisonings from the early 1970's found that blood-mercury levels of 0-100

ng/ml resulted in 9.5% of patients experiencing paresthesia and 5% reporting ataxia, while mercury levels of 1001-2000 resulted in paresthesia in 60% and ataxia in 47% of the cases (Bakir et al., 1973). Also, repeated methylmercury exposure in mice was shown to correlate positively with severity of neuronal degeneration in the corpus striatum, cerebral cortex, thalamus and hypothalamus (Berthoud et al., 1976).

In response to a repeated, homotypic stressor followed by a single heterotypic stimulus, three possible response outcomes are possible: attenuated, sustained, and exacerbated responses. Repeated exposure to stressful stimuli often elicits a stress response that can differ markedly from the response noted to an acute exposure of the same stimulus. A single intraperitoneal exposure to the endotoxin lipopolysaccharide (LPS), a potent stimulator of the innate immune response, results in major increases of stress hormones (ACTH and corticosterone), inflammatory cytokines (eg., TNF), and central induction of immediate early genes (Rivest, 2001; Cohen, 2002; also see introduction to Experiment 3). These responses are significantly attenuated after 3-6 repeated daily injections of LPS (Hadid et al., 1996; Takemura et al., 1997; Navarro et al., 2007). However, acute or repeated administration of hypertonic saline, used as a physiological stressor that is capable of inducing behavioral alterations typically seen after psychogenic stressors, strongly increased ACTH and corticosterone levels in the blood without any associated attenuation of the stress response to repeated injections (Kiss & Aguilera, 1993; Garcia et al., 2000). It is likely, therefore, that the effects of repeated LPS exposure on CNS activation rely on the production of immune mediators, viz., cytokines (reviewed in Herman & Cullinan, 1997). Finally, van Dijken et al. (1993) found that a single foot shock exposure in the rat was enough to behaviorally increase/

sensitize stress and anxiety measures and ACTH responses to a subsequent novel environment stressor. Therefore, the specificity of the systemic stressor plays an important role in the presence of habituation and sensitization. The ability of a stressor to sensitize the response to another different stressor, suggests that similar effects may occur after pre-exposure to methylmercury, and as such will be addressed in the current experiment.

Indeed the literature addressing how repeated exposure to stressful stimuli impact neurophysiological and neurobehavioral function is well documented (Dallman et al., 2004; McEwen, 2007), although relatively little is known regarding whether repeated methylmercury exposure results in habituation of stress-associated parameters. One study has demonstrated in rats that 8-16 weeks of exposure to methylmercury in the drinking water initially induces a stress response by increasing blood levels of ACTH, corticosterone and the cytokine interleukin-6 (IL-6) levels (at week 8), but more prolonged exposure (at week 16) results in a significant attenuation of this response (Ortega et al., 1997). Although this study did not address the response of specific brain regions involved in the stress response, it does suggest that habituation of the central stress response is a viable hypothesis after repeated methylmercury exposure.

In light of this latter evidence, the present Experiment hypothesized that repeated exposure to methylmercury would result in a significant attenuation of the brain c-Fos response in regions forming the stress circuitry of the brain. This experiment will also consider the possibility that methylmercury will sensitize the response to a subsequent heterotypic stressor. Indeed, there is evidence showing that methylmercury produces sensitization to pharmacologic stressors, such as amphetamine, when measuring

stereotypic self-injurious behavioral responses to amphetamine treatment (Wagner et al., 2007).

3.2 - Materials and Methods

The age and strain of mice, behavioral testing and preparation and injection of methylmercury were the same as described in the Materials and Methods for Experiment 1 (See Section 2.2).

Repeated Methylmercury Experimental Procedure

Methylmercury (2 or 4mg/kg) or saline were administered IP every third day over a 15 day period beginning on day zero, for a total of six injections. All injection volumes were 0.2ml. One hour after the final injection mice were either examined in the open field (OF) for 15 minutes or allowed to remain in the home cage (HC), as described in Methods section 2.2.1 and 2.2.2. All mice were transcardially perfused 2h after the injection (see Materials and Methods for Experiment 1). Overall there were six treatment groups: i) Saline/OF, ii) 2mg/kg MeHg/ OF, iii) 4mg/kg MeHg/ OF, iv) Saline/ HC, v) 2mg/kg MeHg/ HC, vi) 4mg/kg MeHg/ HC.

3.3 - Experimental Results

3.3.1 - Open Field Behavioral Testing

Analysis of exploratory behavior in the open field after repeated IP exposure to methylmercury gave discernibly different results when compared to the behavioral results of Experiment 1. A summary of these findings is given below.

Outer Zone

In contrast to acute MeHg treatment in Experiment 1, the percentage time spent in the outer zone of the open field was not significantly altered after a regimen of repeated MeHg treatment (Figures 1 and 2). This was the case whether the novel object was present ($F_{(2,12)}$=0.83, p=0.461) or absent ($F_{(2,12)}$=2.33, p=0.140).

Inner and Novel Object Zones

Repeated exposure to MeHg did not result in statistically significant changes in time spent exploring the inner or novel object zones when compared to saline controls (Figures 3 and 4). Percentages of time spent within the inner zone of the open field, with the novel object ($F_{(2,12)}$=0.87, p=0.443) or without it ($F_{(2,12)}$=2.22, p=0.151), were not significantly different when MeHg treated groups were compared to controls, Analysis of novel object zone data did not reveal any statistically significant differences between mice repeatedly exposed to MeHg and saline controls ($F_{(2,12)}$=0.73, p=0.502).

Total Distance Traveled

With respect to repeated MeHg treatment, total distance traveled in the open field without the novel object was reduced in the 4mg/kg MeHg group when compared to vehicle treated animals ($p<0.05$). However, total distance traveled in the presence of the novel object was not significantly affected by repeated exposure to MeHg ($F_{(2,12)}$=2.03, p=0.174) (Figures 5 and 6).

3.3.2 - Repeated Methylmercury c-Fos Immunohistochemistry

Repeated intraperitoneal exposure to MeHg with and without subsequent exposure to the open field resulted in significant activation of several stress-associated brain nuclei. This occurred in a manner that was unique when compared to acute IP administration of MeHg.

Paraventricular thalamic nucleus

The response of the PVA/PV to repeated MeHg was not significantly different than control levels, while the number of c-Fos immunoreactive cells increased in response to the open field after repeated MeHg treatment ($F_{(1,23)}$=21.39, $p<0.0001$) (Figure 27). Specifically, saline and 2mg/kg MeHg mice exposed to the open field had a more robust c-Fos immunoreactive response when compared to the respective saline and MeHg animals returned to the home cage.

Paraventricular hypothalamic nucleus

An interaction effect was noted in the PVN c-Fos response after repeated MeHg exposure and to the open field ($F_{(2,23)}$=4.68, $p<0.05$) (Figure 28). It follows that this response stems from the MeHg dose-dependent increase in c-Fos cells among home cage groups, while OF groups remained similar to one another.

Central amygdaloid nucleus

Repeated exposure to MeHg led to the final MeHg injection still strongly activating the CeA ($F_{(2,23)}$=8.21, $p<0.01$). However the CeA was not activated in response

to open field alone or in combination with MeHg (Figure 29). The 4mg/kg MeHg-HC treatment created a greater c-Fos response when compared to either the saline or 2mg/kg MeHg-HC treatments.

Dentate gyrus of the hippocampus

In the dentate gyrus there was a reduction of c-Fos-positive cell counts after repeated MeHg treatment ($F_{(2,23)}=4.75$, $p<0.05$), relative to the c-Fos immunoreactivity following repeated saline administration. Alternatively, the number of c-Fos immunoreactive cells increased after OF exposure irrespective of MeHg treatment ($F_{(1,23)}=77.489$, $p<0.0001$) (Figure 30). Saline, 2 and 4mg/kg MeHg-OF treatments were found to have significantly higher c-Fos concentrations than the home cage group, while in response to MeHg the 2mg/kg treatment had significantly lower c-Fos levels than the saline-HC control.

Locus coeruleus

The c-Fos response to repeated MeHg exposure increased significantly in the locus coeruleus ($F_{(2,23)}=4.27$, $p<0.05$), and was similarly increased in response to open field exposure ($F_{(1,23)}=5.54$, $p<0.05$) (Figure 31). Specifically, the saline-HC group had lower c-Fos immunoreactivity than the 4mg/kg MeHg-HC group ($p<0.01$), while the 2mg/kg MeHg-OF group had higher c-Fos levels than saline-OF controls ($p < 0.05$).

Lateral septum

Repeated exposure to MeHg and followed by open field stimulation resulted in an interaction effect ($F_{(2,23)}$=5.42, $p<0.05$). The interaction effect noted in the LS likely resulted from the MeHg-induced dose dependent decrease in c-Fos immunoreactive cell numbers within the open field groups, while the home cage treatments showed lower c-Fos numbers (Figure 32).

Bed nucleus of the stria terminalis

After repeated administration of MeHg and exposure to the OF, the resulting c-Fos stimulation in the BSTm and BSTs differed. Efferent and afferent BSTs projections are in close proximity to the CeA and are thought to share the underlying neural mediation of fear and anxiety following exposure to stressors (Shammah-Lagnado et al., 2000). In addition, medial regions of the BST are believed to have some involvement in mating and sexual arousal (Liu et al., 1997).

Within the BSTm there was a c-Fos interaction effect in response to the repeated MeHg and open field exposure ($F_{(2,23)}$=4.08, $p<0.05$). The repeated MeHg-open field interaction in the BSTm could be due to the MeHg-induced, dose dependent, increase in c-Fos counts in the brains of home cage animals while MeHg-open field treatments remained similar to one another (Figure 33). Significant c-Fos activation in the BSTs resulted from both repeated exposure to MeHg ($F_{(1,23)}$=36.83, $p<0.0001$) and open field ($F_{(2,23)}$=6.04, $p<0.01$). Specifically, repeated MeHg reduced c-Fos cell counts within the 2 and 4mg/kg MeHg-OF groups when compared to saline-OF ($p<0.05$ and $p<0.01$, respectively) (Figure 34).

3.4 - Discussion

Repeated IP exposure to MeHg resulted in several interesting effects on neural stress circuit activity and exploratory behavior in the open field. Behavioral measures revealed a marked attenuation of the dose-dependent reductions in exploratory behavior noted in response to single acute IP exposures to methylmercury (see Experiment 1). In the present experiment, repeated administration of MeHg did not significantly alter the percentage of time spent in the outer, inner, or novel object zones. The only significant differences noted were that mice given 4mg/kg MeHg repeatedly, traveled a shorter distance in the open field when compared to vehicle controls ($p<0.05$).

The lack of a behavioral response to repeated methylmercury within the open field, relative to the dose-dependent decreases in exploratory behavior present in Experiment 1, suggests possible desensitization or habituation to these doses of MeHg. Published data has shown that acute exposure to methylmercury is capable of affecting several sickness-associated behaviors including appetite suppression (Berthoud et al., 1976; Magos, 1982) and reduction in locomotor activity as measured by number of hind limb rearings (Salvaterra et al., 1973) and number of square crossings (Dore et al., 2001) in the open field. It is then quite possible that if sickness-like behaviors are helping to reduce exploratory behavior after acute MeHg exposure, it follows that repeated exposure to MeHg could be resulting in tolerance of the same underlying mechanisms that are responsible for the attenuated behavior in the open field following acute MeHg exposure. Indeed desensitization of sickness-like behaviors to physiological stressors such as LPS can be rapid, occurring after the second and third subsequent exposures (when dosed every two days) (Langhans et al., 1991). The rapid tolerance of behavioral and

physiological stress-associated responses to LPS is due to the underlying pathways contributing to the LPS-induced immune response (Zeisberger & Roth, 1998; Broad et al., 2006); an effect that is mainly attributed to attenuated cytokine levels (He et al., 1992). Also, cytokine-mediated stimulation of the vagus nerve have also been demonstrated to induce both sickness behaviors and reductions in locomotory behavior, and effect that is lost following vagotomy (Wieczorek et al., 2005). This demonstrates the role vagus nerve plays in sickness and locomotory behaviors following exposure to systemic stressors. Finally in light the ability of methylmercury to increase extracellular serotonin levels in astrocytes (Dave et al., 1994), increased serotonin levels have been shown to induce sickness-like anorexic behaviors following acute exposure to dexfenfluramine and a tolerance/ attenuated anorexia following 2-3 repeated exposures (Rowland et al., 2001); this tolerance effect was further correlated with c-Fos immunoreactivity in the PVN, CeA and nucleus tractus solitarius.

Given the multifactorial nature of methylmercury toxicity, it would follow that behavioral responses would be similarly diverse in their origin. Behavioral data from the first two experiments coupled with previously published literature would likely indicate that attenuation of exploratory behavior in response to acute IP MeHg could be due to a combination of effects on neurotransmitter release, cytokine induction and/ or vagus nerve stimulation, whereas the lack of significant behavioral data following repeated MeHg exposure could be due to tolerance effects on these same parameters. To clarify some of these questions resulting from Experiment 1 and 2, it would be of interest to use immunohistochemical techniques to co-localize and measure c-Fos with serotonin reactivity within these stress nuclei and to measure blood cytokine levels to determine to

what extent each of these parameters are related to noted differences in open field behavior after exposure to methylmercury.

In contrast to the loss of behavioral effect observed after repeated MeHg, activity of neural stress circuits continued to be modulated by repeated administration of methylmercury, as measured by c-Fos immunohistochemistry. Methylmercury given repeatedly resulted in an increase in the number of c-Fos positive cells in the PVN, CeC, Lc, and BSTm with a decrease in c-Fos noted in the Dg and BSTs, when compared to saline controls. Published data has demonstrated that while acute exposure to heavy metals, such as methylmercury, are capable of inducing release of neurotransmitters (acetylcholine, serotonin, norepinephrine) within stress-processing neural circuits, repeated administration can result in either a similar increase or diminished response that is dependent on both the neurotransmitter and the metal (Hrdina et al., 1976; Faro et al., 1997). Additionally, increases in c-Fos and corticotrophin releasing factor (CRF) levels have been demonstrated within the PVN of the rat brain in response to a repeated physiological (osmotic) stressor (Brown & Sawchenko, 1997). The c-Fos immunohistochemical analysis from Experiment 2 demonstrated that repeated exposure to MeHg results in continued activation of many of the brain's stress nuclei, albeit with a decrease in activity in a few regions. Chronic MeHg exposure (0.4 and 4.0 mg/kg/day) has been shown to decrease brain acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) levels while increasing norepinephrine (NE) levels (Hrdina et al., 1976). Acetylcholine is a neurotransmitter functioning peripherally within the autonomic nervous system and centrally in the cortex and dentate gyrus (e.g. stress-modulating brain regions); therefore its reduction in response to repeated MeHg exposure may help to explain the reduction in

number of recruited stress nuclei and decreased c-Fos immunoreactivity noted in this experiment.

While significant c-Fos responses to acute intraperitoneal (IP) and intracerebroventricular (ICV) exposure to MeHg were noted in many stress-associated brain regions (PVA/PV, PVN, CeC, Dg, Lc, LS, BSTm) and within each of those regions c-Fos immunoreactivity was increased (see Experiment 1) different patterns of activation were noted in response to repeated MeHg exposure. In the current experiment, repeated administration of MeHg resulted in a fewer number of stress-associated brain regions showing increased c-Fos cell counts (PVN, CeC, Lc, and BSTm) with actual decreases in Fos noted in the Dg and BSTs. From this it would seem that stimulation by repeated exposure to MeHg may be resulting in a habituated c-Fos response. Also, the dentate gyrus has been demonstrated to be preferentially sensitive to MeHg exposure (Yuan & Atchison, 1993; Philbert et al., 2000; Cheng et al., 2006) and therefore the diminished c-Fos response could be resulting from MeHg-associated inhibition of protein synthesis (Yosino et al., 1966; Verity et al., 1977).

When the magnitude of the c-Fos response for each brain nucleus was compared between acute and repeated IP 4mg/kg MeHg exposures (the highest common dose) it was found that the PVA/PV ($F_{(1,8)}$=28.43, $p<0.01$), LS ($F_{(1,8)}$=16.04, $p<0.01$) and BSTs ($F_{(1,8)}$=12.35, $p<0.01$) had higher c-Fos immunoreactivity in response to repeated MeHg exposure when compared to the acutely exposed animals (Figures 35 to 37, respectively); all other comparisons were not significant. This tells us that while the number of recruited regions following repeated IP exposure to MeHg decreased (compared to acute IP doses of MeHg), the thalamus, septum and bed nucleus continued to react with

detectable c-Fos protein in a more robust manner than acutely dosed mice. Given the 2-3 day period it takes to accumulate maximum brain MeHg levels following a single IP injection (Nielsen, 1992), the increase in c-Fos immunoreactivity following repeated MeHg exposure could be due to the increased presence of MeHg in the brain. Indeed, a future point of interest would be to measure brain-MeHg levels to determine if greater magnitudes of c-Fos immunoreactivity correlate with level of methylmercury in the brain.

It is also of interest that while open field behavioral measurements in response to repeated MeHg were not statistically different from controls, the PVA, Dg, Lc, and BSTs had increases in c-Fos positive cell counts in response to the open field stressor. Repeated exposure to homotypic stressors, methylmercury in this experiment, followed by a subsequent acute heterotypic stressor, such as open field exposure, can result in a similarly elevated c-Fos response. Watanabe et al (1994) has revealed increases of similar magnitude (i.e. there was no sensitization) in immediate early gene (c-fos) responses within neural stress circuits (PVN, Lc, raphe nuclei and central gray) to psychogenic stressors (restraint and shaking stress), when one is given repeatedly as a homotypic stressor followed by exposure to the second/ heterotypic stressor. Analogous observations were observed in the present experiment, in that repeated pre-exposure to MeHg as an initial homotypic stressor did not alter the c-Fos response of these brain regions to the heterotypic open field stressor.

In spite of the observation that exploratory behavior in the open field was not altered significantly after repeated MeHg, the open field stressor continued to elicit an effect on the underlying neural stress circuitry. This suggests that while both repeated

MeHg and open field exposures resulted in significant induction of c-Fos protein, c-Fos induction alone is not predictive of altered behavior in the open field.

A potential explanation for the difference in c-Fos profiles between acute and repeated IP MeHg could be due the increased or decreased need for its downstream transcriptional products. The most widely accepted function of c-Fos is as a transcriptional modifier that when bound to the AP-1 protein has been linked to suppression of pro-inflammatory IL-12 (Roy et al., 2000; Dillon et al., 2004) and inducible nitric oxide synthase (Okada et al., 2003). Additionally, mice lacking c-Fos (Fos $^{-/-}$) have enhanced production of pro-inflammatory cytokines TNF-α, IL-6 and IL-12, with a reduction in the anti-inflammatory IL-10 following exposure to LPS (Ray et al., 2006). In light of this information, the anti-inflammatory function of c-Fos protein could help to explain the differences in c-Fos production noted between acute and repeated IP MeHg. While this idea warrants further testing, it remains the case that heterotypic stressors following can induce c-Fos production following repeated pretreatment with a homotypic stressor (Armario et al., 2004).

The observed decrease in the number of stress-associated nuclei recruited in response to repeated MeHg administration, in addition to the decreased c-Fos response noted in other nuclei, suggests the development of a habituated response to the repeated MeHg regimen. How this habituation occurs is not known. However, as noted in the General Introduction, MeHg has demonstrated immunosuppressive effects and is capable of eliciting a range of deleterious effects on the subcellular homeostatsis of neurons. These effects include mitotic arrest (Miura et al., 1978), attenuations of mitochondrial energy production (Verity et al., 1975), and reduced protein synthesis (Yosino et al.,

1966; Verity et al., 1977). It is possible that if MeHg passes into the brain and interacts directly with neurons, these underlying changes may be playing a role in the observed habituation effects.

Experiment 3

Repeated Intraparitoneal Methylmercury Exposure and Lipopolysaccharide

4.1 Introduction

Repeated exposure to MeHg in Experiment 2 revealed several significant and informative findings such as no affect on open field behavioral measures, reduced number of recruited stress nuclei and an increase in magnitude of c-Fos immunoreactivity when compared to acute IP exposed mice; however, it remains to be determined if repeated exposure to MeHg affected the ability of these stress-associated nuclei to respond to a different physiological stressor. The previous experiment used a psychogenic stimulus (open field) as the heterotypic stressor, which was still capable of inducing a c-Fos response in the brain. This provided evidence that the c-fos gene was not suppressed by repeated MeHg treatment. Alternatively, the response to a physiological stressor may continue to reflect the observed reduction in those brain regions where evidence of habituation was evident. Therefore the present experiment will utilize systemic injection of LPS, a widely utilized immunologic stimulus with profound neuromodulatory effects, including induction of central c-Fos responses (reviewed in Cohen, 2002 and Karrow, 2006).

Lipopolysaccharide is a major component of the outer membrane of Gram-negative bacteria and a potent stimulator of the innate immune system (Rivest et al., 2001; Cohen, 2002). It is also responsible for the pathogenicity of several genera of Gram-negative bacteria including *Escherichia* (urinary tract infection, meningitis, septicemia, pneumonia), *Salmonella* (typhoid fever, food poisoning), *Pseudomonas*

(pneumonia, septicemia), *Helicobacter* (peptic ulcers, chronic gastritis, stomach cancer), and *Neisseria* (gonorrhea, meningitis). The term endotoxin is typically used interchangeably with LPS, since LPS is an intramembraneous molecule that is released from the bacterial cell membrane following lysis by phagocytic cells of the host immune system. In recent years the mechanism of cellular activation by LPS has been more fully described, and involves binding of the toll-like receptor (TLR)-4/ cluster of differentiation (CD)-14 receptor complex, present on macrophages, monocytes, neutrophils and endothelial cells (Cohen, 2002). Following LPS binding to the TLR4/ CD14 receptor complex, the signal is transduced within the cell causing increases in transcription factors and proinflammatory cytokines (Cohen, 2002; Karrow, 2006). Proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-6, interferon (INF)-γ, and anti-inflammatory cytokines, including IL-10, are inflammatory signaling proteins/ peptides that function in cell to cell communication after stimulation of the immune system (Rivest, 2001). Release of these cytokines from activated immune cells then causes increased levels of inflammatory mediator prostaglandin E-2 (PGE-2), followed by activation of the hypothalamic-pituitary-adrenal (HPA) axis as reflected in increases in blood levels of corticotropin releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) (Cohen, 2002; Verma et al., 2006; Johnson et al., 2004; Neveu & Liege, 2000).

Experimentally, LPS exposure has been used extensively to better understand how immunological stimuli and their downstream effects influence changes in homeostasis. Exposure to LPS has been used to demonstrate how immunologic stress can exacerbate the neurodegenerative mechanisms associated with multiple sclerosis

(MS) (Buljevac et al., 2002), Alzheimer's disease (AD) (Sly et al., 2001) and prion disease (Cunningham et al., 2005). On a cellular level, LPS exposure results in increased permeability of the blood-brain barrier, increase lymphocyte proliferation, and activation of stress-associated brain circuits (Jaworowicz et al., 1998; Ulmer et al., 2000; Lenczowski et al., 1998). Additionally, LPS is a well known activator of c-Fos production within the murine brain (Akasaka et al., 2006; Brochu et al., 1999, Rossi-George et al., 2005). To date, however, there is little information addressing how repeated treatment of MeHg influences subsequent activity of the brain's stress nuclei to bacterial endotoxin.

The focus of this experiment is therefore to administer LPS following the repeated MeHg regimen used in Experiment 2, in order to examine if pretreatment with MeHg is capable of sensitizing/ desensitizing the response to a known immunologic stimulus (LPS) and if the diminished c-Fos response noted in Experiment 2 was due to the inhibitory effects of MeHg on protein synthesis.

4.2 Materials and Methods

In general, the age and strain of mice and preparation and injection of methylmercury were the same as described in the Materials and Methods for Experiment 1. For further detail regarding materials and methodology not described below please refer to Experiment 1, Section 2.2.

4.2.1 Repeated Methylmercury and Lipopolysaccharide – Experimental Procedure

Injections of saline, 2, or 4mg/ kg methylmercury was administered intraparitoneally (IP) every third day for 15 days beginning on day zero (total of 6 injections), as in Methods section 3.2.1. On day 18 mice were treated with either saline or 10µg LPS (Sigma Aldrich, St. Louis, MO) and transcardially perfused 2h after the injection. All injection volumes were 0.2ml. Overall there were six treatment groups: i) Saline/LPS, ii) 2mg/kg MeHg/ LPS, iii) 4mg/kg MeHg/ LPS, iv) Saline/ Saline, v) 2mg/kg MeHg/ Saline, vi) 4mg/kg MeHg/ Saline.

The 10µg IP dose of LPS administered in this experiment was based upon published data from our laboratory. Rossi-George et al (2005) demonstrated that a 5µg IP dose of LPS was capable of significantly increasing brain c-Fos levels, but given the potential for MeHg-induced inhibition of c-Fos protein production, the dose of LPS was raised to 10µg to ensure that any potential reductions in c-Fos levels after LPS exposure were due to MeHg pretreatment and not due to a lack of an effective LPS dose.

