TARJA TOIMELA

Evaluation of Neurotoxicity of Mercury Compounds and Aluminum in Cell Cultures

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CONTENTS

List of original publications

This thesis is based on the following original articles (referred to in the text by their Roman numerals):

I   Toimela T and Tähti H (2004): Mitochondrial viability and apoptosis induced by aluminum, mercuric mercury and methylmercury in cell lines of neural origin. Arch Toxicol, in press

II   Toimela T and Tähti H (1995): Effects of mercury, methylmercury and aluminium on glial fibrillary acidic protein expression in rat cerebellar astrocyte cultures. Toxicol *in Vitro* 9: 317-325

III   Toimela T and Tähti H (2001): Effects of mercuric chloride exposure on the glutamate uptake by cultured retinal pigment epithelial cells. Toxicol *in Vitro* 15: 7-12

IV   Toimela T, Mäenpää H, Mannerström M and Tähti H (2004): Development of an *in vitro* blood-brain barrier model - cytotoxicity of mercury and aluminum. Toxicol Appl Pharmacol, 195:73-82

Abbreviations

$\Delta\psi_m$	mitochondrial membrane potential
AB-AM	antibiotic-antimycotic solution
ANOVA	analysis of variance
AP7	NMDA antagonist, 2-amino-7-phosphonoheptanoic acid
ARPE-19	human retinal pigment epithelial cell line
ATP	adenosine triphosphate
BBB	blood-brain barrier
BCA	bicinchoninic acid
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CPP32	caspase 3/Yama/apopain
CYP	cytochrome P450 enzyme superfamily
D407	human retinal pigment epithelial cell line
DAG	diacylglycerol
DMEM	Dulbecco´s modified Eagle´s medium
DNA	deoxyribonucleic acid
EC_{50}	effective concentration for half maximal effect
EDTA	ethylene diamine tetra acetate
EGTA	ethyleneglycotetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FBS	foetal bovine serum, also foetal calf serum FCS
GFAP	glial fibrillary acidic protein
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HO-1	heme oxygenase-1
IP_3	inositol triphosphate
KRH	Krebs-Ringer-HEPES buffer
LDH	lactate dehydrogenase
MEM	minimum essential medium
MPTP	mitochondrial permeability transition pore
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form
NFTs	neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NO	nitric oxide
NP40	polyglycol ether (nonionic) surfactant
PBS	phosphate-buffered saline

P-gp	multidrug resistance P-glycoprotein
PHF	paired helical filament
PHFtau	hyperphosphorylated tau
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PTFE	polytetrafluoroethylene
RBE4	rat brain endothelial cell line
RNA	ribonucleic acid
ROS	reactive oxygen species
RPE	retinal pigment epithelium
SC10	PKC activator, 5-chloro-N-heptylnaphthalene-1-sulfonamide
SEM	standard error of mean
SH-SY5Y	human neuroblastoma cell line
U-373 MG	human glioblastoma cell line
WST-1	tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate

Abstract

Mercury and aluminum are neurotoxic metals with diverse effects on cellular functions in the brain. Ultimately exposure to them can lead to neural destruction and degenerative diseases. Although their toxic potency is now widely known, their existence in the environment and in several man-made applications makes human exposure inevitable. There are many mechanisms that cause cellular destruction with a delicate interplay with each other. That is why studies on different adverse mechanisms, and new methodological developments, as applied in this work, broaden the knowledge of the toxicity of these metals. Cell culture systems make such studies possible in strictly defined conditions.

For the experiments on mercuric mercury, methylmercury and aluminum toxicity, several methods and cultures of different neural cell types were used. Cytotoxicity was evaluated in neuroblastoma, glioblastoma and retinal pigment epithelial (RPE) cell lines, with a method based on measuring the mitochondrial integrity, WST-1 assay, and the leakage of lactate dehydrogenase enzyme (LDH-test). To further characterise the mechanism of cell deaths in these experiments, induction of apoptosis, the cellular self-destruction process, was evaluated. The reactivity of glial primary astrocytes as a response to toxic insults was evaluated by measuring the amount of an intermediary filament protein, the glial fibrillary acidic protein (GFAP). The uptake of the main excitatory amino acid glutamate was studied in connection with mercury in primary RPE cells. Evaluations of protein kinase C (PKC)-linked pathways and intracellular calcium level were included to characterise the effect. For assessing how well the study metals can pass the blood-brain barrier (BBB), an *in vitro* BBB barrier model built on transparent membrane filters was established. Furthermore, cellular morphology was one of the aspects monitored throughout the study.

The cytotoxicity studies showed that methylmercury was the most toxic substance in the sense that it exerted its effects at lower concentrations than either mercuric mercury or aluminum. The effect was seen in all culture systems. Apoptotic cell death mechanisms were involved with all metals studied, but with different cell specificity. An unexpected finding was the activation of mitochondrial dehydrogenases, especially in connection with methylmercury and aluminum at low concentrations. The activation as a toxic response may lead to equally significant consequences as deactivations seen in cytotoxicity studies. An important result was that methylmercury toxicity seemed to be irreversible.

Aluminum was not very cytotoxic (did not cause cell deaths), but showed responses that may be equally important, e.g. mitochondrial activation. Formation of fibrillary structures characteristic of aluminum exposure was

especially seen in glial cells, not so much in neuronal cells as had been usual in previous studies.

The induction of GFAP synthesis was adapted for *in vitro* system. The GFAP synthesis was induced with exposure to all the metals studied. The cellular structural filaments may be a sensitive target common to many toxic metals, since mercurial compounds were as active inducers of GFAP production as aluminum.

Mercuric mercury inhibited glutamate uptake in RPE cells. The inhibition was not permanent, since the uptake could mostly be restored by activating the PKC. When glutamate accumulates in the extracellular space of RPE cells, excitotoxic damages in neighbouring neuronal cells may follow. Also another major mechanism that mediates mercury toxicity was shown: Mercuric mercury could increase the intracellular calcium level rapidly from the extracellular calcium pools. Calcium is connected to several toxic cellular reactions.

One of the main outcomes was that an *in vitro* BBB model constructed from human cells was developed. The model was able to distinguish the toxicity differences of methylmercury, mercuric mercury and aluminum. The *in vitro* BBB model can be adapted for the testing of new chemicals and drugs for their potency to cross the BBB and therefore exert adverse effects in the brain.

As a conclusion, mercuric mercury, methylmercury and aluminum showed diverse adverse effects on neural cells. Due to accumulation, the effective concentrations may be exceeded even in human exposures. The study especially emphasised the toxicity of methylmercury, because of its wide potency and irreversibility of the effects. Furthermore, methylmercury is the form of mercury that easily enters the brain. Aluminum seemed to be less cytotoxic, but the specific effects induced by aluminum may initiate or mediate the cellular destruction as well.

Introduction

Mercuric mercury, methylmercury and aluminum are environmental toxicants, whose target organ is the brain (Jope and Johnson 1992, Flaten et al. 1996, Clarkson et al. 2003). Although the characteristics of toxicity and the way of reaching the brain are different with each metal type, there may be some common mechanisms behind their toxicity. That is why comparing the effects of these metals in different study systems is of special interest. Both mercury and aluminum are typically retained and accumulated in the brain (Friberg and Mottet 1989, Yokel et al. 1999). The lipophilic nature of methylmercury helps it through the BBB (Aschner and Aschner 1990). Aluminum crosses the BBB with the help of carrier systems (Bertholf et al. 1989, Julka et al. 1996, Yumoto et al. 1997). Although inorganic mercury cannot normally penetrate the barrier, it still has neurotoxic potential. Metabolic conversions increase the amount of mercuric mercury in the brain, and it is trapped inside the brain, since it cannot cross the BBB the way back, either.

In vitro models are valuable in assessing the mechanisms of chemicals toxic to the brain cells (Walum et al. 1993). Most neural cell types are available for cultivation, either from commercial cell banks or by isolating them from animal or human tissue. Though advantageous in many respects, the use of primary neural cells brings about technical and ethical difficulties. For neuronal cultures, foetal or postnatal tissues are needed, but access to this kind of human material is limited. Although results with animal cells can give important hints of the effects of a specific compound, human cells are indispensable in the final judgement of toxicity. Cell lines of human origin are of great value in this respect. Cell lines usually come from cancerous tissues and can show characteristics that are different from the original tissues. Therefore, careful examination of the cell properties is necessary, especially when specific pathways are studied.

Neurotoxic metals usually resemble or take advantage of the vital metals used in normal metabolism. This is true for both mercury and aluminum. The human body is not adapted to high presence of these metals, because environmental contamination is such a new phenomenon in human history. Molecular mimicry often explains why these metals have access to certain tissues and can replace or block vital processes. It is obvious that several targets can be and are attacked. Cells have specific means for reacting to such attacks. The ultimate response is to get rid of the damaged cell by apoptotic cell death, if the cell is capable to execute the process. It may be advantageous for the organism, but in the brain it causes irreversible damage and may be the cause of the onset of neurodegenerative diseases. Therefore, it is essential to find out

which events precede the major damages. To gain this kind of knowledge, diverse cellular mechanisms, also interrelating ones, need to be studied.

Review of the Literature

Neurotoxicology *in vitro*

Characteristics of the central nervous system cells

The special features of the central nervous system (CNS) make it a sensitive target to toxic insults. The sensitivity of the brain to lack of oxygen and glucose reflects the high-energy demand of the cells for the conduction of electrical impulses (Alberti 1977, Laughlin et al. 1998, Bruckner et al. 1999). Oxygen-utilising aerobic glycolysis produces ATP most efficiently, and glucose is the primary fuel for the glycolysis (Dienel and Hertz 2001). A continuous supply of both oxygen and glucose is needed, because there are no energy stores in the brain. Any toxic agent that interferes with the energy balance causes rapidly severe injury to the brain cells, also in *in vitro* cell culture systems. The demand of signal transduction over long distances also sets morphological challenges to neurons. Long neuronal processes, even though small in diameter, make the cellular volume much larger than that in non-neuronal cells. Protein synthesis and transport of nutrients to all cellular parts have to be efficient (Vallee and Bloom 1991). This emphasises again the high energy requirement of neuronal cells.

The CNS is highly specialised both structurally and functionally. In a network cells work in close cooperation with each other. Various neuronal and glial cells are the major cell types in the brain (Peters et al. 1991). Cells from different brain regions, as well as different cell types (neurons vs. glia) may show strict specificity to neurotoxic agents. Glial cells (astrocytes, oligodendrocytes and microglia) are important in buffering toxic effects. They sequester and metabolise toxins in order to support and maintain the neuronal integrity. However, biotransformation may convert initially harmless and non-reactive compounds to toxic forms. Cytochrome P450 (CYP) is an enzyme superfamily that takes care of most biotransformations of endogenous and exogenous substrates. Various amounts of CYP enzymes are found in the brain cells, including neurons, glial cells, RPE cells and brain capillary endothelial cells (Ghersi-Egea et al. 1993, Lowndes et al. 1994, Morse et al. 1998).

Adult neurons are especially vulnerable to toxins, since they are terminally differentiated and not capable to divide. However, the discovery of neural stem cells, especially in the hippocampus and olfactory system, have shown that there is some potency of neurogenesis in the adult brain (Huang and Lim 1990,

Alvarez and Lois 1995, Weiss et al. 1996, Kempermann et al. 2000, Song et al. 2002, Liu and Martin 2003).

Because of the demand for fast ion conductance, the neurons have high densities of ion channels and ion pumps in their membranes (Eisenberg 1990, Catterall 2000). These are potential targets of toxins. Control of Na^+, K^+, Cl^- and Ca^{2+} ion fluxes is the key for maintaining neuronal functions (Somjen 2002). Uncontrolled trans-membrane ion and water fluxes can be detrimental to cells (Ekholm et al. 1992, Stys and LoPachin 1998).

