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Mercury in the Spinal Cord After Inhalation of Mercury

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Abstract: Amyotrophic lateral sclerosis (ALS) affects anterior horn cells of the spinal cord causing an indolent slow and steady deterioration of muscle strength leading inevitably to death in respiratory failure. ALS is a model condition for neurodegenerative disorders. Exposure to different agents dispersed in the environment has been suggested to cause neurodegeneration but no convincing evidence for such a link has yet been presented. Respiratory exposure to metallic mercury (Hg^0) from different sources may be suspected. Body distribution of metallic mercury is fast and depends on solubility properties. Routes of transport, metabolism, excretion and biological half-life determine the overall toxic effects. Inhalation experiments were performed in 1984 where small marmoset monkeys (*Callithrix jacchus*) were exposed to $^{203}Hg^{0\ vapour}$ mixed into the breathing air (4–5 μg/l). After 1 hr of exposure, they were killed and whole body autoradiograms prepared to study the distribution of mercury within organs. Autoradiograms showed that Hg was deposited inside the spinal cord. Areas of enhanced accumulation anatomically corresponding to motor nuclei could be observed. This study describes a reinvestigation, with new emphasis on the spinal cord, of these classical metal exposure data in a primate, focusing on their relevance for the causation of neurodegenerative disorders. A comparison with more recent rodent experiments with similar findings is included. The hypothesis that long-time low-dose respiratory exposure to metals, for example, Hg, contributes to neurodegenerative disorders is forwarded and discussed.

Degenerative disorders of the nervous system affect an increasing number of individuals throughout the world [1]. Genetic causes for this observed increase have been put forward [2–4]. However, genetics alone cannot explain the observed rise in present prevalence of Alzheimer's disease (AD) [5], Parkinson's disease (PD) [6] and Amyotrophic lateral sclerosis (ALS) [7]. Environmental factors have been suggested as causative in all of these diagnoses, but no convincing evidence for such a link has to date been presented for these multifactorial diseases. ALS has been associated with metal exposure [8, 9], and toxic metals such as mercury (Hg) are possible candidate substances in the complex causation of ALS.

Use of compounds with recognized neurotoxicity in human exposure experiments is not easily justified for ethical reasons. Because of that, animal experiments have been performed to study the possible contribution of toxic agents from different routes of exposure to the nervous system. Exposure data from rodent experiments are, however, in some cases hard to interpret as the metabolism of the rat for certain toxic agents, for example, mercury, is different from the metabolism of primates and absorption routes and elimination patterns may vary. Timing of dosage and exposure is also critical for the outcome. A low-dose exposure over long time of an accumulating toxic agent, albeit naturally occurring in the environment, causes different types of effects in the nervous system than a high-dose short-time exposure to the same substance. Respiratory exposure causes a unique distribution pattern within tissues, when compared to intravenous exposure or dermal exposure; however, the overall systemic effects might be the same.

Route of exposure and chemical species of a toxic compound determines the degree of tissue binding. Distribution to organs depends on solubility properties and oxidation in the blood and is for Hg very fast. The whole body distribution also depends on the rate of blood flow in the organs and the capacity to oxidize Hg intracellulary. Local or systemic toxicity occurs for metallic Hg upon application to the skin. Absorption, distribution, metabolism and excretion and chemical properties of the substance explain the delayed observed toxicity upon exposure to elementary mercury (Hg^0) where effects may be noted several months after exposure. Absorbed metallic Hg vapour is rapidly oxidized in the blood, and Hg ions are transported via blood to the kidney where renal toxicity will develop at critical concentrations. However, mercury vapour that passes the blood brain barrier (BBB) and is not oxidized until after uptake in the brain will remain in this organ for various time and cause adverse effects in this critical organ. The effects are seen after some time.

Among neurodegenerative disorders, ALS is considered the model condition for neurodegeneration. The motor systems are affected, and indolent slow and steady deterioration of muscle strength leading to death in respiratory failure is caused. In ALS, the anterior horn cells of the spinal cord are selectively affected and atrophy of these cells is seen in every case of ALS [10].