4.3 Experimental Results – c-Fos Immunohistochemistry

Repeated MeHg administration (Experiment 2) revealed that by the sixth injection of 2 or 4 mg/Kg MeHg, there was still a significant recruitment of stress-associated brain regions in terms of c-Fos immunoreactivity. The present experiment tested whether 3 days after the final MeHg injection there was a significant response to handling and injection of saline or a neuroactive dose of the proinflammatory stimulus, LPS.

Repeated exposure to MeHg followed by LPS administration 72 hours later did not attenuate the c-Fos response to LPS, which was found to be significant when

compared to controls. Treatment with LPS led to a dramatic increase in the number of c-Fos positive cells present in the PVA/PV ($F_{(1,24)}$=10.35, $p<0.01$), PVN ($F_{(1,24)}$=33.38, $p<0.0001$), CeA ($F_{(1,24)}$=11.83, $p<0.01$), Lc ($F_{(1,24)}$=7.77, $p<0.05$) , and BSTm ($F_{(1,24)}$=10.61, $p<0.01$) when compared to repeatedly treated MeHg or saline groups given vehicle injection (see Figures 38 to 45). Also, repeated administration of varying doses of MeHg did not produce significant c-Fos positive cell count differences in any of the brain regions analyzed after the final injection (see Figures 38-45), suggesting that by the day of LPS administration the c-fos inducing effects of the final MeHg treatment had returned to control levels.

4.4 Discussion

Activity within murine brain stress circuitry to repeated MeHg exposure 72 hours after the last dose demonstrated low c-Fos induction. This reduction in c-Fos was not due to a failure of c-fos induction, since the c-Fos response in these brain regions was strongly induced by subsequent administration of LPS. The lack of c-Fos immunoreactivity 72 hours after the last exposure to MeHg was likely to be in part due to the immediate early gene's transiently active nature. Production of c-Fos within brain nuclei, in response to systemic/ physiological stressors such as LPS and MeHg, has demonstrated that peak levels occur at 2-3 hours after exposure (Wan et al., 1993; Tkacs & Strack, 1997; Cheng et al., 2006). The bell-shaped induction/ degradation pattern for the c-Fos protein has been demonstrated in rat PVN following IP (Wan et al., 1993) or intravenous (Givalois et al., 1995) LPS administration and in the rat CeA after intravenous LPS (Tkacs & Strack, 1997).

Additionally, data from this experiment has demonstrated that there is no significant attenuation of the c-Fos response to LPS exposure after pre-exposure with MeHg. Therefore, repeated exposure to MeHg in this dosing regimen did not inhibit protein synthesis of c-Fos (see Experiment 2), nor did it suppress the ability of these neural stress circuits to become active in response to an immunogenic LPS stimulus. This would also seem to indicate that the reduced number of recruited stress-associated brain nuclei and the reduced c-Fos activation noted in several nuclei following repeated MeHg exposure in Experiment 2 were due to a habituated response that is not dependent on MeHg-induced immunosuppressive or protein synthesis-inhibiting mechanisms within the immune system. This inference is based on evidence that the ability of LPS to activate the HPA axis in rats and mice is dependent on activation of macrophages (Derijk et al, 1991).

This habituation in response to repeated IP administration of MeHg could result from a desensitization of these stress-associated brain regions. It has been well demonstrated that acute administration of LPS strongly activates the HPA axis and stress-associated brain nuclei (Cohen, 2002; Rivest, 2001), whereas repeated injection of LPS produces immunological tolerance after 3-6 daily exposures (Hadid et al., 1996; Takemura et al., 1997; Navarro et al., 2007). While tolerance effects of any immunological effects produced by repeated MeHg has not been addressed, oral MeHg exposure for has been shown to increase ACTH, corticosterone and IL-6 production after short term exposure with attenuation of this response after longer periods of exposure (Ortega et al., 1997). Additionally, tolerance to immunogenic stimulation by LPS has been demonstrated to result in a decreased splenic lymphocyte proliferation and IL-1,

TNF and IL-6 production (Hirokawa & Hayashi, 1980). Habituation to repeated, homotypic MeHg exposure does not affect the ability of these stress circuits to function, as noted by the robust response of these nuclei to the subsequent heterotypic, immunogenic stressor LPS.

Acute and repeated administration of MeHg, given centrally or peripherally, result in unique patterns of activation within the stress-associated regions of the brain when compared to one another. The response of physiological and behavioral stressors mediated by the HPA axis involves both central hypothalamic and extrahypothalamic nuclei and peripheral pituitary and adrenal components. Experiments 1 through 3 have focused on central responses to the neurotoxicant and physiological stressor, MeHg. However, given that each IP dosing regime likely elicited a peripheral stress response prior to its effects on the brain's stress circuitry, examining the peripheral stress response to these different doses of MeHg would be the next research area of interest.

Experiment 4

Splenic Cytokine Production in Response to Acute and Repeated Intraperitoneal Methylmercury Exposure

4.1 – Introduction

The experiments and results reported to this point have focused on the stress response to MeHg exposure within the central nervous system (CNS). However, there is a considerable amount of data on the toxic effects of MeHg outside of the brain. Outside of the CNS, methylmercury has been shown to induce lipid peroxidation in the liver (Lin et al., 1996), epithelial cell lesions of the proximal convoluted tubules in the kidney (Rumbeiha et al., 1991; Yasutake et al., 1990), apoptosis in human blood monocytes (Insug et al., 1997) and T-cells (Shenker et al., 1999), and impairs adrenal function via reduction of blood ACTH and corticosterone levels (Burton & Meikle, 1980). Also, MeHg given to mice has demonstrated immunosuppressive effects characterized by splenic atrophy and reduction in T- and B-cell proliferative responses to mitogen stimulation (Hirokawa & Hayashi, 1980) and increased viral persistence after pretreatment with MeHg (Ilback et al., 2000). The present chapter then focuses on the immunologic effects of MeHg outside of the CNS, using the same repeated administration regimen reported in Experiments 1 and 2. The preceding chapter had already introduced the notion of examining the impact of an immunologic stressor on CNS activation, but had not considered measurement of immune function. Therefore, to begin considering the impact *in vivo* of MeHg exposure, the present study addressed basal levels of cytokines in the spleen.

The peripheral response to physiological stressors frequently involves signaling between circulating cells that monitor changes in homeostasis and organs capable of modulating the stress response. Further, coordination of responses to stress involves bidirectional communication between the immune and nervous systems. Communication from the peripheral immune system to the CNS is currently believed to involve direct cytokine stimulation of the CNS or cytokine-mediated stimulation of centrally-projecting peripheral nerves (Konsman et al., 2000; Ge et al., 2001). Indeed, MeHg treatment activates the PVN and CeC, regions of the brain known to initiate the effector outputs of pituitary-adrenal activation and sympathetic nervous system activity (Herman & Cullinan, 1997), and so it is conceivable that MeHg treatment may alter splenic cytokine function through these mechanisms, since cells of the immune system contain glucocorticoid and adrenergic receptors (Karrow, 2006).

Cytokines are immunologically-derived, signaling molecules produced by activated macrophages, monocytes, dendritic cells, T-helper cells, endothelial cells, and microglia (Rivest 2001; Hickey 2001; Correale & Villa, 2004). Release of cytokines at the site of immunological stimulation and into the circulatory system serves to recruit additional immune cells to the site of infection and alert other immunomodulating systems such as the HPA axis and the autonomic nervous system (Rivest, 2001). Increased levels of circulating cytokines, via peripheral administration of inflammatory cytokines themselves or immunogenic stimuli such as LPS, increase immediate early gene (IEGs) production within stress circuits in the brain (Akasaka et al., 2006; Brochu et al., 1999), induce central cytokine production (reviewed by Cohen, 2002) and activate the HPA axis (Verma et al., 2006; Johnson et al., 2004; Neveu & Liege, 2000). In contrast to

their size and hydrophilic nature, which would prohibit passive diffusion, cytokines are capable of crossing the blood-brain-barrier (BBB) via a receptor-mediated mechanism (Plotkin et al., 1996; Gutierrez et al., 1993; Osburg et al., 2002). Further, cytokine transportation across the BBB increases in response to immune-mediated CNS trauma (eg., autoimmune encephalomyelitis, brain trauma, and LPS) (Pan et al., 1996; Pan et al., 2003; Osburg et al., 2002). Cytokines have also been demonstrated to permeate the brain's circumventricular organs and bind vascular endothelial cells (Blatteis, 1992; Watkins et al., 1995). Increases in brain cytokine levels in response to peripheral immunogenic stimulation, have been repeatedly shown to correlate with activation of the HPA axis (Dunn, 2000; reviewed in Neveu & Liege, 2000 and Kusnecov & Goldfarb, 2005). Further, intracerebroventricular (ICV) administration of the proinflammatory cytokine IL-1β results in increased plasma and splenic ACTH in the rat (Jessop et al., 1997). In summary, peripheral systemic stressors have been demonstrated to increase peripheral production of inflammatory cytokines, activate central stress circuits and cytokine production, which can then further modulate peripheral stress responses.

Typically cytokines are divided into those that display predominantly proinflammatory activity or anti-inflammatory activity. Proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, and IL-2 are frequently associated with immune cell proliferation, differentiation, recruitment and apoptosis, the fever response, increased expression of cell adhesion molecules (CAMs) on endothelial cells and facilitation of antibody production (Correale & Villa, 2004; Rivest, 2001). Proinflammatory cytokines are crucial for initiating and maintaining an effective acute phase immune response following exposure to potential pathogens. Anti-inflammatory

cytokines function to balance the acute phase pro-inflammatory response. Anti-inflammatory cytokines such as IL-10 have been shown to inhibit synthesis of pro-inflammatory cytokines, antigen presentation and phagocytic activity of host immune cells (Hickey, 2001).

Both physiological and psychological stressors are capable of stimulating production of inflammatory cytokines in the periphery, which are then able to activate central stress responses. Physiological stressors such as hypertonic saline (Hashimoto et al., 1990; Kiss & Aguilera, 1993), insulin-induced hypoglycemia (Fuller & Snoddy, 1977; Robinson et al., 1992) and bacterial endotoxin (reviewed in Karrow, 2006) have been shown to increase HPA axis responses as measured by ACTH and corticosterone levels. Systemic, serum cytokine levels also increase in response to psychogenic stress. For example, chronic mild foot shock has been shown to increase splenic levels of the inflammatory cytokines TNF-α, IL-1β, IL-2, IL-10 (Cao et al., 2007). Additionally, exposure to acute restraint stress increases plasma IL-6 levels in rats and mice (Zhou et al., 1993; Takaki et al., 1994). *In vitro* studies utilizing human peripheral blood mononuclear (PMN) cells exposed to hypertonic saline also showed increases in IL-1β and IL-8 (Shapiro & Dinarello, 1997). Similarly, physiological stress induced by hypertonic saline exposure significantly increased IL-10 mRNA levels in murine peritoneal macrophages *in vivo* and endothelial cells *in vitro* (Oreopoulos et al., 2001).

While there are numerous experiments have demonstrated the effects of immunological and non-immunological physiological stressors on circulating cytokines, the effects of mercury compounds on the immune system, and more specifically cytokine production, have not been as widely addressed. One such study has shown that chronic

exposure to MeHg given in drinking water modulates circulating levels of IL-6 (Ortega et al., 1997). Additional work has demonstrated that injections of inorganic and organic mercury have also been shown to induce autoimmune symptomology characterized by increased IL-4 levels, hyperimmunogobulinemia (Haggqvist et al., 2005), lymphocyte (Stiller-Winkler et al., 1988), and T-cell-dependent polyclonal activation of B-cells (Havarinasab et al., 2007[A]). Also, in mice genetically predisposed to autoimmunity and dosed with methylmercury, preferential sequestration of mercury (Hg^{2+}) in peripheral lymphoid tissue was noted; which was associated with an increase in macrophage number (Havarinasab et al., 2007[B]). Further, in vitro methylmercury exposure to human lymphocytes results in genotoxic increases in the number of chromosomal aberrations and polyploidy (Silva-Periera et al., 2005) and induces IL-6 production in human and rat glioma cells (Chang, 2007). From these studies it can be concluded that MeHg is capable of affecting several immunological parameters, including cytokine production, activation of immune cells, and accumulation in secondary immune organs (spleen) in animal and human modeled systems.

Given that the data presented in Experiments 1-3 have shown differing behavioral and central c-Fos responses to acute and repeated IP MeHg exposures, it would be of significant interest to determine how peripheral MeHg exposure affects the peripheral cytokine response. As stated in the previous experiments, given the pattern of activation in the brain produced by acute and repeated MeHg treatment, it is quite possible that that MeHg increased sympathetic and HPA axis activity, which could have then lead to increases in circulating cytokine levels. The current experiment then examined the

effects of acute and repeated intraperitoneal MeHg exposure on splenic production of both pro- and anti-inflammatory cytokines in the mouse.

4.2 - Materials and Methods

All materials and methods were the same as presented in the acute and repeated methylmercury experiments except where noted below. For further detail regarding materials and methodology not described below please refer to Section 2.2 and 3.2, respectively. It should be noted that the spleens for this experiment were taken from Experiments 1 and 2 in accordance with the methodology listed below.

Total protein and ELISA cytokine quantification

Spleens from mice given acute and repeated IP doses of 2 and 4 mg/Kg MeHg were analyzed by enzyme-linked immunosorbent assay (ELISA) for inflammatory cytokines TNF-α, IL-1β, IL-6, IL-2, and IL-10. Immediately after beginning the transcardial saline perfusions in Experiments 1 and 2, spleens were extracted, flash frozen in 2-methylbutane cooled with dry ice, wrapped in labeled aluminum foil and placed into a -70˚F freezer until ready for analysis.

For determination of total protein levels, spleens were dissected and placed in 1ml of 1mM phenylmethanesulfonylfluoride (PMSF) in 0.1M phosphate buffer to inhibit protease activity. The tissue was then homogenized and centrifuged for 30 minutes at 4000rpm, after which the supernatant was collected. A bicinchroninic acid (BCA) protein assay kit (Pierce Biotechnology; Rockford, IL) was used to quantify total protein levels, which were calculated from a standard curve produced from bovine serum albumin

(BSA) standards at an absorbance of 562nm. Total protein levels were expressed as μg/ml. Cytokine data was analyzed as a ratio of total protein.

The supernatants from spleen homogenates were assayed for TNF-α, IL-1β, IL-6, IL-2 and IL-10 with OptEIA ELISA kits in accordance with manufacturer's instructions (BD Biosciences; San Diego, CA). An EL800 universal BioTek microplate reader analyzed samples at 450nm. All samples and standards were run in duplicate. KC Junior software (BioTek) was used to determine the standard curve from which sample cytokine and protein concentrations were calculated. All cytokine data is expressed per microgram of protein.

4.3 – Experimental Results

For easy comparison, summary of the cytokine data from the acute and repeated experiments are presented in the same figure. However, separate statistical analyses were applied on the cytokine data from each experiment, since they were independently executed experiments.

Tumor Necrosis Factor-Alpha

A two-way ANOVA was conducted on the TNF-α values from the acute treatment experiment. The results revealed a significant main effect of open field exposure ($F_{(1,22)} = 4.67$, $p < 0.05$), but no main effect for MeHg ($F_{(2,22)} = 1.15$, $p = 0.33$), nor an open field- MeHg interaction effect ($F_{(2,22)} = 0.29$, $p = 0.74$) (See Figure 46, left panel). It is evident that the main effect of open field was due to a reduction of TNF-α concentration in animals exposed to the open field that had been pretreated with saline or

2 mg/Kg MeHg groups. There was no difference in TNF-α concentration between home cage and open field exposed animals pretreated with 4 mg/Kg MeHg.

Analysis of the TNF-α data from the repeated treatment experiment revealed significant main effects of MeHg treatment $F_{(2,23)} = 4.04$, $p < 0.01$) and open field exposure $F_{(1,23)} = 7.41$, $p = 0.01$). Moreover, a borderline interaction effect was obtained ($F_{(2,23)} = 3.06$, $p = 0.066$). Examination of Figure 46 (right panel), reveals that similar to results from the acute experiment, both saline/OF and 2mg/Kg MeHg/OF animals were markedly lower than corresponding saline and 2 mg/Kg MeHg treated HC animals. The main effect of MeHg treatment is less evident, but was revealed by a post hoc Fisher's LSD test to be due to all (combined open field and home cage) 2 mg/Kg animals being less than all 4 mg/Kg animals ($p < 0.05$).

Interleukin-1β

Analyses of IL-1β cytokine responses to acute MeHg and OF stimulation were similar to those noted for TNF-α. Significant reductions in IL-1β cytokine levels were noted in response to OF exposure (main effect, $F_{(1,22)}= 4.43$, $p< 0.05$) while main effect cytokine responses to MeHg ($F_{(2,22)}= 1.03$, $p= 0.38$) and combined MeHg-OF interactions ($F_{(2,22)}= 0.26$, $p= 0.77$) were not significant (Figure 47, left panel). The reduction in IL-1β levels was noted for the saline control, 2 and 4mg/kg MeHg treatments.

Main effect responses of IL-1β following repeated MeHg exposure were not statistically significant after exposure to the OF ($F_{(1,22)}= 0.17$, $p= 0.69$), MeHg ($F_{(2,22)}= 0.23$, $p= 0.80$) or in response to MeHg-OF interaction effects ($F_{(2,22)}= 0.15$, $p= 0.86$) (Figure 47, right panel).

Interleukin-6

Following acute MeHg administration, splenic IL-6 levels did not respond significantly to main effect OF exposure ($F_{(1,20)}$= 2.91, p= 0.10), MeHg treatment ($F_{(2,20)}$ = 2.10, p= 0.14) or in response to interactions between MeHg and OF ($F_{(2,20)}$= 0.29, p= 0.75) (Figure 48, left panel).

The IL-6 response to repeated MeHg exposure gave several significant findings. Interleukin-6 levels demonstrated main effect decreases in response to open field exposure ($F_{(1,24)}$ = 8.32, p< 0.01) and increases following MeHg administration ($F_{(2,24)}$= 5.16, p< 0.05). The interaction between MeHg and OF was not found to be significant ($F_{(2,24)}$= 1.87, p= 0.18) (Figure 48, right panel). Exposure to the open field resulted in decreases in the 2 and 4mg/kg repeatedly dosed groups, when compared to respective home caged controls (both p< 0.01).

Interleukin-2

Measurement of IL-2 levels in response to acute MeHg exposure and OF exposure gave varying results. Main effect IL-2 responses to acute MeHg were significantly increased ($F_{(2,14)}$= 5.18, p< 0.05), while responses to the OF and acute MeHg-OF interaction effects were not significantly different from controls($F_{(1,14)}$= 1.30, p= 0.27 and $F_{(2,14)}$= 0.34, p= 0.71, respectively) (Figure 49, left panel).

No significant main nor interaction effects were observed for IL-2 in response to repeated MeHg administration[MeHg treatment ($F_{(2,21)}$= 1.29, p= 0.30), OF exposure ($F_{(1,21)}$= 0.0001, p= 0.99), and MeHg-OF interaction ($F_{(2,21)}$= 0.64, p= 0.54)] (Figure 49, right panel).

Interleukin-10

Two-way ANOVA analysis of IL-10 data after acute exposure to MeHg gave both significant and non-significant data. Acute exposure to MeHg resulted in decreased splenic IL-10 levels when compared to saline controls (main effect, $F_{(2,21)}= 3.57$, $p< 0.05$) (Figure 50, left panel). However, levels of IL-10 were not significantly affected by exposure to the open field ($F_{(1,21)}= 0.08$, $p= 0.93$) nor did OF interact with MeHg ($F_{(2,21)}= 0.50$, $p= 0.61$).

Repeated exposure to MeHg resulted in decreases in splenic IL-10 levels as a function of open field exposure ($F_{(1,23)}= 11.32$, $p< 0.01$), while an overall increase was observed in response to MeHg exposure ($F_{(2,23)}= 4.46$, $p< 0.05$) (Figure 50, right panel). No significant MeHg-OF interaction effects were noted for IL-10 in the repeated treatment experiment ($F_{(2,23)}= 2.39$, $p= 0.11$). More specifically, the OF/ 4mg/kg MeHg treatment had lower levels of IL-10 than did the respective HC control ($p< 0.01$).

4.4 Discussion

Splenic cytokine production in response to acute and repeated MeHg gave results that were specific for both treatment and cytokine. Generally, this experiment demonstrated that acute intraperitoneal exposure to MeHg results in no activation of proinflammatory cytokines TNF-α, IL-1β, and IL-6, an increased concentration of IL-2, and decreased levels of the anti-inflammatory cytokine IL-10. The lack of proinflammatory cytokine alteration could be due to MeHg's immunosuppressive action, as it has been shown to reduce *in vitro* activation of human monocytes and T-cells (Shenker et al., 1992), and in turn the circulating cytokines produced by these immune

cells (Gajewski et al., 1989). Previous work has demonstrated that *in vitro* exposure of murine splenic lymphocytes to MeHg increases IL-2 and interferon-gamma (INF-γ) levels (Hu et al., 1997). Acute MeHg exposure has been shown to increase splenic T-cell proliferation in the mouse (Pheng et al., 2003), an event that occurs concomitantly with increases in IL-2 and IL-2 receptors (Johansson et al., 1997). Also, given that IL-2's well established role in T-cell development (Sakaguchi et al., 1995; Thorton et al., 2004), it would then follow that acute MeHg-induced T-cell proliferation noted in the literature occurs with coinciding production of IL-2, noted in this experiment. Given that IL-10 is secreted mainly by activated cytotoxic T-cells (T_c), it would be of interest to determine if circulating T-cell counts were indeed higher after acute MeHg exposure. It would also be worth exploring whether T-cells were activated in greater proportion because while IL-2 increases could be the result T-cell proliferation, the potential lack of activation may also help explain lack of TNF-α, IL-1β, and IL-6 proinflammatory cytokine production noted in this experiment.

Repeated exposure to MeHg tended to increase both pro- and anti-inflammatory cytokine levels with no reductions noted. Specifically, repeated MeHg increased IL-6 and IL-10. Chronic exposure to mercury has been shown to also increase T-cell proliferation and preferentially accumulate in and activate murine macrophages (Hirokawa & Hayashi, 1980; Cunha et al., 2004). Linking these two concepts Kubica-Muranyi et al. (1993) were able to show that in vivo murine models of mercury-induced autoimmunity increased T-cells proliferation and reactivity to antigens released from mercury containing macrophages. Initial T-cell proliferation followed by accumulation in macrophages and later activation of T-cells is a plausible mechanism by which repeated MeHg can be

increasing cytokine levels within this experiment, as activated macrophages and T-cells both produce TNF-α, IL-6 and IL-10 (Cohen et al., 2002). However, in addition to examining T-cell proliferation and activation, given the potential relationship between T-cells and macrophages following MeHg exposure, it would also be worth examining mercury concentrations in splenic and lymphatic macrophages.

Exposure to the OF was conclusively demonstrated to reduce splenic cytokine production, regardless of the frequency of MeHg exposure. Following acute MeHg administration, OF exposure resulted in decreases in TNF-α and IL-1β, while OF exposure following repeated MeHg reduced TNF-α, IL-6, and IL-10 levels. The reduced cytokine response to a psychogenic stimulus could be due to multiple mechanisms that remain to be explored. For example, both glucocorticoids and sympathetically derived catecholamines have been shown to influence the immune system (Elenkov & Chrousos, 2002), and are elevated after stressor exposure (Kvetnansky et al., 1995). Therefore, future research is needed to explore these potential mechanisms under conditions of open field exposure and/or MeHg treatment.

As with many experiments, data from this experiment demonstrate that while cytokine responses to both MeHg and open field yielded significant results, many more questions remain. It would be interesting to repeat this study looking then at blood ACTH, corticosterone and inflammatory cytokine levels to determine how they relate to splenic levels, given that the spleen is an immunologic organ that functions to monitor blood for pathogens and activated host-cells responding to pathogens.

General Discussion

The focus of this thesis was to better understand the extent to which MeHg is capable of affecting activation of brain nuclei typically recruited during a stress response, as measured by c-Fos immunohistochemistry. Movement in the open field and splenic cytokine production were used as measures of the behavioral and immunologic response to MeHg as a physiological stressor. The open field was also used to determine whether neuronal activation seen in stress-associated regions of the brain was associated with behavioral changes, while changes in basal levels of splenic cytokines provided an indication of whether acute and repeated MeHg treatment modified cellular systems outside the brain.

