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

Nathan Jebbett

April 19th, 2011

Advisors: Felix P. Eckenstein and Matthew D. Rand
A. Specific Aims.

Methylmercury (MeHg) is a potent neurotoxin that predominantly affects the developing brain. The effects of low-dose MeHg \textit{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:

\textbf{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.

\textbf{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.

\textbf{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.

\textbf{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

\textbf{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 impairments, and have been hypothesized to involve multiple uncharacterized environmental factors [11, 12].

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 ontology 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 previously been 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 posit 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 O2, 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
HgCl$_2$ poisoning is ameliorated \textit{in vivo} and \textit{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 astroglial 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 \textit{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 morphogenetic 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 \textit{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, 80, 81]. 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.

C. Innovation
Mounting evidence suggests a pivotal role for glia in MeHg toxicity [45, 53-55, 85], but despite nearly three decades of research, only a handful of studies have administered MeHg specifically in the gliogenic period and determined how this process is affected. To our knowledge, the effect of MeHg on gliogenesis has been specifically examined in only one other in vivo study that used a high single dose on PND2 (20 mg/kg) and examined glial markers. This revealed a greater number of BrdU⁺, GFAP⁺, S100β⁺ progenitors concentrated within the sub ventricular zone and white matter in MeHg treated animals, which showed no overt physical manifestations of toxicity. Unfortunately the authors did not address potential mechanisms underlying changes in glial distribution[57]. A 2002 study by the same group administered a similar treatment at E10, E15, and E21 and found that BrdU distribution was altered at E15 and E21, but did not examine markers of cell fate. In both cases, Kakita and colleagues interpreted their results as failed glial progenitor migration, although cells both over- and under- migrated. An alternative explanation might be that enhanced JAK/STAT signaling drove differentiation of glial committed progenitors before they could reach their proper position [58]. Most other studies in the field administered MeHg over different periods of neurogenesis, ignored glial markers, or used broad time-courses[16].

JAK-STAT is important to neurodevelopment and is emerging as a target of metals and metalloids. HgCl₂, CdCl₂ and other chemicals that induce ROS reversibly-inhibit evoked JAK/STAT3 phosphorylation via oxidative stress in neural cell-lines [74-77]. Our approach expands on these findings by testing the idea that JAK/STAT could be involved in MeHg toxicity in vitro and in vivo. Moreover, no studies have determined the role of STAT serine phosphorylation in toxicity (despite interesting newly discovered roles in mitochondrial respiration [86], and few have accomplished comparisons of mercury isoforms using neural cell-lines[87].

NPC and can be cultured from various stages of development making them an increasingly popular models for characterizing neurotoxicity [64, 88]. Several studies have looked at NPC in vitro and found that subcytotoxic levels of MeHg reduced proliferation [15, 60], or inhibited neuronal differentiation [61, 62, 88, 89]; however only two we know of have examined differentiation to glial lineages, in human umbilical cord-generated NPC responding to PDGF-BB and retinoic acid [64], and in PND7 oligodendrocyte type-2 astrocyte (O2A)-cells [42]. None have examined multipotent NPC derived from the gliogenic window of development or NPC differentiated with CNTF or BMP4. In fact, most studies that have looked at NPC differentiation changes in response to MeHg have simply neglected glial fate markers [60-63, 87, 89]. JAK/STAT may be activated by pro-oxidants in non-neuronal cells [90, 91] and it is unclear how it may affect this in astrocytes and NPC being somewhat intermediate between neuronal and non-neuronal.

Although MeHg has been shown to generate ROS, reports have produced conflicting evidence of this at nanomolar concentrations, some showing 120-200% increase in ROS [42, 43], some showing no ROS at these levels [92, 93], and others not determining it [61, 79, 89]. By testing the dose-dependency of both oxidative and cell-signaling events, the experiments in these aims are uniquely designed to provide a comparison between two potentially important mechanisms of action in an important and uncharacterized cell-type.

