Published in final edited form as:

J Toxicol Environ Health A. 2012 ; 75(0): 1091–1101. doi:10.1080/15287394.2012.697840.

GLIA AND METHYLMERCURY NEUROTOXICITY

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Abstract

Methylmercury (MeHg) is a global environmental pollutant with significant adverse effects on human health. As the major target of MeHg, the central nervous system (CNS) exhibits the most recognizable poisoning symptoms. The role of the two major nonneuronal cell types, astrocytes and microglia, in response to MeHg exposure was recently compared. These two cell types share several common features in MeHg toxicity, but interestingly, these cells types also exhibit distinct response kinetics, indicating a cell-specific role in mediating MeHg-induced neurotoxicity. The aim of this study was to review the most recent literature and summarize key features of glial responses to this organometal.

METHYLMERCURY NEUROTOXICITY

Methylmercury (MeHg) is one of the organic forms of mercury (Hg). Over the last century, large outbreaks of MeHg poisoning were reported in Japan (Igata 1993; Tsubaki et al. 1967), Sweden, Pakistan, Guatemala and Ghana (Westoo 1966). In the United States, Hg contamination is widespread. According to the U.S. Environmental Protection Agency (EPA), Hg contaminates 3781 bodies of water across the country, corresponding to 6,363,707 acres of lakes, reservoirs, and ponds (U.S. EPA 2010). More U.S. waterways are closed for fishing because of Hg contamination than any other toxic contaminant (U.S. EPA 2010). Within waterways living organisms rapidly take up MeHg and its concentration is biomagnified through the food chain, reaching at times concentrations 10,000- to 100,000-fold greater in fish than in surrounding waters. As a result, MeHg accumulates at high

concentrations especially in fish-eating populations (Clarkson 1997; Kamps et al. 1972; Spry and Wiener 1991).

The distribution of MeHg from the gastrointestinal tract (GIT) to the bloodstream is completed within approximately 30 h (Kershaw et al. 1980). In the blood, erythrocytes are the major carriers of MeHg, which tightly binds thiol groups (-SH) on cysteinyl residues of the hemoglobin beta-chain (Doi 1991). MeHg is slowly redistributed to virtually all human organs and readily gains entrance into the central nervous system (CNS) (Kershaw et al. 1980). MeHg possesses high affinity for -SH groups in cysteine (Bridges and Zalups 2004; 2012), and as a result of MeHg complexation with cysteine residues, a chemical complex (MeHg-S-Cys) structurally similar to methionine is formed. The complex competes with L-methionine for the L-type large neutral amino acid transporter system (LAT1) on endothelial cells of the blood-brain barrier (BBB) (Yin et al. 2008). Once in the brain, however, MeHg is not evenly distributed, and is preferentially deposited in certain cell types. The largest Hg concentration is found in glial cells, including astrocytes and microglia (Charleston et al. 1994). Neurons have significantly lower levels of intracellular MeHg, and other cell types such as endothelial cells, pericytes, and oligodendrocytes, are rarely observed to have MeHg deposits (Charleston et al. 1994).

Within the brain, MeHg produces injury that is pathologically characterized by atrophy of cerebral cortex and white matter as well as cerebellum (Eto et al. 1992; Howard and Mottet 1986). In the rat cerebellum continuous MeHg exposure via drinking water leads to reduction of total cerebellar cell population. A reduced number of cells in the MeHg-exposed cerebellum was associated with an increased number of mitotic figures in the early stages of mitosis and a decrease in the number in middle and late stages. These studies are consistent with MeHg-induced mitotic arrest in the G2 and early M phases. Clinical symptoms of MeHg intoxication include dyskinesia, primitive reflexes, coordination disturbance, dysarthria, choreoathetosis, and hypersalivation (Harada 1995). Clinically, sensory evoked potentials (EP) and heart-rate variability (HRV) are useful and objective methods for assessing MeHg neurotoxicity (Murata et al. 2007). Children in Ecuador, particularly the Saraguro “Amer-Indians,” affected by Hg exposure as a by-product of the gold-mining process were also found to be at increased risk for neurological impairment (Counter et al. 2002), showing subtle anomalies in brainstem responses (Counter 2003) and acoustic muscle reflexes (Counter et al. 2012). Notably, compared to adult brain the developing brain is more vulnerable to MeHg toxicity, showing diffuse damage. Mental retardation and intellectual disturbances are commonly noted in infants and children exposed to MeHg in utero or postnatally (Harada 1964).