Activation of c-Fos occurs in response to a variety of stressful stimuli. Physiological stressors such as hypertonic saline (Pirnik & Kiss, 2005), insulin-induced hypoglycemia (Niimi et al., 1995), and heavy metals such as lead (Savolainen et al., 1998) and arsenic (Garcia-Medina et al., 2007) have been demonstrated to induce neuronal c-Fos production. Additionally, exposure to psychological stressors such as restraint (Kwon et al., 2006), swim test (Duncan et al., 1993) and open field (Handa et al., 1993) also increase c-Fos levels in stress-associated brain nuclei. Selection of brain regions analyzed in this set of experiments was guided by previous research that demonstrated activation of the hypothalamus, thalamus, amygdala, hippocampus, and lateral septum in response to physiological (Karrow, 2006) and psychological stressors (Senba and Ueyama, 1997). The locus coeruleus was examined as it is an important

region in the response to stress as it projects NE-neurons to nearly all regions of the brain and is implicated in anxiety-like behaviors (Carrasco and Van de Kar, 2003).

As discussed in the general introduction, MeHg exposure results in a range of neurobehavioral deficits, gross histopathological abnormalities and marked effects on cellular homeostasis. Physiological and behavioral stressors can induce improper regulation of stress with subsequent effects on the HPA axis and as a result have been implicated in the neural dysfunction associated with psychological disorders (Kathol et al., 1989; Charney et al., 1993; McEwen et al., 2004), systemic diseases (McEwen & Stellar, 1993) and neurodegenerative disease (Landfield & Eldridge, 1991). An effect that is thought to be due to excessive stimulation of corticotrophin-releasing hormone neurons of the PVN (Raadsheer et al., 1994); a brain nuclei involved in the HPA axis response to stress. Thus the ability of MeHg to broadly affect homeostatic function within the CNS and data indicating that over activation of stress-associated brain nuclei is involved in several neurological diseases, coupled with little experimentation examining the effects MeHg on the activation of these stress nuclei was an underlying reason for conducting the research within this thesis. The general question when beginning this set of experiments was: how does MeHg, a known neurotoxicant, affect the stress response in the brain and in the periphery, and in turn potentially effect behavior. This final chapter synthesizes these observations and will consider the limitations and future possibilities of the research initiated in this thesis.

The function and transiently-active nature of immediate early genes make them a particularly useful tool for analyzing cellular activity, and for this reason, the *c-fos* gene and its protein product c-Fos, have been used to provide neuroanatomical mapping of the

brain following a variety of different treatments, ranging from exposure to psychogenic stressors to exposure to pharmacological agents and physiological stressors (Pirnik & Kiss, 2005; Karrow, 2006; Savolainen et al., 1998; Garcia-Medina et al., 2007; Niimi et al., 1995). While the research reported here has utilized c-Fos in this capacity, some consideration should be given to the additional roles that c-Fos may be playing within the context of these experiments. C-Fos (and other) IEGs are activated in response to extracellular stimuli and play crucial roles in transcriptional regulation and signal transduction. Activation of IEGs is quick (minutes to hours) and their up-regulation lasts for a brief period of time (peaking at 1-3 hours) (Senba & Ueyama, 1997). Transcription of *c-fos* occurs after extracellular stimulation results in receptor-mediated induction of second messengers and Ca^{2+} influx, causing activation of several protein kinases (protein kinase A, C and MAP) (Sheng & Greenberg, 1990). Phosphorylation of the CREB binding domain by these kinases then triggers c-Fos production. After translation, the c-Fos protein then heterodimerizes with the IEG protein c-Jun to produce the activator protein (AP)-1 transcription factor, which binds to regulatory regions of target genes, resulting in further gene transcription (Senba & Ueyama, 1997). Neuronal target genes of AP-1 after stressful stimulation are currently not well characterized.

Genetic mutations of the *c-fos* gene have provided insight into the functional role of this gene and its protein product. Transgenic mice manipulated to under-express *c-fos* show deleterious alterations in neuronal function in response to kainic acid. Deficient mice show a higher degree of seizure severity coupled with an increasing likelihood of death, associated with larger levels of neuropathological damage and TUNEL positive cells, suggesting excitotoxic cell death (Zhang et al., 2002). Also, the induction of c-Fos

protein allows for AP-1 formation, which has been shown to mediate neuronal transmission/ excitability via expression of excitatory amino acid receptors (Hollmann & Heinemann, 1994).

Experiment 1: Effect of Acute Methylmercury Exposure on Activation of Neural Stress Circuitry and Open Field Exploratory Behavior

Following acute intraperitoneal (IP) and intracerebroventricular (ICV) exposure to methylmercury c-Fos data demonstrated that both peripheral and central administration of MeHg can similarly recruit stress nuclei in the murine brain. Data from Experiment 1 also revealed that acute IP MeHg administration results in dose-dependent decreases in exploratory behavior in the open field and that open field stimulation itself was also capable of activating many stress-associated brain nuclei.

Data demonstrating that both peripheral (IP) and central (ICV) acute exposure to MeHg results in recruitment of the same central stress nuclei is of conceptual interest. Acute MeHg given IP is distributed to and interacts with peripheral cells and organs first, prior to exerting any direct effects on the brain. Toxicokinetic data from mouse studies have shown that after a single peripheral (oral or IP) exposure, MeHg concentrations in the kidney and liver peak hours after exposure while brain levels peak 2-3 days after exposure (Nielsen & Andersen, 1991; Nielsen, 1992).

Given that in Experiment 1 mice were sacrificed 2 hours after injection, the robust c-Fos response in the brain was likely due to peripherally-modulated pathway(s) and not due to the direct central effects of MeHg. Communication between peripheral, immune

and organ systems and the central nervous system is believed to be via cytokine-mediated mechanisms and/or by stimulation of centrally projecting nerves (Konsman et al., 2000; Ge et al., 2001). Production of pro-inflammatory cytokines can occur rapidly, as noted in response to LPS exposure, with peak serum TNF-α levels found around 1.5 hours after administration and undetectable at 4-6 hours (reviewed in Gifford & Flick et al., 1987), which is temporally similar to the pattern of c-Fos activation (Wan et al., 1993; Tkacs & Strack, 1997). Thus it is very possible that the MeHg-induced increases in c-Fos immunoreactivity in the brain and attenuated behavior in the open field noted in Experiment 1 occurred as a function of initial changes that occurred in the periphery.

As detailed in Chapters 4 and 5, peripheral production of cytokines occurs in response to many physiological (Fuller & Snoddy, 1977; Kiss & Aguilera, 1993) and psychological (Zhou et al., 1993; Cao et al., 2007) stressors. Mercury exposure has been demonstrated to increase in vivo and in vitro cytokine production of interleukin (IL)-1 and tumor necrosis factor (TNF)-α in murine macrophages (Zdolsek et al., 1994; Kim et al., 2002). Cytokines such as IL-1β, TNF-α, IL-6 are capable of crossing the blood brain barrier (Banks et al. 1993; Gutierrez et al., 1993; Banks et al., 1994, respectively). Further, peripheral administration of these cytokines is capable of inducing HPA axis activation (reviewed by Dunn, 2000) and neuronal c-Fos production (Senba & Ueyama, 1997; Rivest, 2001).

Therefore the increases in c-Fos noted in stress-associated nuclei of the murine brain in response to acute doses of MeHg could be result of MeHg-induced immunogical stimulation of macrophages followed by increases in circulating inflammatory cytokines. Consistent with this idea was the notion that increased production of IL-2 was revealed in

the spleen in response to acute IP MeHg exposure, noted in Chapter 5. There is evidence to suggest that IL-2 increases in the neurotransmitter NE in the PVN and cortex of the mouse brain (Zalcman et al., 1994). Thus the peripheral increases in IL-2 demonstrated in this experiment could be increasing activity of the PVN and modulating activity to afferently projecting stress nuclei. To better substantiate the claim that circulating IL-2 is affecting the central c-Fos response to acute peripheral MeHg, it would be useful to run an experiment with the same methodology as Experiment 1, but instead of perfusing two hours after MeHg exposure, trunk blood and brains could be taken for cytokine analysis. If cytokine levels were increased, it would be useful to administer similar IP MeHg doses to IL-2 or IL-2-receptor knock-out mice. Indeed, data for other cytokine deficient mice has revealed inhibition of HPA axis stress responses to the cytokine-inducing stimulus LPS (Kozak et al., 1998; van Enckenvort et al., 2001; Hayley et al., 2004).

In addition to cytokine-mediated communication between the immune system and the CNS, studies have demonstrated that the vagus nerve is a potential link between the immune and central nervous systems (Dantzer, 1994). Anatomically, the vagus nerve extends from the brain stem (innervating the area postrema and nucleus tractus solitarius) to the abdominal cavity and functions in maintaining proper heart rate, gastrointestinal peristalsis and breathing. Peripheral immuno-modulated information is thought to enter dorsal vagal complex via the vagus nerve, where it can be relayed afferently via central stress-circuits, including the hypothalamus, leading to activation of the HPA axis (Ge et al., 2001). Research has demonstrated that subdiaphragmatic vagotomy followed by intraperitoneal administration of LPS results in a decrease in plasma adrenocorticotropic hormone (ACTH) and corticosterone levels (measures of HPA axis activity), c-Fos

protein production in limbic and autonomic regions (PVN, CeA, BST, nucleus tractus solitarius), and attenuation of behavioral suppression in response to LPS (Konsman et al., 2000; Marvel et al., 2004; Wieczorek 2005). If the vagus nerve may also be playing a role in communicating the MeHg stimulatory response from the periphery to the CNS, an additional experiment looking at the central c-Fos response to a similar dosed of MeHg in vagotomized mice would shed light on this issue. In such an experiment, one could hypothesize that vagotomy would lead to markedly decreased c-Fos responses in the brain when compared to non-vagotomized animals. Whereas if there were no difference in c-Fos profiles, it would lead me to speculate that other mechanisms, such as cytokines may be playing more of a role in communicating information from the periphery to the brain.

To take this idea further, exposure to metallic salts is also thought to play a role in creating a malaise-like behavioral response that is coupled with activation of neural stress circuitry. Intraperitoneal lithium chloride (LiCl) exposure has been demonstrated to induce internal malaise, as measured in conditioned taste aversion tests, and to induce c-Fos production in brainstem nuclei (AP, NTS), an effect that was dependent on the vagus nerve (Yamamoto et al., 1992). Additional central c-Fos responses to LiCl-induced malaise were noted in the PVN and CeA (Lamprecht & Dudai, 1995). Peripheral exposure to arsenic was shown to increase c-Fos activation in hindbrain and forebrain regions likely via nerves projecting from the gastrointestinal (GI) tract to the brain stem (Garcia-Medina et al, 2007). Interestingly, this effect was shown to be independent of the vagus nerve (Garcia-Medina et al., 2007). Therefore it is also plausible that IP MeHg in

this experiment could be stimulating non-vagal afferent GI nerves, such as the splanchnic nerves.

It is apparent from Experiment 1 that several stress-associated nuclei in the murine brain were activated in response to acute IP and ICV MeHg, but how these nuclei afferently and efferently transfer synaptic information between one another (and potentially activate one another) is a point of interest. The most posterior stress-associated brain nucleus examined for activity in these experiments was the brain stem region, the locus coeruleus (Lc). The Lc is a major source of noradrenergic neurons, and responds to stress and panic. Major efferent projections from the Lc lead to nearly all major brain regions (spinal cord, additional brain stem nuclei, cerebellum, thalamus, hypothalamus, hippocampus, amygdala and cortex), and in turn receive input from the hypothalamus, amygdala and the cingulate gyrus (Saper, 1987; Dunn et al., 2004). The Lc has been shown to play an important excitatory role in stimulating the paraventricular hypothalamic nucleus (PVN) and HPA axis in response to psychological restraint (Ziegler et al., 1999) and in response to physiological cytokine-induced stress (Lacosta et al., 1998). Additionally, the function of the circumventricular organ, the area postrema, includes detection of toxins in the blood and the vomit reflex/sickness behavior (Sutton et al., 1988). Interconnectivity of other brainstem nuclei, notably between the area postrema (AP) and nucleus tractus solitarius (NTS), which connects anteriorly to the PVN, contributes to PVN activation after peripheral IP exposures to systemic interleukin-1 (Buller et al., 1998), a cytokine that increases in response to peripheral MeHg exposure (see above, Zdolsek et al., 1994 and Chapter 5). The notion that systemic/ physiological stressors stimulate brainstem regions, which then relay this stimulation directly to the

PVN for modulation of the HPA axis has been well demonstrated in the literature (reviewed by Herman & Cullinan, 1997). In addition to those regions already identified above, the Lc also innervates additional stress-associated nuclei such PVA/PV, CeA, Dg and cortex, all of which are capable of further stimulating or inhibiting stress-induced stimuli to regions such as the LS and BST (Saper, 1987; Dunn et al., 2004). For instance the gamma amino butyric acid (GABAergic) circuits between the CeC and BST are so strongly linked that the BST is considered by many to be an extension of the CeA; in this way stimulation of the CeA is often coupled with stimulation of the BST (Cassell et al., 1999). Also, the hippocampus receives noradrenergic stimulatory input from the Lc and BST, GABAergic projections from the PVA and has strong GABAergic inhibitory efferent projections to the PVN as a means for regulating its activation (Forray & Gysling, 2004). It could be hypothesized that strong activation of the anteriorly-projecting afferents from the Lc after MeHg exposure might then activate the hippocampus to help limit the stress response of the PVN (and HPA axis). This notion is conceivable in light of Lc lesion studies that result in an inhibited PVN response to neural hemorrhage (Rodovalho et al., 2006), while hippocampal lesions are associated with increased corticotropin releasing hormone (CRH) mRNA in the PVN (Herman et al., 1995[B]).

Given previous studies demonstrating the interconnectivity of the brain's stress nuclei noted above and examined in Experiment 1, one could hypothesize that systemic exposure to MeHg results in vagus or GI-innervating nerve mediated activation of brainstem nuclei followed by the Lc, which both can then activate the PVN and the additional stress nuclei. As both IP and ICV administration of MeHg similarly activated

these neural stress nuclei, it would be interesting to see how lesioning of the Lc or the PVN affect the c-Fos response to similarly administered acute injections of MeHg. Additionally, given the strong neurotoxic nature of MeHg it would be of interest to examine how measures of toxicity, such as TUNEL, ROS production, ATP and Ca2+ levels, co-localize with c-Fos production. Given the pleiotropic nature of the c-fos and its broad effect as a transcription factor, taken with what has been reported in the literature and this thesis, it is unlikely that c-Fos measures are predictive of MeHg toxicity within stress-associated brain nuclei. Instead it is likely that c-Fos induction by neural stress nuclei is likely a protective effect used to maintain a regulated stress response to MeHg, acting as a physiological stressor (Ray et al., 2006). However, Cheng et al. (2006) were able to demonstrate that low to moderate levels of methylmercury (0.5 and 5.0 mg/kg, respectively) induced increases in c-fos mRNA levels which were correlated with increases in nitric oxide in both the hippocampus and cortex.

Literature has shown that exposure to MeHg is capable of affecting different measures of exploratory behavior (Salvaterra et al., 1973; Morganti et al., 1976; Goulet et al., 2003; Weiss et al., 2005). Consistent with these findings, Experiment 1 was able to further demonstrate that IP administration of MeHg resulted in a dose-dependent decrease in exploratory behavior as measured by increased time spent in the outer/ peripheral zone, decreased time spent exploring the inner zone and a reduction in total distances traveled (see Experiment 1 for further detail).

In Experiment 1, exposure to the open field also served to significantly activate central stress nuclei in injected mice. Exposure to psychogenic stressors such as restraint (Chowdhury et al., 2000), forced swim (Duncan et al., 1993), and open field (Rossi-

George et al., 2005) have been shown to increase c-Fos in the PVN, CeA and Lc stress nuclei. In this experiment open field stress also induced c-Fos production in the PVN and in the PVA, Dg, LS, and BSTm/s indicating that these additional areas could be involved in the stress-response to the open field. Psychological stressors have been shown to modulate activation of the BST (Choi et al., 2007), Dg (Cullinan et al., 1995), LS and PVA (Martinez et al., 1998). Given that methylmercury is capable of both inducing c-Fos and strongly altering behavior in the open field it would be interesting to examine how, behaviorally, c-fos knockout mice respond to similar doses of MeHg, as this would allow one to determine how important the c-fos gene is in mediating behavior following MeHg exposure.

Alterations in exploratory behavior after acute IP MeHg exposure may be resulting from a malaise or sickness-like behaviors. Doses of LiCl used to induce malaise in taste-aversion experiments have also been shown to result in open field reductions in approaches to the novel object and time spent within the inner zone (Crawley, 1983) and distance traveled (Gray et al. 1976), which is quite similar to the decreases in exploratory behavior noted in response to MeHg in Experiment 1. In addition to these behavioral similarities, as mentioned previously, exposure to MeHg results in recruitment of similar central stress nuclei, providing further support for the notion that MeHg is perhaps acting in similar fashion to LiCl. Further, lithium chloride induced malaise is attenuated by dopamine (DA D_1 and D_2) receptor antagonists as measured by saccharine consumption, alluding to the role of this neurotransmitter in malaise/ sickness behaviors (Fenu et al., 2005). Exposure to MeHg does induce spontaneous efflux of dopamine (McKay et al.,

1986; Kalisch & Racz, 1996), which could also be contributing to the open field behavior noted in this experiment.

Sickness behavior may also be contributing to the effects on open field behavior as MeHg exposure in mice has been shown to result in immediate appetite suppression (anorexia) (Berthoud et al., 1976; Magos, 1982), which may result from the underlying stress response to MeHg as revealed by activation of central stress nuclei. Anorexic responses to LiCl and cholecystokinin-induced gastric stimulation have been shown to occur along with increases in PVN c-Fos production, a response that is attenuated by lesioning of the NTS brainstem nuclei (Portillo et al., 1997; Rinaman, 2003). This suggests that activation of central stress-mediating nuclei after stimulation of GI projecting nerves is occurring through the brainstem. Increases in c-Fos production in the BST and CeA were also noted in response to cholecystokinin-stimulated anorexia (Li & Rowland, 1994).

Experiment 2: Effects of Repeated Methylmercury Exposure on Central Stress Circuit Activation and Exploratory Behavior

After discussing the implications of data from Experiment 1, which addressed single peripheral and central doses of MeHg as strongly stimulating central stress circuits, it became apparent that it would be important to document how repeated administration of MeHg affects activation of neural stress circuitry and exploratory behavior in the open field.

Single acute human exposures to organic (dimethyl) mercury have been documented within the scientific literature (Nierenberg et al., 1998); however, the majority of human MeHg poisonings have resulted from repeated exposures occurring over a period of weeks to months (Takeuchi, 1968; Bakir et al., 1973). Many of the gross histopathological and cellular abnormalities characterized in response to human MeHg exposure (Takeuchi, 1968; Bakir et al., 1973) have been also demonstrated in mouse models (Berthoud et al., 1976; Morganti et al., 1976; Goulet et al., 2003). With this in mind, the objective of Experiment 2 was to determine how repeated IP MeHg exposure (given every 3rd day, for 15 days) would affect the neuroanatomical and behavioral measures described in Experiment 1.

Repeated exposure to MeHg resulted in neural c-Fos activity patterns that were similar to those found in Experiment 1, but behavioral endpoints that were markedly different. Repeated IP MeHg administration resulted in increased c-Fos immunoreactivity in the PVN, CeA, Lc and BSTm, while decreasing the total number of recruited regions when compared to the acute IP MeHg exposure. Additionally c-Fos production was decreased in the Dg and BSTs when compared to repeatedly exposed vehicle controls. The increase in magnitude of c-Fos response following repeated MeHg exposure, when compared to acute exposure, noted in this experiment could be due to the long half life (~208 hours) of the 37kDa deltaFosB protein which increases in response to repeated chronic induced-seizures and drug treatments (Chen et al., 1997), and would therefore allow for long-lasting and robust Fos responses after repeated MeHg stimulation.

As noted in the general discussion of Experiment 1, projections from the Lc innervate both the brain stem and more anterior regions such as the PVN, CeA, and BST

(Saper, 1987; Dunn et al., 2004). Thus activation of the brain stem by the vagus nerve or through detection of toxic entities in the blood by the area postrema could relay MeHg-induced nervous stimulation to the Lc. Strong activation of the Lc by repeated MeHg could then afferently stimulate the PVN, CeA and BST, resulting in the increases in c-Fos immunoreactivity in these nuclei. Previous studies have demonstrated that increased neuronal activity can result in response to continued MeHg exposure. For example, repeated MeHg exposure has been shown *in vivo* to result in sustained increases in striatal neurotransmitter levels (HVA, DOPAC) (Faro et al., 1997). Also, in the rat brainstem NE levels increase in response to repeated treatment with MeHg (Hrdina et al., 1976). The increases in brainstem NE after repeated exposure to MeHg is certainly consistent with findings from this experiment, given that the activated Lc can then project excitatory NE afferents directly to the PVN and CeA and to the BST via the CeA (Dunn et al., 2004). These four nuclei were the only nuclei with increased c-Fos cell counts after repeated MeHg treatment. Indeed colocalized increases of c-Fos and neurotransmitter levels have been demonstrated in response to a variety of conditions; including increases in dopamine following psychological stimulation (Bharati & Goodson, 2006) and increases in tyrosine hydroxylase in the nucleus tractus solitarius following sickness-associated behaviors (emesis) (Rodgers et al., 2003).

However, this also tells us that the overall decrease in the number of activated stress nuclei (with the PVA/PV and LS not significantly affected) and the decrease in c-Fos in the Dg and BSTs could be the result of habituation of these nuclei to repeated treatment with MeHg. The LS relays information to and from the hippocampus and receives input from the PVA, which also innervates the hippocampus (Swanson, 1977);

therefore the lack of significant c-Fos production in the LS after repeated MeHg exposure could be resulting from decreased PVA stimulation when compared to acute MeHg treatment. Additionally, reductions in efferent stimulation from the LS and PVA could explain the overall decrease in activity within the hippocampus. It should be noted that the Dg is well documented to be preferentially sensitive to MeHg-induced toxicity (Annau and Cuomo, 1988, Kakita et al., 2000, Andersson et al., 1997) with cell death occurring through a caspase-dependent apoptotic pathway (Falluel-Morrell et al., 2007). In addition to affecting cell viability, the 15 days of repeated exposure to MeHg in this experiment could be affecting the ability of this nucleus to produce the c-Fos protein, via direct inhibition of protein synthesis (Verity et al., 1977) through reductions in phenylalanyl-tRNA sythetase activity (Cheung & Verity, 1985). Further, thalamic lesioning has been shown to decrease c-Fos immunoreactivity in the Dg in response to behavioral stimulation (Jenkins et al., 2002), thus demonstrating the PVA/PV's role in modulating Dg activity.

Experiment 3: Effects of Intraparitoneal Methylmercury Pretreatment and Subsequent Lipopolysaccharide Exposure on Neuronal c-Fos Production

In Experiment 2, repeated exposure to MeHg was shown to continue to elicit CNS reactivity as judged by detection of increased numbers of c-Fos immunoreactive cells. The introduction of a heterotypic stressor (open field exposure) shortly after the final MeHg injection did not reveal distinct evidence for what might be considered a sensitization effect. However, sensitization is typically assessed at longer periods after

the initial or last stressor exposure. For example, exposure to novel heterotypic stimuli results in a sensitized ACTH and corticosterone response when preceded by repeated homotypic foot shock stress (van Dijken et al., 1993). Additionally, pre-exposure of mice to MeHg has been shown to sensitize/ increase stereotypic self-injurious behavior after subsequent amphetamine exposure (Wagner et al., 2007). Pretreatment of mice to tail shock stimulation desensitizes the HPA axis and cytokine response to glucocorticoids following exposure to LPS (O'Connor et al., 2003).

It was therefore decided to conduct an experiment that examined potential sensitization several days after the final MeHg injection. In so doing, the immunologic stimulus LPS was used as the heterotypic stressor, rather than opting for open field exposure. This allowed for a determination of whether another systemic stressor, operating through different mechanisms (viz., immunological) produces an altered response as a function of prior MeHg exposure. Furthermore, the decision to give LPS several days after the final MeHg treatment was based on the results of Experiment 2 which showed that there was still a c-Fos response to the final injection. Waiting three days increased the likelihood that this response had waned, thereby providing an opportunity for LPS to impact on a CNS that had recovered from the last response to MeHg.

The results of this experiment revealed several intriguing findings. Pretreatment with 15 days of repeated MeHg did not significantly affect the c-Fos response to a subsequent LPS dose (on day 18, 3 days after the final MeHg injection). That is, increases in c-Fos were noted after LPS exposure, regardless of pretreatment. LPS activation of central stress-associated nuclei is via initial activation of innate immune

cells, followed by production of proinflammatory cytokines (reviewed in Cohen, 2002; see the introduction to Chapter 4). From Experiment 2 it was postulated that while habituation was likely causing the decrease in numbers of c-Fos immunoreactive cells when compared with Experiment 1, it might also be the case that repeated MeHg could be affecting the synthesis of the c-Fos protein. Indeed, as mentioned in the general introduction and in the conclusion for Experiment 2, MeHg has been demonstrated to inhibit protein synthesis (Yosino et al., 1966; Verity et al., 1977).