Cell/tissue culture models for studying neurotoxicity

Neurotoxicity can be studied *in vitro* in several cell culture systems (Figure 1) depending on what kind of information is to be obtained (Fedoroff and Richardson 2001).
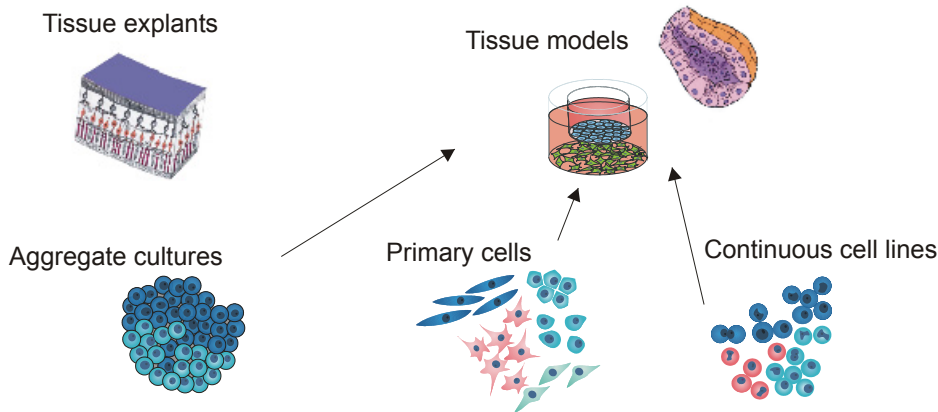
Figure 1.   *Cell culture models*

Explant cultures or brain slices resemble closest the structure *in vivo*. Cellular contacts and tissue architecture is maintained and neural cells retain their differentiation (Tominaga et al. 1994, Bahr et al. 1995, Wilhelmi et al. 2002). Since the structure is not disrupted, special features, e.g. synapses and axons, remain relatively intact (Li et al. 1994, Launey et al. 1997, Xiang et al. 2000). Different brain regions can be maintained in culture to study region-specific effects (Connelly et al. 2000). Nevertheless, an important feature of the tissue *in vivo* is missing: there is no nutrient supply through the vascular system, and nutrients have to reach cells by passive diffusion. This limits the size of applicable tissue pieces to the thickness of 1 mm. However, Moser et al. (2003) claimed that brain capillaries may persist in organotypic brain slices.

In suspension and reaggregate cultures the three-dimensional architecture and morphological differentiation of the cells can be reconstructed. When different foetal neural cell types are cultured together in suspension, they tend to form spontaneously an architecture resembling the stucture *in vivo*, and they

differentiate and start synaptogenesis and myelination (Seeds and Vatter 1971, Sheppard et al. 1978, Stafstrom et al. 1980, Bruckner 1985, Loughlin et al. 1994). The drawback with suspension cultures is that microscopic examination is difficult. Because of the interaction and differentiation of cells, these types of cultures are frequently used in basic toxicity studies (Atterwill 1987).

Primary cells from different brain areas can be cultivated (McCarthy and Partlow 1976, Cardozo 1993, Ray et al. 1993, Tucker and Morton 1995, Kivell et al. 2000). The advantage of primary cells is that they may still express the characteristics, e.g. receptors and ion channels, of the *in vivo* cells (Hertz et al. 1984, Rossie and Catterall 1987, Boehm and Huck 1997, Janssens and Lesage 2001). Maintaining these special features in culture conditions for long times requires special manipulations and is not always possible, because cells tend to shut down and downregulate any unnecessary functions. The tissues utilised in setting up primary neuronal cultures usually come from embryonic or foetal animals (Svenningsen et al. 2003). The development of adult stem cell culture techniques may change this concept in future (Davis and Temple 1994, Gritti et al. 1996, Song et al. 2002). Cell cultures of supporting cells that are able to divide, e.g. glial and brain endothelial cell cultures, can be started from older animals as well. Growth medium specificity can be useful if it is necessary to favour one cell type over another (Needham et al. 1987, Xie et al. 2000), e.g. different glucose and glutamine concentrations favour neuronal cells and glial cells differently. Also antimitotic agents or technical manipulations can be used to select a specific cell type (Orr and Smith 1988, Hammond et al. 2002), but even purified primary cultures are almost always a mixture of different cell types.

Continuous cell lines are easy to handle and serve a constant supply of cells, and furthermore, cells of human origin are available (Murayama et al. 2001). This is an advantage since the access to human cells may be limited and cause ethical difficulties. Many different neural cell types are commercially available. The obvious drawback is that usually continuous cell lines originate from cancer tissue and can exert different characteristics compared to corresponding intact cells *in vivo*. The results obtained with these cells should be always evaluated in respect to whether the characteristics of the cells are appropriate for the study subject.

BBB models

Even though the brain is vulnerable to toxic insults, it is protected best of all organs against xenobiotics. All nutrient and chemical transport to the brain is strictly controlled. A special structure of brain capillaries forms a protective barrier (Figure 2). This structure is called the blood-brain barrier (BBB) and is formed by brain capillary endothelial cells that are in close contact with astrocytic endfoots (Goldstein 1988, Prat et al. 2001, Engelhardt 2003). Astrocytes and pericytes contribute to the formation of a tight barrier (Tontsch

and Bauer 1991, Bauer and Bauer 2000, Mi et al. 2001). The inductive capabilities of astrocytes have been shown both *in vivo* and *in vitro*. *In vitro,* the induction can be gained by adding astrocyte-conditioned medium or astrocytes into the culture system (Rubin et al. 1991, Tontsch and Bauer 1991, Igarashi et al. 1999). Pericytes associate closely with the endothelium and exhibit gap junctions with endothelial cells (Larson et al. 1987). The pericytes in BBB possibly originate from microglia (Coomber and Stewart 1985). Similarly to astrocytes, pericytes regulate the proliferation and differentiation of endothelial cells and induce the tightness of BBB (Minakawa et al. 1991, Hellstrom et al. 2001). Tight junctions that connect the endothelial cells to each other prevent the paracellular passage of molecules between the cells (Rubin and Staddon 1999, Huber et al. 2001). In this way the brain is much better protected against foreign compounds than peripheral tissues are (Figure 2).
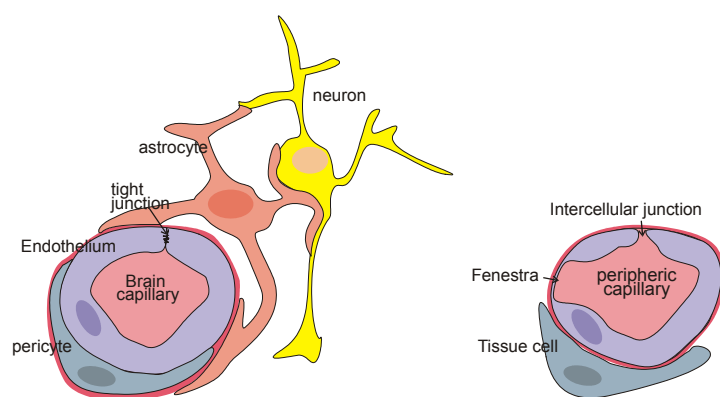
Figure 2. *Structural differences of brain and peripheral capillaries*

The BBB prevents most hydrophobic chemicals from reaching the brain. Even the transport of vital nutrients and ions through the barrier requires active receptor-mediated transport mechanisms (Pardridge 1999, Gloor et al. 2001). The endothelial cells of the brain capillaries have additional qualities, e.g. they show metabolic activities and exhibit the multidrug resistance P-glycoprotein (P-gp) (Cordon-Cardo et al. 1989, Lowndes et al. 1994, El Hafny et al. 1997, Regina et al. 1998), a transporter that helps to exclude the entrance of a wide range of xenobiotics from the brain. P-gp especially works with lipophilic and amphipathic compounds, which are normally easily transported through plasma membranes. Sometimes, the existence of P-gp is a harmful feature, because it prevents the entry of beneficial drugs to the brain (O'Brien and Cordon-Cardo 1991, Demeule et al. 2002).

Culturing of BBB cells is possible, and several *in vitro* models have been published (Rubin et al. 1991, Reinhardt and Gloor 1997, Lundquist et al. 2002). In the simplest BBB models, a single layer of endothelial cells, usually isolated

from animal brains, is let to grow to confluency on membrane filters. Others utilise other endothelial or epithelial cells capable of forming tight cell layers (Veronesi 1996, Scism et al. 1999). The obvious drawback of using non-neural tissues is that their cells do not necessarily express similar transport systems to those used by brain capillary endothelial cells. On the other hand, culturing brain primary endothelial cells raises problems about how to ensure the similarity of cultures each time the cultures are set up. Passaging primary cultures further is possible for some time, but the cells tend to lose their differentiation in culture (Panula et al. 1978). That is why primary cultures have to be restarted, and each time they are different. These problems can be avoided by using continuous cell lines. A further advantage is that brain endothelial cell lines from different species are commercially available, though unfortunately not many of human origin.

Neurotoxicity of mercury

Mercury is a natural element in the earth's crust (Nriagu 1979). Mercury can exist in elemental, inorganic and organic forms, and in different oxidation states, each of which shows different toxicological characteristics. Biological ecosystems are exposed to mercury to some extent, because mercury is degassed into the atmosphere from the earth´s crust both naturally and as a consequence of human activity (e.g. mining, smelting) (WHO 1990, WHO 1991).

Mercury has been utilised for various purposes, e.g. in devices measuring pressure (thermometers, barometers), electrical apparatuses (batteries, switches), paints, pharmaceuticals, and in the production of chemicals (WHO 1990, WHO 1991). The increasing awareness of the toxic capabilities of mercury has led to replacing mercury with less toxic substances in many of these applications. Human exposure is usually of occupational or environmental origin. Environmental exposure includes dietary intake, especially from fish (Salonen et al. 1995, Nakagawa et al. 1997, Castro et al. 2002, Hightower and Moore 2003). Environmental accidents involving large amounts of people in Iraq and Japan in the 60's and 70's have clearly shown various manifestations of methylmercury toxicity including paresthesia, ataxia, dysarthia and loss of vision. (Bakir et al. 1973, Mizukoshi et al. 1975, Takeuchi et al. 1989). The contribution of dental amalgams to neurotoxic symptoms has also been discussed. It remains controversial, although there is strong evidence for it in many recent studies (Johnson and Pichay 2001, Larose 2001, Stringer 2001, Sweeney et al. 2002).

The brain is one of the principal targets of mercury toxicity. Mercury's passage into the brain is highly dependent on the chemical form of mercury (Aschner and Aschner 1990). Lipid-soluble forms of mercury, mercury vapour and methylmercury pass easily the BBB cell membranes and enter the brain. Inorganic mercury is mainly accumulated in the kidneys and, in general, it passes poorly the intact BBB. Nevertheless, inorganic mercury deposits have been

found in the brains of human beings and animals (Farris et al. 1993, Vahter et al. 1994, Charleston et al. 1995, Pedersen et al. 1999). Molecular mimicry with endogenous substrates may play a role in how mercury reaches different tissues (Clarkson 1993, Ballatori 2002). Cells try to scavenge mercury with small thiol-containing molecules, such as glutathione (Reed 1990). Both mercuric mercury and methylmercury can react with glutathione (Aschner et al. 1994). The degradation product of the Hg^{2+}-glutathione complex, dicysteinyl-Hg, resembles the endogenous compound cystine, and that of methyl-Hg, methyl-Hg-Cys, resembles methionine. This may be an explanation for the tissue-specific accumulation of these two mercury species, since there is a selective amino acid transport system for cystine in the proximal tubular epithelia of kidneys (Zalups 2000), and for methionine in the endothelial cells of the BBB (Kerper et al. 1992, Mokrzan et al. 1995).