Neurons, as the major target of MeHg toxicity in the CNS, have been extensively investigated. Direct neuronal injury was visualized by electron microscopy of human autopsy specimens, demonstrating ribosome aggregation, loss of rough endoplasmic reticulum, and shrunken neurons (Eto et al. 1992). MeHg exerts antimitotic effects on neurons, inhibiting the polymerization of tubulin (Sager et al. 1982), as well as promoting microtubular fragmentation formation (Choi et al. 1980). MeHg (1) inhibits neuronal DNA and RNA synthesis and repair by selectively binding to the “zinc finger” core of DNA repair enzymes (Asmuss et al. 2000) and (2) disrupts intracellular calcium homeostasis by

increasing sodium and decreasing potassium concentrations in the synaptic clefts (Komulainen and Bondy 1987; Oyama et al. 1994). As a result of altered intracellular calcium levels, the release of neurotransmitters, including dopamine, glutamate, γ -aminobutyric acid (GABA), and acetylcholine is altered (Juang 1976). MeHg is also known to induce oxidative stress in neurons (Ali et al. 1992; LeBel et al. 1992; Yee and Choi 1994), which may be partially ameliorated by antioxidants, such as vitamins (A, E and C) (Li et al. 2011), tocopherols, tocotrienols (Shichiri et al. 2007), pyrroloquinoline quinone (PQQ) (Zhang et al. 2009), and ebselen (Yin et al. 2011).

GLIAL CELLS

Glia, including astrocytes and microglia are important cellular components of the CNS. Although they are not electrically excitable, glial cells possess diverse and important functions, such as providing nutrition and physical support to neurons (Hamilton et al. 2007). Glial cells phagocytize cellular debris and mediate CNS immune responses (Beyer et al. 2000; Huizinga et al. 2012). Astrocytes and microglia share several common features, but are fundamentally different in terms of cellular function and their roles in MeHg-induced toxicity (see later discussion).

Astrocytes are characterized by their star shape and expression of glial fibrillary acidic protein (GFAP) (Ni et al. 2011). Astrocytes carry out many critical functions in the normal brain, including formation of BBB (Walz 1989) and expression of transporters for neurotransmitters, such as glutamate and γ -aminobutyric acid (GABA) (Santello and Volterra 2009). Blockage of the astrocytic glutamate uptake via the specific astrocytic glutamate transporter-1 (GLT-1) inhibitor dihydrokainic acid (DHK) was shown to impair spatial memory (Bechtholt-Gompf et al. 2010). There are also two astrocytic GABA transporters, GAT1 and GAT3. Each has different regulatory functions of GABA-mediated inhibitory postsynaptic currents (IPSC). GAT1 regulates GABA near synapses and selectively modulates peak IPSC amplitude, while GAT3 expression is broader and includes distal extrasynaptic regions. Astrocytes also participate in the formation of the brain's microcirculation via neuron-to-astrocyte signaling (Zonta et al. 2003). Pathologically, astrocytes fill in the space upon CNS injury and form the glial scar (Frontczak-Baniewicz et al. 2011).

Microglia are sensitive to pathological changes (Dissing-Olesen et al. 2007). Microglia are the resident macrophages of the CNS, thus contributing to innate immune defenses. Microglia secrete interleukin-4 (IL-4), which triggers neuronal repair and promotes regeneration. Microglial IL-4 also induces neurogenesis and oligodendrogenesis in vitro (Napoli and Neumann 2009). Microglia are distributed throughout the brain and survey the parenchyma for any foreign material, damaged or apoptotic cells, DNA fragments, and plaques (Aloisi 2001). They fulfill their cytotoxic role by releasing various cytotoxic substances, including superoxide anion (O_2^-), hydrogen peroxide (Zhang et al. 2007), and nitric oxide (NO) (Ryu et al. 2000). Microglia are also critical components of the adaptive immune system; they express major histocompatibility complex II (MHC-II) and function as the brain's antigen-presenting cells (APC) (Roth et al. 2012). Furthermore, microglia modulate complex cell-cell networks by secreting interferon- γ (INF- γ) into the extracellular