Possible exposure routes for noxious metal agents that might give rise to ALS consist of dermal exposure [11],

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gastrointestinal exposure [12], mercury vapour from dental amalgam fillings [13], axonal routes [14] and respiratory exposure, all of which are valid in the discussion of possible environmental impact in causing neurodegenerative disorders. Long-time low-dose respiratory exposure to a toxic agent with long biological half-life absorbed and accumulated in specific cells will give rise to cellular toxicity which will have an impact on the detoxification systems and release of the agent from those cells. Respiratory exposure is unique in this context as it comprises high turnover of large volumes of possibly contaminated air during life-time. To what extent low doses of inhaled mercury vapour can be transported to the spinal cord remains to be elucidated.

This study describes a reinvestigation, with new emphasis on the spinal cord, of some classical metal exposure data in a primate, focusing on their relevance for the causation of ALS. A comparison with more recent rodent experiments with inhaled Hg is included. The hypothesis that long-time low-dose respiratory exposure to metals contributes to ALS is forwarded and discussed with Hg^0 as an example.

Methods

Respiratory exposure of monkey. In 1984, metal inhalation experiments were performed in a male marmoset monkey (*Callithrix jacchus*). It was exposed to radioactive elemental mercury vapour mixed into the breathing air. After 1 hr of exposure to $^{203}Hg^0$ at 4–5 μg/l air, the monkey was killed immediately by gaseous carbon dioxide and whole body autoradiograms prepared [15] by cryosectioning. The monkey received a $^{203}Hg^0$ dose of 480 μg/kg b. wt. Distribution of Hg within organs and between organ systems was studied in the primate [16]; however, the spinal cord received no specific attention at that time. Ethical permission was given following the rules for performing experiments in the 1980s. This study is to be regarded as an archive study of material already granted permission.

The original autoradiograms had been stored in a dark, dry environment since exposure in the 1980s. As far as can be judged, there was no decay in the images and they were easily readable with no apparent loss of detail in silver staining as the autoradiographic technique used is very stable [15]. All autoradiograms covering the nervous system in one animal were selected for further analysis. Among these, four sections with the best representation of the central canal of the spinal cord in the monkey were chosen for investigation. Some sectioning artefacts were present skewing the spinal cord, however, not affecting the precision of anatomical localization. Autoradiograms were examined in a digital photograph reproduction system (Medical photography service, Oslo University Hospital) with many levels of contrast and brightness and enlargement allowing for detailed study of the spinal cord.

Respiratory exposure of mouse. In a more recent inhalation study performed by Stankovic in 2006, 8-week-old wild-type 129/Sv mice were exposed in a closed chamber to Hg^0 in a single dose of 500 μg/m^3 for 4 hr [17]. The animals were anaesthetized with pentothal and killed. Cardiac perfusion with 4% performaldehyde in 0.1 M phosphate-buffered saline was performed. Transverse sections of mouse cervical spinal cord were prepared using autometallographic technique [18] and counterstained with 0.5% aqueous cresyl violet to detect heavy metal deposits (fig. 1). The study group consisted of six exposed mice and six controls, a number sufficient for the autoradiographic or autometallographic methods.