Experiment 4: Cytokine Production in the Murine Spleen in Response to Acute and Repeated Intraperitoneal Methylmercury Exposure

Experiments 1 through 3 focused on central stress nuclei responses to peripheral and central administration of MeHg. When discussing proposed mechanisms for production of c-Fos in Experiment 1 and after examining the literature it became clear from toxicokinetic data that MeHg was less likely to be affecting the brain directly (Nielsen, 1992) but rather may be resulting in stimulation of peripheral nerves which are then likely transmitting this information through the brainstem to specific stress nuclei in the brain (for further detail see the Experiment 1). Given that a peripheral response was likely occurring after IP dosing in Experiments 1 and 2, it was of immediate value to have a peripheral measure of stress.

Cytokine profiles from mice acutely and repeatedly dosed with MeHg showed significant responses with respect to exposure to physiological and psychological stressors. Responses to acute MeHg increased T-cell associated cytokine Il-2, led to no

change in the response of proinflammatory cytokines TNF-α, IL-1β, and IL-6, and lead to a decrease anti-inflammatory IL-10. As pro-inflammatory cytokine levels were at control levels and with the decrease in IL-10, the overall splenic cytokine response to acute MeHg could be described as marginally anti-inflammatory. Also, increases in IL-2 levels following acute MeHg administration could be part of a mounting response to induce T-cell maturation, which occurs following increases in IL-2 (Pheng et al., 2003; Johansson et al., 1997). The lack of proinflammatory cytokine production could also be explained by the initial immunosuppressive effects of MeHg, as noted on activation of T-cells (Shenker et al., 1992) and a downstream reduction in cytokine production (Gajewski et al., 1989).

Repeated exposure to MeHg gave a vastly different splenic cytokine profile when compared to acutely dosed mice. Increased levels of the proinflammatory cytokines TNF-α and IL-6 and anti-inflammatory production of IL-10 were noted following repeated administration of MeHg. These data and literature reports led to the hypothesis that repeated exposure to MeHg may be inducing cytokine production through a macrophage and T-cell dependent mechanism. Indeed, repeated exposure (15 days) to MeHg has been shown to result accumulation of MeHg in lymphatic macrophages and is followed by macrophage-dependent stimulation of T-helper cells (Kubica-Muranyi et al., 1993). Mercury has also been shown to activate murine macrophages (Cunha et al., 2004). Activated T-cells and macrophages are well-known sources of these inflammatory cytokines (Cohen, 2002), and in this way repeated IP exposure to MeHg could then result in increased levels of TNF-α, IL-6 and IL-10 inflammatory cytokines within the spleen.

The reductions in cytokine levels in response to open field stimulation after both acute and repeated MeHg exposure could be resulting from yet undetermined influence by sympathetic catecholamines and/or glucocorticoids (Elenkov & Chrousos, 2002), which are known to affect immune function during the stress response (Kvetnansky et al., 1995).

Data from this experiment demonstrate that following acute exposure MeHg is eliciting an immunosuppressive effect on splenic cytokine production, while inducing production of both pro- and anti-inflammatory cytokines after more prolonged, repeated exposure. The increase in cytokine production following longer periods of exposure to MeHg could be resulting from increases in cell death within the spleen. Pheng et al. (2000) have demonstrated that 48 hours after low-level exposure (0.001-1.0 μM) to methylmercury, the number of TUNEL-positive splenic T-cells increased significantly, with MeHg-induced cell death occurring through a Fas-dependent pathway. Induction of Fas-mediated cell death has been demonstrated following increases in pro-inflammatory TNF-β, lymphotoxin-β, and IL-2 (Lombard et al., 2003) and after inhibition of IL-12 (Marth et al., 1998) in the spleen. In this way repeated exposure to MeHg from Experiment 2 could be inducing cell death and production of inflammatory cytokines in the spleen.

Future Directions

The motive for the experiments within this thesis was to address if and to what extent methylmercury affects immediate early gene production in the brain, exploratory behavior in the open field and cytokine production in an immunologic organ, the spleen. And while each experiment shed light onto how the dose, frequency and route of

administration of MeHg affected each of these measured parameters, after each experiment it became clear that there was a great deal that the data could not say without additional experiments. It would be useful to highlight what future areas of interest could be used to help make each experiment a more complete story.

Experiment 1: Effect of Acute Methylmercury Exposure on Activation of Neural Stress Circuitry and Open Field Exploratory Behavior

Given the robust c-Fos response within the brain following both acute IP and ICV MeHg exposure, it would be interesting to see how hindbrain lesions of the AP, NTS, and Lc or forebrain PVN, CeC, or BST lesions also affect the c-Fos response to MeHg and open field exposure to determine which brain nuclei play the most crucial role in responding to physiological and psychological stressors. Lesioning of the Lc and PVN results in marked reductions of stress-associated vigilance and predation behavior (Delagrange et al., 1993; Canteras et al., 1997, respectively). PVN lesions also result in reductions of sensory behavior in the open field (Herman et al., 1991). Further lesions of the rat BST similarly result in retarded behaviors in the forced swim test, characterized by reduced escape behavior and decreased movement (Schulz & Canbeyli, 2000). From this body of literature, lesioning of the Lc, PVN, or BST would perhaps result in a reduction of the c-Fos response following acute IP MeHg exposure and increase movements in the open field due to deficits in vigilance and sensory behaviors.

Experiment 2: Effects of Repeated Methylmercury Exposure on Central Stress Circuit Activation and Exploratory Behavior

Repeated exposure to homotypic systemic stressors (MeHg in this experiment) have been shown to result in attenuated behavioral and physiological stress responses (reviewed in Amario et al., 2004). For example, acute exposure to systemic immunological stressors, such as LPS, results in robust production of c-Fos protein in the brain, increases in circulating inflammatory cytokines and CRH, and activation of host immune cells and alterations in exploratory behavior, measures of which are drastically reduced in response to subsequent doses (Langhans et al., 1991; Zeisberger & Roth, 1998; Kusnecov & Goldfarb, 2005; Broad et al., 2006). As mentioned previously, repeated exposure to metals can result in diminished neurotransmitter (ACh, NE) release within brain stress nuclei (Hrdina et al., 1976; Faro et al., 1997), inhibit both adrenergic reuptake and turnover in presynaptic neurons and inhibit postsynaptic adrenergic binding sites (Slotkin & Bartolome, 1987). The effects of MeHg on neurotransmission could account for some of the decrease in c-Fos activation in the Dg and BSTs and attenuated behavioral effects noted after response to repeated MeHg. Future studies might look at neurotransmitter immunohistochemical localization and in situ hybridization mRNA levels within the same stress nuclei in response to both the acute and repeated MeHg exposure outlined in Experiments 1 and 2. From this colocalization paradigm and from previously mentioned studies, it would be anticipated that exposure to MeHg would likely induce an increase (efflux) in neurotransmitter levels following a single, acute exposure and an attenuated/ reduced response following repeated doses.

Experiment 3: Effects of Intraperitoneal Methylmercury Pretreatment and Subsequent Lipopolysaccharide Exposure on Neuronal c-Fos Production

Given the robust response to LPS in Experiment 3 following MeHg pretreatment it would seem that repeated administration of MeHg is not affecting the ability of c-Fos protein to be synthesized. The biological half-life of MeHg in the mouse ranges from 6-13 days (Nielsen, 1992), so repeated administration in Experiments 2 and 3 would result in a steady state even 3 days after the last exposure, thus ensuring that MeHg is present at relatively constant levels even on day 18. Additionally, repeated MeHg exposure followed by saline treatment on day 18 resulted in low c-Fos immunoreactivity that was not significantly different from saline pretreatments, which is likely a function of the transient nature of c-Fos (typically peaking at 2-3 hours) and hence, not being expressed 72 hours after the last exposure to MeHg.

Follow up studies to Experiment 3 should include analysis of central (brain) and peripheral (blood, spleen) cytokine levels to better understand whether the preceding exposure to systemic (IP) MeHg affects the inflammatory response to LPS. In situ hybridization for pro- and anti-inflammatory cytokine mRNAs within each of the stress nuclei examined in Experiments 1 through 3 could be correlated with c-Fos induction and hence linked to it's response to methylmercury.

Experiment 4: Cytokine Production in the Murine Spleen in Response to Acute and Repeated Intraperitoneal Methylmercury Exposure

Given that acute and repeated exposure to methylmercury produced differing levels of both pro- and anti-inflammatory cytokine levels in the murine brain, and that

repeated exposure produced the largest cytokine response, it would of interest to see if cytokine levels correlated with MeHg levels within these same spleens, and if so which cytokines were most strongly correlated. Radio labeled MeHg (^{203}Hg) would be once such way to quantify splenic mercury levels.

Summary

The behavioral, neuronal and splenic responses to acute and repeated administration to methylmercury examined within this thesis demonstrated the multifactorial effects that methylmercury exposure can have on a biological system. Acute exposure to IP MeHg resulted in strong, dose-dependent attenuation of behavior in the open field, increased activation of neural stress nuclei, and reduced splenic cytokine production. Acute ICV exposure to MeHg demonstrated that MeHg infused directly into the brain results in similar recruitment and c-Fos induction within these stress-associated nuclei. Induction of c-Fos following IP MeHg could be assisted by activation of centrally-projecting peripheral nerves such as the vagus, which could also be partially attributed to the noted alterations of behavior in the exploratory behavior, manifested as malaise or sickness-like behaviors. Repeated exposure to MeHg exposure demonstrated that MeHg did not sensitize or desensitize the response to LPS and showed that the reduction in c-Fos immunoreactivity noted in after repeated MeHg was not due to inhibition of c-Fos production. Finally, MeHg given acutely resulted in decreases or no change in splenic cytokine levels, while repeated MeHg exposure increased inflammatory cytokine levels, perhaps as a result of increased cell death within the spleen.

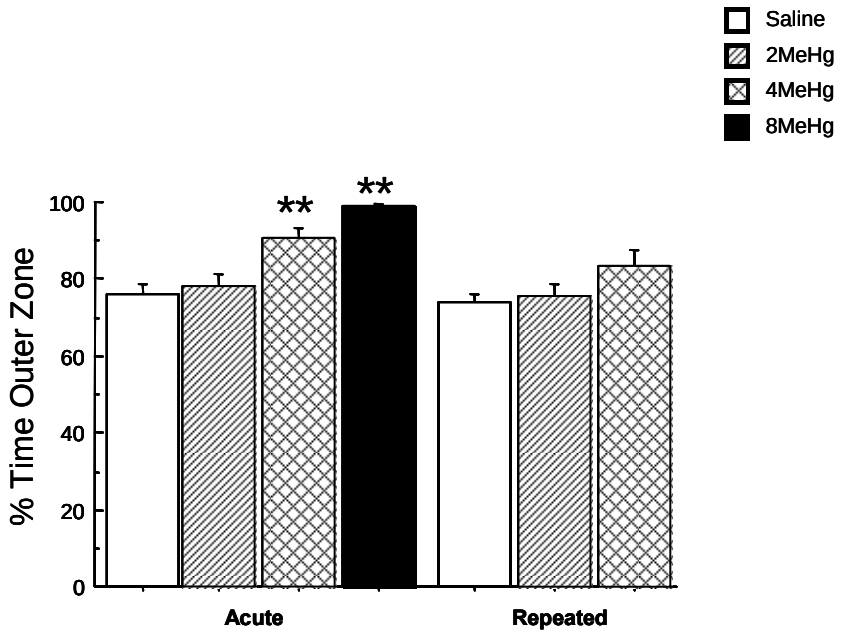

Figure 1. Impact of acute and repeated IP exposure to MeHg on behavior in the outer zone of the open field without a novel object. Each bar represents the mean value (+/- standard error) of N = 3-8/group. All MeHg treatments are mg/kg. * and ** represent $p < 0.05$ and 0.01 respectively when compared to corresponding saline injected controls.

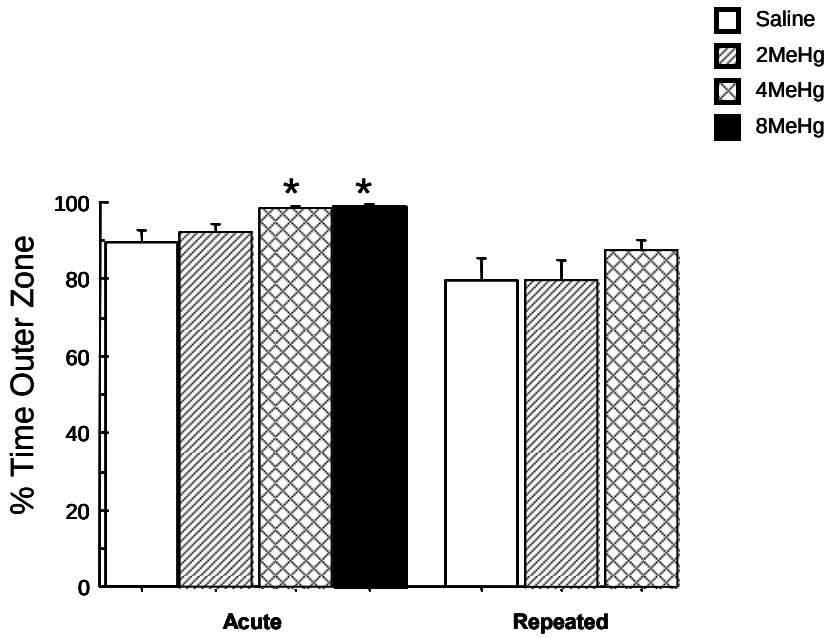

Figure 2. Impact of acute and repeated IP exposure to MeHg on behavior in the outer zone of the open field with a novel object. Each bar represents the mean value (+/- standard error) of N = 3-8/group. All MeHg treatments are mg/kg. * and ** represent $p < 0.05$ and 0.01 respectively when compared to corresponding saline injected controls.

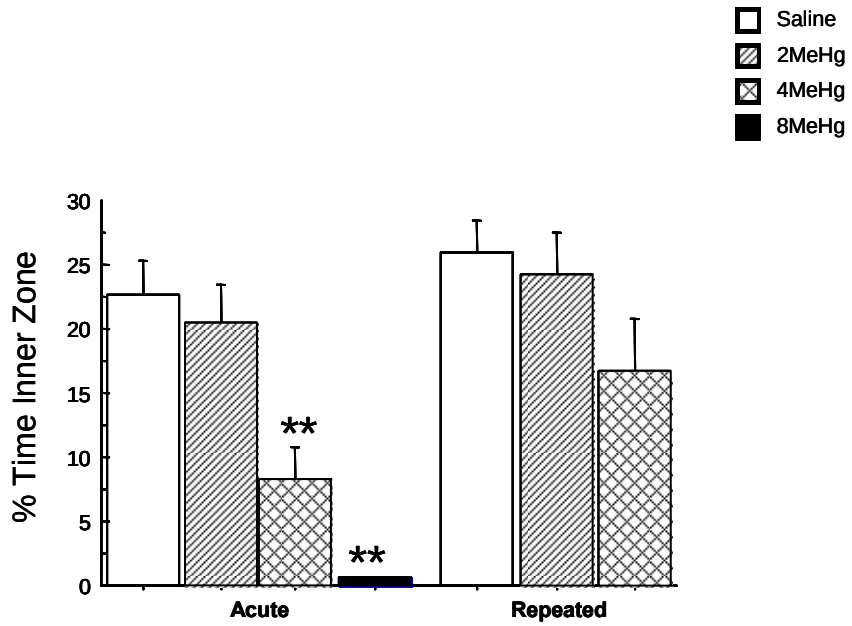

Figure 3. Impact of acute and repeated IP exposure to MeHg on behavior in the inner zone of the open field without a novel object. Each bar represents the mean value (+/- standard error) of N = 3-8/group. All MeHg treatments are mg/kg. * and ** represent $p < 0.05$ and 0.01 respectively when compared to corresponding saline injected controls.

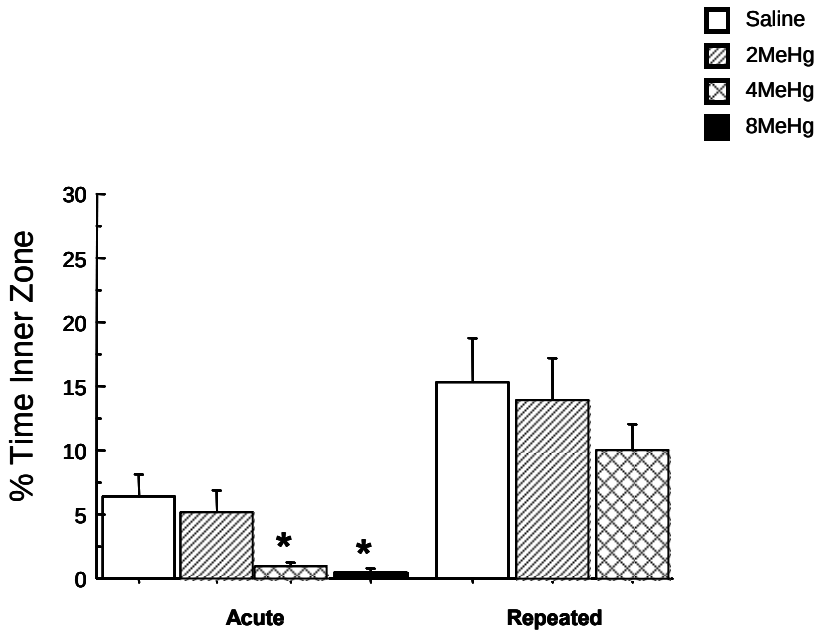

Figure 4. Impact of acute and repeated IP exposure to MeHg on behavior in the inner zone of the open field with a novel object. Each bar represents the mean value (+/- standard error) of N = 3-8/group. All MeHg treatments are mg/kg. * and ** represent $p < 0.05$ and 0.01 respectively when compared to corresponding saline injected controls.

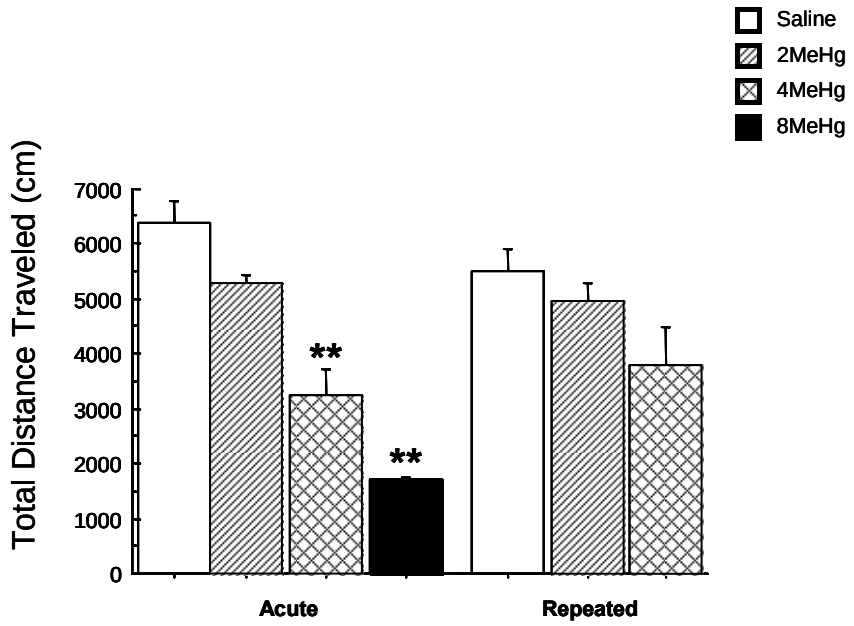

Figure 5. Impact of acute and repeated IP exposure to MeHg on total distance traveled (cm) without a novel object. Each bar represents the mean value (+/- standard error) of N = 3-8/group. * and ** represent $p <$ 0.05 and 0.01 respectively when compared to corresponding saline injected controls.

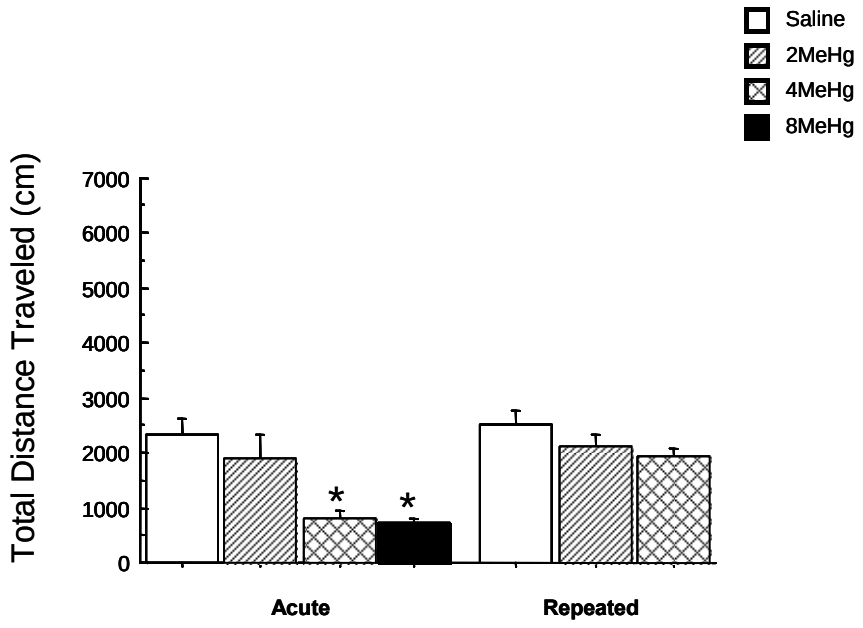

Figure 6. Impact of acute and repeated IP exposure to MeHg on total distance traveled (cm) with a novel object. Each bar represents the mean value (+/- standard error) of N = 3-8/group. * and ** represent $p < 0.05$ and 0.01 respectively when compared to corresponding saline injected controls.

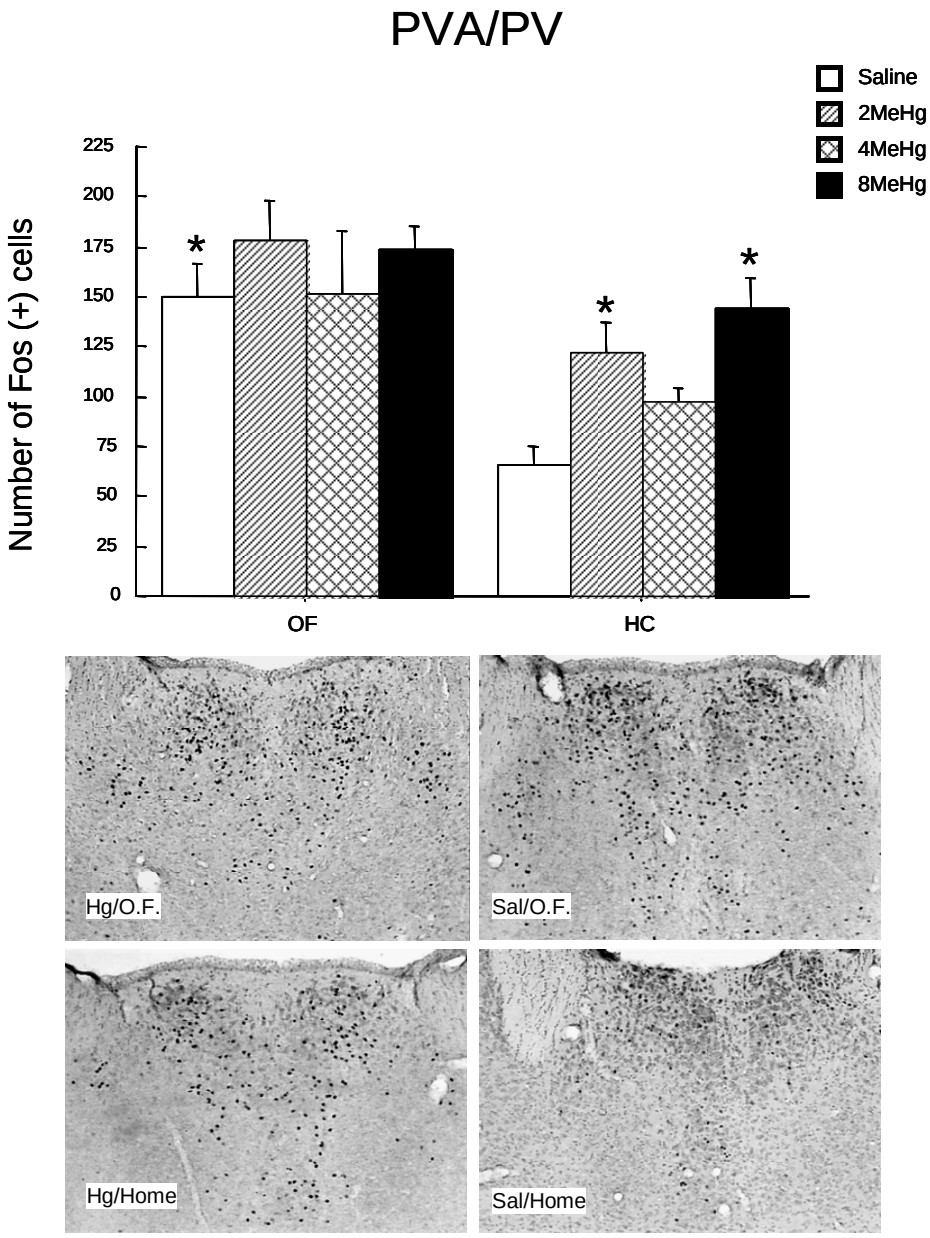

Figure 7. Effect of open field stress and/or acute MeHg exposure on c-Fos immunoreactivity in the PVA of the thalamus. Each bar represents the mean number of c-Fos immunoreactive cells of N = 4-10/group. Representative photomicrographs indicate c-Fos immunoreactivity for the various treatment groups as described in materials and methods. All MeHg treatments are mg/kg. * and ** represent $p < 0.05$ and 0.01 respectively when compared to corresponding saline injected controls.