space (Wang and Suzuki 2007). Microglia also release additional cytokines, such as tumor necrosis factor- α (TNF- α) (Benveniste 1997) and interleukin-8 (IL-8), promoting B cells differentiation and antibody formation (D'Aversa et al. 2008). More recently, microglia and astrocytes were cultured from the brains of neonatal BALB/c mice and stimulated with PAM(3)CSK(4) (PAM(3)), a toll-like receptor (TLR) ligand, after MeHg exposure (Bassett et al. 2012). MeHg led to a concentration-dependent reduction in IL-6 secretion but failed to alter tumor necrosis factor (TNF)- α and IL-1 β . These findings are consistent with the inability of PAM(3) to induce the secretion of IL-1 β from glial cells. Consistent with our studies, these studies also corroborate that microglia are a target of MeHg and that the organometal might modify the response of glial cells and their interactions with astrocytes, characterized by observations that the ratio of microglia/astrocyte exerted an effect on the secretion of IL-6 but not TNF- α (Bassett et al. 2012; Chang 2007).

Pathologically, microglia are also involved in neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and HIV-associated dementia. Uncontrolled microglial activation leads to neuronal damage via *N*-methyl-D-aspartate (NMDA) receptor-mediated processes. Microglial glutamate is produced via glutaminase, which may be inhibited by 6-diazo-5-oxo-L-norleucine (DON). Inhibition of this enzyme resulted in decreased microglial-induced neuronal death (Takeuchi et al. 2008). In Alzheimer's disease microglia exhibit dual functions: (1) elimination of β -amyloid aggregates via phagocytosis and (2) killing of nearby neurons by producing inflammation and release of neurotoxic proteases (Eikelenboom and Veerhuis 1996; El Khoury et al. 1998; Streit 2004). Other evidence of the importance of microglia in mediating neurodegenerative diseases was shown in human immunodeficiency virus type 1 (HIV) dementia. The virus does not directly infect neurons, but produces damage indirectly through the activation of microglia (Gupta et al. 2010). The infected microglia release neurotoxic mediators, including both cellular activation products and viral proteins, which possess neurotoxic properties (Yadav and Collman 2009).

MeHg TOXICITY IN GLIA

Astrocytes and microglia share some common features in MeHg-induced toxicity, but their responses have distinct dynamic profiles. These common features include the following key points:

1. MeHg is deposited in both cell types. Previous studies addressed intracellular Hg levels in astrocytes and microglia with ^{14}C -MeHg (Ni et al. 2011). Direct evidence for MeHg uptake in microglial cells is still lacking (but likely reflects analogous functional transporters to those described in other cells, such as astrocytes). Yin et al. (2008) indicated the presence of the neutral amino acid transporter (LAT1) system on astrocytic membranes. This transporter is capable of selectively mediating cysteine-MeHg uptake, mimicking the structure of L-methionine, an endogenous substrate for this transporter. The substrate specificity and high affinity of this transport system resemble the properties of the L-type large neutral amino acid transporter system (LAT) on the BBB (Aschner et al. 1990).

2. MeHg produces concentration-dependent oxidative stress in both cell types, which leads to cell death. Ni et al. (2011) showed that MeHg produced increased intracellular reactive oxygen species (ROS) generation in both cell types, as detected in a lipophilic dichlorodihydrofluorescein diacetate acetylestear (H_2DCFDA) based assay. The intracellular ROS are detoxified by the reduced form of glutathione (GSH) to the oxidative form of GSH, GSSG. Consistent with the changes in ROS levels, MeHg also produces concentration-dependent decrease in the GSH/GSSG ratio (Ni et al. 2011). In addition to the direct consumption of intracellular GSH via ROS generation (Figure 1), MeHg also inhibits the biosynthesis of GSH by inhibiting the uptake of cysteine, a crucial precursor for GSH, which further contributes to lower GSH levels (Shanker et al. 2002), thus setting forth a vicious toxic cycle.
3. Another common feature shared by both cell types is the upregulation of NF-E2-related factor 2 (Nrf-2) protein levels in response to MeHg treatment (Ni et al. 2011) (Figure 1). As a key protective protein against oxidative stress, Nrf-2 increases in both cell types after MeHg exposure (Ni et al. 2010; Wang et al. 2009). In the absence of oxidative stress, Nrf2 is bound to Kelch-like ECH-associating protein 1 (Keap1) protein in the cytoplasm and the complex is degraded by proteasomes (Kensler and Wakabayashi 2010). However, the bound form rapidly dissociates upon oxidative stress and the oxidation of cysteine groups on Keap1, forming free Nrf2 (Figure 1). The latter is resistant to proteasomal degradation (Chen et al. 2009), and de novo Nrf2 accumulates and undergoes nuclear translocation in both cell types (Li and Kong 2009). In the nuclei, Nrf2 functions as a transcription factor that increases downstream genes expression. Upregulated genes include heme oxygenase 1 (*Ho1*), NAD(P)H dehydrogenase, quinone 1 (*Nqo1*), and x- C-type transporter (*xCT*), whose protein products are used to detoxify xenobiotics and endogenous reactive electrophiles (Itoh et al. 1999; Prester et al. 1995; Prester et al. 1993) (Figure 1).