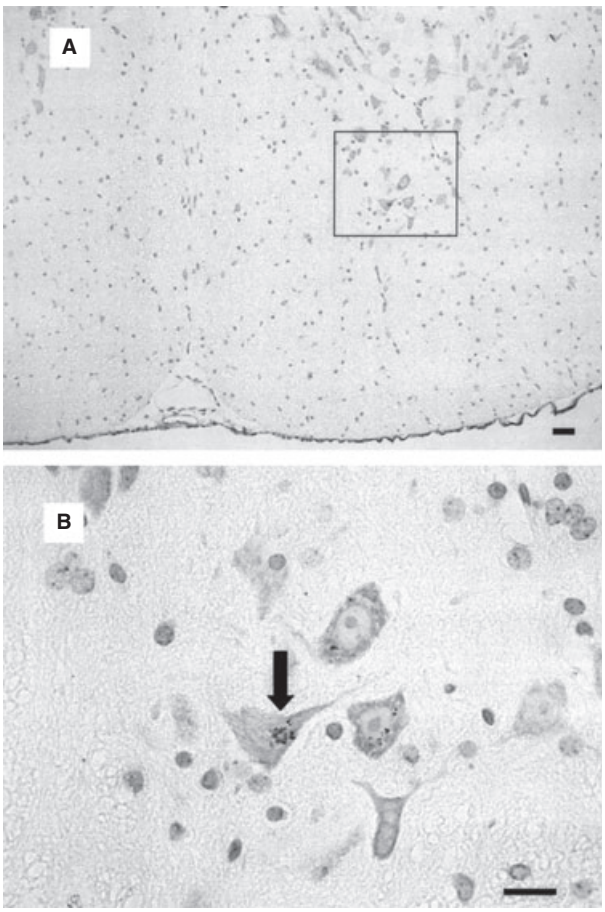

Fig. 1. Transverse section of mouse spinal cord. Arrow pointing at metal in anterior horns cells after exposure to Hg^0. (A) Depositions indicating Hg^0 detected in cytoplasm of anterior horn cells but not in astrocytes or other motor neurons that are not from the anterior horn. (Scale bar = 40 μm). (B) Enlarged section. Metal deposits are seen throughout the cytoplasm of the motor neuron (Scale bar = 30 μm). From Stankovic *et al.* 2006, with permission.

Results

Mercury in spinal cord of monkey.

Relatively high concentrations of radioactive mercury were found in the spinal cord of the primate after inhalation of radio-labelled $^{203}Hg^0$ metallic mercury vapour (fig. 2).

The staining can be followed in the spinal cord as a black undulating line (fig. 3) from the cerebellum via brain stem structures though the cervical spine down to approximately second thoracic vertebral level where it is lost because of skewed sectioning. There is a sharp contrast between the spinal cord with relatively heavy staining and the surrounding pale fat tissue not accumulating the metal. The undulating course of the cord may be apprehended as an effect of the freeze fixation procedure and should not be considered abnormal [19]. These form changes are not seen in chemically fixed nerve structures. One section shows 3–6 spinal nerves (fig. 4) leaving the spinal cord, and in the spinal area adjacent to the branching of the cord into spinal nerves, accumulations