Conversion to different forms and oxidation states is characteristic of mercury. Thus, it is not always possible to assess the exact contribution of the original form of mercury to tissue distribution. Inorganic mercury deposits in the brain are suspected to originate mostly from methylmercury, elemental mercury vapour or metallic mercury as a consequence of cellular metabolism. In the cells, elemental or metallic mercury is oxidised to divalent mercuric mercury and, methylmercury is demethylated to mercuric mercury by cleavage of the carbon–mercury bond. The other way round, formation of organic forms from mercuric compounds has not been found in mammalian tissues. In the brain, glial cells are suspected to be responsible for the demethylation (Hansen et al. 1991, Pedersen et al. 1999), but there is also contradictory evidence of no demethylation taking place (Petersson et al. 1991). The rate of demethylation depends on the exposure time and developmental as well as on the physiological state of the subject.

On the cellular level, the effects of mercury are diverse, and all chemical forms cause toxic effects. One of the characteristics of mercury may play a major role behind the toxicity: mercury binds with high affinity to thiol groups (Kromidas et al. 1990, Atchison and Hare 1994). Many enzymes and proteins contain thiol groups that mercury can bind to. This results in inhibition of the activity of a vast variety of enzymes, interference with transporter proteins or disturbance of structural integrity, such as microtubular formation. With longer exposures, inhibition of RNA and protein synthesis leads to retardation of cell growth, and cell division is ultimately inhibited (Syversen 1982, Sarafian and Verity 1985, Ponce et al. 1994). Methylmercury has been shown to be a more efficient inhibitor of mitotic spindle formation than colchicine (Miura et al. 1998).

Cell death is the ultimate endpoint of mercury-induced toxic injury (Eto et al. 1999). Necrosis and apoptosis are two common death mechanisms, which any cell can encounter (Fawthrop et al. 1991). The cellular energy status, as well as duration and severity of the insult determine which death mechanism is activated in mercury exposure (Nicotera et al. 1998), (Figure 3).

Necrosis is the form of cell death that occurs if cells are exposed to high concentrations of mercury or if the duration of exposure is prolonged (Gallagher

et al. 1982, Pathak and Bhowmik 1998, Berntssen et al. 2003). Necrosis is the passive unregulated form of cell death that does not require ATP energy. All events that lead to interference of the cell energy balance, including oxidative stress, mitochondrial damage and ATP depletion, can cause necrotic cell death upon mercury exposure (Boti et al. 1981, Nicholson et al. 1985, Stohs and Bagchi 1995). Charasteristic of necrosis is that it can spread to adjacent cells or to large tissue areas. Mercury is able to bind to ion pumps that maintain the ion balance inside the cells. As a consequence of the collapse of cellular ion balance the cells swell, the cytoskeleton is detached from the plasma membrane, and eventually the cell membrane is ruptured.
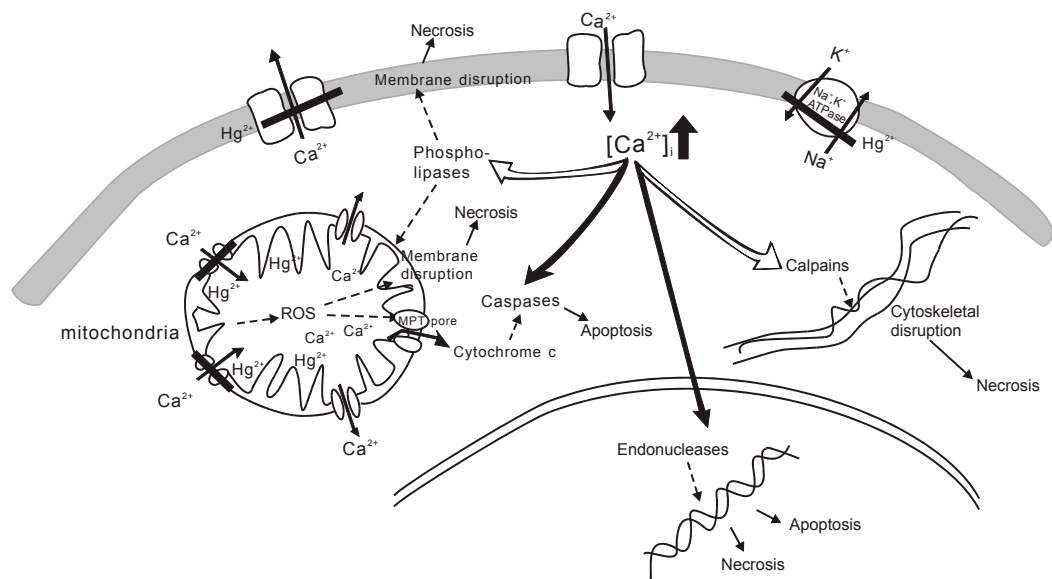
Figure 3. *Cellular targets and signaling pathways which mercury may attack*

Apoptosis is a common response to milder mercury intoxications before severe cytotoxicity makes it impossible. Apoptosis is sometimes called programmed cell death because it requires the orderly activity of a complex cascade of sequential enzymes (Wadewitz and Lockshin 1988). Cysteine-aspartate proteases called caspases are the key excutioners of apoptosis (Cohen 1997, Zhivotovsky et al. 1999). Apoptosis is the natural form of cell death and it is normal during development, immune system function, and when the body wants to get rid of damaged (and thus hazardous) cells, such as cancer cells (Bär 1996, Clarke et al. 1998, Sun and Shi 2001). Because apoptosis requires the activity of cellular systems, it is an energy-dependent process (Nicotera et al. 2000). If a toxin depletes the cell's energy supplies, apoptosis is not possible. Because each cell is triggered to go to apoptosis, it does not spread in the same way as necrosis. Usually adjacent cells take up the remnants of the dead cell and no external signs of the late cell remain. Morphological signs of apoptosis include membrane blebbing as well as shrinkage and condensation of organelles.

By biochemical analysis, DNA is broken to distinct pieces by endonuclease enzymes, which enables the visualisation of typical DNA ladders. In necrosis, the breakdown of DNA is unspecific (Majno and Joris 1995).

Apoptosis can be started after different signals, and there are several checkpoints before the phase of no return is reached. The loss of mitochondrial membrane potential and the release of cytochrome c into the cytoplasm from mitochondria is one of such signals (Hampton et al. 1998, Green and Reed 1998, Gorman et al. 1999, Slee et al. 1999, Ott et al. 2002). Cytochrome c complex is a mediator of mitochondrial electron transport, and it is normally attached to the mitochondrial inner membrane. It is large in size and cannot normally cross the mitochondrial membrane. A hypothesis of how it can cross the membrane involves the opening of the mitochondrial permeability transition pore (MPTP) (Crompton 1999), (Figure 3). MPTP normally regulates calcium (and other ion) concentrations in mitochondria by letting extra ions out. MPTP is blocked by cyclosporin A, a fact that has been utilised in MPTP studies (Bernardi et al. 1994, Araragi et al. 2003). Opening of MPTP causes depolarisation of mitochondrial inner membrane and collapse of the mitochondrial membrane potential $\Delta\psi_m$. As protons stream to matrix, the osmolarity rises and mitochondria swell due to water influx (= permeability transition). Mitochondrial permeability transition has been reported in connection with mercury intoxications (Chavez et al. 1989, Bondy and McKee 1991, Levesque et al. 1992, InSug et al. 1997, Shenker et al. 1999, Belletti et al. 2002, Araragi et al. 2003), and it is most probably one of the major mechanisms causing mercury-associated apoptosis.

An initiative event leading to cell death may be the mercury-caused oxidative stress in cells (Yee and Choi 1994, Hussain et al. 1997, Ercal et al. 2001, Belletti et al. 2002), (Figure 3). Oxidative stress follows after excessive formation of reactive oxygen species (ROS), such as hydroxyl radical (HO.), superoxide radical (O_2.-), or hydrogen peroxide (H_2O_2), which are often derived from the incomplete reduction of molecular oxygen. Normally, cells take advantage of the capability of molecular oxygen to be easily reduced and activated (Davies 1995). Reactive molecules are produced in many physiological processes, e.g. electron transport chain in mitochondria, and CYP-mediated oxidation (Voeikov 2001, Droge 2002). Cellular antioxidants prevent these prooxidants from causing any damage to the cells, and the overall effect of the increased ROS depends on how well the cells are protected with antioxidant molecules (Gate et al. 1999, Nordberg and Arner 2001). Mercury, however, disturbs this delicate balance. Mercury depletes especially the thiol-containing antioxidants and enzymes, e.g. glutathione (Sarafian et al. 1996, Shenker et al. 2002). The overproduced ROS acts on several cellular targets such as lipids, proteins and DNA, and interferes with vital events such as signal transduction and gene regulation (Farber 1994). Therefore, increased ROS may cause many of the defects seen in mercury intoxication. Oxidative stress has been connected to the pathogenesis of many neuronal diseases, such as Parkinsonism and Alzheimer's disease (Christen 2000, Fiskum et al. 2003).

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Another major cellular pathway that mercury interferes with is calcium signalling (Figure 3). Mercury has been shown to increase the intracellular calcium level (Tan et al. 1993, Gasso et al. 2001). The importance of calcium comes from its pivotal role in regulating normal cellular metabolism (Berridge et al. 2000). Furthermore, calcium acts as a second messenger, and activates several enzymes and proteins. As calcium is such a strong mediator and activator of cellular processes, the concentration of intracellular Ca^{2+} is normally strictly regulated. At the same time, there must be a supply of Ca^{2+}, from where it can be fast and appropriately mobilised. The cells store calcium in the endoplasmic reticulum (ER) and transiently in mitochondria (Gill et al. 1989, Marhl et al. 2000). If the intracellular calcium level is high, the traffic in and out of mitochondria increases. The transporter antiport system, used to transport calcium, eventually depletes mitochondrial NAD^+ resources and leads to energy crisis, because ATP is depleted as well. This may be the reason for the mercury-induced energy crisis.

The actual calcium export pumps are also targets of mercury (Figure 3). Cells keep the intracellular Ca^{2+} concentration at a low level with the aid of calcium-binding proteins and calcium export pumps in plasma, ER, and mitochondrial membranes. Mercury can bind to these proteins just like to other proteins. By inhibiting the calcium export, intracellular $[Ca^{2+}]_i$ is retained high. Several calcium-linked proteases, endonucleases, lipases and calpains are activated (Frandsen and Schousboe 1993). The outcome of the activation varies. As mentioned earlier, caspase activation can lead to apoptosis. On the other hand, phospholipase activation can lead to necrotic cell death due to damages to plasma membrane or mitochondrial membrane. Calpains are especially interesting since they act on the cytoskeletal protein components (Fischer et al. 1991, Yokota et al. 2003). Methylmercury has been shown to cause microtubular disassembly (Graff et al. 1993, Miura et al. 1998, Castoldi et al. 2000), which can lead to various morphological changes seen in mercury exposure.

Neurotransmitters such as glutamate, the major excitatory transmitter of the brain, control and regulate cellular ion fluxes. One of the consequences of the activation of glutamate receptors is Na^+ and Ca^{2+} influx into the cells. If glutamate is not efficiently removed from the extracellular space, the neuronal activation is prolonged, and excess ion influx causes several toxic consequences (Kimelberg et al. 1989, Choi 1992). Glutamate uptake is an essential process in safeguarding the neuronal environment from the excitotoxic effects (Olney 1982, Choi 1992, Savolainen et al. 1995). Glutamate transporters are in part responsible for removing glutamate from outer cellular space, and they are themselves targets of mercury (Brookes 1992, Kim and Choi 1995, Nagaraja and Brookes 1996, Aschner et al. 2000).