D. Approach.
Our overall strategy is to test our central hypothesis at the molecular, cellular, and organism levels and determine a potential mechanism. The first aim will accomplish a molecular description of MeHg’s effects on key components of JAK/STAT and on ROS in both well-characterized neural models and, for the first time, in NPC derived from the gliogenic phase. The second aim will explore the mechanism by low-doses of MeHg enhance JAK/STAT signaling. Next, we will characterize downstream effects on gene expression and cell fate decision in NPC undergoing glial differentiation, determining whether increased JAK/STAT3 signaling results in enhanced generation of astrocytes. Lastly, we will test whether these changes are sufficient enough to enhance gliogenesis in vivo.

AIM 1: To document effects of sub cytotoxic concentrations of MeHg upon cytokine-evoked STAT3 phosphorylation in cell culture.

AIM1: Strategy. We will use a well-characterized human neuroblastoma line, SH-SY5Y, for comparing MeHg vs. HgCl₂’s effects on CNTF-mediated JAK/STAT activation/phosphorylation using a dosing regimen from previous toxicology studies with JAK/STAT [74]. Additionally, we will test for these effects in a novel system, PND1 mouse cortical NPC. Viability tests will be done first, as they are essential to this aim. It is believed that low-dose effects occur in absence of neurodegeneration in vivo, as does inhibition of STAT by HgCl₂ and CdCl₂ in vitro [15, 74, 76]. We will determine inhibition/ activation of JAK/STAT3 in the sub-cytotoxic range by pretreating with MeHg, HgCl₂ (to support our hypothesis that MeHg acts differently and replicate previous data), and H₂O₂ (to determine the effect of pure-ROS on STAT3), then probing for phosphorylation of STAT3 in lysates harvested after 30min CNTF-stimulation. To determine if any changes represent a homogenous cellular response or larger-magnitude response by a sub set of heterogeneous cells, we will also detect PY-STAT3 by immunocytochemistry.

AIM1: Methodology. PND1-NPC will be obtained cultured in vitro as has been described[94], and SH-SY5Y cells obtained from ATCC. Cell Viability. The methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay [95] has been used extensively to characterize the cytotoxicity of mercury compounds [60, 64, 74]. We will generate kill curves for 5, 24, and 48h exposure periods for both MeHg and HgCl₂ in order to ensure that amounts used in subsequent treatments fall within the subcytotoxic range. To do this cells growing at 75-85% confluence will be exposed to low nM- to high μM concentrations of HgCl₂, MeHg or vehicle (DMSO) for the times indicated, 1% Triton X-100 being used as a control for 100% cell death. Complimentary methods of cell death detection such as calceinAM/ ethidium homodimer co-labeling (LIVE/DEAD assay) and cell counts (CyQuant- Sigma) will be used to confirm the validity of the MTT assay results, controlling for effects of toxicants on mitochondrial function. Sub-cytotoxicity will be defined as doses lower than the lowest dose producing a statistically significant decrease in viability from positive controls by ANOVA followed by post hoc tests. Linear regression using a four-parameter logistic function will be used to determine LC values and associate 95% confidence intervals.