Despite the common features shared by astrocytes and microglia, the two cell types exhibit distinct sensitivity to MeHg, which leads to differential temporal adaptive responses. Recent results from our laboratory suggest that microglia are the early responders while astrocytes are late responders to MeHg exposure (Ni et al. 2011). Key differences in the cell-specific responses are summarized next.

1. Microglia are more sensitive to endogenous as well as exogenous stimuli, and therefore are the first line of cellular defense against MeHg toxicity. Several studies established in vivo microglial activated response to MeHg exposure (Charleston et al. 1994; Sakamoto et al. 2008). Long et al. (2007) demonstrated that microglia mount immediate oxidative stress upon TiO_2 exposure. Similarly, MeHg produced a significant increase in ROS generation in microglia commencing at one min post exposure (Ni et al. 2011). It is noteworthy that at this time point elevated ROS generation is absent in astrocytes (Wang et al. 2009; Yin et al. 2007); ROS generation is not detected until 1 h post MeHg treatment.

As stated earlier, GSH detoxifies ROS and in the process is converted to the oxidized form of GSH, GSSG. As a result of the rapid and almost immediate ROS elevation in microglia (Figure 1), GSH levels in these cells decrease as early as one min post MeHg exposure, well before discernable changes are noted in astrocytes (Ni et al. 2011). In addition to the faster fall in GSH levels, microglia also inherently possess lower baseline intracellular GSH levels compared to astrocytes (Ni et al. 2011). GSH plays a key role in offsetting MeHg-induced toxicity (Mullaney et al. 1993; 1994), forming a MeHg-SG complex, which is readily pumped out of cells by the multidrug resistance proteins (MRP). As a result, GSH decreases intracellular Hg concentrations and limits its toxicity (Konig et al. 1999). Microglial GSH levels are approximately 25% of those previously reported in astrocytes (Ni et al. 2011), reflecting the diminished ability to buffer MeHg-induced ROS generation. In addition, lower microglial GSH levels likely produce reduced MeHg efflux and elevated intra-cellular Hg levels (Ni et al. 2011). This may explain previous findings establishing the earliest and highest accumulation of Hg deposits in rat (Garman et al. 1975) and nonhuman (Charleston et al. 1995) microglia. In agreement with these findings, Miura and Clarkson (1993) reported a significant inverse correlation between GSH levels and MeHg toxicity in an MeHg-resistant rat pheochromocytoma PC12 cell line. The levels of GSH in the resistant cells were fourfold higher compared to nonresistant cells, likely leading to greater efflux and attenuated intracellular retention of MeHg (Miura and Clarkson 1993; Miura et al. 1994).

2. As the critical regulator of the antioxidant machinery, upregulation of Nrf2 and its downstream antioxidant genes, including *Ho1*, *Nqo1*, and *xCT*, parallels the MeHg-induced rise in ROS and decreased GSH levels (Ni et al. 2011). Nrf2 protein in microglia increases within 1 min of MeHg treatment and its subsequent nuclear translocation commences shortly thereafter (10 min post treatment) (Ni et al. 2011), closely approximating the changes in intra-cellular GSH levels and enhanced ROS generation (Figure 1). In comparison, increased Nrf2 and its nuclear translocation were not observed in astrocytes until 90 min post MeHg treatment (Wang et al. 2009).

