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Methylmercury: Recent Advances in the Understanding of its Neurotoxicity

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Framing the Problem

Methylmercury (MeHg) is a major environmental neurotoxicant. Though recognized for its toxicity for generations, it continues to pose appreciable risk to human health even contemporarily. Most recent large scale epidemics include the MeHg poisoning in Japan and Iraq (Takeuchi et al., 1972; Bakir et al., 1973). Issues related to exposure to MeHg via food consumption in fish eating populations are at the forefront of health risk assessment. Anthropogenic sources resulting in the acidification of freshwater streams and lakes, and the impoundment of water for large hydroelectric schemes continue to lead to higher MeHg concentrations in fish. Given that fish adulterated with MeHg represent a common source for human MeHg consumption, it is not surprising that public health agencies have continuously directed attention and resources to health policies focused upon the question as to whether MeHg intake from a diet high in fish is associated with alterations of brain function. It is noteworthy that all sources of environmental mercury represent a potential risk for human poisoning given the methylation of inorganic mercuric in aquatic bodies by methanogenic bacteria to MeHg. Subsequently, the MeHg bio-concentrates and accumulates in aquatic food chain. The upper hierarchy of the aquatic biota, namely predator fish, such as shark, swordfish, tuna and alike, tend to accumulate the highest MeHg concentrations per gram body weight. MeHg-adulterated fish represent the most prevalent source for human exposure to this MeHg.

It is well established that excessive MeHg ingestion from a diet high in certain fish species is associated with aberrant central nervous system (CNS) functions (Kjelstrom et al., 1989; Grandjean et al., 1997, 1999). The developing nervous system is the most vulnerable and sensitive to MeHg toxicity. Recent studies in human populations support the earlier findings that the maternal mercury exposure during pregnancy is associated with neurological as well as neuropsychological deficits detectable at 6 to 7 years of age (Steuerwald et al., 2000; Cordier et al., 2002; Grandjean et al., 2003). Another recent study has pointed to MeHg's selective detrimental effects on neurogenesis (Faustmann et al., 2002). However, despite

these observations, the ability of MeHg derived from food consumption to affect cognitive and behavioral functions children remains controversial as exemplified by other studies (Myers et al., 1997; Davidson et al., 1998; Huang et al., 2003; 2005) in which no association was noted between MeHg and neurodevelopmental outcomes in children at 66 months of age. Thus we still do not know the effect of MeHg in the human population and clearly there remains a need to understand the mechanisms and consequences of MeHg exposure on central nervous system (CNS) function. The current literature suggests that no single mechanism can explain the multitude of effects observed in MeHg-induced neurotoxicity. This review will specifically focus on the aberrant effects of MeHg on the CNS, and it will emphasize the need for distinction between mechanisms of neurotoxicity associated with developmental vs. adult MeHg poisoning. In addition, attention will be directed at potential mechanisms associated with MeHg neurotoxicity.

Mercury and Thiols

Thiol groups play fundamental structural and functional roles in protein chemistry, being located mainly within the active catalytic sites of many enzymes. Mercury compounds react specifically with active sulfhydryl (-SH) groups forming complexes of defined stoichiometry, -S-Hg-R. The high affinity of MeHg for the anionic form of -SH groups is responsible for the toxicological behavior of this compound (Hughes, 1957). The affinity of MeHg for the anionic form of -SH groups ($\log K$, where K is the association constant) is extremely high, on the order of 15–23, whereas its affinity constants for oxygen-, chloride-, or nitrogen-containing ligands such as carboxyl or amino groups are about 10 orders of magnitude lower (Carty and Malone, 1979). Indeed, wherever a MeHg compound has been identified in biological media, it has been complexed to -SH-containing ligands. However, MeHg rapidly redistributes when novel sulfhydryl groups become available indicating that although the equilibrium chemistry favors the Hg-S-complex, the complex can be easily dissociated (Hughes, 1957; Carty and Malone, 1979).

Complexes with cysteine and GSH have been identified in blood (Naganuma and Imura, 1979; Rabenstein and Fairhurst, 1975), and complexes with GSH have been identified in brain (Thomas and Smith, 1979), liver (Omata et al., 1978), and bile (Refsvik and Norseth, 1975).