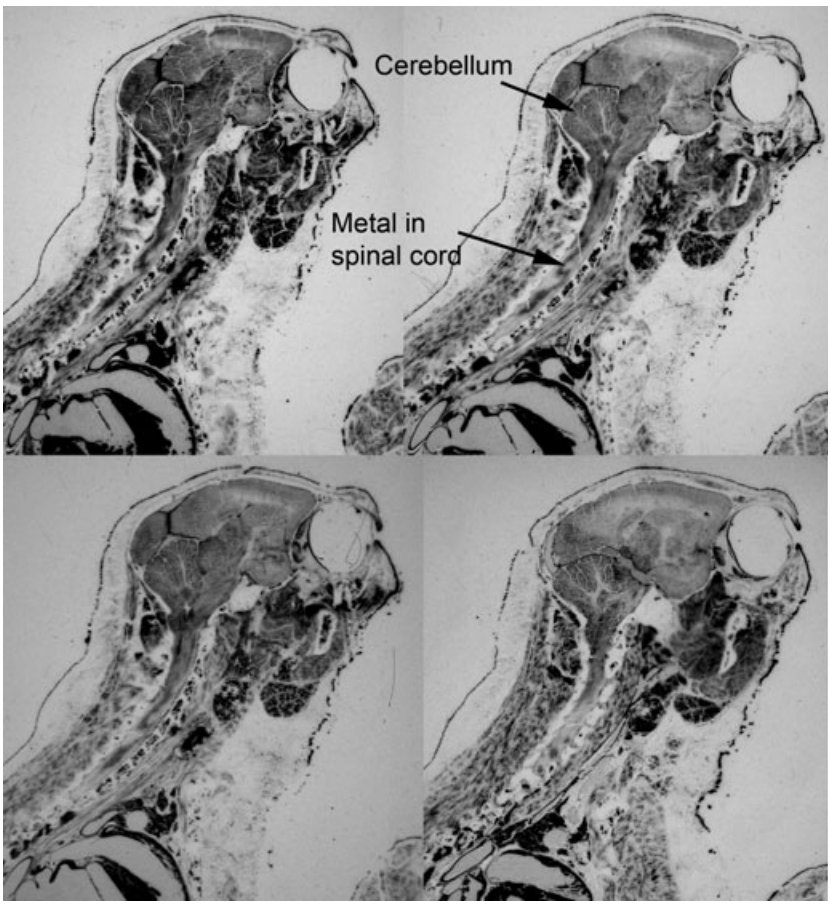

Fig. 2. Mercury deposition in spinal cord and brain of Marmoset monkey following respiratory exposure to metallic mercury vapour. Four adjacent sections showing different aspects of the spinal cord are shown.

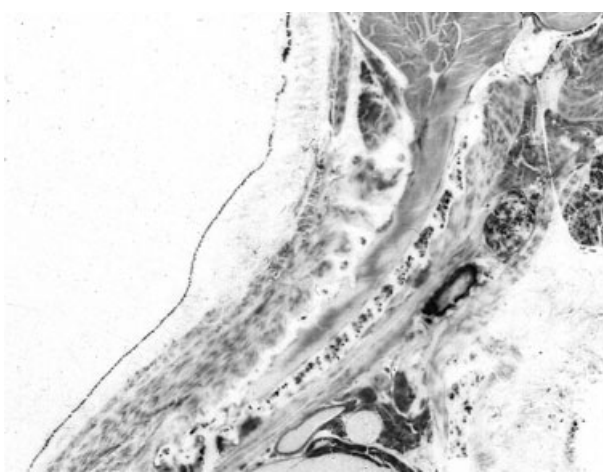

Fig. 3. Enlargement showing cerebellar and brain stem regions. The striking contrast in staining between central parts of the spinal cord (dark) and peripheral parts of the cord (light grey) can be observed.

of staining can be observed as dark rounded structures (black arrows) probably representing motor nuclei. The most prominent uptake of inhaled Hg is in the lung, observed as black staining in the lower part of the picture. When the whole animal was analysed [16], lungs were showing the most prominent accumulation of Hg followed by kidney, heart and liver. Brain and spinal cord concentrations were lower and comparable to the intensity of staining in spleen and testes.

Mercury in spinal cord of mouse.

In the study [17] performed by Stankovic in 2006, the cytoplasm of ventral horn motor neurons showed granular deposits (fig. 1) corresponding to the presence of Hg^{0} in the mice receiving mercury vapour by inhalation but not in the control mice. Astrocytes and neurons outside of the ventral horn did not contain granular deposits corresponding to the presence of inorganic Hg [17]. Simultaneously performed studies of forelimb grip strength showed a decrease in strength and atrophy of large myelinated motor axons.

Discussion

Respiratory exposure to toxic agents has been suggested as a possible cause of neurodegenerative disorders, and such environmental exposure of the general population would provide one explanation to nerve cell deterioration in these probably multifactorial disorders. Mercury, including several Hg species, is a well-known neurotoxicant and some reports [11, 12] indicate that Hg intoxication contributes to motor neuron