Determining JAK/STAT Activity. Cells growing at 75-85% confluence will be starved for 1h, and then pretreated for 5h with toxicant. This paradigm is in consideration of previous studies demonstrating toxicant-induced JAK/STAT inhibition [74, 76, 77]. The time course may be varied in subsequent experiments to explore any noteworthy results. Following this cultures are stimulated for 30min with +/- 20ng/mL rat recombinant CNTF followed by cell lysis and processing for SDS-PAGE. This time-point corresponds with maximal phosphorylation levels of STAT [96]. We will focus on CNTF stimulated STAT3 activation, as STAT3 has been demonstrated to serve a crucial role in the generation of astrocytes in the later stages of neurogenesis and in adult repair [65], and is about 10-times more sensitive to CNTF than STAT1 [96]. Western Blotting for phosphorylated STAT3. Total cellular lysate will be obtained with inhibitors and separated with 8% SDS-PAGE. The following antibodies will be used to assess STAT3 activity: PY705-Stat3, PS727-STAT3, Total STAT3 and Actin for normalization. Tyrosine phosphorylation is the primary indicator of activity, although serine phosphorylation may be necessary to the full activity of STATs [97]. Activation of CNTF-receptors may result in costimulation of other signaling pathways including the extracellular signal-regulated kinases (ERK), and Akt pathways [98]. To determine if the effects of toxicant treatment are specific to altering JAK/STAT activity, the following antibodies will be used to probe other pathways: P-Ser473 Akt, Total Akt, P-Thr202/Tyr204 p42/44ERK, Total ERK. Results for all western blot experiments will be scored for integrated band density, scaled, and presented as phosphorylated/total and total/actin. ANOVA and pertinent post hoc analyses will determine if >20% differences between the various treatment groups and the positive vehicle control are significant. Immunocytochemistry for PY705-STAT3. In three independent experiments, cells will be grown as described for the western blot studies, but will be fixed for immunocytochemistry 30 m following the addition of CNTF and processed for...
immunocytochemistry. PY705-STAT3 will be assessed alongside DAPI to label all nuclei. In random fields of view, cells (nuclei) will be counted and scored as not-staining (-), weak/some staining (+) or densely staining (++), 200 cells being counted for each condition/experiment. mRNA expression as determined by quantitative real-time reverse transcriptase polymerase chain reaction (qRT2PCR). Because STAT3 functions as a transcription factor, meaningful changes in its activation by phosphorylation should be accompanied by related changes subsequent in gene expression, which was not determined in previous investigations [74-77]. Using cell culture and treatment paradigms described above for NPC, determining gene expression at 12 and 24 h time-points following cytokine addition. mRNA will be specifically converted to cDNA by reverse-transcription with poly(T) primers and levels of selected transcripts determined by qRT2PCR. JAK/STAT targets assayed will include GFAP, gp130 (testing for positive regulation) SOCS1, and SOCS3- their expression levels determined by the deltaCt method, using beta-actin as a housekeeping gene. Suppressors of cytokine signaling (SOCS) are gene targets of JAK/STAT that accomplish negative regulation by binding to JAKs and targeting them for proteasomal degradation [67]. Results are scaled according to determination of primer efficacy and will be expressed in ppm relative to actin and significance determined by comparison to untreated controls with ANOVA.

**AIM1: Analysis.** At equimolar concentrations, HgCl2 has previously been reported to be ~5 times less cytotoxic that MeHg [88], which we expect to confirm in SH-SY5Y and NPC. NPC are expected to be more sensitive to MeHg than HgCl2. In SH-SY5Y, EC50 for cell death after 24 h was ~1-2μM and proliferation as determined by BrdU incorporation was inhibited over a similar range [99]. In NPC derived from E14.5 mice, the observed LC50 after 2 d was 56 nM[38]. Therefore, we expect to find that NPC are more sensitive to the toxic effects than SH-SY5Y or other cell lines, as has been previously reported [42, 89]. Although we expect to find that H2O2 and HgCl2 inhibit Y705-STAT3 phosphorylation based on previous reports, we are unsure how patterns of S727-STAT3 phosphorylation change in response to HgCl2, as this has not been examined previously. HgCl2 produced an observable drop in PY705-STAT3 in BE-2-C cells at 300 nM [74] and it is plausible that MeHg could also affect JAK/STAT at nanomolar concentrations. Low-concentration (20 nM-50 nM) MeHg has been shown to induce ROS [42, 43], which might produce inhibition of JAK/STAT3 signaling [75], although many other compounds that are also capable of generating ROS (i.e. manganese chloride, lead acetate, and iron (II) chloride) do not inhibit JAK/STAT, even at toxic concentrations [74].

Despite these observations, our own preliminary data show enhanced STAT3 phosphorylation independent of ROS in MeHg, and suggest HgCl2 behaves as previously reported in both SH-SY5Y and mouse PND1 NPC (see Figure 1+2). It is expected that gene expression will directly vary with STAT3 activity in NPC; however, several studies have failed to demonstrate any changes in gene expression resulting from CNTF treatment of SH-SY5Y and we may choose not pursue gene expression changes in these cells further.

With regards to cytotoxicity measurements, dependence of the MTT-assay on mitochondrial integrity [95] and possible inhibition of mitochondria by MeHg [100], could result in false positive signal and over-estimation of cell death. Complimentary methods of cell death detection such as calceinAM/ ethidium homodimer co-labeling (LIVE/DEAD) and cell counts will be used to confirm the validity of the MTT assay results. Additional experiments incorporating a washout and recovery time may also prove useful.