In the brain, glial cells bear the main responsibility for the uptake of glutamate, and several types of glutamate transporters have been found in them (Rothstein et al. 1994, Kondo et al. 1995, Swanson et al. 1997, Sonnewald et al. 1997, Ye and Sontheimer 1998). This also applies to the retina, as the retinal glial cells, Müller cells, efficiently take up glutamate (Izumi et al. 1996, Lehre et

al. 1997). RPE cells, located between the choriocapillaries and the neuroretina, are other regulators of retinal glutamate concentration (Miyamoto and Del Monte 1994). The RPE cells are in close contact with photoreceptor outer segments and actually help to regenerate photoreceptors by phagocytising the old outer segments of photoreceptors (Kennedy et al. 1994). Glutamate regulates the phagocytosis of rod outer segments (Lopez-Colome et al. 1993). By interfering with the glutamate uptake of RPE cells, mercury disturbs the vital maintenance functions of vision. It may lead to vision defects reported in connection with mercury intoxications (Fox and Sillman 1979, Gobba 2000).

Neurotoxicity of aluminum

Aluminum is an abundant element, comprising 8% of the earth's crust. It is not an essential element for mammals, and until now it did not cause any major hazard to humans and other species, because it used to exist in a non-bioavailable form. During the last decades, acid rain has increased the bioavailability of aluminum, which has resulted in various destructive effects on biological systems (Driscoll 1985).

Furthermore, the characteristics of aluminum have made it a suitable material for various industrial uses. Human exposure comes mainly from drinking water (remnants of water purification processes), foods (food preparation and containers), cosmetics (antiperspirants) or pharmaceuticals (antacids). The absorption of aluminum from the gut depends on the chemical form of the aluminum compound and on the pH of the environment. In cell cultures, lipophilic Al species have been more toxic than hydrophilic ones (Kawahara et al. 2001). Generally, aluminum is poorly absorbed due to its tendency to form insoluble salts, especially with phosphates (Martin 1992). In the blood, aluminum binds avidly to the iron-transfer protein transferrin (Trapp 1983, Cochran et al. 1984, Roskams and Connor 1990, Hemadi et al. 2003). This fact strongly affects the distribution of aluminum to tissues, since there are receptors for transferrin in most tissues. The interaction with iron has many implications to aluminum toxicity.

Aluminum can pass the BBB and reach the brain (Bertholf et al. 1989, Julka et al. 1996, Yokel 2002). Aluminum crosses the BBB with the help of carrier systems, presumably with transferrin-receptor-mediated endocytosis and by using monocarboxylate transporters (Yokel et al. 1999). The accumulation of aluminum in the brain has been connected to the onset of various neurodegenerative diseases, such as Alzheimer´s disease (Henderson and Finch 1989, Exley 1999), Parkinsonism dementia (Hirsch et al. 1991, Altschuler 1999), dialysis encephalopathy (Brun and Dictor 1981) and amyotrophic lateral sclerosis (Gadjusek and Salazar 1982). The contribution of aluminum to these diseases remains controversial. There are epidemiological studies failing to

reveal a connection with the intake of aluminum (Savory et al. 1996). But even if aluminum is not the causative element, it may initiate events leading to diseases or accentuate the pathogenesis.

In the diseased brain, typical findings seen along with aluminum deposits are neurofibrillary tangles (NFTs) and senile plaques (Brun and Dictor 1981, Landsberg et al. 1993, Jellinger and Bancher 1998, Jones et al. 1998, Murayama et al. 1999). The major component of senile plaques is β-amyloid protein, which is aggregated abnormally in aluminum exposure (Kawahara et al. 2001). The principal constituent of NFTs is the paired helical filament (PHF), comprised of tau phosphoprotein (Scott et al. 1993, Li et al. 1998). One of the functions of tau is to stabilise the microtubule network inside the cell (Cleveland et al. 1977, Billingsley and Kincaid 1997). Both *in vivo* and *in vitro*, aluminum has been shown to induce hyperphosporylation of tau (Shin et al. 1994, Zatta 1995, Singer et al. 1997). Aluminum binds to hyperphosphorylated tau (PHFtau) proteins, and forms bridges between the phosphate groups of PHFtau, and as a consequence, PHFtau aggregates. In this way PHFtau cannot bind and stabilise microtubules anymore. Interestingly, beside aluminum, iron has been reported to accumulate in neurons with NFTs and induce aggregations of soluble PHFtau (Yamamoto et al. 2002). Iron has to be in the oxidised state (III) to induce the aggregation of PHFtau, just like Al^{3+}. Aluminum also enhances the ability of iron to cause ROS formation by β-amyloid (Bondy et al. 1998). Oxidative stress and the lipid peroxidation of membranes caused by this may be one of the causative events in neuronal destruction.

Beside the disruption of the cytoskeletal system, mechanisms underlying aluminum neurotoxicity are likely to involve many common pathways, partly similar with those of mercury. Inhibition of the activities of enzymes, such as choline acetyltransferase (Bilkei-Gorzo 1994), Na^+, K^+, Ca^{2+} ATPases (Rao 1992), glucose-6-phosphate dehydrogenase (Cho and Joshi 1989), and mitochondrial monoamine oxidase (Rao and Rao 1994), as well as increased activities of proteases (Guo-Ross et al. 1998), have been reported in connection with aluminum. Activation of cysteine proteases plays a role in the onset of apoptosis, and thus it is not surprising that aluminum has induced apoptosis in different cell systems (Scott et al. 1993, Li et al. 1998, Savory et al. 1999, Tsubouchi et al. 2001, Ghribi et al. 2002). Suarez-Fernandez et al. (1999) postulated that glial apoptosis may be one of the factors leading to neuronal destruction in neurodegenerative diseases.

Neurotransmitter signalling is yet another major pathway disturbed by aluminum. Glutamate-nitric oxide (NO)-cGMP pathway is one of the targets of aluminum (Canales et al. 2001, Rodella et al. 2001), and it may play an important role in glutamate-associated neurotoxicity. Aluminum activates NMDA channels and the increased Ca^{2+}, after binding with calmodulin, activates NO synthase. NO activates guanylyl cyclase and increases cellular cGMP content (Fedele and Raiteri 1999). Several important processes, such as the release of neurotransmitters (Provan and Yokel 1992) and synaptic long-term potentiation (Platt et al. 1995), are inhibited. Contradictory evidence has also

been found. Llansola et al. (1999) postulated that by inhibiting NO synthase aluminum may attenuate glutamate toxicity. However, in long-term exposures the glutamate-NO-cGMP pathway is severely impaired, which may connect aluminum with neurobehavioral deficits and impaired memory formation (Zheng and Liang 1998, Canales et al. 2001).

Aims of the study

In the present study, the neurotoxic effects of mercury and aluminum were assessed in cell cultures. In addition, new *in vitro* models were developed to evaluate the neurotoxic mechanisms.

The aims of the present study were

1   to evaluate, with several methods (WST-1, LDH leakage, apoptosis measurements), the basic cellular toxicity caused by mercuric mercury, methylmercury and aluminum in different cell culture models composed of neural cells

2   to study mercuric mercury toxicity in connection with glutamate uptake and to characterise the possible regulation pathway involving PKC

3   to evaluate the usefulness of GFAP induction as a marker of neurotoxicity in astrocyte cultures

4   to establish a novel *in vitro* BBB model constructed of human cells, and to evaluate the BBB-crossing properties of mercurial compounds and aluminum

Materials and methods

Cell cultures

Origin of the cells

Human RPE cell line ARPE-19, human neuroblastoma cell line SH-SY5Y, and human glioblastoma cell line U-373 MG were purchased from American Type Culture Collection, USA. Rat brain capillary endothelial cell line RBE4 was a gift from Dr. Michael Aschner, Winston-Salem, NC, USA, and human RPE cell line D407 was a gift from Dr. Hunt, Columbia, SC, USA. Eyes for setting up pig primary RPE cultures were obtained from a local slaughterhouse. Sprague-Dawley rat pups for setting up primary astrocyte cultures were supplied by the Laboratory Animal Unit of the Tampere University Medical School.

Primary astrocytes (II)

Primary astrocyte cultures were started from the cerebellar tissue of newborn rats (Holopainen et al. 1987). First, the isolated tissue was cleaned of the meninges and capillary structures. The tissue fragments were dissociated by passage through 80 µm nylon mesh. The resultant cell suspension was plated at the density of 1.6-2.4 x 10^4 living cells/cm^2. The isolation and growth medium was Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % foetal bovine serum (FBS), 2 mM glutamine and 50 µg/ml of antibiotic gentamycin. The cultures were allowed to grow and mature for two weeks in standard cell culture conditions (5% CO_2, humidified atmosphere, temperature + 37 °C). The cells were verified as GFAP-positive astrocytes by an immunofluorescence staining technique.

Pig RPE cells (III)

RPE cells were isolated from fresh pig eyes obtained from a slaugterhouse (Toimela et al. 1995). The opening of the eyes and the revealing of RPE cell layer were performed aseptically under a dissection microscope. The RPE cells were dissociated with trypsin incubation (trypsin was added into the eyecup). The isolated cells were counted and plated at the density of 5 x 10^4 cells/cm^2 in

DMEM supplemented with 20 % FBS, 5 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. The cells were cultured in standard cell culture conditions. Once they had settled and spread, the 20 % FBS was replaced with 10 % FBS. Reaching confluency usually took 11-14 days, after which the cells could be subcultured. Passages 2-4 were used in the studies.
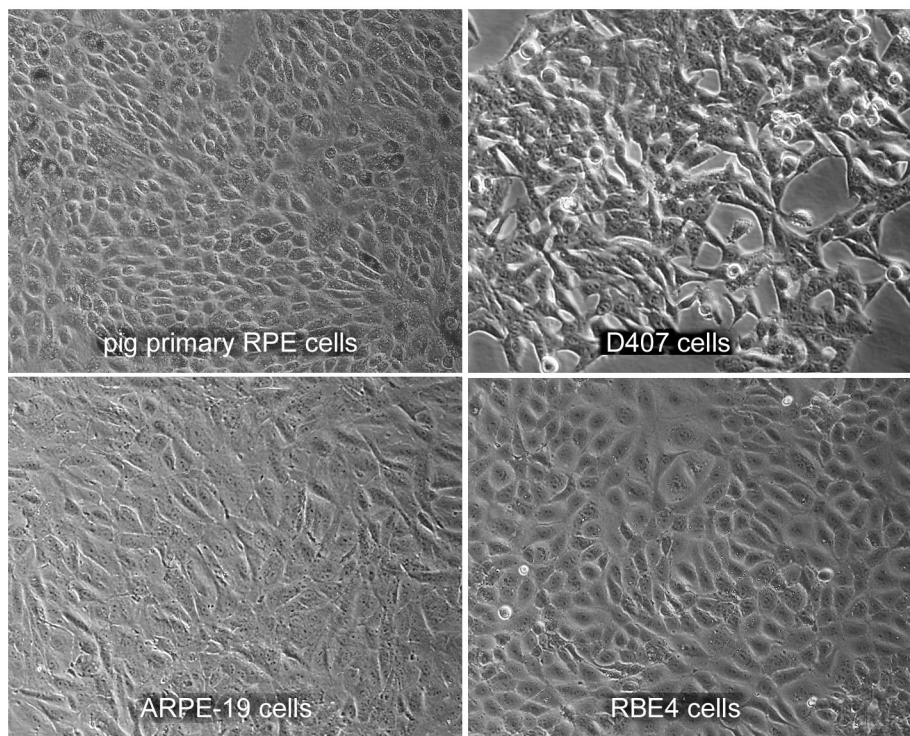
Figure 4. *The morphology of primary RPE cells and D407, ARPE-19 and RBE4 cell lines*

Human RPE cell line D407 (I,III)

Human D407 pigment epithelial cells were cultured in a high-glucose DMEM with 3% foetal bovine serum (FBS) in standard cell culture conditions. The cells were split 1:4 just before reaching confluency (usually once a week). Passages 70-79 of the cell line were used.