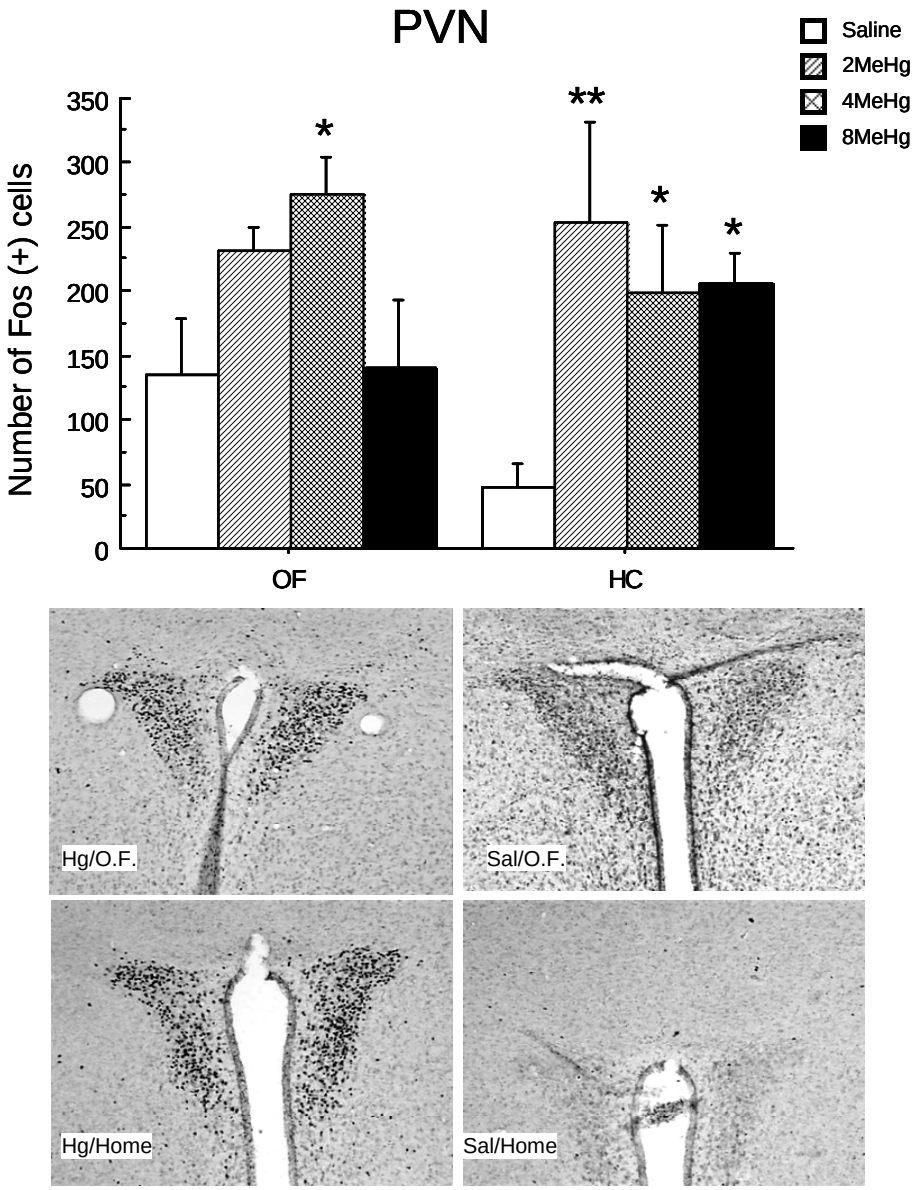

Figure 8. Effect of open field stress and/or acute MeHg exposure on c-Fos immunoreactivity in the PVN of the hypothalamus. Each bar represents the mean number of c-Fos immunoreactive cells of N = 4-10/group. See Figure 7 for further details.

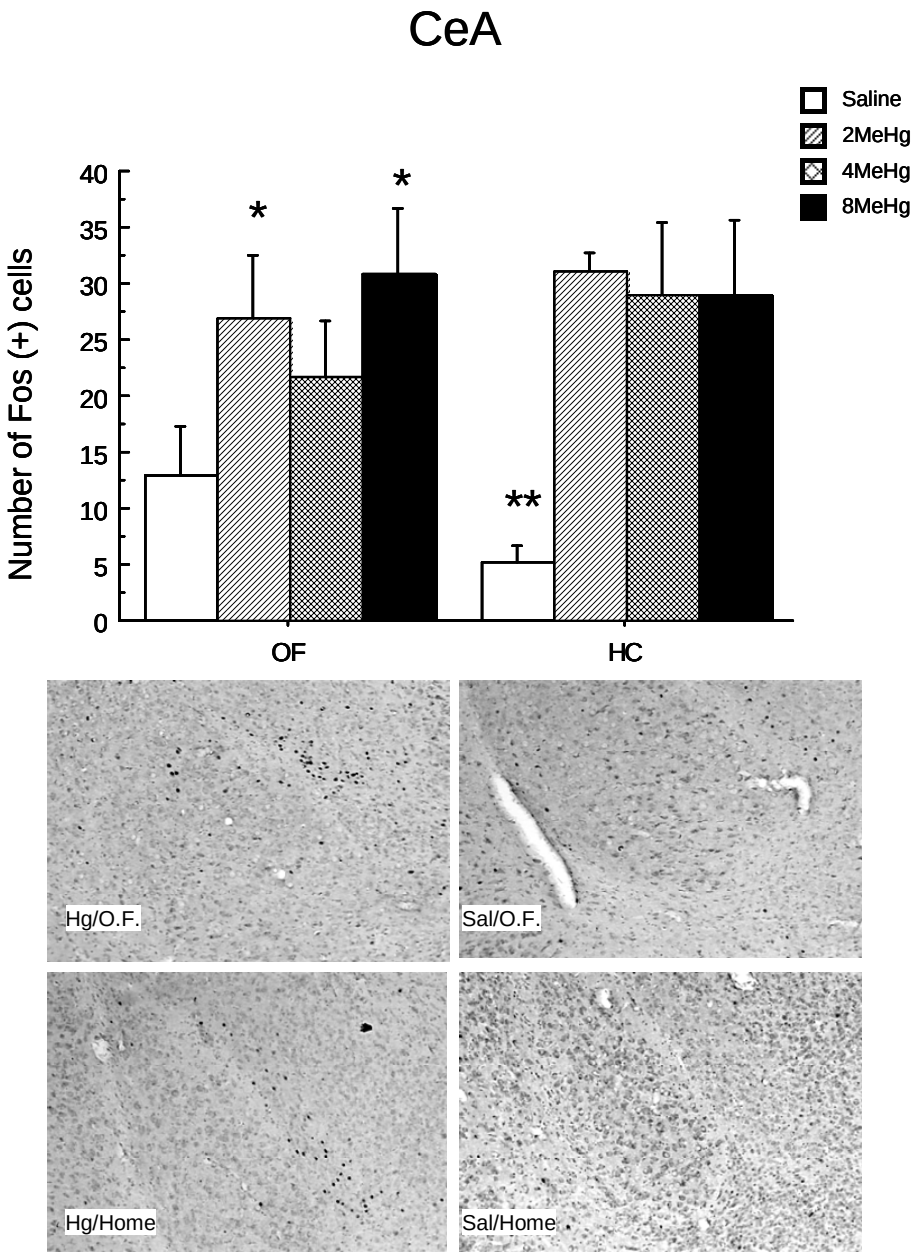

Figure 9. Effect of open field stress and/or acute MeHg exposure on c-Fos immunoreactivity in the central amygdaloid nucleus (CeA). See Figure 7 for further details.

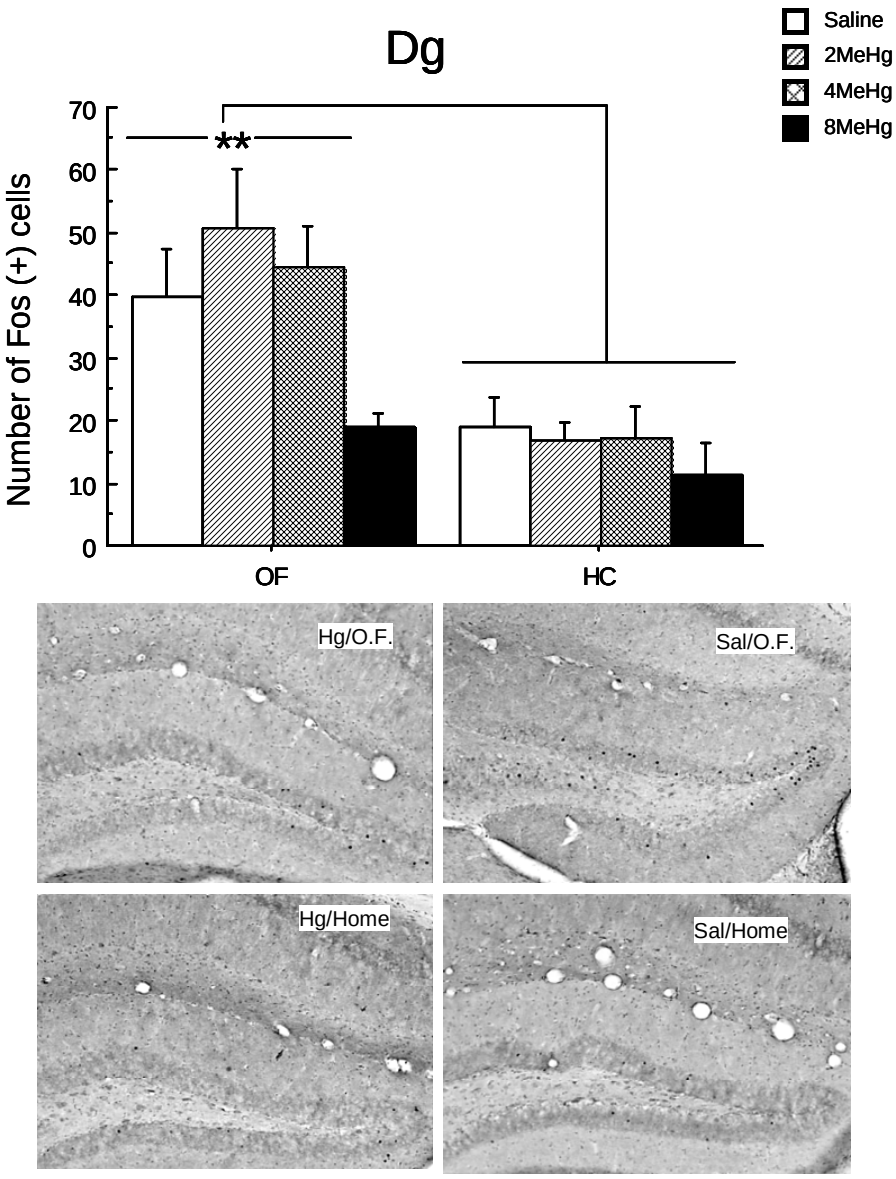

Figure 10. Effect of open field stress and/or acute MeHg exposure on c-Fos immunoreactivity in the dentate gyrus (Dg) of the hippocampus. See Figure 7 for further details.

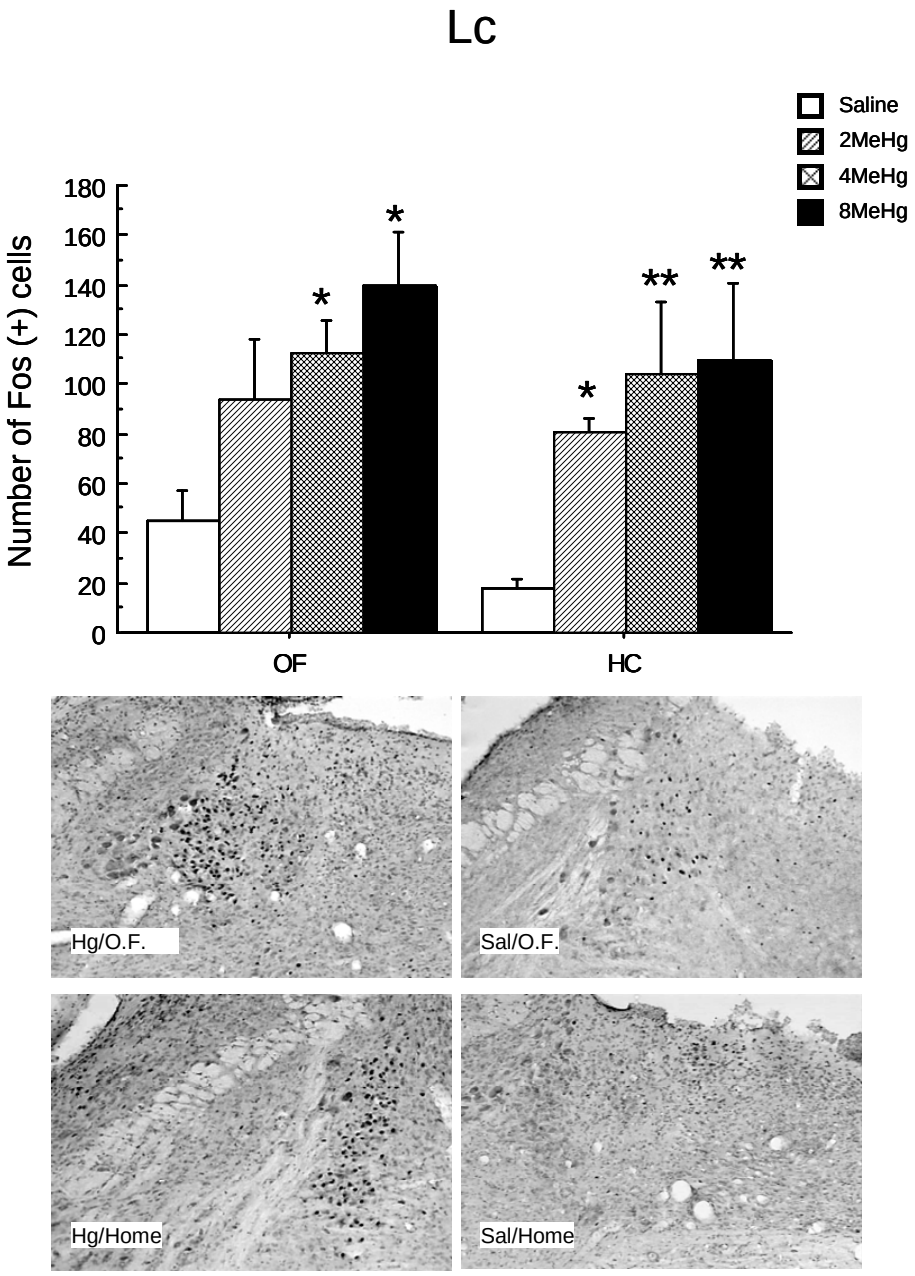

Figure 11. Effect of open field stress and/or acute MeHg exposure on c-Fos immunoreactivity in the locus coeruleus (Lc). See Figure 7 for further details.

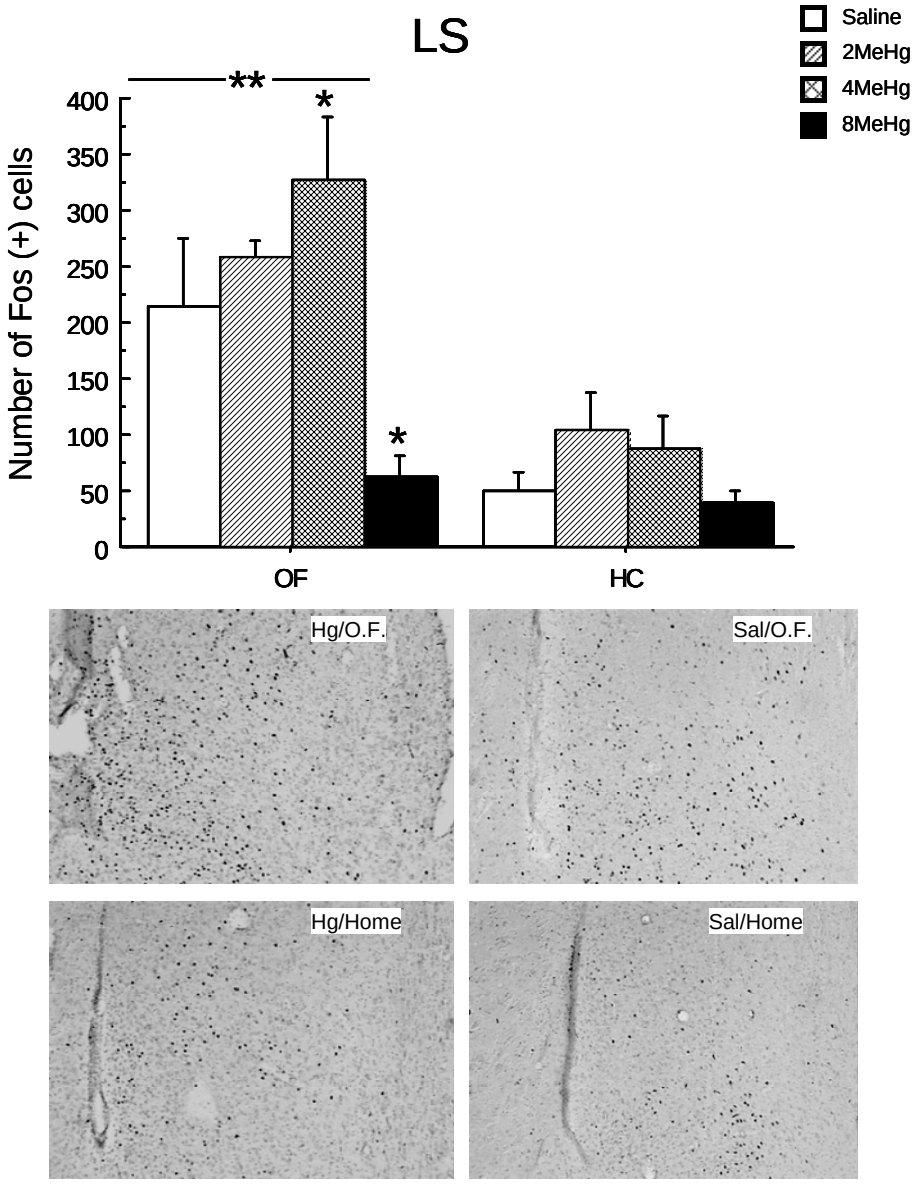

Figure 12. Effect of open field stress and/or acute MeHg exposure on c-Fos immunoreactivity in the lateral septum (LS). See Figure 7 for further details.

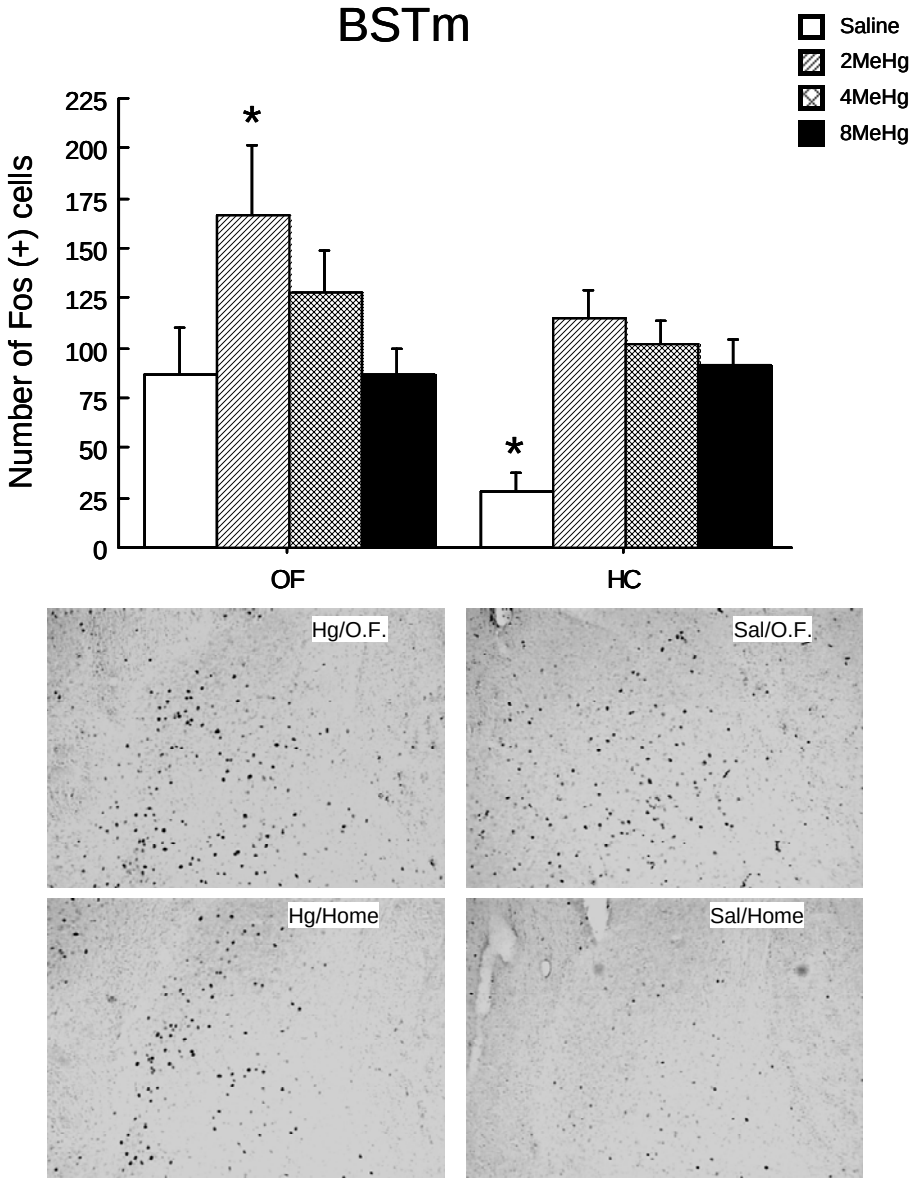

Figure 13. Effect of open field stress and/or acute MeHg exposure on c-Fos immunoreactivity in medial bed nucleus of the stria terminalis (BSTm). See Figure 7 for further details.

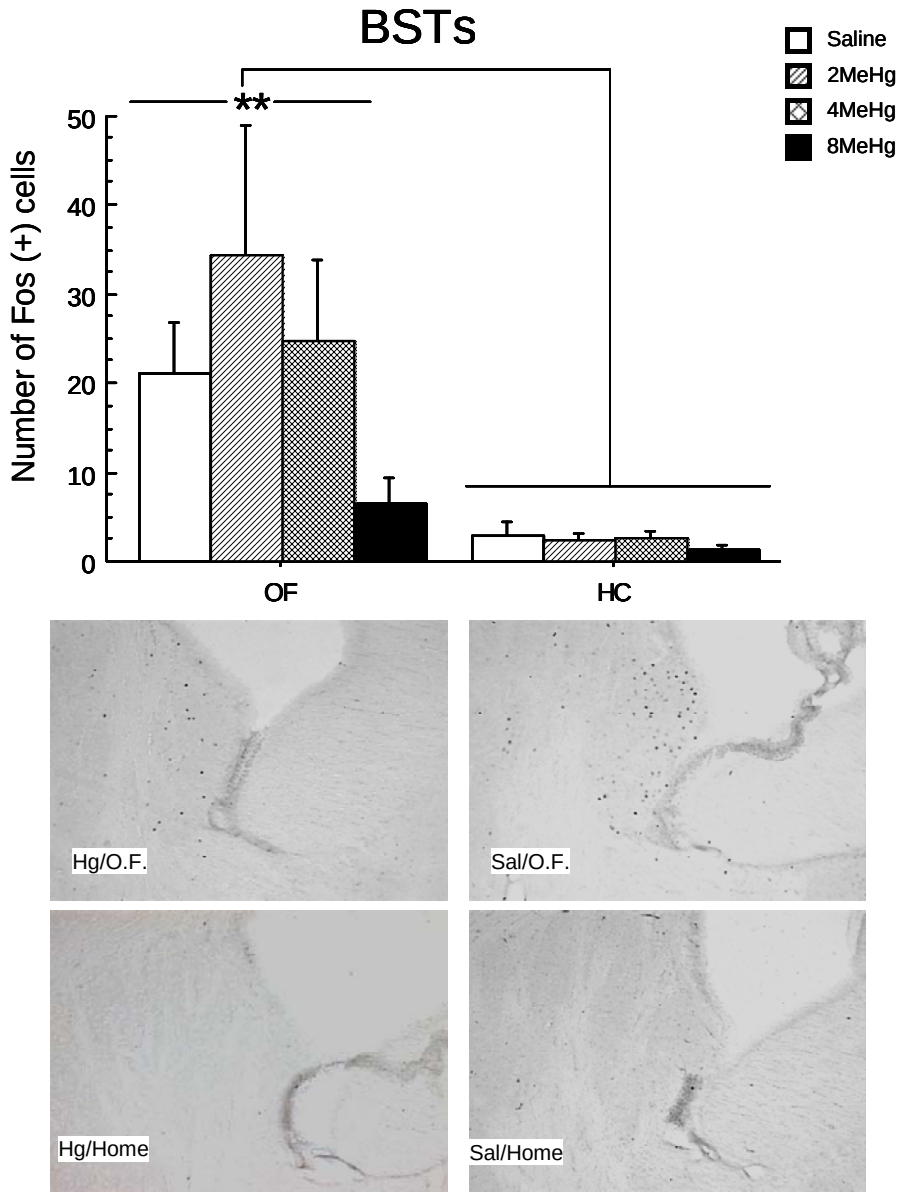

Figure 14. Effect of open field stress and/or acute MeHg exposure on c-Fos immunoreactivity in the supracapsular bed nucleus of the stria terminalis (BSTs). See Figure 7 for further details.