**AIM2: To determine the mechanism underlying low-dose enhancement of cytokine signaling.**

**AIM2: Strategy.** Our preliminary results suggest low-amounts of MeHg can lead to ~160% increased cytokine STAT3 phosphorylation in gliogenic NPC (30 nM) and in SH-SY5Y (1 μM). What is not clear is how MeHg’s unique chemical properties translate into an effect on JAK/STAT. Based on our review of the literature, we hypothesize that several alternative mechanisms could explain this observation:
oxidants. HgCl2 will serve as a basis for comparison with MeHg during these experiments, as it is known that the degree to which MeHg-induced changes are blocked with antioxidant treatment and enhanced with pro-oxidant/Antioxidant Treatment. We will address both of these possibilities with separate sub aims and will also determine if ROS are involved in changes to cell signaling.

**AIM2A: To test for enhanced JAK-activity.** MeHg enhances phospho-STAT3 upon cytokine stimulation and therefore may also activate JAKs. JAKs are the canonical regulators of STATs-phosphorylation although STATs can become phosphorylated by kinases such as SYK, [101] leading to activity by non-JAK routes. Differentiating between canonical and non-canonical pathways would help distinguish MeHg’s effects on JAK/STAT from those of HgCl2, which reportedly inhibits JAK activity through a ROS mediated protein nitrosylation [75].

**AIM2B: To determine if negative regulators of STAT3 are inhibited by MeHg.** Protein tyrosine phosphatases have a redox-sensitive active site cysteine [102, 103], making them a plausible target for MeHg’s purported strong thiol affinity. Furthermore, MeHg’s low-dose alterations in ERK signaling appear to involve increased deactivation [60]. Changes in phosphatase activity have previously been connected with reactive metal electrophiles [104] and are integral to normal JAK/STAT activation and deactivation [105].

Several phosphatases are involved in regulating JAK/STAT. SHP1 and SHP2 negatively regulate JAK/STAT by dephosphorylating JAKs and receptors. Protein tyrosine phosphatases PTP1B and PTP1C negatively regulate Jak2 and Jak2/Tyk2 respectively [97] and PTP1B, PTP, PTEN, and SHP2 contain reactive site cysteine residues and are candidates for inhibitory S-nitrosylation [103], a modification that has been noted to directly inhibit JAK in response to brief subcytotoxic ROS in the Halverson group’s research [75].

The involvement of protein phosphatases will be determined as a mechanism for either increased activity JAKs and/or STAT3 depending on the specific JAKs/STATs results of AIM2A. Specific antibodies directed against active conformations of phosphatases will allow us to determine if MeHg treatments increase their activity. Specific inhibitors to these phosphatases are available, and will be used to confirm their involvement if they are found to be regulated by MeHg.

**AIM2C: To determine ROS levels and effect on STAT3.** Because oxidative damage is the chief mechanism underlying JAK/STAT inhibition by several toxicants [75], we will test for ROS generation parallel to determining JAK/STAT activity using a popular multi-plate fluorescence assay. Additionally, we will determine the degree to which MeHg-induced changes are blocked with antioxidant treatment and enhanced with pro-oxidants. HgCl2 will serve as a basis for comparison with MeHg during these experiments, as it is known that subcytotoxic concentrations of HgCl2 inhibit JAK/STAT by increasing ROS [74].

