The effects of methylmercury on the cytokine-signaling and glial differentiation of neural progenitors.

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A. Specific Aims.

Methylmercury (MeHg) is a potent neurotoxin that predominantly affects the developing brain. The effects of low-dose MeHg in utero, stemming from seafood consumption during pregnancy, are not fully understood. At a cellular level, higher doses of MeHg can lead to apoptosis and necrotic cell death in certain vulnerable neural populations. Moderate levels of MeHg exposure can affect cell migration and division during neural development. These observations have evoked hypotheses that MeHg specifically alters neural progenitor cell (NPC) proliferation and differentiation. It is well documented that glia play an important role in sequestering MeHg and protecting vulnerable neurons. Yet, effects of MeHg on gliogenesis have not been explored. Inorganic mercury has been documented to induce oxidative stress that in turn inhibits the activation of JAK/STAT3, a key signaling events in the production of astrocytes. The effect of MeHg on the JAK/STAT3 pathway has not been investigated. Our preliminary observations show that unlike mercury chloride, low-dose MeHg can enhance JAK/STAT3 activity resulting from cytokine stimulation in a neural derived cell line. Our overall hypothesis is that MeHg alters JAK/STAT3-signaling in neural progenitors, resulting in perturbation of gliogenesis. We will test important components this hypothesis at the molecular, cellular, and organismal levels via the following specific aims:

**Aim 1: To document effects of sub cytotoxic concentrations of MeHg upon cytokine-evoked STAT3 phosphorylation in cell culture.** We will employ both neural cell lines and primary NPCs to examine if the effect of MeHg in enhancing STAT3 phosphorylation is conserved. Dose-dependent effects of MeHg will be monitored by quantitative analyses using phospho-STAT3 specific antibodies. We anticipate these findings will illustrate a novel activity of MeHg and will provide insight into differential toxicity of mercury species in neural progenitors.

**Aim 2: To determine the mechanism underlying low-dose enhancement of cytokine signaling.** We hypothesize that MeHg induced changes to STAT3 signaling will enhance phosphorylation by one of two mechanisms: inhibiting phosphatase activity or, alternatively, activating kinases, such as JAKs. To differentiate between these two alternatives we will differentiate the effects of MeHg on JAK versus phosphatase activity using pharmacological inhibitors. Additionally, we will assess free radical production by MeHg, and anticipate that these findings will clarify the low-dose mechanism of MeHg by relating novel and well-established modes of action.

**Aim 3: To determine whether MeHg promotes glial differentiation of neural precursors.** We hypothesize that MeHg will increase astrogliogenesis via JAK/STAT3 activation. We will test this hypothesis by characterizing the ratios of neurons, astrocytes, and oligodendrocytes generated by CNTF-differentiation of NPCs treated in the presence and absence of MeHg. Additional experiments will contrast these effects with inorganic mercury treatment. We anticipate these findings will provide novel evidence of low-dose MeHg effects on astrocyte differentiation in a previously uncharacterized system.

**Aim 4: To determine if MeHg promotes astrocyte differentiation in vivo.** We hypothesize MeHg can promote JAK/STAT3 mediated astrocyte differentiation of dividing NPC during the gliogenic period. In order to test this, neonatal mice will be subjected to acute MeHg treatment together with lineage tracing. We predict that immunohistochemical study will reveal a greater proportion of NPC differentiated into astrocytes in the presence of MeHg. We anticipate these experiments will greatly add to the current understanding of MeHg toxicity by providing novel observations of glial specific perturbations in the developing brain.
B. Significance

A1 Importance of the problem

A1.1 Methylmercury’s (MeHg) impact on human health.

The developing nervous system is exquisitely vulnerable to certain higher molecular weight transition metals including lead, mercury, arsenic, and cadmium, which are widespread in the environment [1-4]. Methylmercury (MeHg) is an organic derivative of mercury that is amongst the most dangerous because of its persistence in the environment, bioaccumulation via aquatic food chains, and ability to readily cross physiological barriers to damage nervous tissue [3, 5]. The increasing use and deposition of Hg in the environment therefore represents a serious and growing threat to human health.

Methylmercury’s impact may be underestimated. According to National Health and Nutrition Examination Survey data, roughly 6% of American women of childbearing age have blood MeHg concentrations above 3.5μg/L [6], which could lead to concentrations in the cord blood that have been linked to cognitive impairment of some 300,000 children born every year [7]. The aggregate cost of MeHg toxicity on American children is estimated to range from $2.2 billion and $13.9 billion dollars yearly (estimated from impact of decreased intelligence on loss of lifetime earnings) [7]. Major birth defects occur in 2-8% of human infants and fetuses representing a major health concern [8-10]. Additionally, cognitive and emotional problems, attention/learning impairment of some 300,000 children born every year [7]. The aggregate cost of MeHg toxicity on American children is estimated to range from $2.2 billion and $13.9 billion dollars yearly (estimated from impact of decreased intelligence on loss of lifetime earnings) [7]. Major birth defects occur in 2-8% of human infants and fetuses representing a major health concern [8-10].