Human RPE cell line ARPE-19 (IV)

ARPE-19 cells were grown in standard cell culture conditions in 1:1 DMEM/Ham's F12 medium supplemented with 10% FBS, 1% antibiotic-antimycotic (AB-AM) solution containing 100 units/ml penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B, and the final concentration of 1.2 g/l of

27

sodium bicarbonate. The cells were split just before reaching confluency. Passages 35-38 were used.

Rat brain endothelial cell line RBE4 (IV)

The RBE4 cell line was grown in 1:1 Alpha Minimum Essential Medium (MEM)/Ham´s F10 medium supplemented with a final concentration of 1 ng/ml of basic fibroblast growth factor, 10 % FBS, 1 % AB-AM, 25 mM HEPES and 2 mM L-glutamine. The cells were split just before reaching confluency. Passages 5-7 (after receiving the cell line) were used.

Human SH-SY5Y neuroblastoma cells (I,IV)

The SH-SY5Y neuroblastoma cells were grown in standard cell culture conditions in 1:1 MEM/Ham´s F-12K medium supplemented with 1 % AB-AM, 10 % FBS, 2 mM L-glutamine and 0.1 mM non-essential amino acids. The cells were always split to low cellular density to prevent favoring epithelial-like morphology. Passages of 31-39 were used.
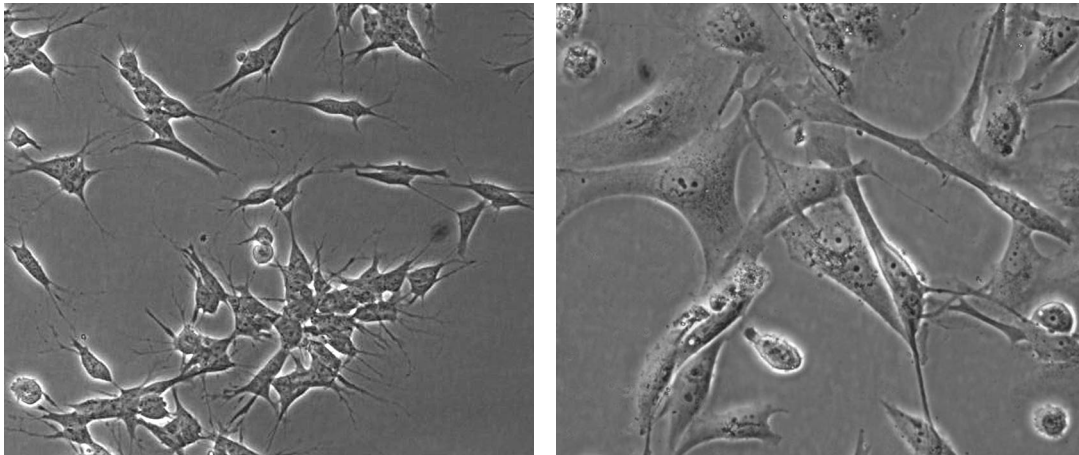
Figure 5. *SH-SY5Y neuroblastoma cells(left) and U-373 MG glioblastoma cells (right) in culture*

Human U-373 MG gliobastoma cells (I,IV)

The U-373 MG gliobastoma cells were cultured in standard cell culture conditions in MEM supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 % AB-AM, 1.5g/l sodium bicarbonate, and 10 % FBS. The cells were split just before reaching confluency. Passages 189-201 were used in the studies.

Preparation of metal solutions and exposure (I-IV)

Mercuric chloride was dissolved in distilled water to 20 mM concentration, and aluminum chloride was dissolved to 200 mM concentration. These stock solutions were then sterilised by filtering with 0.2 μm filter. Methylmercury chloride was dissolved in ethanol at 20 mM concentration. In the exposures, the same amount of the appropriate solvent was added into control cultures as into exposed ones. Special care was taken that the ethanol concentration in the cultures did not exceed 0.1% (v/v). The dilutions were made in the serum-free growth medium of each cell type. As an exception, in studies with BBB model, 1% FBS was used in the exposure medium, and with GFAP determination 10 % FBS in normal growth medium. The final metal concentrations in the exposures varied and depended on the exposure time and the type of experiment. Exposure concentrations for methylmercury and mercuric mercury varied between 0.01 – 100 μM, and for aluminum 1μM – 5 mM. When a recovery period was used, the exposure medium containing the study metals was replaced with a fresh medium with no metals, and the cells were further incubated until measurement.

Mitochondrial enzyme activity (WST-1 measurement) (I,III)

The cells were subcultured in 96 plates for a day before the experiment. Cell density for SH-SY5Y cells was 4×10^4 cells/well, for U-373 MG cells 0.6×10^4 cells/well, and for D407 RPE cells 1.5×10^4 cells/well. 0.1-500 μM methylmercury, 0.1-500μM mercuric mercury and 1-5000 μM aluminum were added into the wells, and incubation continued for the exposure time (15min, 24 h or 48 h). If cellular recovery was to be assayed, the medium was changed into a fresh one after 24 h exposure, and incubation continued for one more day. Mitochondrial cell viability was assayed by using WST-1 reagent. The WST-1 reagent was added into the wells. The cells were incubated with the reagent to let the enzyme reaction develop. The formazan dye thus formed was measured at the wavelength of 450 nm using Labsystems Multiskan MS microplate reader.

The results of WST-1 studies were used to calculate basic dose-response curves for each metal (when possible).

Apoptosis measurement (activation of caspase 3)(I)

Activated caspase 3 (CPP32) was determined with a fluorescent CaspACE Assay System. SH-SY5Y, U-373 MG and D407 cell cultures at similar densities as in the mitochondrial assays were exposed to 0.1-100 μM mercuric, methylmercury

and aluminum chloride for 6 h, 1 day and 2 days. After the exposure, the cells were collected by scraping. Cellular contents were lysed and extracted with a hypotonic lysis buffer containing 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM EDTA, 5 mM dithiothreitol, 2mM phenyl methyl sulfonyl fluoride, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. In the measurement of the activated caspase 3, the CPP32 substrate Ac-DEVD-AMC was added to the wells and incubated for 60 min. To assess the specific contribution of CPP32 enzyme in the reaction, part of the assays were performed in the presence of a selective inhibitor for CPP32 (Ac-DEVD-CHO). The fluorescence was monitored with Labsystems Ascent Fluorometer at the excitation wavelength of 355 nm and at the emission wavelength of 460 nm. The protein contents of the samples were determined with bicinchoninic acid (BCA) assay. The results were normalised and expressed as percent of control activity.

Protein measurement (I,II,III)

The protein content of the samples was determined by a bicinchoninic acid (BCA)-based modification of the Lowry method (BCA Protein Assay, Pierce, USA). Alkaline BCA-Cu^{2+} reagent was applied into wells containing protein samples. After appropriate incubation times (depending on the temperature and protein concentration), the colour formed was measured at 560 nm with Labsystems Multiskan MS microplate reader. Bovine serum albumin was used for calculation of standard curves. The sample dilution was chosen so that the linear area of the resultant regression line was used.

GFAP determination with ELISA (II)

After two weeks in culture, primary astrocytes were exposed to 0.01-10 μM methylmercury, 0.1-10 μM mercuric mercury and 10-1000 μM aluminum chloride, and then incubated for one week before the measurement of GFAP. In part of the exposures a four-day exposure was followed by a three-day recovery period. While stopping the exposure, the cells were treated with hot sodium dodecyl sulfate. GFAP was measured using a modified sandwich ELISA method (O'Callaghan 1991b). First, microtiter plates were coated with a polyclonal rabbit anti-GFAP. Unbound sites of wells were blocked with 1 % non-fat dry milk. Study samples were diluted and incubated in the wells for an hour. Next, a monoclonal mouse antibody was bound to the GFAP captured by the first antibody, and an enzyme-linked anti-mouse antibody was bound to the monoclonal antibody in the next step. After thorough washing, *p*-nitrophenylphosphate substrate was added, and the reaction was stopped 10 minutes later. The result was read at 405 nm using a Labsystems Multiskan ®

MCC/340 microplate reader device. For standard curve, pure GFAP samples were prepared at dilutions between 0,5 - 10 ng GFAP/100 µl.

LDH leakage (II)

The LDH results were collected from primary astrocyte cultures after one-week exposure to mercuric mercury, methylmercury and aluminum. In half of the experiments, this week included a recovery period. The recovery period was started after four days of exposure by changing the medium to a fresh one containing no study metals. The incubation still continued for three more days and was stopped at the same time as the continuous exposures.

Lactate dehydrogenase enzyme activity was measured from the cell growth medium of primary astrocytes in GFAP determination after exposure to mercuric mercury, methylmercury and aluminum. In the reaction (*pyruvate + NADH+H$^+$ ⇔ L-lactate + NAD$^+$)*, NADH + H$^+$ coenzyme was first preincubated with the sample. The reaction was started by adding the substrate pyruvate. The enzyme reaction was followed at 340 nm for 3 minutes, and the change in the enzyme activity per minute was calculated.

GFAP Immunocytochemistry (II)

The astrocytes were grown on plastic chamber coverslips for two weeks. The cells were exposed to 1 µM mercuric mercury for four days with a three-day recovery period. For staining, the cells were fixed in 4 % paraformaldehyde and in freezing acetone, and dried in air. The cultures were incubated with a dilution of monoclonal mouse GFAP antibody in PBS containing 1 % bovine serum albumin and 0.15 % Triton X-100. Anti-mouse IgG conjugated with fluorescein isothiocyanate was applied to the cell layer. The coverslips were mounted with 1:1 glycerol-PBS with an anti-bleaching agent.

Glutamate uptake (III)

Pig primary RPE cells were let to grow close to confluency before the glutamate uptake test. The test was performed in Krebs-Ringer-Hepes (KRH) buffer. The test compounds, 0.1-100 µM HgCl$_2$, 200 µM SC10, 100 µM chelerythrine chloride, 1.5 µM manoalide, 10 µM lavendustin A, 30 µM AP7, and 1770 U/ml heparin were incubated for 10 min before radioactive [^{3}H]-glutamate solution was added, and the incubation was stopped after further 10 min with ice-cold KRH. The cells were then dried and broken with 0.4 N NaOH. The radioactivity

inside the cells was counted by using LKB Wallac liquid scintillation counter, and the results were calculated against the protein content of the cells (expressed as specific activity).

Intracellular calcium determination (III)

D407 RPE cells were subcultured at 2×10^5 cells/96-well and allowed to grow for one day. For the measurement of intracellular Ca^{2+}, the cells were loaded for 1 hour with Fluo-3 AM under red light. After loading, the cells were left in dark for 30 minutes to metabolise the dye to fluorescent membrane-inpermeant free acid. The baseline fluorescence was measured before starting the measurements. 1-1000 µM concentrations of mercuric chloride were added into the wells with a built-in dispenser, and the signal was measured immediately. The reaction was monitored for further 3 min. Maximum signal (F_{max}) was obtained by adding the ionophore NP40 to the wells, and minimum signal (F_{min}) was obtained by adding the calcium chelator EGTA to the wells. The baseline and peak $[Ca^{2+}]_i$ were calculated according to the formula: $Ca^{2+}(nM) = (\Delta F-F_{min}/F_{max}-\Delta F)K_d$; $K_d= 390$ nM for fluo-3. Labsystems Ascent Fluorometer at excitation wavelength 485 nm and at emission wavelength 538 nm was used for the measurements.