**AIM2: Methodology.** SH-SY5Y will be used to initially characterize protein involvement to avoid potential complexities in interpreting data from NPC. Western blots. Cells will be cultures and samples obtained after MeHg-treatment as described in AIM1. We will first determine the activity of the JAKs immediately upstream of STAT3, which are JAK1, JAK2, and Tyk2 [98], and phosphatases SHP2, PTP1B, PTP1C, and PP2A using active site phospho-antibodies and the methodology for quantitating western blot band density described in AIM 1. Inhibitors to kinases or phosphatases will be added to the cell culture media 2 h prior to the addition of CNTF, and Y705-STAT3 phosphorylation measured as previous. Concentrations of inhibitors will be determined in preliminary experiments. Measuring ROS using nitro blue tetrazolium (NBT). Preliminary data obtained using 5-(and-6)-carboxy-2',7'-dichlorodihydro-fluorescein diacetate (CMHDCFDA), a fluorescein-analog dye that has been extensively employed in MeHg studies measuring ROS [43, 74, 76, 77, 92], suggested ROS formation is not elevated at low-concentrations of MeHg that produce STAT activation (Figure 2). In order to determine if ROS are elevated at the time of CNTF addition using a more sensitive assay specific to superoxide anion, NPC and SH-SY5Y will be grown and treated with HgCl2 and MeHg as before, having 3 mg/mL NBT in Hank’s balanced salt-buffer added at the 5h time point. Cells are incubated 30 m, fixed in 100% methanol, solubilized in DMSO and KOH, and read at 620 nM on a multi-well plate spectrophotometer. Data will be expressed as +/- % control, n=3 independent experiments. Differences from positive control will be determined by ANOVA with post hoc tests. To determine if changes represent a homogenous cellular response or are specific to a subset of cells NBT staining will also be observed with confocal microscopy. Pro-oxidant/Antioxidant Treatment. In order to be certain that ROS are not involved in MeHg’s enhancement of JAK/STAT, we will add antioxidants with no intrinsic antioxidant activity such as procysteine [106] 2h before toxicant addition and determine the outcome on Y705-STAT3 as prev. To assess the effect of pro-oxidative compounds on JAK/STAT3 signaling, cells will be starved for 5.5h, and then pretreated for 0.5h with 1 mM H2O2 to mimic oxidative stress [77].
AIM2: Analysis. If MeHg enhances JAK activity (phosphorylation) to be important to MeHg’s activation of STAT3 as determined by inhibitors AG490 or P6, we will determine the activity of several upstream regulators noted in the MeHg literature. These include gp130, ERK, Src, PKC, p38 MAPK [66, 107-109], and aforementioned phosphatases. Implication of unknown phosphatases could be addressed with a broad spectrum and specific phosphatase inhibitors such as NaF or PMSF.

If the MeHg does not lead to increased phospho-JAK over positive control, JAK may not be involved and we will look to other enhancers of STAT3 function implicated in MeHg toxicity including ERK1/2 [110] and the Notch pathway[80, 81]. Notch intracellular domain (NICD) triggers S727 phosphorylation [111], and Hes-binding facilitates STAT3 by JAKS [112]. Interestingly, NICD increases in response to amounts as low as 2.5 nM MeHg in cultured E15 NPC [61]. If these pathways are not altered at nanomolar concentrations several other routes lead to STAT3 activity including EGF and PDGF [113] could be investigated. If ROS are involved, it would suggest they are able to change cellular signaling patterns and STAT3 activation, which will be tested with antioxidant treatments. One potential candidate that could mediate such an effect, SYK, is a cytoplasmic STAT3 tyrosine kinase that can be activated by ROS [101].

We anticipate that we will confirm HgCl2 blocks JAK/STAT signaling through generation of ROS and will be the first report of this effect in SH-SY5Y and NPC. Significant rises in NBT signal should be observed after 5.5h toxicant treatment with subcytotoxic doses in accordance with previous observations [74]. Since our preliminary observations suggest that subcytotoxic doses of MeHg have an opposite effect on STAT3 (Figure 1), it could be possible that ROS are elevated (yet uncoupled from STAT3 effects), unchanged, or paradoxically decreased. Additional preliminary experiments examining this in the SH-SY5Y line suggest that CMHDCFDA signal is not elevated at doses of MeHg which elevate CNTF evoked STAT3 Y705 phosphorylation (Figure 2), but this bears testing in the NPC cells, and confirmation with NBT and Western blot experiments examining H2O2 and antioxidant modulation of this effect.

Studies typically examine a given pathway because of its role in neurodevelopment and not an unbiased determination of its involvement in toxicity in vivo per se. We may determine that JAK/STAT is modulated by a series of events that are not specific to this signaling cascade, although these changes would nonetheless prove informative. Inhibition of proteasomal and transport systems is one example of a mechanism not addressed by these aims and if the mechanism is not determined by the present aim for such reasons, several other unbiased approaches are available.