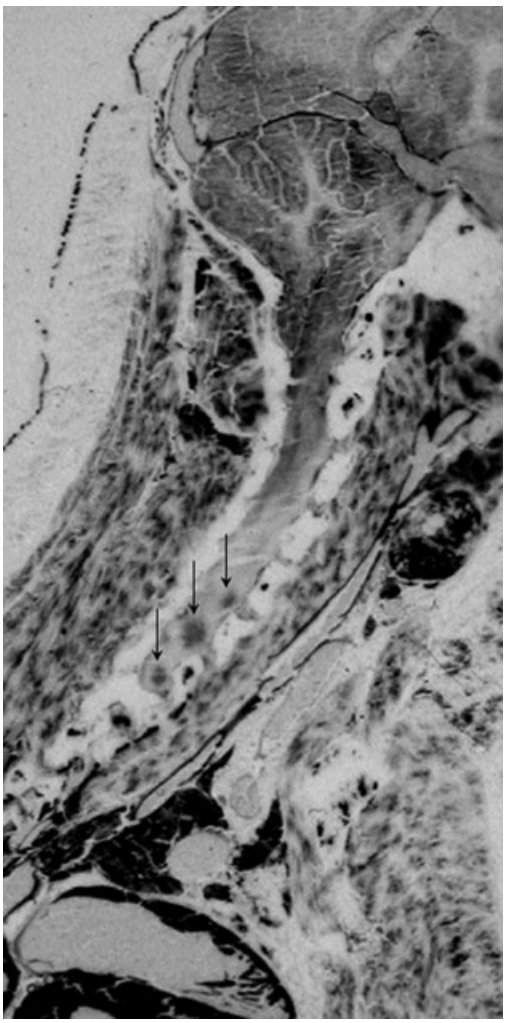

Fig. 4. Spinal nerves leaving the spinal cord approximately at cervical levels C3–C6. Within the cord at the level of the motor nuclei accumulations (arrows) of mercury can be seen as black dots. Scattered staining is also noted in the spinal nerves.

deterioration. Several entry routes for Hg exposure have been described [14, 20, 21], and inhalation of mercury vapour over time gives rise to toxic effects of Hg on the nervous system. Inhalation gives rise to tenfold higher concentration in the central nervous system than i.v. injection of the same amount of Hg in the form of mercury chloride [22]. The absorption from lung is rapid, and widespread distribution occurs where Hg is retained in organs such as lungs, liver, myocardium and nervous system. Mercury vapour, once taken up, is readily distributed via blood to these organs and part of the absorbed Hg^0 is rapidly oxidized to Hg^{2+} by catalase, an enzyme with individual differences in activity [23].

Mercury.

There are considerable differences in metabolism between metallic mercury vapour and mercuric mercury. Because of its lipid solubility, mercury vapour penetrates cell membranes and is easily absorbed and rapidly oxidized to Hg^{2+}. This ion is likely to bind to sulphydryl or selenohydryl groups on proteins and has limited mobility. Mercury binding to sulphydryl groups can change the tertiary structure of proteins and block receptor binding [24].

The most abundant form of Hg is methylmercury, and the most common route of exposure is enteric [25]. In the context of motor neuron degradation and in relation to the possible uptake of Hg into the spinal cord, we are interested in how, and to what extent, inhaled Hg can pass barrier systems and enter the central nervous system in patients with ALS. Higher concentrations of Hg were found in CSF from ALS patients compared to controls, however, not statistically significant.

Barriers.

The main entry route for metal into the nervous system is via the endothelium between the blood vessels and the brain tissue known as the BBB. The cerebrospinal fluid volume is normally protected against toxic metals by the choroid plexus [26]. Several metal transport systems exist [27] but no specific Hg transporters have to date been identified.

The spinal cord seems to receive no protection from Hg entry neither from the barriers between brain tissue and bloodstream nor from the barriers between spinal cord and bloodstream [28], as the staining in these inhalation experiments [16] is distributed within the structures at the inside of the barriers. Hg^0 penetrates both barriers before oxidization, and comparable concentrations in spinal cord and brain can be expected. The possibility of injury by Hg to the protective capillary membranes themselves or to the choroid plexus must also be taken into consideration [28]. Barriers may be circumvented by uptake of Hg into the anterior horn cells of the spine through the mechanisms of retrograde transportation [14] either directly via the nose and olfactory nerve [20] or through the neuromuscular junctions where no protective barrier exists [29].