Construction of barrier model (IV)

Transparent membrane filters were coated with rat-tail collagen on both sides. The ARPE-19 or RBE4 cells were plated on the filters at the densities of $3.5-4 \times 10^5$ cells/filter and the cultures were placed to grow in a standard cell culture incubator. In the model with glial cells, 2×10^4 glial U-373 MG cells were attached on the opposite side of the filter. The culturing continued with cells on both sides of the membrane filter. The development of confluency was microscopically observed and confirmed by the measurement of electrical resistance. In the resistance measurement, Ag/AgCl electrodes (Millicell-ERS) were first sterilised with ethanol and then equilibrated with the cell growth medium. For the measurement, the electrodes were placed so that one electrode was inside the filter cup and the other in the outside liquid. For calculating the results in Ω cm^2, the resultant value was multiplied by the membrane area after subtracting the blank values.
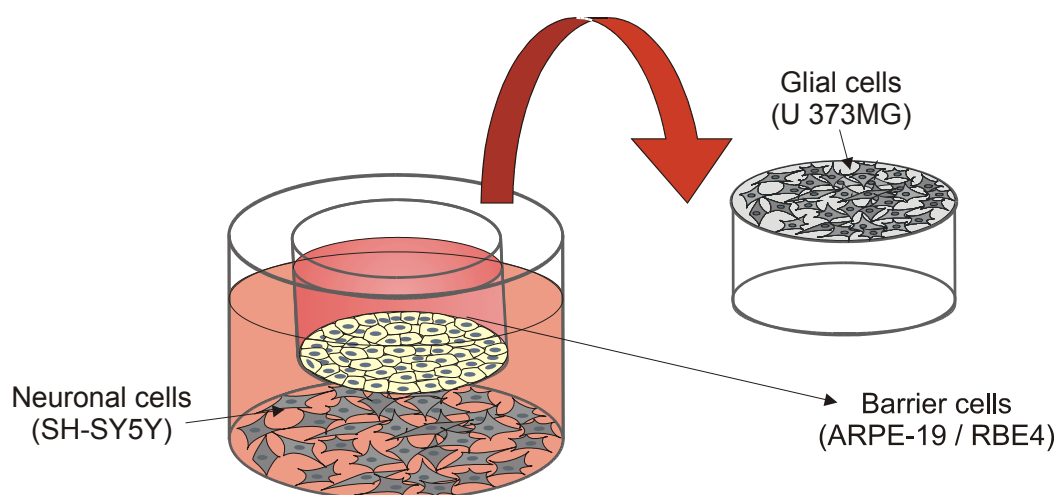
Figure 6. *Construction of the BBB model*

The target cells, SH-SY5Y neuroblastoma cells, were plated onto the bottom of culture plates at the density of 10 x 10^4 cells/cm^2. When the cell layer on the filters was confluent, the filter was placed on top of the SH-SY5Y cells. The medium was changed into one with low serum-content (1%), and the cultures were left to adapt for one day before the exposure to test chemicals, 0.1-100 µM methylmercury and mercuric mercury, and 1-1000 µM aluminum. Into the exposure medium, a fluorescent tracer was added along with the test metals. The leaking fluorecence was measured from the bottom well after 24 h with Thermo Labsystems Fluoroskan Ascent fluorometer at excitation wavelength of 485 nm and emission wavelength of 538 nm.

Total ATP determination (IV)

Total ATP was measured from all the cell layers of the *in vitro* barrier models to evaluate the amount of living cells. The initial procedure was different for each layer, but the measurement of the total ATP was performed in the same way. The filter cups containing ARPE-19 cells or RBE4 cells were moved to unused wells and handled separately. In the glial coculture models, the glial cells were first detached from the filter with trypsin treatment, and then treated separately.

To stop cellular activity before the measurement of the total ATP, ice-cold TCA was added and the plates were put into a -75ºC freezer. The freezing/thawing was repeated three times to release all ATP from the cells. For ATP measurement, a specimen of the thawed cell suspension was moved to a black microtiter plate, and luciferase reagent in appropriate buffer was added into all wells. Luminescence indicating the amount of ATP in the specimen was

measured with Thermo Labsystems Luminoskan Ascent luminometer with the integration time of 1000 ms.

Photographing (I)

Microscopic photographs were taken from neuroblastoma (SH-SY5Y), glioblastoma (U-373 MG), and SH-SY5Y/U-373 MG cocultures after 24-hour exposure to selected concentrations of methylmercury, mercuric mercury and aluminum. SH-SY5Y, U-373 MG cells, as well as cocultures of SH-SY5Y and U-373 MG cells were subcultured to 8.8 cm^2 dishes. The plating density for SH-SY5Y cells was 1 x 10^5 cells/cm^2 and that of U-373 MG cells 1.57 x 10^4 cells /cm^2, respectively. The density of both cells was halved for cocultures. After one day in culture, the cells were exposed to a fresh medium containing HgCl$_2$ (1 and 15 μM), CH$_3$HgCl (0.1 and 1 μM) and AlCl$_3$ (1 μM and 1 mM). Photographs were taken after 24 h incubation with inverted Olympus CK40 phase contrast microscope using Olympus DP10 digital camera and 200 x magnification.

Data analysis and calculations (I-IV)

Each biochemical experiment was repeated three independent times with 3-6 parallel samples at each concentration level. The results were collected and means and standard error of mean (SEM) of each sample were calculated with Microsoft Excel 97 or 2000 software. The statistical significances of the differences between controls and exposed cultures were calculated with Student's unpaired *t*-test or with ANOVA using Tukey's multiple comparison post test. The statistical analysis software used was GraphPad Prism 3.0.

EC$_{50}$ values were calculated with GraphPad Prism 3.0 with four-parameter logistic equation:
Y=Bottom + (Top-Bottom)/[1+10^((logEC50-X)*HillSlope)]
X = the logarithm of concentration
Y = the response
Y starts at the bottom and goes to top with a sigmoidal shape
Hillslope = the slope factor describes the steepness of a curve

Results

Cytotoxicity and apoptosis after exposure to mercury and aluminum

Mitochondrial WST-1 activity

The results with WST-1 showed a clear dose response after exposure to mercurial compounds in all the cell lines studied (SH-SY5Y neuroblastoma, U373 MG glioblastoma, D407 RPE). The toxicity also depended on the exposure time so that shorter times were less cytotoxic. The EC_{50} values suggested that 48 h exposure to methylmercury was the most toxic to neuroblastoma cells, with an EC_{50} value near to 0.8 µM. Glioblastoma cells could resist methylmercury and mercuric mercury toxicity up to markedly higher concentrations (48 h EC_{50} values around 7.5 and 14.0 µM, respectively). D407 cells were an exception in the sense that both mercurial species were equally toxic in 24 h exposures (EC_{50} ~10 µM), but in 48 h exposure methylmercury was markedly more toxic. The D407 cells were more sensitive than glial cells to methylmercury (48 h EC_{50} 7.5 µM for glia, and 2.2 µM for D407). A notable result was that methylmercury-induced changes were irreversible in all cells. The mercuric mercury effect was reversible; the decrease in EC_{50} stopped and some recovery of the enzyme activity/cell growth was evident. At low mercury concentrations there often was an increase in the enzyme activity before the decrease started. Especially methylmercury could activate the cells in both 15 min and 24 h exposures. With longer exposures the activation vanished.

Aluminum behaved in a different way, and no dose response curves with EC_{50} values could be calculated. There was a clear activation of mitochondrial enzyme activity, especially in U-373 MG glioblastoma and D407 pigment epithelial cells. The activation already started after 15 min exposure in these cells. The main difference was that the activity started at lower concentrations in D407 cells (1 µM) than in U-373 MG cells (1 mM). The activity was still abnormally high in 24 h exposure, after which it started to approach normal levels, most slowly in glial cells. In 48 h exposures there was no activation seen. On the contrary, there was a significant decrease in the activity at high concentrations.

LDH leakage

The activity of LDH enzyme was measured from the growth media of primary astrocyte cultures after exposure to 0.01- 10 μM methylmercury, 0.1-10 μM mercuric mercury and 10–1000 μM aluminum. LDH released from the cells indicates cell membrane damage or other cytotoxic injury influencing the integrity of cell membranes, also necrotic cell death. The results showed marked concentration-dependent release of LDH after one-week exposure. There was a clear difference between the two mercurial species, methylmercury showing more damage. Most striking was that the cells were not able to resist the methylmercury-induced leakage even when they were allowed to recover, which indicates permanent damage in the early days of the exposure. In 0.1-1 μM mercuric chloride exposure, the recovery period lessened the leakage, but at 10 μM concentration the leakage was higher in spite of the recovery. Aluminum was a totally different case. The recovery enhanced the LDH leakage a great deal. When present, aluminum did not induce high leakage up to 100 μM concentration.

Apoptosis

The activity of caspase 3 enzyme was studied in human neuroblastoma (SH-SY5Y), human glioblastoma (U-373 MG) and human RPE (D407) cell lines as a marker of apoptosis. Methylmercury, mercuric mercury and aluminum all affected the activity of caspase 3, but differently in both different cells, concentrations and exposure times.

In neuroblastoma cells, the apoptosis was evident at 0.1-1 μM concentrations of mercuric chloride, but not at higher concentrations. 0.1 μM methylmercury showed some apoptotic response after 24 h, but higher concentrations failed to yield any significant activation. Aluminum was totally inactive in neuroblastoma cells.

Glioblastoma cells were turned to go to apoptosis by all three metals, but with different time courses. The effect of 1 μM aluminum started after 6 h and was at its peak after 24 h. Higher aluminum concentrations were not as effective, although there was increased activity at 100 μM concentration. Also 1 μM mercuric mercury and 10 μM methylmercury seemed to induce the enzyme activity, both after 24 h incubation.

In D407 pigment epithelial cell line, both mercurial species were efficient apoptosis inducers; 1 μM (24 h) mercuric chloride induced the highest activity. Aluminum could induce apoptosis more slowly, and the activation was most prominent at 100 μM concentration in day two.

Beside activation, also inactivation of the caspase 3 enzyme was seen in the exposures. The phenomenon was most clear in 48 h exposures and happened with all study metals. D407 cells were most prone to great variations in caspase 3

activity; e.g. 10 μM mercuric mercury first induced profound activation after 24 h exposure, then the activity collapsed in 48 h exposure.

GFAP as a marker for neurotoxicity

The amount of GFAP was analysed with ELISA method in rat primary astrocyte cultures after one-week exposure to methylmercury, mercuric mercury and aluminum. Before the exposure, the presence of GFAP-positive astrocytes was verified with immunocytochemistry. The experiments were set up so that some of the cultures were allowed to recover (cells were cultured without the toxin).

The two experimental setups gave clearly different results. The induction of GFAP synthesis was evident with all study metals, but clearly only when there was a recovery time. In the 10 μM concentration of both mercury species, the GAFP level returned to control levels. This indicates that there was already irrecoverable damage in protein synthesis after the four-day exposure time at this concentration. Similarly, if the toxic agents were present for the whole time, the synthesis of new GFAP was not marked. The amount of GFAP naturally increases when the cells proliferate. To reveal the relative portion of GFAP in the cells, the calculations of GFAP were performed against the whole protein content of the cells.

Glutamate uptake and intracellular calcium after mercury exposure

Acute exposure of mercuric chloride reduced glutamate uptake dose-dependently in the pig primary RPE cell line. At 10 μM concentration the uptake was at its lowest. The effect was specific and not caused by cytotoxicity, since at the same concentration of $HgCl_2$, the cellular viability was still over 90 %. Glutamate uptake was investigated in the presence of the mediators and inhibitors of PKC–linked pathways. The direct activator (SC10) and the inhibitor (chelerythrine chloride) of PKC showed a clear effect on the uptake. The PKC activator could even overcome most of the 10 μM mercuric effect. The PKC inhibitor only reduced the uptake to 75% of control. The inhibitors of phospholipase A_2/C, tyrosine kinase, and inositol triphosphate (IP3) receptor, failed to give any major response to the uptake. The competitive antagonist (AP7) of the ionotrophic NMDA receptor did not show any effects on the glutamate uptake. The level of intracellular calcium increased dose-dependently after mercuric mercury exposure in D407 RPE cells. The increase came mostly from outside of the cell, since in calcium-free buffer the increase was not so prominent. However, at 1 μM concentration there was a statistically significant rise also in calcium-free buffer. This indicates calcium release from intracellular pools. At higher

concentrations the intracellular release was diminished. Heparin sulfate, supposed to inhibit Ca^{2+} release from intracellular pools, was able to inhibit gluamate uptake slightly, but the effect may have been overcome by the simultaneous larger influx of calcium from outside pools.