The Differential Toxicity of MeHg in the Young vs. Adult

Though the brain is not the only target for MeHg poisoning, and cardiovascular disease has been associated with MeHg exposure (reviewed by Clarkson et al., 2003), emphasis in this chapter will solely be directed at CNS effects associated with exposure to this metal.

The developmental sequelae of human MeHg exposure are relatively well characterized (Myers et al., 1998). At the morphological level, the neuropathological consequences of MeHg exposure have also been quite well delineated due to the unfortunate exposures to MeHg in the two best known epidemics in Japan and Iraq (Harada 1995; Inskip and Piotrowski, 1985; Bakir et al., 1973). In both epidemics a range of developmental effects have been noted, both in children exposed in utero and at young age.

As mentioned above, a clear distinction needs to be made when characterizing the consequences of MeHg in the developing vs. adult brain. There are considerable differences in the distribution of pathological changes in the young brain compared to adults (Takeuchi et al, 1979). In the adult brain damage associated with MeHg exposure is confined to specific areas, such as the granule layer of cerebellum and the visual cortex of cerebrum. This selectivity has been puzzling given the ubiquitous distribution of MeHg in the brain, and it has yet to be rationally explained.

In utero exposure to MeHg, in the neonatal period or at early age leads to damage throughout the CNS. The damage is pervasive, and commonly, the earlier the exposure, the more generalized damage (Choi, 1989). Such age-related differences are seen also in other mammals although the detailed areas damaged may differ. The nature and pervasiveness of the damage in the young vs. the mature brain has been accounted for by the presence of an immature blood-brain barrier in the young, and by inference a more leaky, and generalized distribution of mercury. However, as noted above, it seems that after adult exposure MeHg is also found throughout the brain and localization per se does not correlate with the pathological changes, and is clearly not a determining factor in the toxic outcome. It also needs to be considered that a more generalized damage in the young compared to older brain may be a sequelae of differential expression of toxicity but this is well has yet to be experimentally proven.

To examine the underlying bases of the differential expression of MeHg toxicity in the young vs. mature brain additional information on potential molecular targets for MeHg will be required. MeHg readily binds to sulfhydryl groups and the free concentration of the metal in a biological systems is extremely low (Carty and Malone, 1979). Noting the importance of sulfhydryl groups to the proper function of proteins, e.g. enzymes, one would predict that MeHg might cause a range of effects at many locations throughout the body. However, reflecting upon its ubiquitous distribution. Yet, as mentioned above, studies in adult animals demonstrate not only specificity for damage that may not be equated with distribution, but also establishes that the primary target of MeHg's is the CNS, despite organ concentration of MeHg that are considerably higher in liver and kidney. Thus, most cells have a capacity for resisting or repairing the damage inflicted by MeHg. If so, the targets of MeHg would be cells that cannot meet the metabolic challenge of damaged proteins and/or does not have the ability to sequester MeHg through e.g. the synthesis of metallothionein.

In the adult brain it has been shown that MeHg accumulates in astrocytes (Figure 1) and interfere with the glutamate uptake resulting in high extracellular glutamate which neurons may not tolerate. Such mechanisms may also be in effect in the developing brain, but there are a number of other processes which are unique to the developing brain. Some examples with possible relevance to MeHg would be how neurons move from site of germination to the final site of function, the formation of interneuronal contact and the apoptotic death of neurons. From animal studies we have seen that immature neurons, e.g. cerebellar granule and Purkinje cells, are arrested before or during their transport to their permanent site of function (Kumimoto and Suzuki, 1997). Cell movement is a highly complex process involving major cytoskeletal changes in the neurons as well as the proper function of guiding cells. The interaction of MeHg with the microtubular element of the cytoskeleton has been

extensively studied (Miura et al, 1999). The observed interference of MeHg with neuronal movements may very well be a result of MeHg hindering the repolymerization of microtubules. The formation of dendrites and axons depend on the proper function of a growth cone. Studies using primary neurons in culture have shown that one of the earliest effects of MeHg in such cultures is the retraction of growth cones and extensions (Miura et al, 2000). Apoptosis is a necessary process during the development of the brain. MeHg will also induce apoptosis and could thus interfere with cells that are “primed” for apoptosis so that the process occurs at an inappropriate time (Nagashima et al, 1996; Nagashima, 1997). There certainly are several mechanisms by which MeHg may cause developmental damage to the central nervous system making the fetus and the young brain far more vulnerable than the adult brain.