Rodent experiments have demonstrated increased uptake in the nervous system after inhalation of different metals [30] including Hg [31,32]. However, data from primates are scarce, and the present investigation [16] represents the only controlled radio-labelled Hg respiratory exposure experiment performed in primates where the distribution of Hg in the nervous system is visualized. In these experiments [16], the monkey was exposed to 4–5 mg Hg per m^3 which is a high dose that may be compared to the 1982 WHO maximum recommended allowable concentration for industrial workers, which was 25 μg Hg per m^3 inhaled air. The Swedish occupational threshold limit value for Hg is 0.03 mg/m^3 [33]. The different possible respiratory exposures of human beings to inorganic Hg in daily life are complex [34] and the actual exposure dose varies considerably.

Are these high experimental concentrations of metal vapour relevant to human low-dose long-time exposure from different sources? Exposure to Hg^0 results in the presence of Hg in the spinal cord of marmoset monkey after one hour of vapour inhalation. The BBB does not seem to protect the spinal cord against this exposure. The results may be compared to the

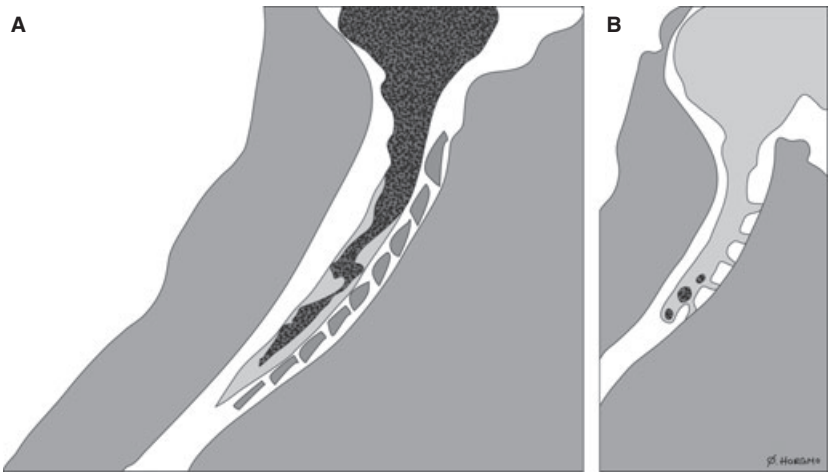

Fig. 5. Schematic drawing showing regions of Hg^0 accumulation (black dots) in spinal cord of exposed monkey. (A) Spinal cord and brain stem regions, corresponding to fig. 3. (B) Motor nuclei, corresponding to fig. 4.

more recent rodent Hg inhalation experiments [17] with a similar outcome. In these experiments, mice were exposed to Hg^0 in a concentration of approximately 500 μg Hg^0/m^3 for 4 hr and then monitored for 7 months. Atrophy of large myelinated axons could be quantified. A steady decline in forelimb grip strength as measured with a digital force metre at 4-week intervals was noted when the mice grew older, after 22 weeks of age. Spinal cord autometallography showed granular metallic depositions in the cytoplasm of the anterior horn cells, not present in the control mice. Interestingly, these depositions were confined to anterior horn cells exclusively, as the surrounding astrocytes contained no metallic granule (fig. 5).

Mice with the presence of metal in their anterior horn cells showed progressive weakness in the forelimbs and atrophy of large myelinated axons, features known from clinical and electrophysiological investigations of patients with motor neuron disease [35]. The degree of axonal atrophy can be estimated with neurographic methods. Motor amplitudes in motor neurography correspond in a linear fashion to degree of axonal atrophy. Reduced motor amplitudes are consistent findings in motor neuron disease [35] and correlate with reduced strength.

Storage of mercury.

Experiments with inhaled metal mercury vapour in rodents have shown distribution of Hg to the lung, liver and spinal cord [29]. Within the nervous system, the most pronounced Hg accumulation is found in the motor systems, in the brain stem motor nuclei and in the spinal cord motor nuclei of the anterior horn which receive the bulk of inhaled Hg [36]. At the ultrastructural level, Hg is stored in lysosomes [36]. The spinal cord may also be reached in spite of a normal and intact BBB by the mechanism of retrograde axonal transportation [14] via the neuromuscular junction causing indefinite accumulation into motor neurons. The anterior horn cells of the spinal cord being the most cranial part of the lower motor neurons are large end stations for this retrograde transportation, and Hg stored in anterior horn cell lysosomes is not excreted and persists in the nerve cell [37]. Lysosomal storage capacity for the metal might eventually be exceeded, and Hg will then cause damage to the cytoplasm resulting in nerve cell death.