A1.2 Methylmercury in the environment: How little is too much? At doses much lower than those affecting the adult CNS, MeHg can induce cell-cycle arrest, oxidative-stress, and cell death during neurodevelopment while sparing the ontogeny of other organ systems. Severe in utero exposures result in profound developmental delays, cerebral palsy, deafness, blindness, and seizures. Until recently, most mechanistic studies have employed doses that model severe cases of developmental neurotoxicity where concentrations of mercury in the brain are in the micromolar range and cell death is evident [5, 13-16]. Evidence exists for a spectrum of nanomolar (50-900nM) - dose effects manifesting as general disturbances of cognition in domains such as language, perception, attention, and motor coordination [5, 7, 17-20].

Animals studies have largely supported these observations, suggesting that motor, memory, and stress systems are affected at lower concentrations (nanomolar to low micromolar) than those producing obvious neurological signs (micromolar), and that these changes persist over the life of the adult [21-24]. The EPA’s current reference dose is 0.1 mg/kg/day, which is associated with average maternal cord blood concentrations of is 58 μg/L or 270 nM; [25] which is higher than the lowest observable effect level.

Even moderate consumption of contaminated seafood can produce levels of MeHg in pregnancy-aged women that have been previously associated with learning disorders and altered cellular function and cognitive processes. On average single 7 oz. (198 g) can of tuna contains 0.35 ppm or 64 μg [26] MeHg, considerably more than the EPA suggested reference dose of 6.2 μg/day for a 62 kg person [25]. MeHg’s half-life in the body ranges from 45-90 days [27]; meaning that consecutive meals of contaminated fish can lead to increasing body burden over time, which is especially alarming given that swordfish and shark can contain as much as 4-times MeHg as a can of tuna, 0.32 μg/g versus 1.2 μg/g [26].

A1.3 The Unknown Molecular Mechanism of Low-Dose MeHg. In addition to establishing a concentration-range for the lowest observable effects, a critical barrier in progress has been difficulty in determining a molecular mechanism for low-dose methylmercury. Findings from animal and human studies suggest that nanomolar concentrations can change a variety of neurodevelopmental processes without neurodegeneration, but it is not known how MeHg’s chemical properties accomplish such effects.

The affinity of mercurials for sulfhydryl (-SH) bonds [28] is believed to be central in MeHg’s interaction with biological systems. MeHg interact strongly, yet reversibly with cysteine and disruption of these –SH bonds has previously thought to disrupt microtubules [29], and alter Ca^{2+} homeostasis [30]. It is unclear how specific effects arise when virtually all cellular processes depend on cysteine residue containing proteins in some fashion. Another well-supported series of hypotheses posits that MeHg interacts with –SH moieties to disrupt signaling cascades integral to neurodevelopment.

Formation of reactive oxygen species (ROS) has also been ascribed as a dominant mechanism in toxicology and is undoubtedly involved in transition metal toxicity [31]. Singlet-electron oxides, such as O, OH, NOO and O_2, can damage cellular lipids, DNA, and proteins. The majority of cellular cysteine is contained in glutathione (GSH), the primary small molecular antioxidant in cells, which is involved in detoxifying MeHg [32]. MeHg indirectly induces oxidative damage by binding to cysteine’s –SH group and depleting cellular reserves
[33, 34] and may also disrupt mitochondrial function [35, 36] and inhibit antioxidant enzymes [37]. MeHg and HgCl₂ poisoning is ameliorated in vivo and in vitro with exogenous antioxidants and thiol-chelating agents [31, 38].

New findings suggest that the process of neural precursor cell (NPC) differentiation is mediated by molecules that are highly sensitive to the redox state of the cell, 15-20% changes in the red/ox balance of the cell can alter processes such as division, differentiation, and growth [39-41]. Several in vitro studies have demonstrated MeHg can cause oxidative-stress at nanomolar concentrations [37, 42, 43].

**A2 Novel Concepts In Methylmercury Toxicity**

**A2.1 Gliogenesis as a target of MeHg.** Astrocytes, once though to be merely supportive cells, are integral to normal nervous system function and are the most numerous cells in the brain out-numbering neurons 10-fold. To suggest that 100% of a toxin’s effects are entirely mediated by 10% of the cells in this organ seems equally dubious. They are undoubtedly involved in MeHg toxicity, given their roles in coordinating neuronal activity, mediating glutaminergic neurotransmission, detoxifying and eliminating toxic compounds (such as MeHg), buffering ROS, and coordinating changes in blood flow [44]. Despite these important roles, the ontogeny of astrocytes has not been studied extensively in cases of developmental MeHg poisoning [16, 45].

In humans, the differentiation and maturation of NPC into astrocytes follows the generation of most neural types[46]. This occurs during the third trimester, corresponding with the perinatal and early postnatal developmental stage in rodents ~embryonic day (E)17- postnatal day (PND)15 [47-49]. NPC migrate tangentially through the white matter and then radially, populating all layers of the cortex. Here, they undergo morphological changes as they mature into astrocytes, going from simple bi-or-tri polar cells to those with highly branched and radially distributed processes contacting blood vessels and synapses and containing glial-fibrillary acid protein (GFAP) a marker for immature astrocytes and astrocyte-like cells [50, 51].