With the devastating and irreversible effects of MeHg on the developing brain it becomes important to establish a safe dose of exposure. Animal experiments may provide us with guidance in this question, but it does require epidemiological assessment of exposed populations. For low dose exposure two such study areas are of prime importance here; in Faeroe Islands (Steuerwald et al, 2000) and on the Seychelles (Axtell et al, 2000; Huang et al., 2003; 2005). On both these locations there are no industrial sources. The MeHg would come entirely from diet and mainly from pelagic marine animals. In Faeroe Islands a correlation was found between levels found in the mother at time of birth (measured in cord blood) and neurological deficits in the off-spring. However, such a relation was not found in the Seychelles study. There might be many technical reasons for arriving at opposing results (methods, testing, age profiles etc). However, we do know that whale meat and blubber is part of the Faeroe Island diet and that such food from the North Atlantic will contain e.g. PCB. This is not the case in the Seychelles. PCB-exposure might be an important confounder because it may interfere with estrogen-receptors and thus may affect the development of spines on neuronal dendrites during development. Thus, PCB and MeHg may have independently caused neurological effects in the Faeroe population, as has indeed been suggested by (Rice, 2000). There may be several other reasons for the different results in the two studies: (1) the measurement instruments differ in the 2 studies. Instruments that were used in the Faeroe Islands (where an effect on child development was found in the cohort) were not used in the Seychelles Islands. (2) The oldest children in the Seychelles study were 5 years old at the time of testing, while children studied in the Faeroes Islands were 7 years of age. (3) The cognitive tests used in the Faeroe Islands are better suited to older children and it is easier to do neurocognitive testing in school-aged children. (4) The study in the Faeroe Islands utilized cord blood Hg levels as the correlative exposure indicator. This approach may miss high first trimester fetal exposure to Hg.

In the Iraq study hair samples were collected. By measuring MeHg in longitudinally sections of hair the maximum blood concentration can be estimated. From these studies it was concluded that the threshold level for prenatal exposure should be 10 ppm in maternal hair. The threshold value for adults was suggested to be 50 ppm indicating the relative susceptibility of the developing brain. Although the studies of fish-eating populations in coastal areas does not entirely convert on a single value – it seems that taking them as a whole the proposal from the Iraq-studies is reasonable and still a valid threshold value (Myers et al, 2003).

MeHg and Aberrant Glutamatergic Innervations: A Possible underlying Mechanism of Neurotoxicity

There is abundant evidence in support of the pivotal role of astrocytes in mediating neurotoxicity (Figure 1). There are clearly distinct and direct neuronal effects of MeHg, which are beyond the scope of this proposal. However, the neuronal effects are unlikely to solely account for the neurotoxic consequences of MeHg neurotoxicity, given the pattern of its accumulation, and especially under conditions of chronic exposure, where the majority of MeHg accumulates in astrocytes and microglia (see below). Numerous studies have established astrocytes as a unique and relevant experimental model for the assessment of mechanisms underlying MeHg-induced cytotoxicity.