The role of Nrf2 was further tested with respect to affording protection to both microglia and astrocytes by knockdown Nrf2 experiments (with shRNA). Notably, in both cell types MeHg increased frequency of cell death in the absence of optimal Nrf2 response (Ni et al. 2011). Prosurvival features of Nrf2 were also observed in other cell types. Toyama et al. (2007) reported that primary mouse hepatocytes isolated from *Nrf2*-deficient mice are highly susceptible to MeHg-induced cytotoxicity, and *Nrf2* overexpression attenuates MeHg-induced cytotoxicity in SH-SY5Y neuroblastoma cells. In human lymphoma cells, gallium nitrate upregulated *Ho-1* mRNA levels as a result of Nrf2 activation (Wang et al. 2009; Yang and Chitambar 2008). Similarly, Nrf2 in renal epithelial cells upregulated the *Nqo1* reporter construct after exposure to hypoxia/reoxygenation (Leonard et al. 2006). In addition to the upregulation of *Ho1*, *Nqo1*, and *xCT*, the *Nrf2*-afforded protection against MeHg is likely related to upregulation of *GCLC* gene, which encodes

glutamate cysteine ligase, an important enzyme catalyzing the first and rate-limiting step of GSH biosynthesis (Figure 1). This hypothesis is based upon the fact that *GCLC* is a downstream gene that is regulated by *Nrf2*, and GSH plays a key role in MeHg detoxification.

As the components of complex cell–cell networks, astrocytes and microglia affect additional cell types post MeHg exposure. In the case of astrocytes these cells provide cysteine, the GSH precursor, to neurons. Therefore, the MeHg-induced decrease in astrocytic cysteine uptake contributes to neuronal oxidative damage and reduced ability to maintain optimal redox status (Allen et al. 2002). MeHg also inhibits glutamate uptake in astrocytes and stimulates its efflux, resulting in excessive glutamate concentrations in the synapse and, consequently, neuronal excitotoxicity (Yin et al. 2007; Aschner et al. 1993). Moreover, MeHg increases intracellular sodium (Vitarella et al. 1996), aspartate uptake (Yao et al. 1999; 2000), and excessive production of excitatory neurotransmitters, which eventually may affect astrocyte–neuron interactions (Schousboe et al. 1992).

As the first cell type to respond to MeHg, the microglial response to this metal may influence other cell types via secretion of interleukins, prostaglandins, and other cytokines. Chang (2007) demonstrated that MeHg exposure leads to IL-6 release, which exerts various effects on different cell types. Eskes et al. (2002) found that microglial IL-6 induced astrogliosis, leading to a glial scar. Microglial IL-6 may also exert a neuroprotective function by preventing MeHg-induced degeneration of the neuronal cytoskeleton (Eskes et al. 2002).

In summary, MeHg is toxic to both astrocytes and microglia, producing increased intracellular ROS generation and decreased GSH levels. Both glial cell types utilize similar cellular antioxidant machineries to counteract MeHg-induced toxicity by uncoupling of Nrf2, its nuclear translocation, and the upregulation of *Ho1*, *Nqo1*, and *xCT*. However, these two cell types demonstrate different response kinetics to MeHg exposure, with astrocytes responding on a protracted time axis, while microglia appear to respond almost instantaneously (Figure 1). Future studies on the biological significance of the kinetic differences are clearly warranted, as these may facilitate the development of potential therapeutic modalities to ameliorate MeHg-induced CNS damage.

Acknowledgments

We are grateful for support by NIEHS R01ES07331 and the Center in Molecular Toxicology NIH grant P30ES00267.

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FIGURE 1.

Differences between microglial and astrocytic responsiveness to MeHg. When microglia or astrocytes are exposed to hypothetically similar amounts of MeHg, the formation of the excretable GS–MeHg complex is higher in astrocytes due to their higher amounts of GSH. In astrocytes, lesser amounts of MeHg are prompt to interact with other sulfhydryl-containing biomolecules, such as Keap1–Nrf2 complex. Conversely, in microglial cells, which present lower GSH levels when compared to astrocytes, higher amounts of MeHg are prompt to activate Keap1–Nrf2 complex, producing Nrf2 translocation into the nucleus and increasing the expression of phase 2 (*Ho1*, *Nqo1*, *xCT*) enzymes. In both cell types, Nrf2 activation seems to present protective effects against MeHg-induced toxicity. Greater letter size represents a greater abundance of respective molecules in astrocytes versus microglia. GSH, reduced glutathione; +HgCH₃, methylmercury; GSHgCH₃ = methylmercury–glutathione complex; MRP = multidrug resistance proteins (color figure available online).