In vitro BBB models

Mercury and aluminum toxicities were investigated in three different BBB models. The toxic response was evaluated with the measurement of the total ATP level of the cells. This measurement was performed from all cell layers, although the actual target cells were neuroblastoma (SH-SY5Y) cells. A model with rat capillary endothelial (RBE4) cells was considered as a reference model. The models with human ARPE-19 cells were constructed with or without the presence of glial (glioblastoma U-373 MG) cells. For testing the integrity of the barrier, the leakage of a fluorescent marker and transepithelial resistance were also measured.

The electrical resistance measurements yielded dubious results and did not seem to be reliable in evaluating the barrier integrity. Therefore, visual inspection and fluorescein leakage were paramount in assuring the barrier quality. The fluorescent marker leakage measurements showed that glial cells help ARPE-19 cells to form a tighter cell layer, and when the RBE4 and ARPE-19 barriers were compared, the ARPE-19 barrier seemed to be more resistent to mercury and aluminum-induced loss of barrier integrity.

The results with total ATP meaurements showed that glial cells induced the growth of SH-SY5Y cells as well. RBE4 and ARPE-19 barrier models with glial cells gave quite similar results, when the effect of the different ATP levels of the cells was taken into account. I was most interesting was that methylmercury could go through the barrier before causing any major cytotoxicity in the barrier cell layers and that, apparently, also aluminum could go through the ARPE-19 cells (slight activation seen in SH-SY5Y cells). Inorganic mercury seemed to be retained in the barrier cell layer, and toxicity in the target cells only followed at high concentrations.

Morphology of cells in culture

Methylmercury, mercuric mercury and aluminum induced different morphological changes that were concentration-dependent. Most striking was the formation of fibrillary structures in glioblastoma cultures. After 1 µM aluminum exposure, the fibrilles were more oriented than after 0,1 µM methylmercury exposure. 1 µM methylmercury seemed to induce apoptosis-like membrane blebbings and cell shrinkage. 15 µM concentration of mercuric mercury incuded

clear swelling in glioblastoma cells. Photographs of GFAP-stained primary astrocytes exposed with 1 µM mercuric mercury also showed heavy fibrillary structure formation.

Discussion

In this thesis, mercuric mercury, methylmercury and aluminum were studied in different neural cell culture models. The aim was to assess their toxic potential and the mechanisms that could mediate the effects leading to neurotoxicity.

A special focus was set on methodological aspects. Novel *in vitro* models for studying the neurotoxicity of the study metals were established: an *in vitro* BBB model and an *in vitro* GFAP induction model.

Cell viability

Mitochondrial viability

The cytotoxicity results obtained with WST-1 assay were reviewed in connection with related experiments, e.g. in studies on apoptosis, LDH leakage, and morphological changes. The mitochondrial dehydrogenases that WST-1 measures are only active in living cells. Therefore, the WST-1 test method has commonly been used for the evaluation of the amount of living cells. However, the WST-1 test is based on a specific metabolic process, and toxins that disturb mitochondrial integrity influence the results. In short exposures, the results reflected mitochondrial activity and not so much a change in the amount of cells. In the longer exposures, there is the possibility that some cells divide and some die in the same culture. The cytotoxicity seen in longer cultures may also come from the inhibition of growth, not from actual cell deaths. Data of unexposed control cultures and knowledge of the characteristics of cells were invaluable in evaluating the results.

The mitochondrial aspect is interesting, since many toxins, including mercury and aluminum, accumulate inside the mitochondria (Chavez et al. 1989, Bondy and McKee 1991, Levesque et al. 1992, InSug et al. 1997, Shenker et al. 1999, Belletti et al. 2002, Araragi et al. 2003). The negative transmembrane potential of the inner mitochondrial membrane tends to draw small cationic metals towards it. Succinate dehydrogenases that the WST-1 test measures are localised in the mitochondrial inner membrane and are tightly coupled to the electron transport in the ubiquinone-cytochrome b site of the respiratory chain. This site of mitochondrial membrane has been shown to be the target of Hg^{2+} and methylmercury action in the studies of Lund et al. (1991) and Sone et al. (1977).

Succinate dehydrogenases may also be connected with aluminum toxicity. These enzymes exhibit iron-sulfur centers that can undergo Fe(II)-Fe(III) valence changes. Iron and aluminum are functionally alike, and aluminum has been shown to enhance the activity of iron-involving processes (Bondy et al. 1998, Atamna et al. 2002). Metals that can cycle in different valence states are especially deletoriuos, because they can start redox cycling and cause oxidative damage in the mitochondria (Campbell et al. 1999).

The interaction of aluminum and mercury with the mitochondrial enzymes may explain the puzzling results found in the WST-1 assay (also seen in Publication III). Sometimes, a clear and statistically significant activation was found. The activation was already evident after a short exposure time (15 min), which suggests that the cause was really the activation of enzymes and not cellular proliferation. The significance of the activation is obscure. Sone et al. (1977) claimed that mitochondrial succinate dehydrogenase resisted well the inhibitory effect of methylmercury. However, mercury has been shown to induce the transcription and activity of other enzymes, such as heme oxygenase-1 (HO-1) (Brawer et al. 1998, Kaliman et al. 2001). At the same time mercury triggers the uptake of iron into mitochondria (Brawer et al. 1998). Interestingly, also aluminum is connected to iron-involving processes and to HO-1 induction (Chmielnicka et al. 1994, Fulton and Jeffery 1994). The increase in the enzyme activities may be a protective effort against oxidative stress. Whether the mitochondrial activation seen in this study leads to increasing ATP production is not clear, since the total ATP level of the neuroblastoma cells increased with aluminum exposure (seen in the barrier model in Publication IV), but no significant mitochondrial activation was seen in these cells. However, the barrier model consisted of several cell types, whose interaction may play a role in the result.

Of the EC_{50} values obtained in this study, methylmercury was the most toxic, followed by mercuric mercury and aluminum. This was in agreement with the result of lactate dehydrogenase leakage in Publication II and with the results of other groups (Ng and Liu 1990, Costa et al. 1991, Voccia et al. 1994, Bohets et al. 1995, Kehe et al. 2001). The individual cell lines behaved in different ways, as could be expected from their different nature. In general, the neuroblastoma cell line was the most susceptible to methylmercury toxicity. Miura et al. (1994) postulated that neuronal cell lines are more susceptible to methylmercury toxicity than nonneuronal cell lines because they contain lower glutathione levels. The RPE cell line was an exception in that the toxicity was about the same for methylmercury and mercury in short exposures. One of the functions of RPE cells is to collect and accumulate toxic ions to protect the neural retina. The RPE cells have to tolerate high concentration levels of toxins that accumulate over time. The melanin pigment of RPE cells binds foreign compounds (Schraermeyer et al. 1999, Eves et al. 1999). RPE forms an outer blood-retinal barrier and functions in the same way as the BBB elsewhere in the brain (this ability of RPE cells was utilised in the development of the *in vitro* BBB).

In the brain, glial cells collect methylmercury and protect neurones (Aschner 1996). Methylmercury can be demethylated to mercuric mercury in the glia. An important finding was that neuroblastoma cells were not able to recover after methylmercury exposure. This implies that methylmercury, the form of mercury that usually reaches the brain, may be toxic to neuronal cells in a way that is irreversible.

The leakage of LDH

Leakage of the intracellular enzyme LDH from primary astrocytes was measured as a general indicator of cell damage in connection to the GFAP studies. LDH is released to the outside of cells when a toxic agent destroys membrane integrity. This can happen after severe cellular injury (necrosis) or earlier after membrane damage. When LDH activity was determined from cell growth medium, some of the toxic agent was present in the enzyme reaction. Mercury is known to inhibit the activity of enzymes. The concentration of mercury was diluted in the LDH measurement, but at high concentrations the result may be somewhat attenuated.

Primary astrocytes could keep their membrane integrity relatively well in low-level exposures. It may reflect the task of glial cells to sequester toxic metals. The astrocytes resisted mercuric chloride toxicity especially well. For glial cells it is advantageous to be able to tolerate mercuric mercury, since they metabolises methylmercury to mercuric mercury and store it inside them (Hansen et al. 1991, Pedersen et al. 1999). The results were in agreement with the findings of Publication I, where U-373 MG glioblastoma cells tolerated mercuric mercury much better than methylmercury. Also, methylmercury induced an LDH leakage that was even higher after the recovery time, which suggests irreversible damage, like in the WST-1 test. The effect of aluminum was also interesting. Even though these concentrations of aluminum are not supposed to be highly cytotoxic, significant increase of LDH activity was found, which indicates severe membrane damage. Similarly, in the studies of Suarez-Fernandez et al. (1999), aluminum needed longer exposure times to be effective.

Apoptosis

Apoptosis is a mechanism of cell death that calls for active participation of the dying cell (Wadewitz and Lockshin 1988, Bär 1996). Protein synthesis and sequential enzyme activity of several enzymes, such as cysteine-aspartate proteases, caspases is needed (Cohen 1997, Zhivotovsky et al. 1999). That is why the cells cannot be damaged to the utmost to go to apoptosis. The concentration of the toxin is important, as the same toxin can cause apoptosis at lower concentrations, and necrosis, i.e. uncontrolled cell death, at higher concentrations. Mercury acts just like that. The mechanism by which mercury may work in apoptosis is obscure, since mercury is a known inhibitor of enzyme

activity. It has a high affinity to the sulfhydryl groups of enzymes and proteins, which inhibits enzyme activity (Ozaki et al. 1993, Brawer et al. 1998). Direct enzyme inhibition may explain the results obtained in this study, since no clear dose-response (in that high concentrations induce more apoptosis) with either of the mercurial compounds was found. Part of the obvious cytotoxicity according to the WST-1 test may still originate from apoptosis, but because of the enzyme inhibition the result may be underestimated. The exact ratio of apoptosis and necrosis in the cell deaths cannot be concluded from the results. A test that measures a different parameter, e.g. the formation of DNA ladders, would be useful in determining the amount of apoptotic cells, but if caspases, the key executioners of apoptosis, are inhibited, the reaction cascade will not necessarily go to the end.

A probable cellular mechanism of apoptosis after mercury exposure starts from mitochondrial dysfunction (Nath et al. 1996, InSug et al. 1997, Matsuoka et al. 1997, Shenker et al. 1999, Castoldi et al. 2000, Makani et al. 2002, Belletti et al. 2002, Issa et al. 2003). Mitochondrial permeability transition has been reported in connection with mercury intoxications (Levesque et al. 1992, Shenker et al. 2000, Araragi et al. 2003). The interference of the mitochondrial transmembrane potential $\Delta\psi_m$ leads to cytochrome C release into the cytoplasm and thus causes the onset of apoptosis (Kluck et al. 1997, Ott et al. 2002, Lemasters et al. 2002, Wigdal et al. 2002, Chandra et al. 2002). The key factors in the process are the concentration and duration of the exposure. With longer exposure times, the interaction of mercury with proteins leads to protein synthesis inhibition, cell cycle arrest and cessation of cellular growth, and apoptosis is then no more the main death process (Zucker et al. 1990, Ponce et al. 1994). Also in this study, with longer exposures (48 h) and high concentrations, the decreased activity of caspase 3 enzyme may suggest that protein synthesis (e.g. production of caspases) had ceased.