- a. Chronic exposure to MeHg in primates is associated with preferential accumulation of MeHg in astrocytes (and to some extent in microglia) (Charleston et al., 1994; Charleston et al., 1996; Garman et al., 1975).
- b. MeHg inhibits astrocytic glutamate uptake and stimulates its efflux (Aschner et al., 1993; Aschner, 1996; Aschner et al., 2000; Allen et al., 2001b; Brookes and Kristt, 1989), increasing in vivo glutamate concentrations in the extracellular fluid (ECF) and sensitizing neurons to excitotoxic injury (Juarez et al., 2002). Damage associated with MeHg correlates with brain areas with dense glutamatergic innervations (Miyamoto et al., 2001). The ionotropic glutamate receptor N-methyl-D-aspartate (NMDA) antagonist, dizocilpine (MK801) protects against neuronal damage induced by in vivo MeHg (Miyamoto et al., 1999; Miyamoto et al., 2001; Lafon-Cazal, et al., 1993).
- c. MeHg selectively inhibits astrocytic (but not neuronal) uptake systems for cystine and cysteine transport (Allen et al., 2001a; Shanker et al., 2001a; 2001b), compromising glutathione (GSH) synthesis and the CNS redox potential (Aschner et al., 2002). Compared with astrocytes, neurons have lower levels of GSH as well as of a second putative antioxidant, metallothionein (MT) (Philbert et al., 1991; Maret, 1994) making them more susceptible to the effects of increased ROS. Since GSH synthesis is dependent upon precursors derived from astrocytes (Dringen et al., 1999; Wang and Cynader, 2000), MeHg-induced inhibition of cystine transport and astrocytic GSH production would ultimately lead to decreased neuronal GSH levels and increased glutamate toxicity.
- d. MeHg-induced ROS formation in astrocytes can be attenuated by antioxidants (Shanker et al., 2002), reversing its functional effects on glutamate uptake inhibition (Allen et al., 2001b).
- e. Co-application of non-toxic concentrations of mercury with glutamate results in the appearance of typical neuronal lesions associated with excitotoxic stimulation (Matyja and Albrecht, 1993).
- f. In human and non-human primates chronic in vivo exposure to MeHg is associated with swelling of astrocytes (Oyake et al., 1966; Charleston et al.,

1994; Charleston et al., 1996; Vahter et al., 1994), a process associated with release of glutamate into the extracellular fluid (Aschner et al., 1990).

- g. In the absence of glutamate, neurons are unaffected by exposure to mercury, suggesting that neuronal dysfunction is secondary to disturbances in astrocytes (Brookes, 1992).
- h. Additional data invoke astrocytic cPLA₂ as a target for MeHg toxicity, supporting the notion that cPLA₂-stimulated hydrolysis and release of arachidonic acid (AA) play a role in MeHg-induced neurotoxicity (Shanker et al., 2002).

Reactive Oxygen Species and MeHg Neurotoxicity

Oxidative stress has been implicated in a variety of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS) (Kaltschmidt et al., 1993; Hunot et al., 1997; Kitamura et al., 1997; Lukiw and Bazan, 2000). It also plays an important role in other degenerative conditions such as autoimmune and inflammatory diseases (i.e. ischemia and rheumatoid arthritis), cancer, diabetes mellitus and atherosclerosis (Kehrer and Smith, 1994; Halliwell, 1994; Betteridge, 2000), as well as in metal induced toxicity (LeBel et al., 1992; Bush, 2000). The balance between oxidative and reductive cellular processes is known to be adversely affected in these various disorders (Betteridge, 2000; Sayre et al., 1999). Oxidative stress is associated with the accumulation of high levels of toxic reactive species, such as, reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive nitrogen oxygen species (RNOS), as well as unbound metal ions (Betteridge, 2000; Bush, 2000; Davis et al., 2001). Typical ROS include oxygen radicals such as superoxide radical (O₂^{•-}), hydroxyl radical (*OH), as well as non radical derivatives of oxygen including hydrogen peroxide, H₂O₂). Examples of RNS include nitric oxide (NO) radical, and that of RNOS include a highly reactive oxidant species peroxynitrite (ONOO⁻), which is a product of reaction between NO and the superoxide radical. These reactive species are highly oxidizing and potentially damaging to cellular redox-sensitive proteins, enzymes and DNA, and they also cause peroxidation of membranes.

ROS are known to mediate MeHg-induced neurotoxicity in multiple experimental models. For example, MeHg induces ROS formation in vivo (rodent cerebellum), and in vitro (isolated rat brain synaptosomes) (Ali et al., 1992), as well as in cerebellar neuronal cultures, a hypothalamic neuronal cell line and in mixed reaggregating cell cultures (Sarafian, 1994; Park et al., 1996; Sorg et al., 1998; Sarafian et al., 1999; Gasso et al., 2001). In addition, an increase in ROS has been observed in (a) mitochondria isolated from MeHg-injected rat brains; (b) mitochondria isolated in vitro from rat brain and then exposed to MeHg (Myhre and Fonnum, 2001); (c) mitochondria from Hg and glutamate exposed astrocytes and neurons (Dugan et al., 1995; Brawer et al., 1998). This ROS formation resulting from MeHg and glutamate induced oxidative stress contributes to mitochondrial dysfunction. Overproduction of ROS is mediated, at least in part, by glutamate, since this toxicity can be attenuated by N-methyl D-aspartate (NMDA) receptor antagonists (Park et al., 1996). The source of glutamate is likely to be astrocytic given the effect of MeHg on glutamate uptake and release from astrocytes (Aschner et al., 1994; Allen et al., 2001a; 2001b). Therefore,

although, the toxic damage of MeHg might be most prevalent in neurons, a large body of literature suggests that neuronal damage in response to MeHg, most likely represents aberrant control by astrocytes of the extracellular milieu.