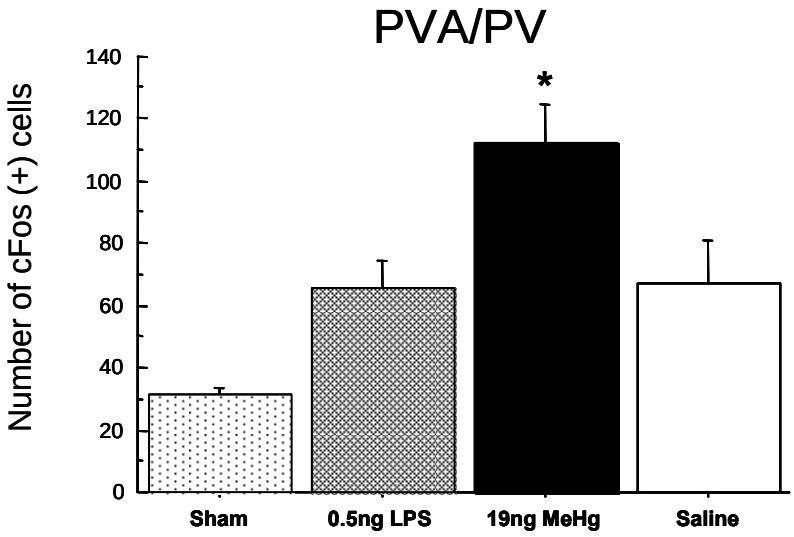

Figure 15. Effect of intracerebroventricular (ICV) MeHg exposure on c-Fos immunoreactivity in the PVA of thalamus. Each bar represents the mean number of c-Fos immunoreactive cells of N = 4-9/group. * $p < 0.05$ relative to saline infused animals.

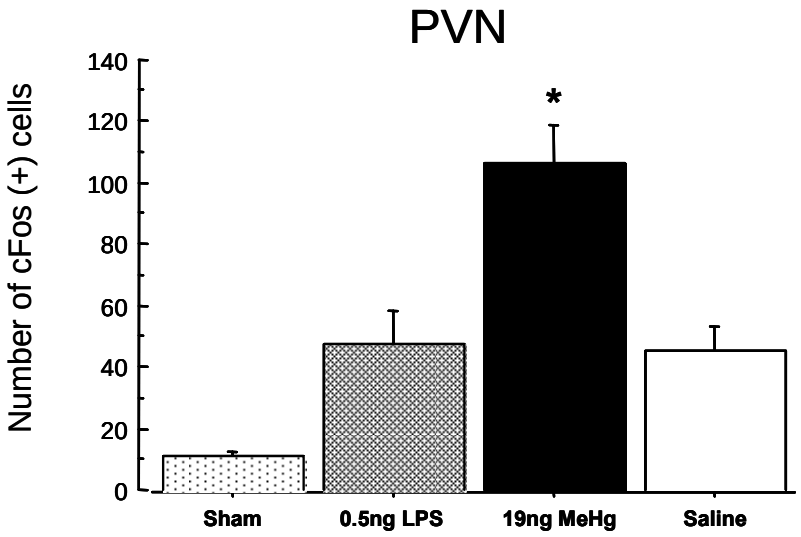

Figure 16. Effect of intracerebroventricular (ICV) MeHg exposure on c-Fos immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN). For further detail see Figure 15.

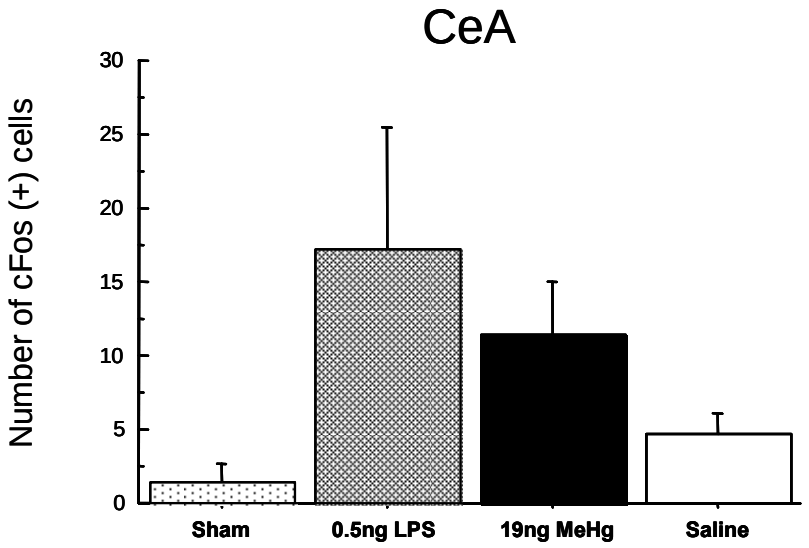

Figure 17. Effect of intracerebroventricular (ICV) MeHg exposure on c-Fos immunoreactivity in the central nucleus of the amygdala (CeA). For further detail see Figure 15.

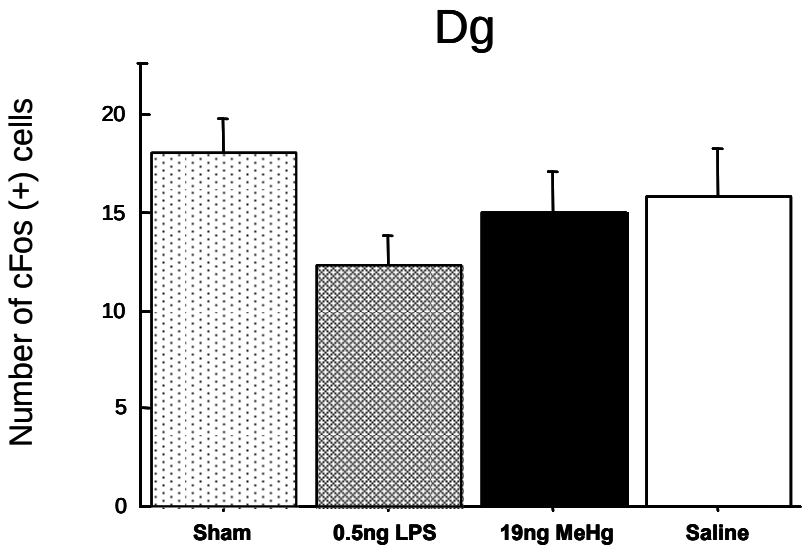

Figure 18. Effect of intracerebroventricular (ICV) MeHg exposure on c-Fos immunoreactivity in the dentate gyrus (Dg) of the hippocampus. For further detail see Figure 15.

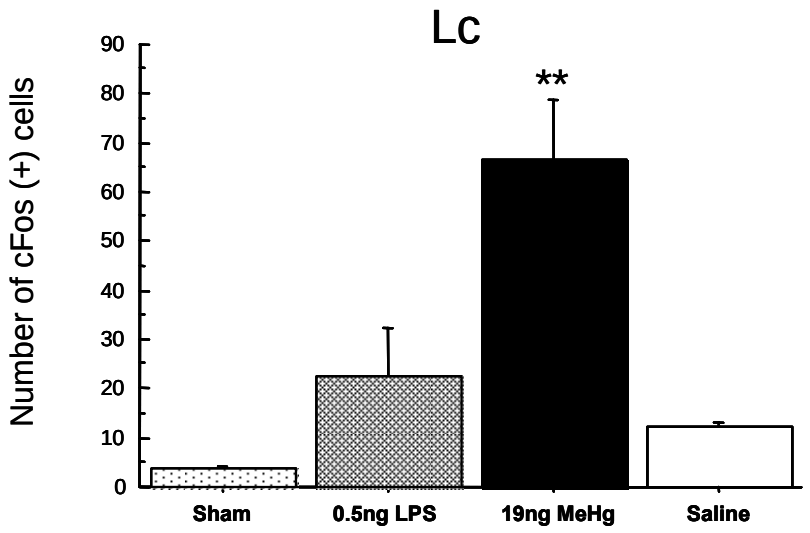

Figure 19. Effect of intracerebroventricular (ICV) MeHg exposure on c-Fos immunoreactivity in the locus coeruleus (Lc). For further detail see Figure 15.

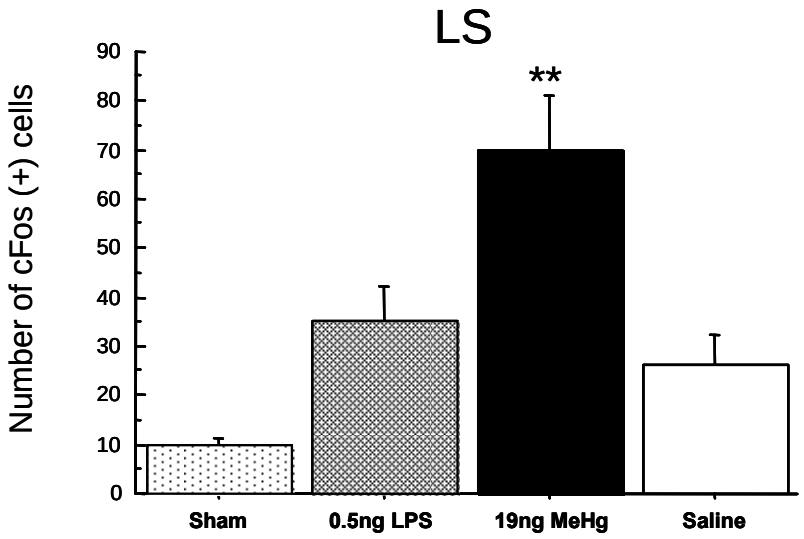

Figure 20. Effect of intracerebroventricular (ICV) MeHg exposure on c-Fos immunoreactivity in the lateral septum (LS). For further detail see Figure 15.

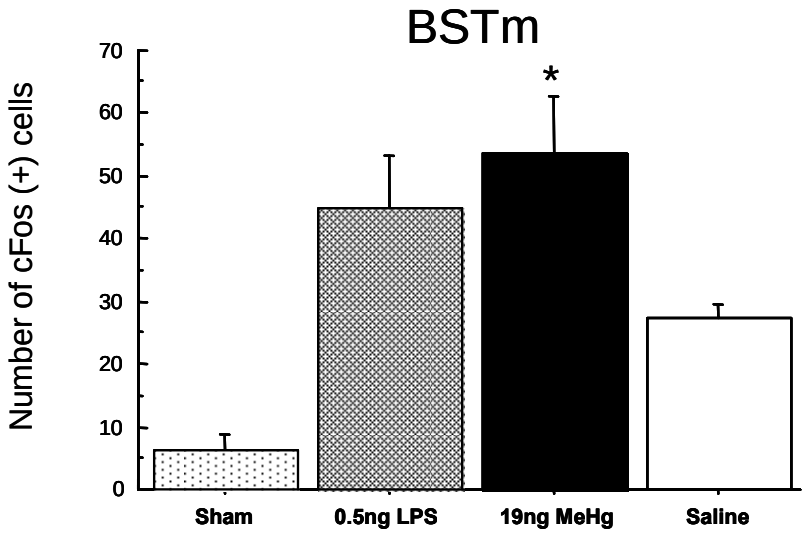

Figure 21. Effect of intracerebroventricular (ICV) MeHg exposure on c-Fos immunoreactivity in the medial bed nucleus of the stria terminalis (BSTm). For further detail see Figure 15.

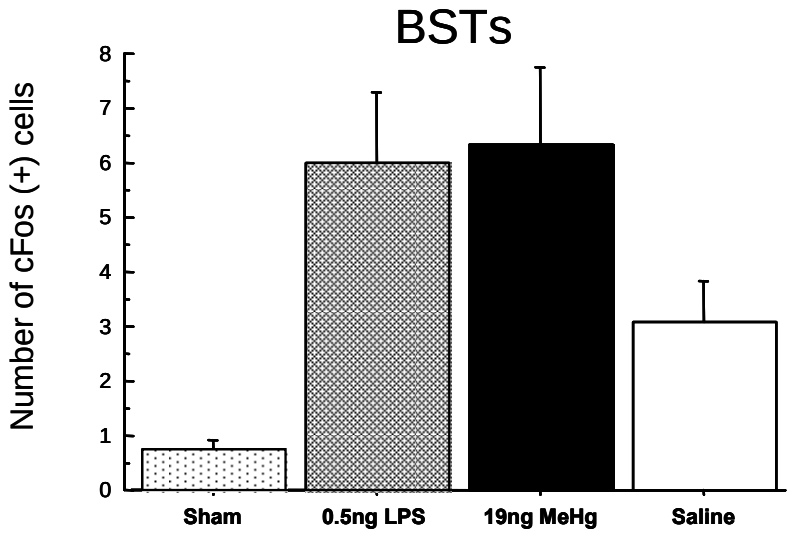

Figure 22. Effect of intracerebroventricular (ICV) MeHg exposure on c-Fos immunoreactivity in the supracapsular bed nucleus of the stria terminalis (BSTs). For further detail see Figure 15.

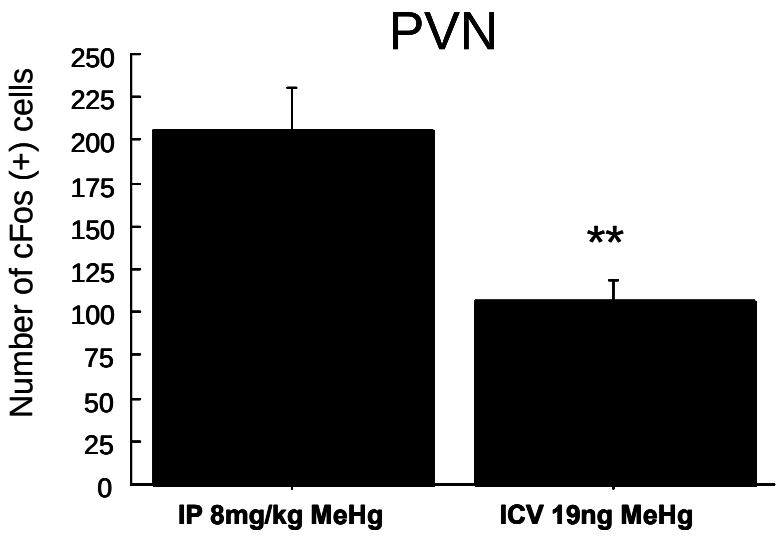

Figure 23. Comparison of magnitude of c-Fos immunoreactivity in the paraventricular nucleus of the hypothalamus (PVN) following acute IP or ICV MeHg. N=5 for IP MeHg and N=9 for ICV. Each bar represents the mean (+/- SE) with * and ** represent $p < 0.05$ and 0.01 respectively.

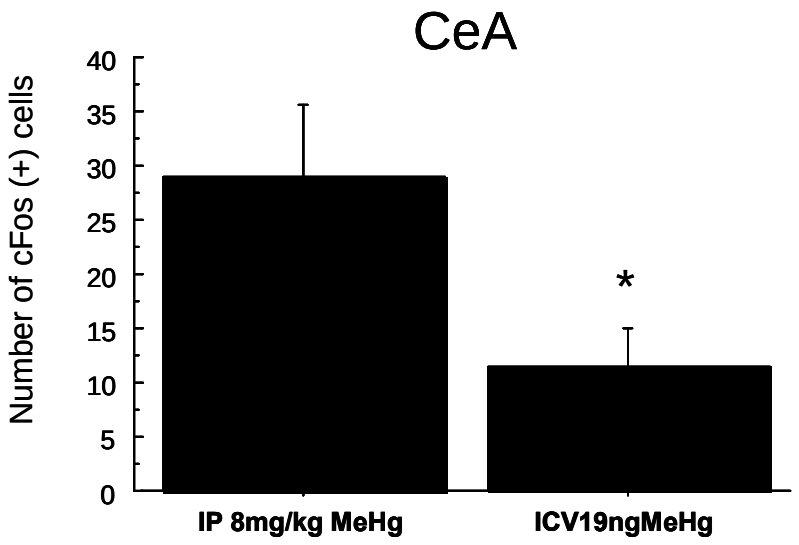

Figure 24. Comparison of magnitude of c-Fos immunoreactivity in the central amygdaloid nucleus(CeA) following acute IP or ICV MeHg. N=5 for IP MeHg and N=9 for ICV. For further detail see figure 23.

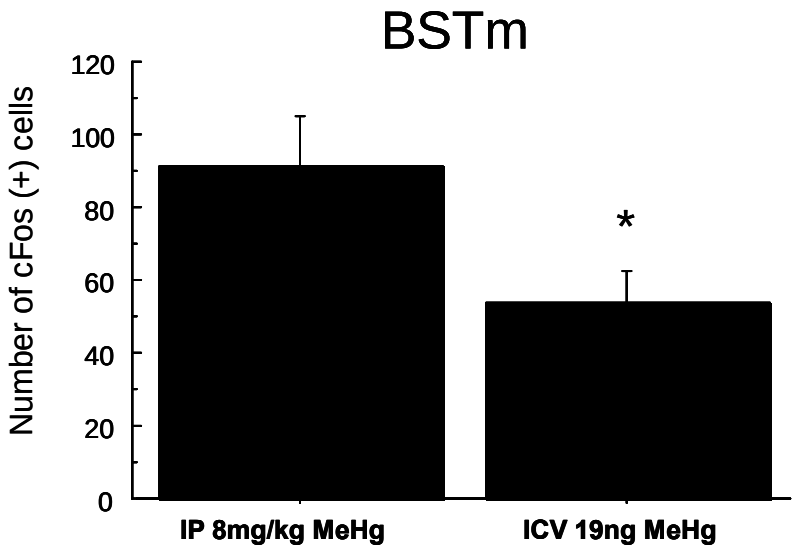

Figure 25. Comparison of magnitude of c-Fos immunoreactivity in the medial bed nucleus of the stria terminalis (BSTm) following acute IP or ICV MeHg. N=5 for IP MeHg and N=8 for ICV. For further detail see figure 23.

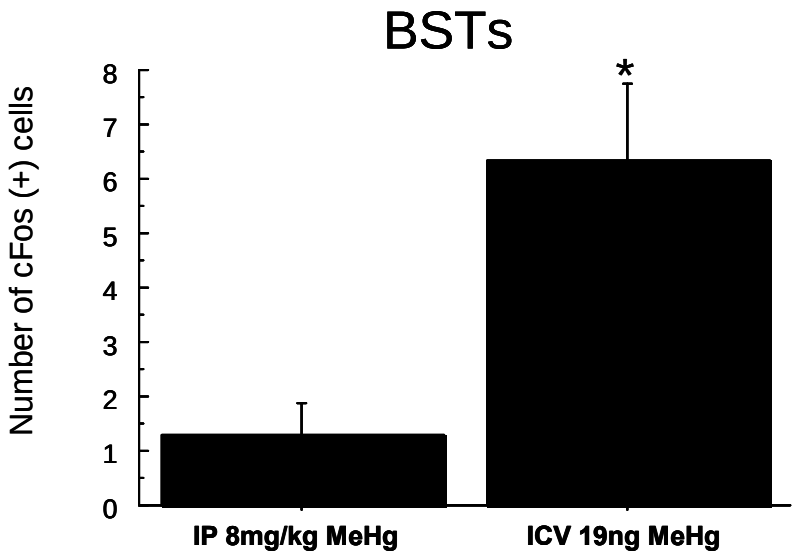

Figure 26. Comparison of magnitude of c-Fos immunoreactivity in the supracapsular bed nucleus of the stria terminalis(BSTs) following acute IP or ICV MeHg. N=5 for IP MeHg and N=9 for ICV. For further detail see figure 23.

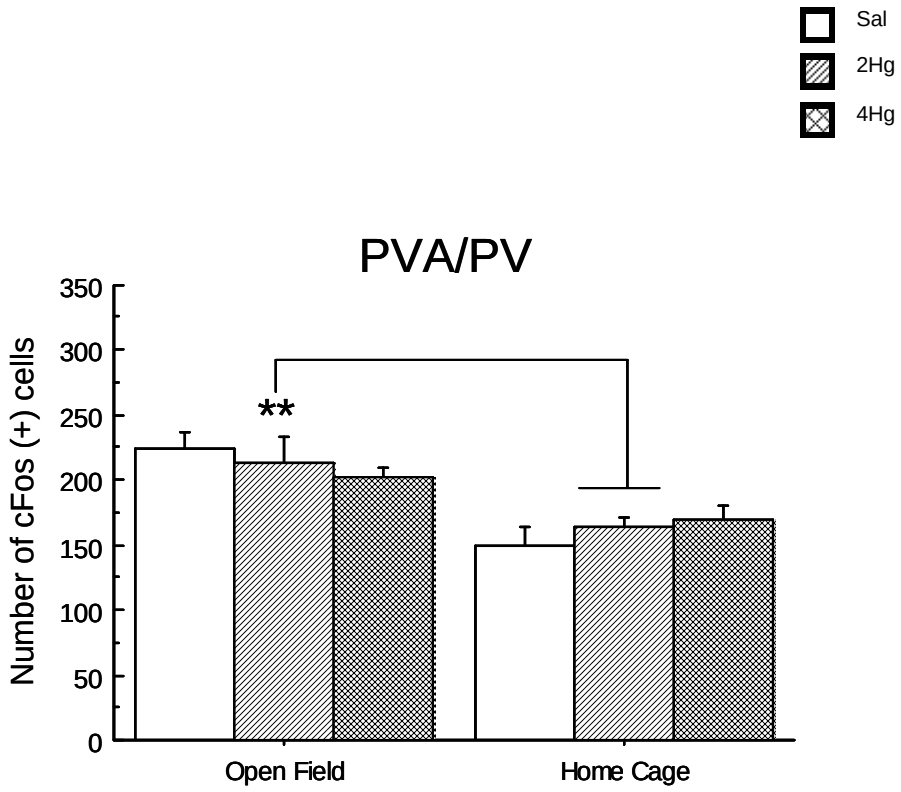

Figure 27. Effect of open field stress and/or repeated MeHg exposure on c-Fos immunoreactivity within the paraventricular thalamic (PVA/PV). Each bar represents the mean number of c-Fos ir cells of N = 4-10/group. All MeHg treatments are mg/kg. * and ** represent $p <$ 0.05 and 0.01 respectively when compared to corresponding saline injected controls or where indicated as combined group comparisons.