Interestingly, fetal cord blood is one of the most sensitive indicators of MeHg consumption at this time and the most sensitive indicator of later cognitive deficit, compared with early-term blood levels and levels in breast milk [18]. Rodent models administering MeHg specifically during this period are lacking, but generally support vulnerability at this time showing subtle yet persistent cognitive deficits at low-level exposure[24, 48, 52] and increased astrocyte markers are found in the cerebrospinal fluid of MeHg exposed neonates [53].

At the cellular level, glial cells (myelin-forming oligodendrocytes and astrocytes) are abnormally distributed to the sub ventricular zone, white matter and the leptomeninges with human MeHg exposures. There is also evidence for astrogial hypertrophy [54-56]. These findings have been replicated in animal studies that employed high nanomolar-low micromolar doses of in or near the gliogenic period [52, 53, 57, 58]. One explanation for these results may be that NPC are prematurely differentiated along an astrocyte lineage in response to MeHg. Alterations in the balance of neuronal proliferation and differentiation can result in neurotoxicity and developmental disorders [59], and multiple studies have shown these effects in non-gliogenic NPC cultured in vitro occurring at nanomolar concentrations [60-64].

**A2.2 JAK/STAT signaling as a target of MeHg.** In neurodevelopment, Janus kinase/signal transducers and activator of transcription (JAK/STAT) mediates the functions of various cytokine family signaling protein-receptors such as interleukin 6 (IL-6), IL-11, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), cardiotrophin-1 (CT-1), interferon-gamma (IFN-γ), and oncostatin-M [65]. JAKs (JAK1, JAK2, and TYK2) are cytosolic tyrosine kinases that associate with receptor components, become activated by trans-phosphorylation and phosphorylate the receptors and STATs. In the simplest view, this causes cytosolic STATs to dimerize and localize to the nucleus where they bind to consensus regions in the promoters of a variety of target genes [66, 67].

JAK/STAT is reported as the main regulator of astrocyte gliogenesis [68, 69] and STAT3 increases glial-fibrillary acid protein (GFAP) expression [70]. CT-1 mainly, but also LIF and CNTF promote differentiation of astrocytes between mouse E17 and PND14 through JAK/STAT signaling, after becoming competent to generate astrocytes by an epigenetic mechanism [71]. Other pathways, e.g. bone morphogenic protein 4 (BMP4), can accomplish GFAP expression independent of STAT3 but may generate different astrocyte phenotypes [72, 73].

Several publications suggest that JAK/STAT is potentially involved in other forms of metal poisoning, including mercury chloride and cadmium [74-77]. Inhibition with these metals was specific to neurons and occurs at the level of JAK1 and JAK2, possibly via modification of JAK-kinases by ROS and nitrosylation [75]. Another –SH reactive compound, sodium arsenite has been found to inhibit JAK/STAT signaling via a covalent modification of the JAK-homology domain [78].

The role of JAK/STAT in MeHg toxicity and its contribution to a mechanism for producing patterns of glial progenitor disruption in vivo is unknown. Several other signaling cascades have previously been implicated in low-dose methylmercury toxicity have intersections with JAK/STAT including, ERK [60, 79] and Notch [61,
Disrupted JAK/STAT signaling is believed to underlie overproduction of GFAP+ astrocytes in Down Syndrome patients [82] whose extra copy of amyloid precursor protein may lead to enhanced JAK/STAT3 signaling [83]. JAK/STAT may be sensitive at this time, as positive feedback might lead to significant changes in development from small changes in signaling [69], perturbations leading to aberrant acceleration/deceleration of astrocyte differentiation. GFAP mutations are causative in Alexander Disease, a progressive degenerative disease with an early-life onset (<2 mo.) and characterized by white matter destruction and progressive neuronal death, leading to loss of developmental milestones and seizures[84], similar to what is observed in less-severe cases of MeHg poisoning (albeit more progressive).

**A2.3 Hypothesis: MeHg enhances JAK/STAT signaling in NPC with downstream consequences on astrocyte differentiation.** The observations summarized above lead us to propose the novel hypothesis that MeHg acts in a similar manner to HgCl₂, inhibiting JAK/STAT via ROS at subcytotoxic concentrations. Preliminary experiments led us to refine this hypothesis further, as MeHg increased CNTF-evoked STAT3 activity in the SH-SY5Y cell line without increasing ROS. Therefore, we hypothesize that MeHg activates JAK/STAT3-signaling in neural progenitors at doses too low to cause oxidative stress, in contrast to the activity of inorganic mercury. Testing this hypothesis with the following aims will benefit public health by expanding the body of knowledge regarding the neurotoxic mechanism of MeHg. Additionally, these AIMs will test a novel and potentially translational mechanism in cell lines and in vivo.