Even in identical cell-lines, the amount of ROS resolved with fluorescein-analog probes depends the amount of probe, treatment times, cell-density, cell cycle, and culture media composition [92]. To explore this potential confound, a dose-response curve will be performed with NBT and H2O2. Successful use should allow us to consistently resolve significant increases following 0.5h 0.1-1 mM H2O2 [77]. If our efforts do not reach this benchmark, several alternative means of measuring ROS are available such as dihydrorhodamine in conjunction with flow cytometry.

AIM3: To elucidate whether MeHg promotes glial differentiation of neural precursors.

AIM3: Strategy. In order to provide a clearer description of MeHg’s effects on astrocyte differentiation and gliogenesis, we will determine if subcytotoxic concentrations of MeHg (see AIM1) affect CNTF-induced and BMP4-induced differentiation of NPC. We will superimpose MeHg treatments over this paradigm, which typically generates GFAP⁺ and S100β⁺ astrocytes from Nestin⁺ undifferentiated NPC, and determine the effect on commitment by obtaining ratios of cell expressing given fate-markers. This will be done using PND1 derived cortical NPC, which will be compared with NPC generated from early-neurogenic (E10) and late-
neurogenic/early gliogenic (E16) time-points in mouse development in order to determine any specificity with regards to developmental stage. Gene expression changes accompany differentiation and we will determine the effect of MeHg on patterns of mRNA expression to provide additional evidence of an effect of MeHg on gliogenesis.

**AIM3: Methodology.** Mouse PND1 NPC cells will be obtained, plated, grown, and serum-starved as previously described in AIM1. Cryopreserved NPC from E10 and E15 will also be obtained from UVM’s stem cell core facilities and will be grown and treated in an identical manner. 5 h pretreatment with MeHg will be followed by differentiation in the presence/absence of 20 ng/mL rat CNTF or 10 ng/mL rat BMP4 and/or MeHg for 12, 24, or 48 h. These ligands for inducing astrocyte differentiation will be studied because we hypothesize that this effect is specific to JAK/STAT signaling and that BMP4-mediated GFAP expression will not be changed by nanomolar concentrations of MeHg. **Immunocytochemistry and morphological analyses.** Nervous system cells possess lineage-specific morphologies that could hold clue to fate decisions marker expression could miss. We will measure total process number, mean process length, number of branch points of NPC after periods of differentiation with and without coexposure to MeHg or oxidants. Phase-contrast images will be taken and analyzed using digital-morphometric software. Additionally, fixed cells will be labeled for markers of cell type: astrocytes- GFAP and S100β, neurons- βIII-tubulin (Tuj1), uncommitted NPC- Nestin, and oligodendrocytes- Sox10 in parallel with PY705-STAT3, and DAPl to label nuclei. Results will be expressed as the % of nuclei labeling for each given marker, and the extent of co-labeling for PY705-STAT3 with nuclei as well as each of the fate markers (n=3 separate determinations) with comparisons done by one or two-way ANOVAs. **Marker gene expression, qRT-PCR.** Total cellular RNA will be extracted from each condition following 12, 24, or 48h treatment, and will be converted into cDNA and measured as outlined in AIM1. Genes analyzed will include markers increased in astrocyte-like cells (GFAP, S100β, GLAST), expressed in various stages of neuronal differentiation (βIII-Tubulin, MAP2), in oligodendrocytes (Sox10, PDGFRα) and in uncommitted NPC (Nestin, RC2). JAK/STAT targets SOCS3 and Bcl-2 will also be assayed to further indicate JAK/STATs involvement. Comparisons of 3 separate determinations will be done with one or two-way ANOVA.