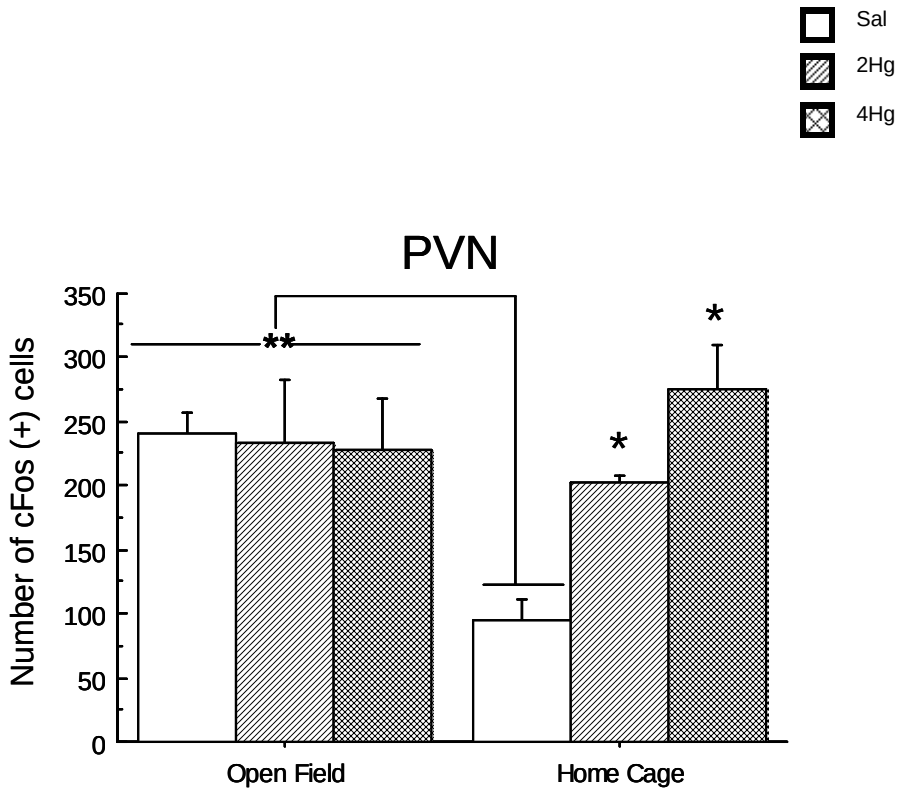

Figure 28. Effect of open field stress and/or repeated MeHg exposure on c-Fos immunoreactivity within the paraventricular hypothalamic nucleus (PVN). See Figure 27 for further details.

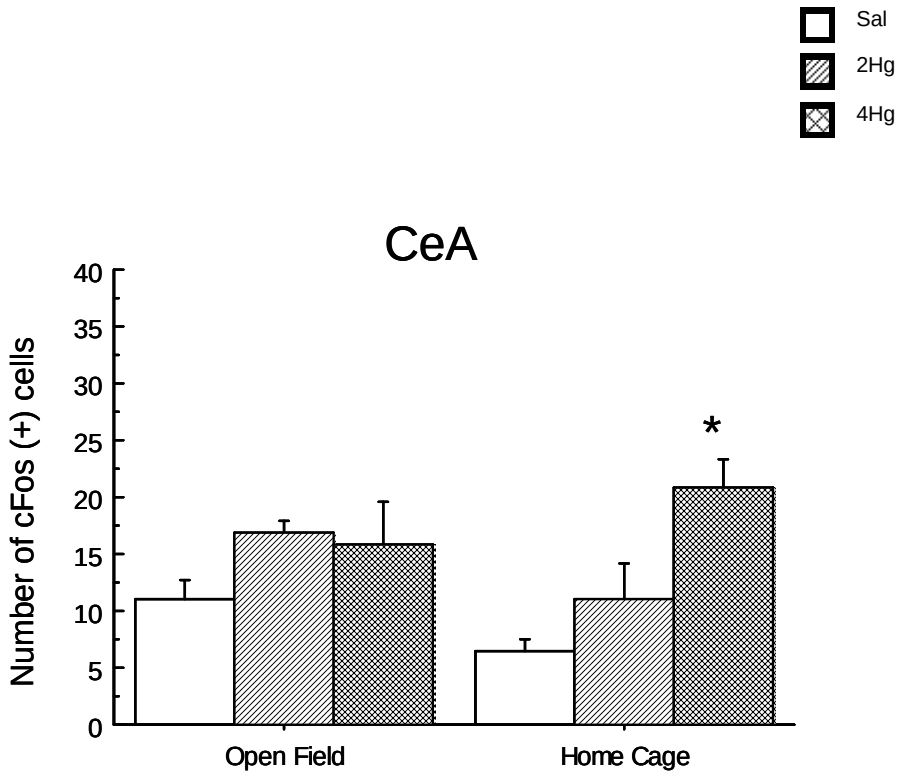

Figure 29. Effect of open field stress and/or repeated MeHg exposure on c-Fos immunoreactivity in the central amygdaloid nucleus (CeA). See Figure 27 for further details.

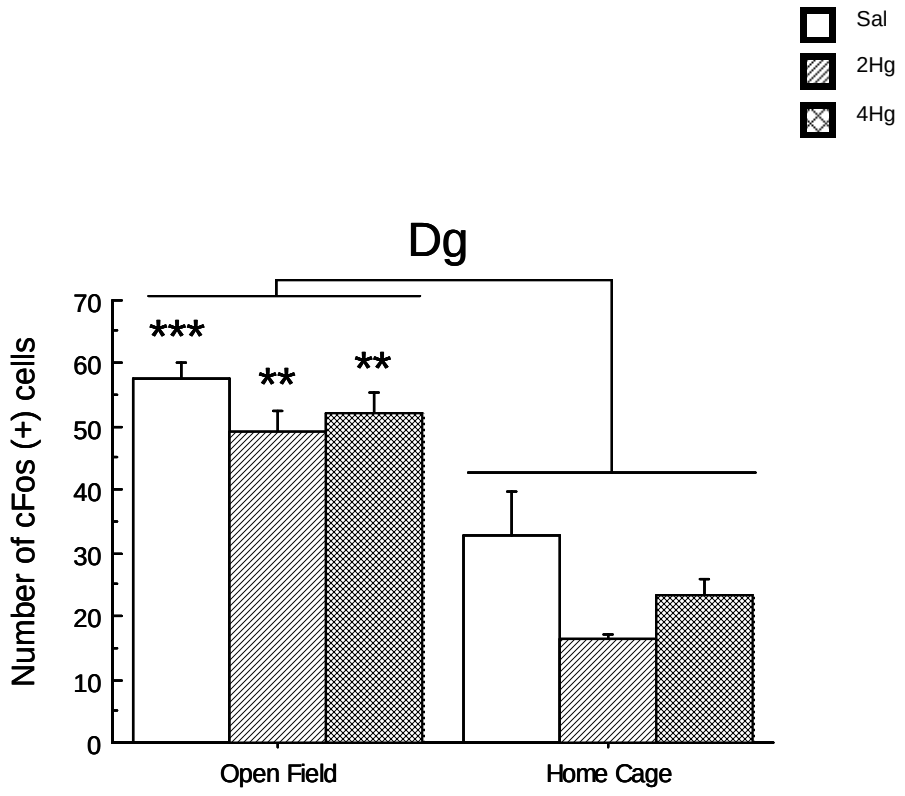

Figure 30. Effect of open field stress and/or repeated MeHg exposure on c-Fos immunoreactivity in the dentate gyrus (Dg) of the hippocampus. See Figure 27 for further details.

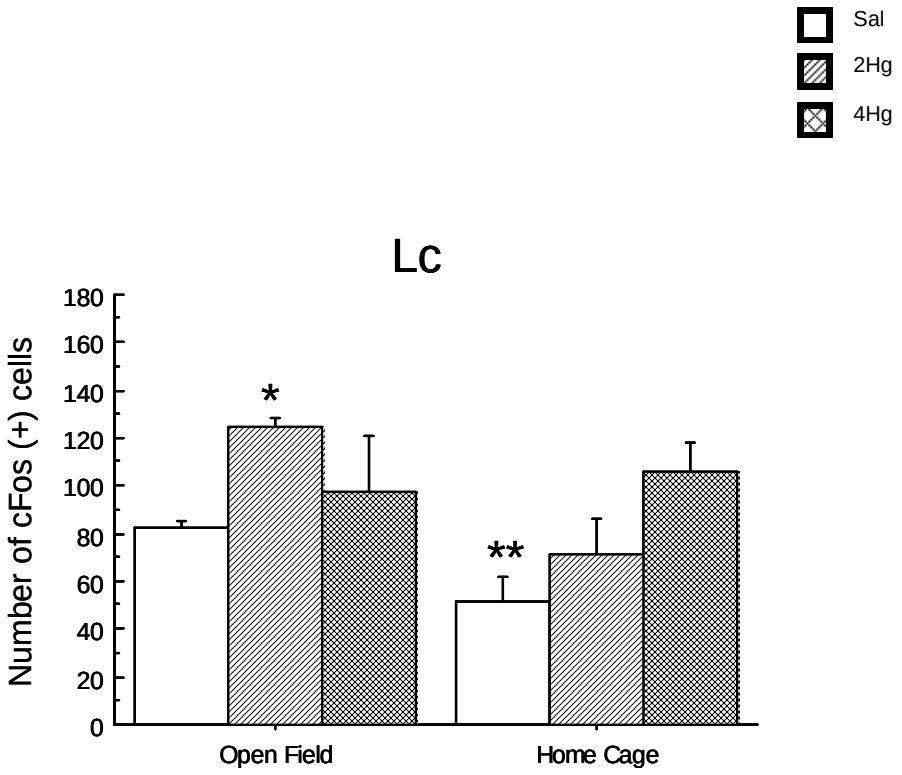

Figure 31. Effect of open field stress and/or repeated MeHg exposure on c-Fos immunoreactivity in the locus coeruleus (Lc). See Figure 27 for further details.

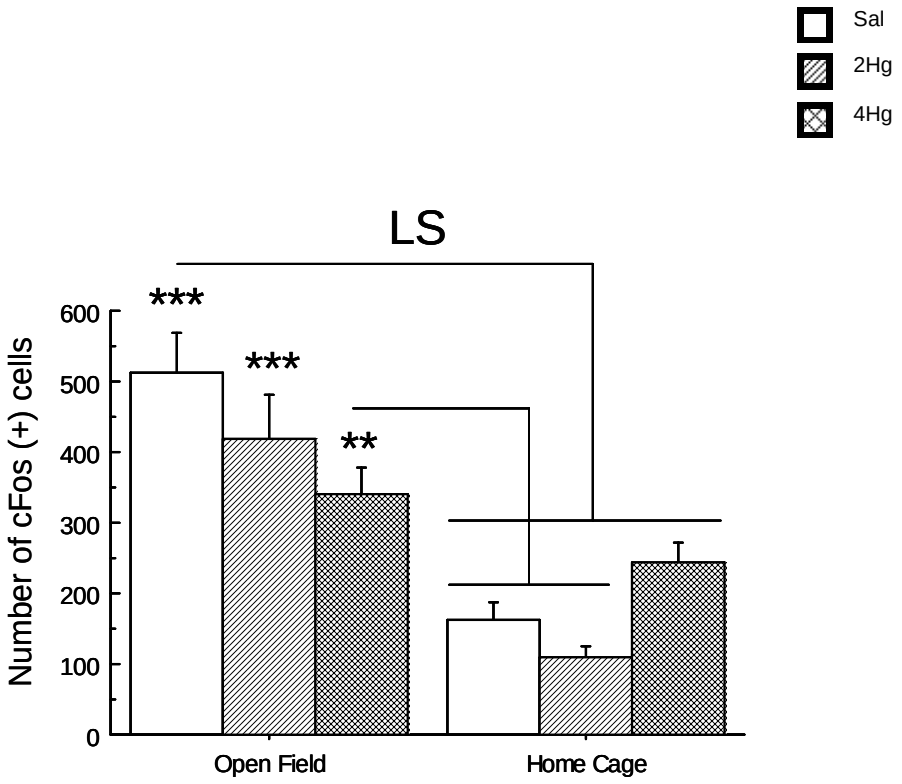

Figure 32. Effect of open field stress and/or repeated MeHg exposure on c-Fos immunoreactivity within the lateral septum (LS). See Figure 27 for further details.

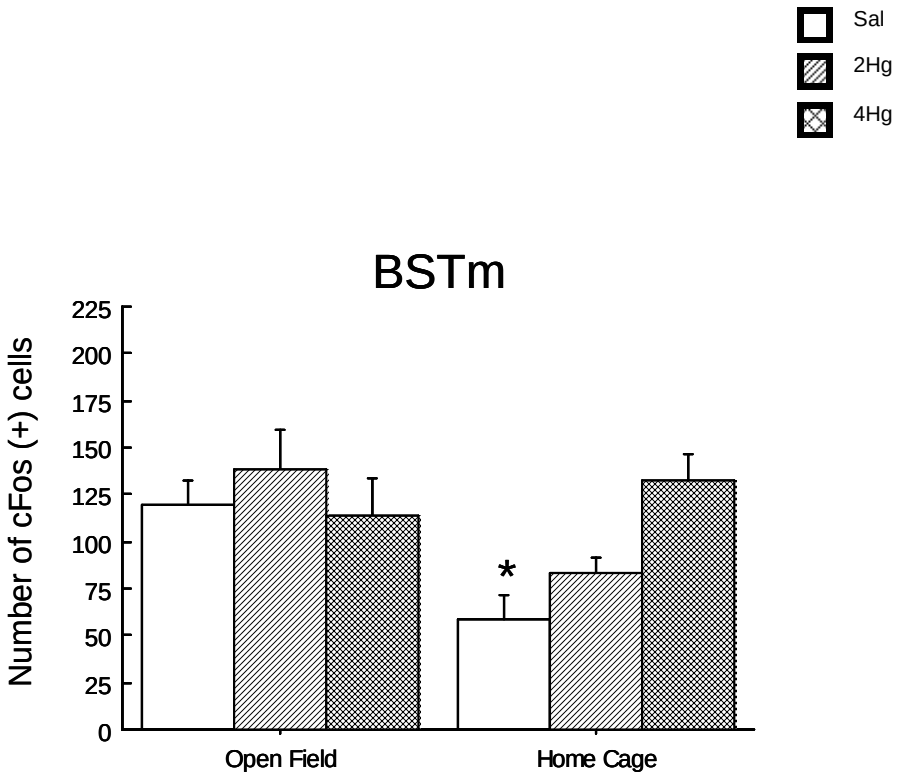

Figure 33. Effect of open field stress and/or repeated MeHg exposure on c-Fos immunoreactivity within the medial region of the bed nucleus of the stria terminalis (BSTm). See Figure 27 for further details.

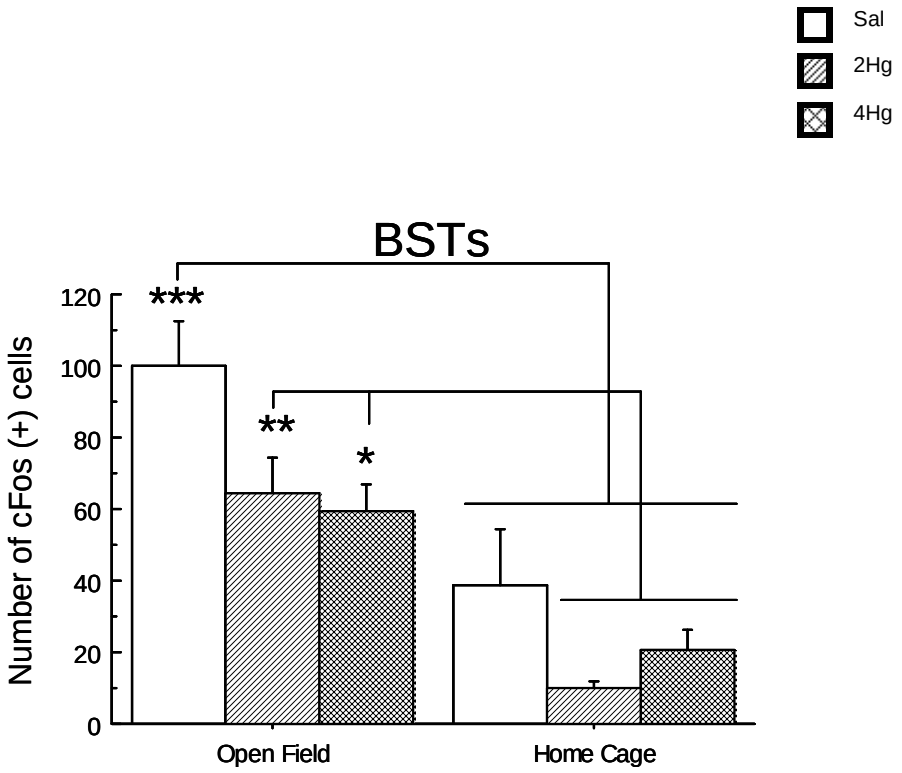

Figure 34. Effect of open field stress and/or repeated MeHg exposure on c-Fos immunoreactivity within the supracapsular region of bed nucleus of the stria terminalis (BSTs). See Figure 27 for further details.

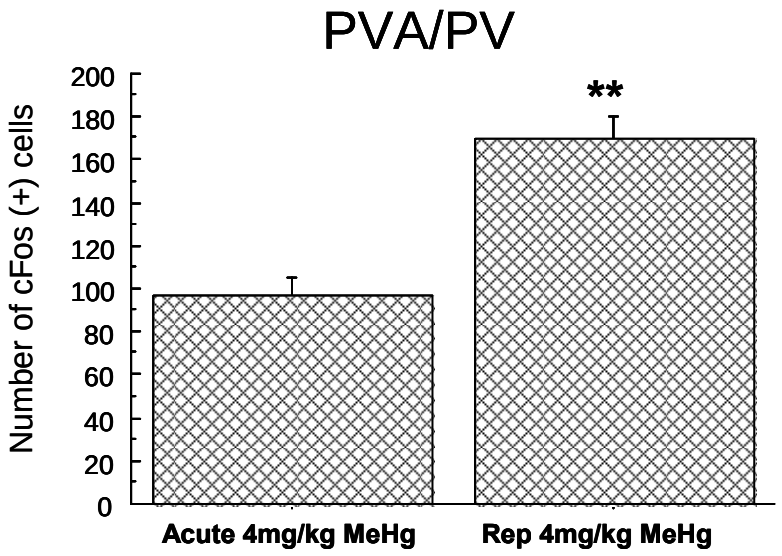

Figure 35. Comparison of magnitude of c-Fos immunoreactivity in the paraventricular thalamic nucleus (PVA/PV) for acute and repeated intraperitoneal 4mg/kg MeHg (all n=5). Each bar represents the mean (+/- SE) with * and ** represent $p < 0.05$ and 0.01 respectively.

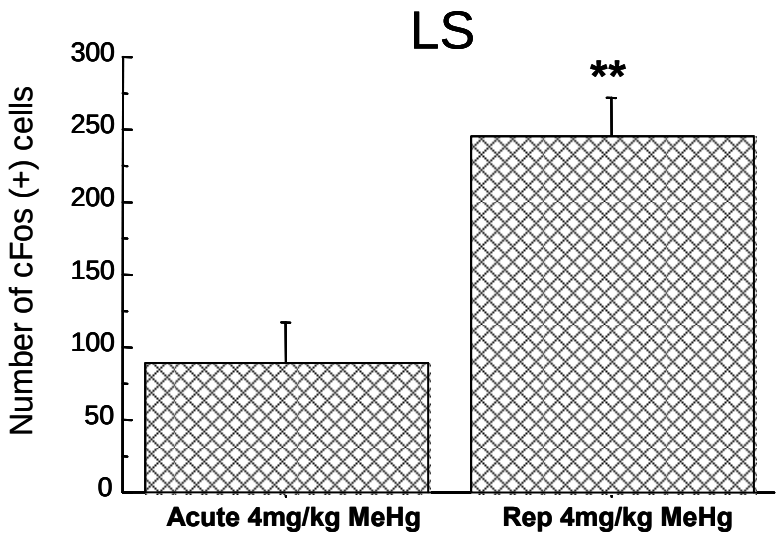

Figure 36. Comparison of magnitude of c-Fos immunoreactivity in the lateral septum (LS) for acute and repeated intraperitoneal 4mg/kg MeHg home caged treatments (all n=5). See figure 35 for further details.

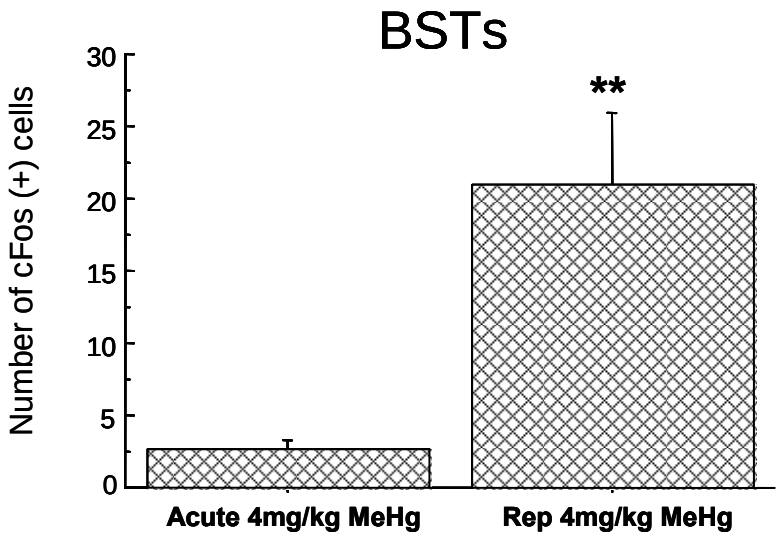

Figure 37. Comparison of magnitude of c-Fos immunoreactivity in the supracapsular bed nucleus of the stria terminalis (BSTs) for acute and repeated intraperitoneal 4mg/kg MeHg home caged treatments (all n=5). See figure 35 for further details.

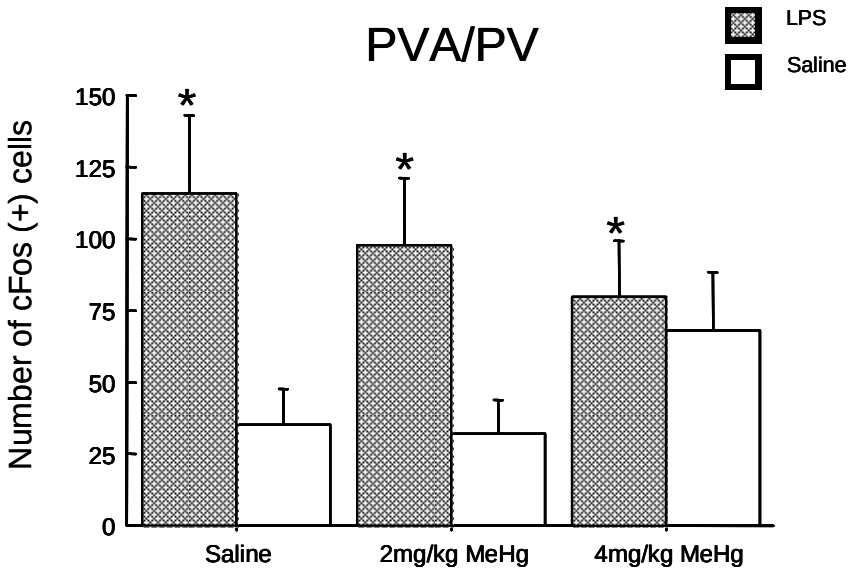

Figure 38. Effect of LPS treatment on c-Fos immunoreactivity in the PVA of thalamus in mice previously exposed to repeated injections of MeHg. Each bar represents the mean (+/- SE) of N=5/group. All MeHg treatments are mg/kg. * and ** represent $p < 0.05$ and 0.01 respectively when compared to corresponding saline or MeHg pretreated groups injected with Saline on the day of sacrifice.

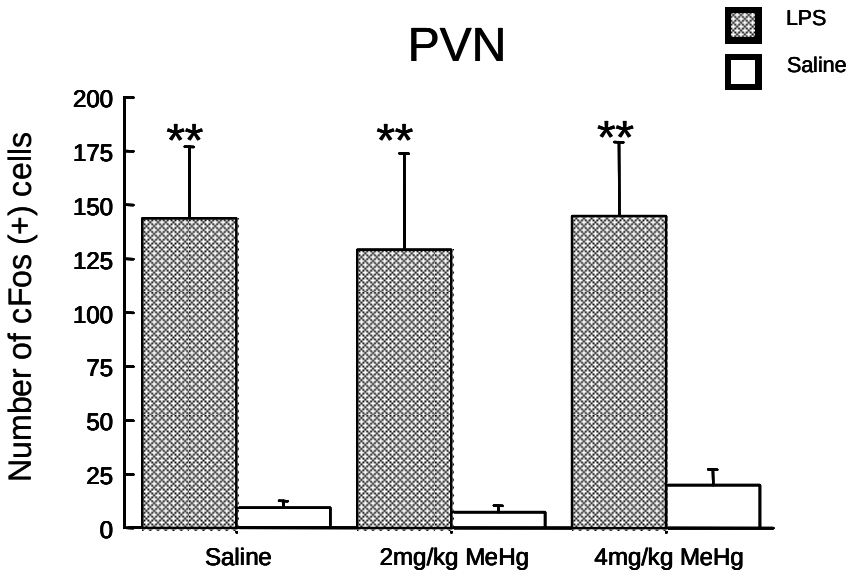

Figure 39. Effect of LPS treatment on c-Fos immunoreactivity in the paraventricular hypothalamic nucleus (PVN) in mice previously exposed to repeated injections of MeHg. See figure 38 for further details.