The uneven distribution of Hg as seen in Stankovic *et al.* 2003 in the rodent spinal cord and in these studies of the marmoset spinal cord (fig. 4) needs further explanation. Deposits are found in anterior horn cells of the spinal cord but not elsewhere in the cord. Uneven distribution of catalase activity may explain such accumulations, as it has been demonstrated [38] that catalase activity is elevated in astrocytes after exposure to Hg. Protective mechanisms in astrocytes and other glial cells may prevent an accumulation of Hg outside of the anterior horn cells. Certain metalloproteins such as metallothionein (MT) provide such protection. MT induction in mouse cells *in vitro* is higher in astrocytes than in neurons [39,40]. The unprotected anterior horn cells seem to receive more exposure from Hg^0 than their surroundings possibly explaining the selective degeneration of anterior horn cells.

Metallothionein.

Protective mechanisms against toxic effects of Hg on nerve cells include certain metalloproteins, the most important being metallothioneins (MT) [41]. These proteins are inducible and provide zinc for the regulation of gene expression, contribute to the redox stability of the cell and bear the potential of binding metal atoms [28]. Metallothioneins have a protective function towards nerve cells as indicated by the fact that MT knockout mice show more pronounced myelinated axon atrophy upon respiratory exposure to Hg^0 compared to wild-type mice [42]. In motor neuron disease, MT is up-regulated in the spinal cord [43].

In Hg^0 respiratory exposed MT knockout mice [29], there were more pronounced congestive changes in liver and lung compared to wild-type mice, however, no pathological changes in motor neurons of the spinal cord. Noteworthy, no MT expression was noted neither in the motor neurons of the spinal cord nor in the axons of the ventral roots of Wt mice or

MT knockout mice [29]. Axons in the ventral root of Wt mice did not stain for MT.

Metallothionein proteins are highly inducible in response to metals but also to other stimuli such as glucocorticoids. They occur throughout the brain and spinal cord and the main cell type expressing MTs is the astrocyte, especially the reactive astrocyte [44]. Several other metalloproteins with relevance for different neurodegenerative disorders have recently been identified [45], and further investigations into the mechanisms of toxic nerve cell injury as an effect of respiratory metal exposure are warranted.

Respiratory exposure.

Inhalation experiments such as the two Hg vapour inhalation studies described in this retrospective discussion show accumulation of Hg in anterior horn cells of the spinal cord after respiratory exposure to Hg vapour. The spread of Hg from respiratory organs to the spinal cord seem to be rapid and passing protective barriers. Case reports on accidental [11] or occupational [46] respiratory exposure to Hg have described a clinical picture of widespread muscle atrophy and weakness as in ALS. To what extent low-dose respiratory Hg exposure contributes to ALS is still an open question. Small size particulate Hg might add to the respiratory exposure of Hg vapour [47] and ALS-like clinical pictures have been described following inhalation of mercuric oxide dust [48]. Mercury alone may not be responsible for the invariably deadly course in ALS but the possible contribution of inhaled Hg in the causation of this multifactorial disease cannot be neglected.

Conclusions

Inhalation experiments in rats and primates show deposition of Hg in spinal cord following single high-dose short-time exposure. Mercury accumulation in anterior horn cells is followed by axonal atrophy and distal weakness similar to the clinical picture in human ALS. Protective mechanisms against central nervous system Hg toxicity include induction of MT and catalase in astrocytes supporting anterior horn cells in the spinal cord, however, lack of MT expression in anterior horn cells *per se* may leave these structures unprotected from Hg toxicity. Respiratory Hg exposure could contribute to elevated concentrations of Hg found in cerebrospinal fluid from patients with ALS. Further studies into the contribution from inhaled metal dust or vapour in the causation of neurodegenerative disorders are warranted.

Acknowledgements

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