**AIM3: Anticipated Results.** Treatments which cause ectopic activation of gp130/JAK/STAT3 have previously been shown to induce glial differentiation of NPC [83]. Thus, we predict that MeHg treatment of CNTF-treated NPC will lead to increased glial marker expression, possibly at the cost of fewer uncommitted or neuronal cells. By obtaining morphological data, we will be able to determine if MeHg effects an important feature astrocyte development not represented in immunocytochemistry cell counts. Furthermore, we anticipate that MeHg exposure during CNTF differentiation will enhance GFAP, SOCS1, and SOCS3 by qRT-PCR. We will consider a 20% change significant if validated by ANOVA w/ post hoc corrections. MeHg is not expected to alter BMP4-mediated astrocyte marker expression, although a positive result might suggest a broader effect on signaling through a common messenger and a more general inhibition of gliogenesis. We would then tailor our investigation into the mechanism of MeHg’s affects gliogenesis with this in mind. There are several difficulties commonly associated with maintaining NPC and producing replicable data. A problem common to use of NPC is variation; from animal to animal, clone to clone, or passage to passage (DIV), cell drift can occur and affect reproducibility. Expanding and freezing a large clonal population of cells prior to our experiments and utilizing low-passage number 3-10 cells will aid with this concern. If difficulties in obtaining reproducible data are encountered several commercially available glial-progenitor multipotent cell lines exist.

**AIM4: To determine if MeHg promotes astrocyte differentiation in vivo.**

**AIM4: Strategy.** To test our hypothesis that NPC exposed to MeHg during the peak of the gliogenic period excessively activate JAK/STAT and are more likely to generate astrocytes, we will assess the effect of low-dose MeHg given on PND2 along with BrdU injections to label dividing cells. To determine effects on JAK/STAT, animals will be sacrificed shortly afterwards and their brains sectioned colabeled with phospho-STAT and markers of progenitor subtypes to determine changes in STAT3 activity and what populations may be sensitive. To determine if astrogial fate is upregulated following any changes in STAT3 signaling, a separate cohort will be sacrificed 1 week later and their brains examined for astrocyte markers.

**AIM4: Methods.** PND2 C56/BL6 mice pups will be administered MeHg by gavage (vehicle or 10mg/kg, est. 700nM acute brain concentration) followed 2h later by i.p. injection of BrdU for cell lineage tracing as previous [57]. The Kakita studies employed twice this does and saw overt no physical or developmental defects, whereas cumulative doses over 30 mg/kg produce outward manifestations of toxicity such as ataxia, motor incoordination,
and memory problems [56]. Animals are returned to their home-cages and 12 hours (short-term) and 1 week (long-term) following this, animals (n=6/group, treatments being balanced across litters and genders) will be weighed, sacrificed and their brains processed for serial coronal sections at coordinates corresponding to the level of the primary motor and sensory cortices as previous[57, 58]. **Immunohistochemistry.** In the short-term groups, BrdU incorporation will be assessed alongside PY705-STAT3, polysialic acid neural cell adhesion molecule (PSA-NCAM), doublecortin (markers for immature neuronal progenitors), and GFAP distribution, the number of labeled/celabeled progenitors being counted in 5 sections for each data point. In the 1 week groups, distribution of neural (NeuN) and astrocyte markers GFAP, S100β, ALDH1L1[114], assessed alongside BrdU label in a similar manner.

**AIM4: Analysis.** We expect to see that a greater proportion of cells that were dividing at the time of injection will show activated STAT (after 12h) and later differentiate into astrocytes in the MeHg treatment groups (after 1 week). PND2 exposure has previously induced an altered distribution of GFAP and S100β immunoreactivity, with more labeled cells located near the sub ventricular zone at PND28. Dissociations between GFAP and S100β immunoreactivity in MeHg groups might be observed at 1 week and finding different distributions would suggest JAK/STAT regulation of individual genes but not overall patterns of differentiation. Observing the brains at one week will also permit immediate effects of MeHg to be observed before BrdU label is diluted in faster dividing cells.

We would expect to observe fewer proliferating cells, fewer neurons, and fewer oligodendrocytes in the 1-week exposed brains, as the NPC may ectopically differentiate into astrocytes if JAK/STAT signaling is enhanced during this time. Unfortunately, testing this hypothesis by determining all cell-types generated would require a large number of sections to label with different antibodies and would complicate analysis and interpretation in a study of this size. Instead we’ve chosen to focus on Results in vivo might greatly differ than results which would be predicted from cell culture experiments. If a reduction in GFAP is found, SOCS production, or compensatory long-term overexpression of negative regulators may be involved and we will pursue further investigation into this line of reasoning. Additionally, if additional financial support becomes available, a third cohort could be introduced to address what behavioral outcomes are evident at this dose.

**E. References**


84. NINDS, NINDS Alexander Disease Information Page. 2010.