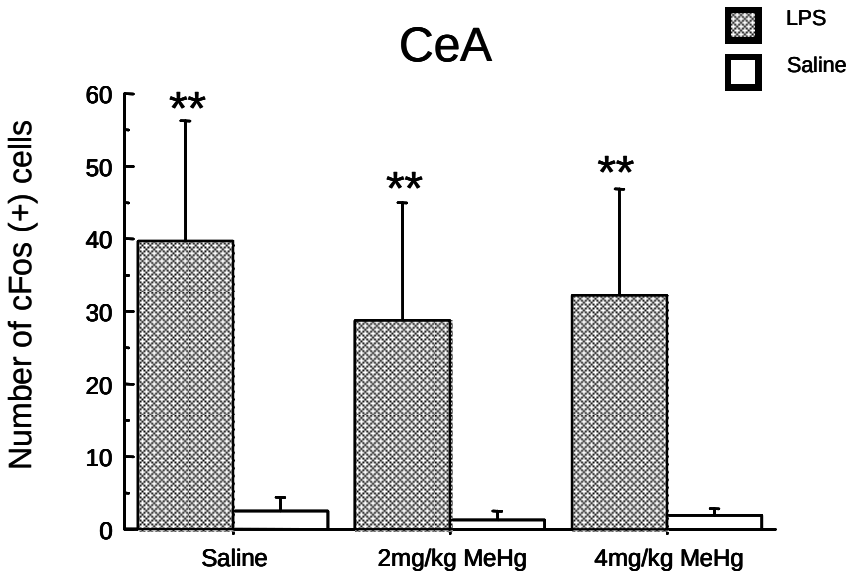

Figure 40. Effect of LPS treatment on c-Fos immunoreactivity in central nucleus of the amygdala (CeA) in mice previously exposed to repeated injections of MeHg. See figure 38 for further details.

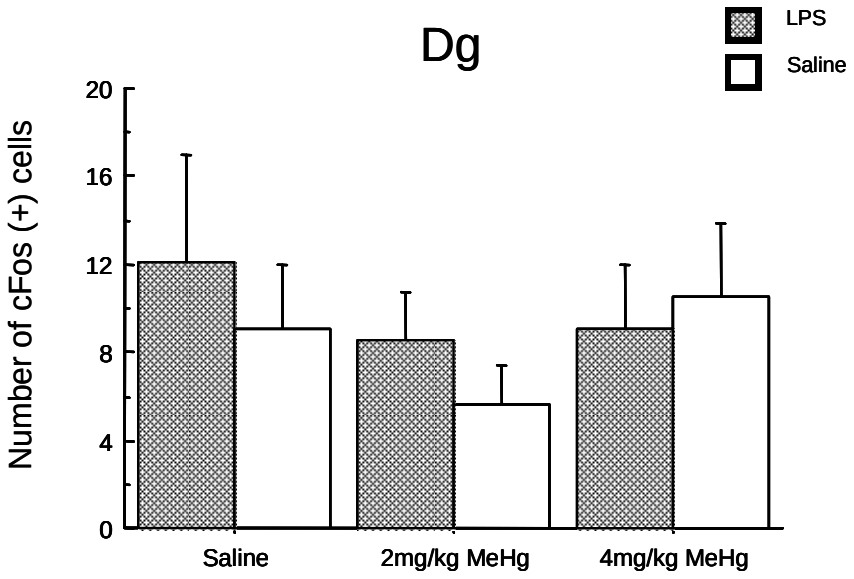

Figure 41. Effect of LPS treatment on c-Fos immunoreactivity in the dentate gyrus (Dg) of the hippocampus in mice previously exposed to repeated injections of MeHg. See figure 38 for further details.

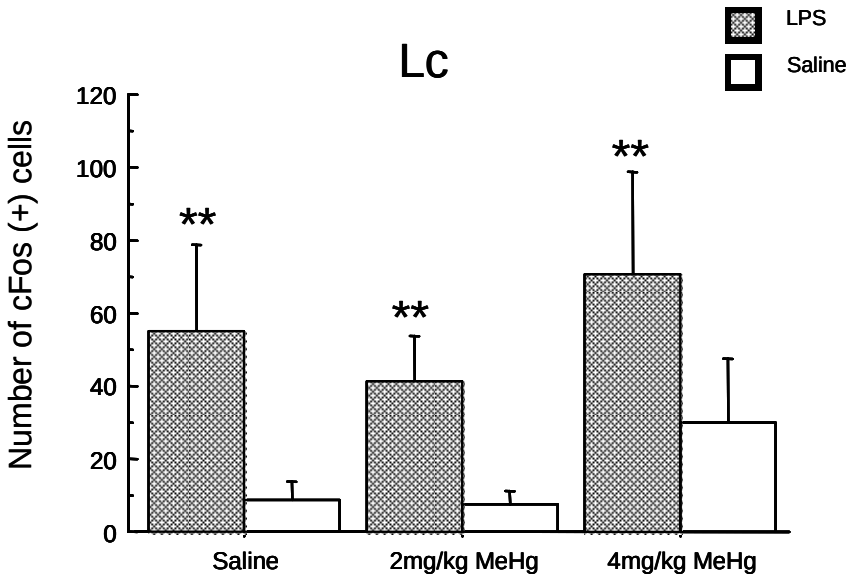

Figure 42. Effect of LPS treatment on c-Fos immunoreactivity in the locus coeruleus (Lc) in mice previously exposed to repeated injections of MeHg. See figure 38 for further details.

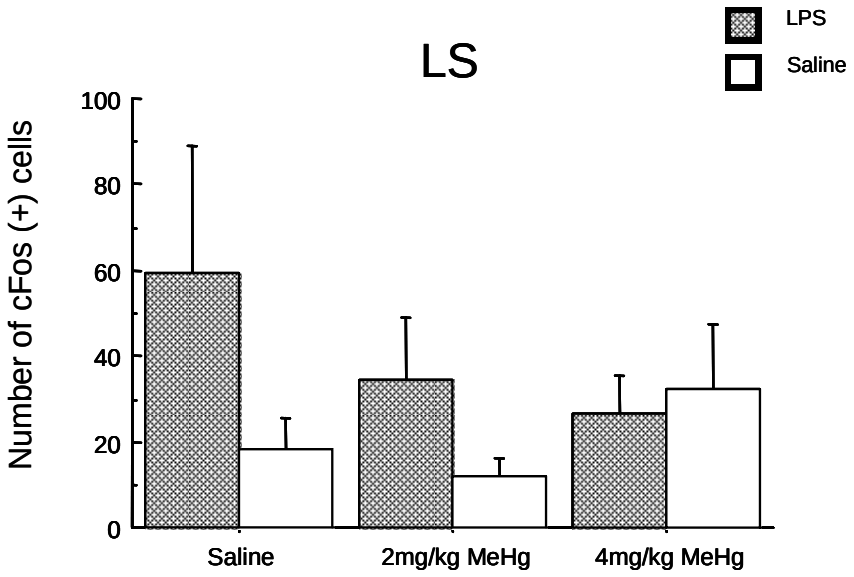

Figure 43. Effect of LPS treatment on c-Fos immunoreactivity in the lateral septum (LS) in mice previously exposed to repeated injections of MeHg. See figure 38 for further details.

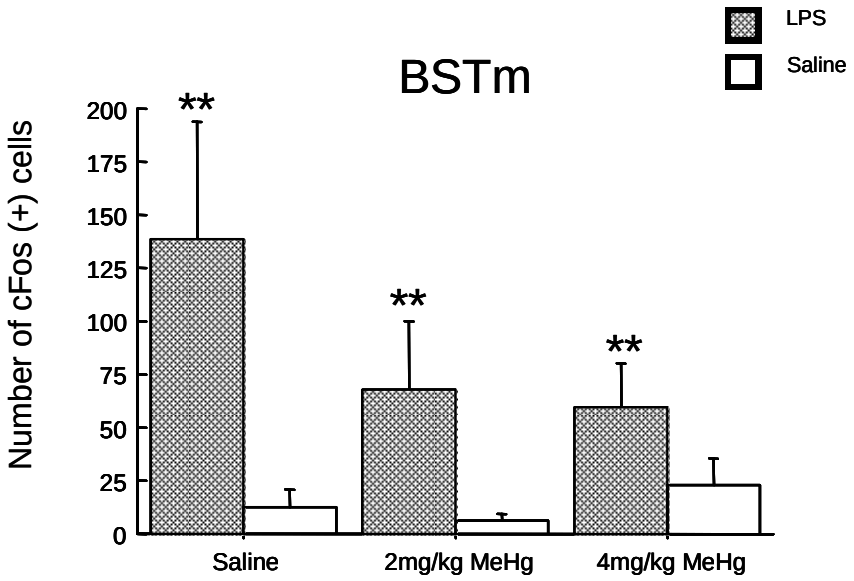

Figure 44. Effect of LPS treatment on c-Fos immunoreactivity in the medial bed nucleus of the stria terminalis (BSTm) in mice previously exposed to repeated injections of MeHg. See figure 38 for further details.

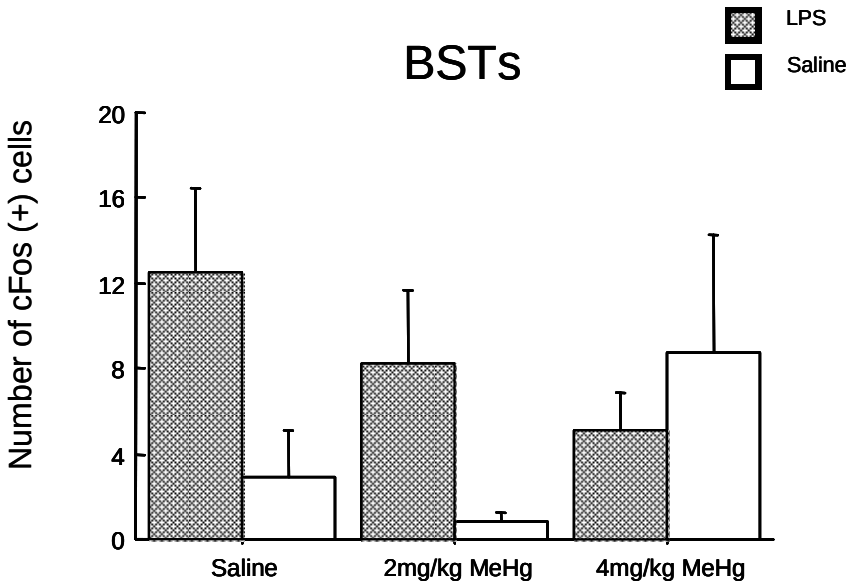

Figure 45. Effect of LPS treatment on c-Fos immunoreactivity in the supracapsular bed nucleus of the stria terminalis (BSTs) in mice previously exposed to repeated injections of MeHg. See figure 38 for further details.

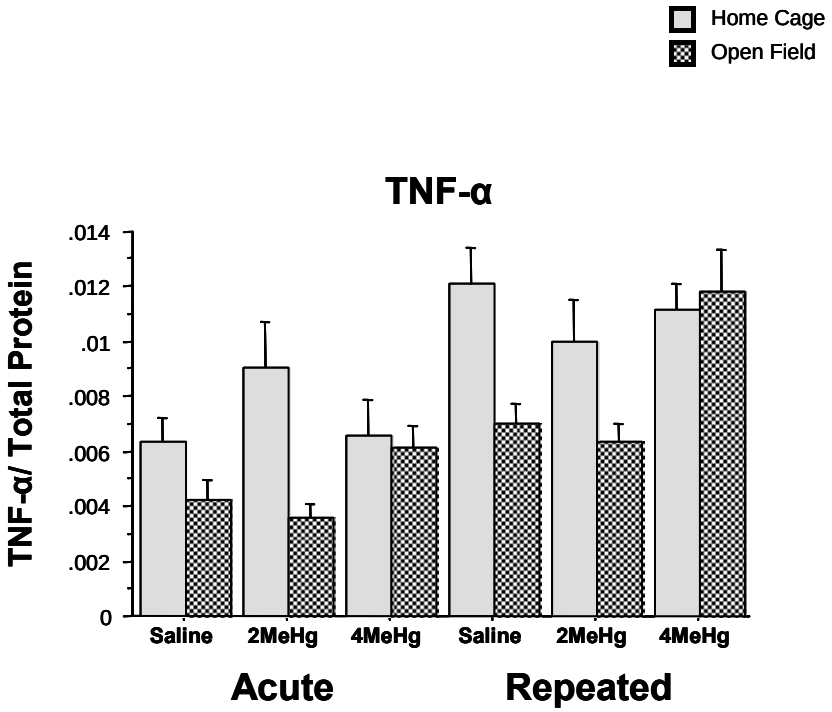

Figure 46. Total protein levels (µg/ml) of splenic tumor necrosis factor alpha (TNF-α) in response to open field and/or acute or repeated exposure to MeHg. Each bar represents the mean (+/- SE) of N=4-5/group. All MeHg treatments are mg/kg. * and ** represent $p < 0.05$ and 0.01 respectively when compared to corresponding groups home cage controls.

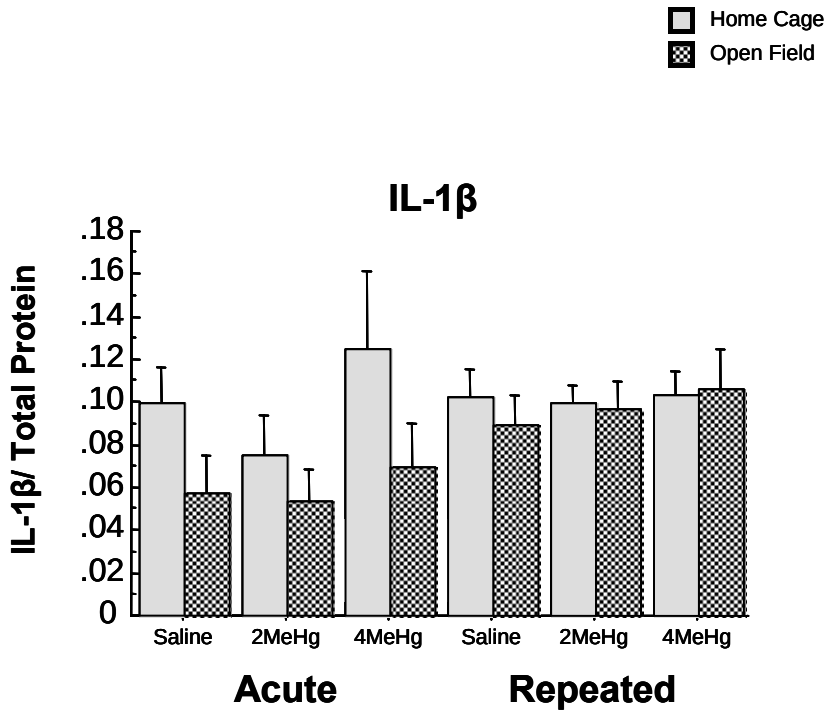

Figure 47. Total protein levels of splenic interleukin-1 beta (IL-1β) in response to open field and/or acute or repeated exposure to MeHg. For further detail see Figure 46.

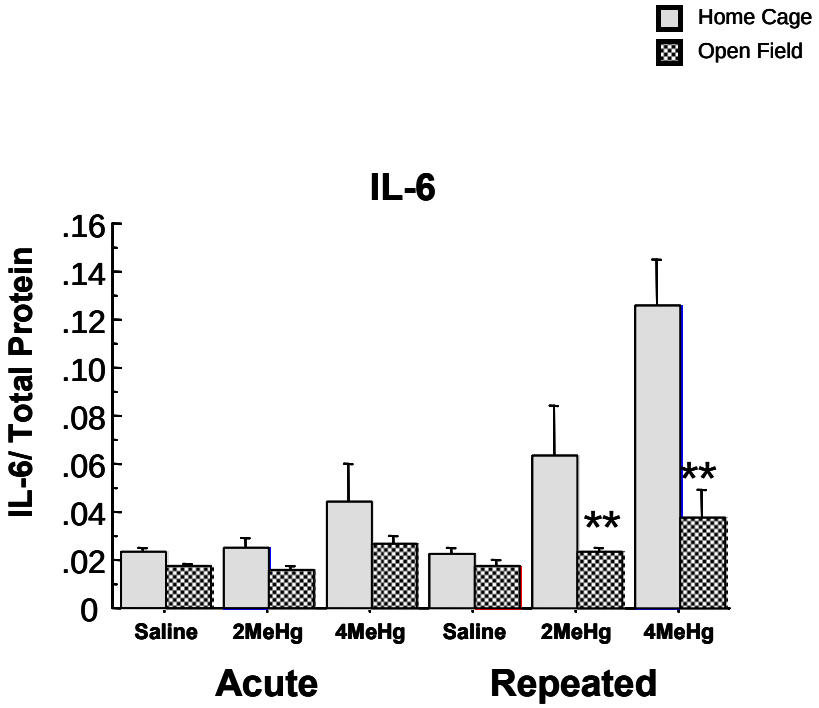

Figure 48. Total protein levels of splenic interleukin-6 (IL-6) in response to open field and/or acute or repeated exposure to MeHg. For further detail see Figure 46.

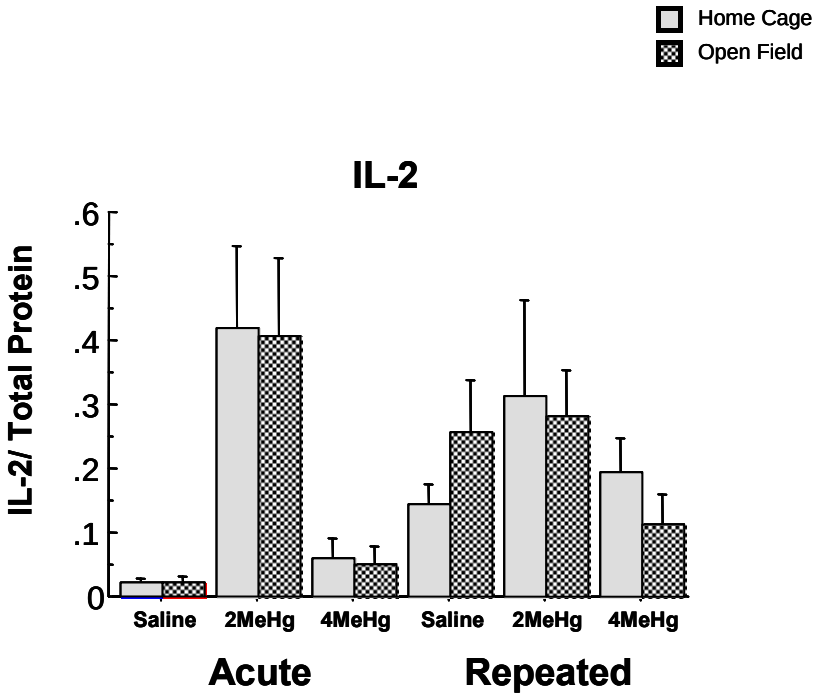

Figure 49. Total protein levels of splenic interleukin-2 (IL-2) in response to open field and/or acute or repeated exposure to MeHg. Each bar represents the mean (+/- SE) of N=2-5/group. For further detail see Figure 46.

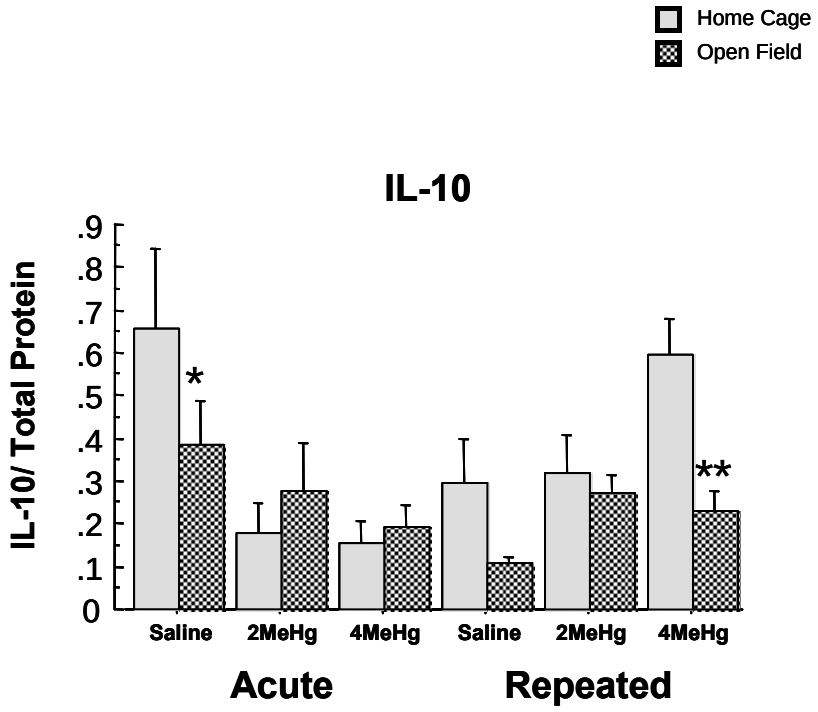

Figure 50. Total protein levels of splenic interleukin-10 (IL-10) in response to open field and/or acute or repeated exposure to MeHg. For further detail see Figure 46.

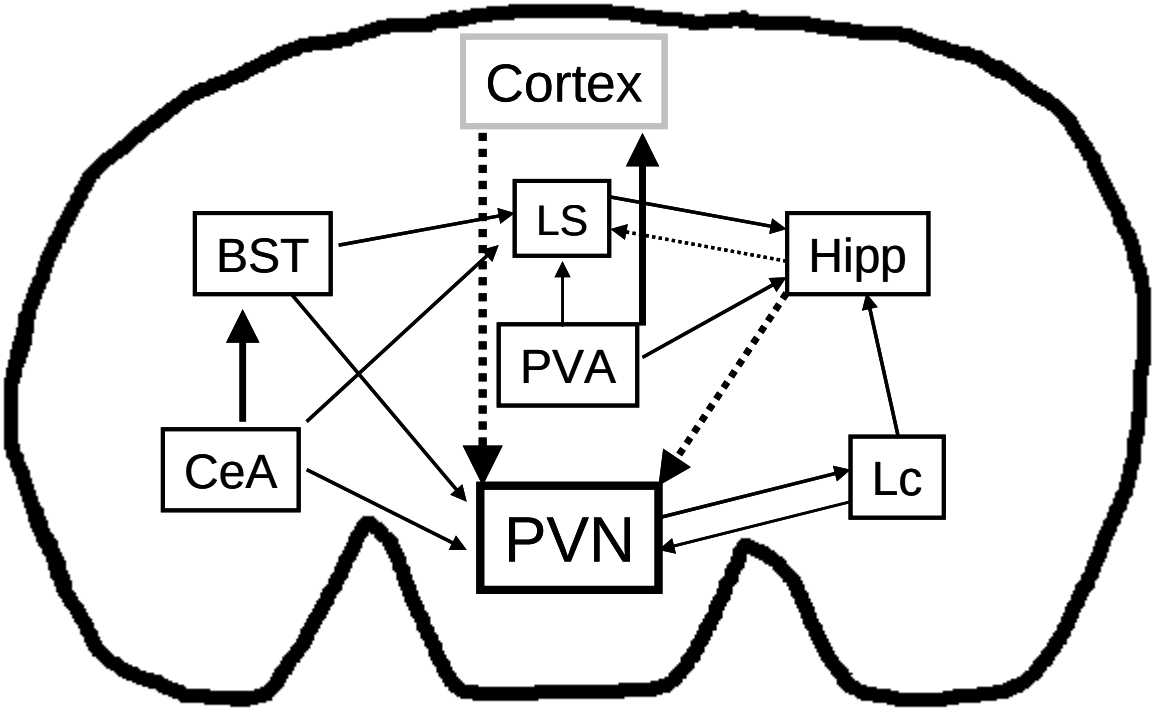

Figure 51. Summary of c-Fos activation within the murine brain in response to acute methylmercury exposure.

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CURRICULUM VITAE

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PUBLICATIONS

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TECHNICAL EXPERIENCE

***In vivo* Techniques:**

- Intracerebroventricular (ICV) stereotaxic surgery and associated sterile technique
- Administration of both chronic and acute ICV, intraparitoneal (IP), and subcutaneous dosing regimes
- Saline and formalin-based intracardial perfusions, in addition to flash-freezing preservation of tissue samples for use in histochemical analysis
- Surgical skills for gross necropsy retrieval of tissue specimens for histologic and subcellular analysis, including isolation of specific brain regions
- Psychological (open field) and neuromotor (rotorod) functional testing associated with neurotoxic insults
- Management, housing, breeding, and handling of transgenic and knockout murine species

***In vitro* Techniques:**

- *Histologic tissue sectioning* on freezing microtome and cryostat
- Free-floating and slide-mounted *immunohistochemical tissue staining*, in addition to routine H&E staining techniques
- *Cell Culture* of immortalized cell cultures
- *Immunoassays:* SDS-PAGE, western blots, ELISA, immunocytochemistry
- *Subcellular fraction preparation*: cytosols and microsomes of rat brain and liver
- *Protein purification*: ion-exchange and affinity HPLC
- *Handling and management of radioactive samples*: α-, β-, γ- emitters
- *Instrumentation:* HPLC, mass spectrometer, gas chromatograph, NMR, spectrophotometers, spectrofluorometers, ultracentrifuges, microscopy (light, fluorescent, confocal), β- and γ- radioactivity counters, microtomes, and cryostats