The excitatory neurotransmitter glutamate has been shown to play a crucial role in neuronal damage associated with trauma, ischemia, toxins and chronic neurodegenerative disorders. Glutamate neurotoxicity takes place as a result of glutamate binding to the *N*-methyl-D-aspartic acid (NMDA) receptor and to a minor extent to other receptor subtypes. Glutamate neurotoxicity is marked by acute neuronal swelling and depends on extracellular Na^+ and Cl^- uptake by the cell that causes plasma membrane depolarization. This causes Ca^{2+} channel opening that triggers massive influx of extracellular Ca^{2+} and release from intracellular Ca^{2+} stores, resulting in calcium overload that initiates a cascade-like effect leading to cell death (Atlante et al., 2001). A causal relationship between glutamate neurotoxicity and ROS production was first proposed by Dykens et al. (1997) who observed that neuronal damage caused by the excitotoxin, kainate, is prevented by SOD. Conversely, the depletion of the antioxidant GSH was found to exacerbate glutamate neurotoxicity. Electron paramagnetic resonance spectroscopy provided direct evidence that NMDA receptor activation leads to the generation of O_2^- (Lafon-Cazal et al., 1993). Glutamate stimulates production of O_2^- via both NOS- and AA-dependent mechanisms (Culcasi et al., 1994). Schulz et al. (2000) provided the first *in vivo* evidence that excitotoxic neuronal injury is linked to free radical generation from mitochondria.

It is noteworthy that astrocytes also play cardinal roles in the developing nervous system. Astrocyte-neuron cell-to-cell contact mediated by adhesion molecules is well exemplified by the migration of cerebellar granule cells along Bergmann fibers. Translocation of neuronal cell bodies requires the coordinated temporal and spatial expression of different adhesive molecules, e.g., N-CAM, astrotactin, and L1 (Rakic, 1990; Stitt and Hatten, 1990). Similarly, neurite outgrowth along astrocytic cell surfaces and the extracellular matrix is characterized by a specific spatiotemporal elaboration of a number of adhesive molecules including L1, N-CAM, N-cadherin and integrin-class extracellular matrix receptors (Smith et al., 1990). A prominent feature of prenatal MeHg poisoning is a reduction in CNS mitotic activity (Rodier et al., 1984), and interference with neuron migration (Choi, et al., 1981; Choi, 1983). Effects of MeHg on neuronal migration in culture reveal cessation of cell movement at 10 μM MeHg. Abnormalities of neuronal migration are also prominent pathological features of MeHg-affected human brains (review, Reuhl and Chang, 1979). Since neurons migrate along radial glial processes, it is possible that a major effect of MeHg is damage to the astrocytes. Indeed, effects on astrocytes have been shown. The initial site of MeHg injury appears to be the astrocytic plasma membrane with a marked shift in the distribution of anionic groups and loss of filopodial activity. Electron spin resonance studies on cultured astrocyte membranes exposed to MeHg indicate a significant reduction in membrane fluidity following exposure to this organometal (Choi, 1988).

Conclusions and Future Directions

The pathophysiology of MeHg and related organic mercury species bears upon exposure from fish consumption. However, one would be remised to ignore other contemporary issues

in mercury poisoning, including another issue of public concern, namely, the adverse effects of thimerosal, a vaccine preservative that is metabolized to ethylmercury (EtHg) and thiosalicylate. Given that cumulative infant exposure to EtHg through immunizations may exceed federal safety guidelines for MeHg (Ball et al., 2001), establishing the biologic basis for adverse neurological outcomes following exposure to any organic Hg species is deemed highly meritorious. There remains a clear mandate to study the consequences and mechanisms of mercurial-induced CNS deficits both in developing and adult animals, shedding novel information on the mechanisms of mercurial associated perturbations in CNS function.

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