In the brain, the induction of apoptosis depends on the interaction of glial cells and neuronal cells, e.g. the efficacy of glial cells in sequestering methylmercury. Glial cells act as mediators of toxicity, even though the actual targets are the neurons (Young 1992, Ohgoh et al. 2000). If glial cells die of apoptosis, buffering against toxic agents and support for neurons disappear. At the same time, the toxic ions that glial cells have accumulated are released into the environment. The consequence is that neurons are even more affected. The resistance of glial cells against apoptosis would be beneficial in preventing the propagation of neuronal destruction. In our apoptosis studies, glial cells did not go easily to apoptosis in mercury exposure. The highly cytotoxic 10 μM concentration of methylmercury was able to induce a statistically significant increase in the caspase 3 activity. Also in the WST-1 test, glial cells tolerated methylmercury quite well. Similarly, RPE cells buffer the toxicity of metals and act as a protecting barrier. The toxicity of mercury in the brain and retina is dependent on its ability to cross the BBB. Therefore, the integrity of the barrier is of great importance. Unfortunately, already 1 μM concentrations of both

mercurial species induced apoptosis in D407 cells. Any further conclusions are difficult to draw, because the results were highly varying.

In the neuroblastoma cells, methylmercury was not as great an inducer of apoptosis as mercuric mercury. However, the WST-1 test showed that neuroblastoma cells were extremely sensitive to methylmercury. Because no marked caspase 3 activity was seen, the death mechanism was likely to be non-apoptotic. The caspase 3 activity seen at low (0.1 μM) methylmercury concentration was interesting, since, at the same concentration, increased mitochondrial activity was seen in the WST-1 test. However, evidence for the suggestion that mitochondrial activation might predict the onset of apoptosis was not convincing.

Aluminum did not induce cell deaths in the WST-1 test, so it was not expected to induce much apoptosis either. In the WST-1 test, 1 μM aluminum concentration suggested toxicity in glial and D407 cells, and a significant induction of apoptosis at this same concentration was found, especially in glial cells. Also higher aluminum concentrations were effective. Glial cells seemed to be most prone to go to apoptosis in aluminum exposure. Apoptosis and aluminum have been connected in many earlier studies (Guo and Liang 2001, Savory et al. 2001, Ghribi et al. 2002), and there are suggestions that glial cells mediate neuronal degeneration by going to apoptosis (Young 1992, Suarez-Fernandez et al. 1999, Ohgoh et al. 2000).

GFAP as a marker for neurotoxicity

GFAP is the main subunit of the intermediate filaments of astrocytes (Eng 1988). When the brain encounters destructive damage, glial cells are activated and start to proliferate and fill the empty spaces (differentiated neurons are not able to do so). As GFAP is the main constituent of the glial microtubular structures, its amount in the brain naturally increases after reactive glial response (O'Callaghan 1991a, Eng and Ghirnikar 1994, O'Callaghan et al. 1995), but GFAP concentration inside the cells increases as well (O'Callaghan 1991a). This fact was utilised in developing a new *in vitro* GFAP induction model (Publication II), where the amount of GFAP was evaluated in astrocyte cultures after exposure to mercury compounds and aluminum. Primary astrocytes used in the study need time to mature, and immunoreactive GFAP can be seen after two-week culture. The exposure time was extended to one week to allow a *de novo* protein synthesis for GFAP to take place. Furthermore, the study was arranged so that in part of the study the toxic substance was removed after 4 days. This proved to be essential for the formation of GFAP. If the exposure continued for one week without withdrawal of the metal, no profound induction of GFAP synthesis was seen. This suggests that the presence of both aluminum and mercury may interfere with protein synthesis. Interestingly, both mercury species as well as aluminum could induce GFAP formation. Typically of primary cultures, the

variance was high between independent experiments (the newly started cultures always differ from each other). At 1 µM mercuric mercury concentration the induction was statistically significant. Photographs taken at this concentration also revealed a significant increase in GFAP immunoreactivity. Extensive formation of fibrillary structures was seen, as in the photographs of Publication I. This leads to the conclusion that fibrillary structures also appear after mercury exposure, and that glial cells are reactive in this sense. The postulation that GFAP induction is a sensitive marker for toxicity was shown to be true also *in vitro*. However, it is not clear how specific the effect is, since the study metals possibly influence microtubules anyway.

Possible mechanisms of mercury neurotoxicity

Glutamate is the major excitatory neurotransmitter of the brain and the retina. It is essential that the extracellular glutamate concentration is precisely regulated. Glutamate transporters keep the glutamate concentration at a desired level by taking up the excess glutamate (Olney 1982, Choi 1992, Savolainen et al. 1995). Glutamate transport has been studied extensively in the glial cells (Rothstein et al. 1994, Kondo et al. 1995, Swanson et al. 1997, Sonnewald et al. 1997, Ye and Sontheimer 1998). Other cell types also take part in the glutamate uptake. In the retina, RPE cells exhibit glutamate transporters (Miyamoto and Del Monte 1994, Mäenpää et al. 2004).

Mercuric mercury was shown to be a very efficient inhibitor of glutamate uptake in RPE cells. Similar effects have been reported earlier in glial cells (Brookes 1992, Kim and Choi 1995, Nagaraja and Brookes 1996, Aschner et al. 2000). The exact mechanism is not known, but it may again reflect the ability of mercury to bind thiol groups of glutamate transporters (Killinger et al. 1995). Glutamate regulates its own concentration by a mechanism that may involve metabotrophic glutamate receptors (Gegelashvili et al. 1996), (Figure 7). Metabotrophic glutamate receptors are functionally coupled with PKC, which can phosphorylate glutamate transporters directly (Casado et al. 1993). The present study gave support to the role of metabotrophic glutamate receptors in regulating the glutamate uptake in RPE cells. The evaluation of the interaction of PKC-connected intermediates revealed that PKC was involved in the regulation of glutamate uptake. By inducing PKC with its activator SC10, the glutamate uptake was mostly restored even in the presence of mercuric mercury. However, the results imply that PKC is not the only regulator of glutamate uptake, since the PKC inhibitor could not hinder glutamate uptake totally. On the other hand, mediators acting on the phopshoinositide pathway, e.g. inhibitors of PLC/A$_2$, IP3 receptor and tyrosine kinase, had no effect. Similarly, no effects were found with the NMDA receptor antagonist AP7, which suggests that NMDA receptors do not play a major role there. Nevertheless, RPE cells have been shown to contain NMDA receptors (Lopez-Colome et al. 1994, Fragoso and Lopez-Colome 1999).

One of the functions of the NMDA receptor is to transport Ca^{2+} into the cells (Figure 7).
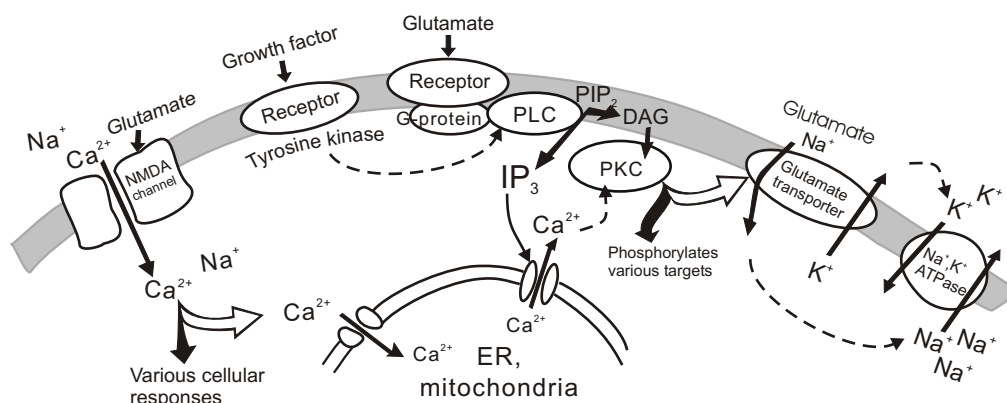
Figure 7. *Signaling pathways that affect glutamate uptake.*
PIP2= phosphatidylinositol 4,5-bisphosphate, DAG=diacylglycerol, IP3=inositol triphosphate

Intracellular calcium measurements showed that mercuric mercury could increase the calcium level inside the cells. This may be a major mechanism behind the mercury-caused toxicity, because of the importance of calcium as a second messenger and activator of various cellular processes. The elevation of calcium concentration was due to influx of extracellular Ca^{2+}, since no marked elevation was found in a calcium-free buffer. Nevertheless, at low concentrations intracellular calcium stores may also have played a role. Stout et al. (1998) suggested that mitochondrial calcium uptake is involved in the regulation of glutamate toxicity. The effect of mercury on the activation of calcium channels is controversial (Blazka and Shaikh 1991, Badou et al. 1997). In the study of Yallapragada (1996), both mercuric mercury and methylmercury inhibited calcium pumps.

Development of human cell BBB model

The significance of *in vitro* BBB models is a result of the need for testing the vast amount of new chemicals and drugs in industry (and eventually in the environment). Human neurotoxicity evaluation is especially difficult, since *in vivo* testing cannot be extensive, at least with the most toxic agents. That is why an *in vitro* BBB model built up with human cells is especially valuable for basic neurotoxicity testing. This was the basis for developing a BBB model of our own. There are no human brain endothelial cell lines commercially available. ARPE-19 cells used in this study are of human origin and have properties that suggest they are able to form a tight layer of cells in culture (Dunn et al. 1998).

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Also their functional properties may be suitable for the BBB model, since RPE forms a natural blood-retinal barrier in the retina (Dunn et al. 1996).

Transparent PTFE membrane filters were chosen as a substratum for the barrier cells, to enable visual inspection of the cells. As for barrier cells, ARPE-19 cells were compared with rat brain endothelial cells (RBE4), (Regina et al. 1998). The function of glial cells was to induce the formation of a tighter ARPE-19 layer, which was confirmed with the fluorescence leakage studies. The glial cells were cultured on the opposite side of the membrane filters. This fact caused some technical difficulties in the implementation of the studies and also necessitated the use of coating material on both sides of the filter. The greatest concern was how to ensure the integrity of the barrier layer, since transmembrane resistance results were very varying and did not seem reliable. Visual inspection of the cell layer in parallel with fluoresence leakage was utilised.

The cytotoxicity parameter measured from all cell layers was the total ATP content of the cells. The amount of ATP in various cell types is constant in normal conditions and thus reflects the cell amount (Lundin et al. 1986). The total ATP measurement usually gives results that are well comparable to WST-1 test. Under certain conditions, where ATP is depleted or the synthesis of new ATP is inhibited, the ATP measurement shows toxic metabolic response.

ARPE-19 cells proved to be as good a cell type for the barrier as RBE4 cells. The toxicity results gained with mercury compounds and aluminum were in good agreement with the other findings in this thesis, although direct comparison of the effective concentrations is hard due to differences in exposure systems. Methylmercury could easily cross the barrier and exert its effects on target cells before cytotoxicity was seen in the barrier. The barrier cells also seemed to retain mercuric mercury, and the toxicity in the target cells was only apparent at high concentrations, even when the barrier cells already seemed to suffer from the mercury effects and their level of total ATP was comimg down. Aluminum, on the other hand, could not induce cytotoxicity in the barrier cell layer, but activation of target cells was seen. This suggests that aluminum can somehow cross the barrier. A possible mechanism is the transferrin receptor-mediated endocytosis using clathrin-coated transport vesicles, a mechanism shown to be active in ARPE-19 cells (Tugizov et al. 1999). Aluminum is avidly bound to transferrin, and in this experimental setup some serum (and the transferrin therein) was present in the culture medium. Intrestingly, increased levels of ATP were seen at low concentrations of mercuric mercury and methylmercury in RBE4 barrier model. It suggests that the mitochondrial activation seen in WST-1 studies may also lead to increased ATP production.

The ARPE-19 cells appeared to be promising candidates for an *in vitro* BBB model. For further cytotoxicity studies, some technical improvements can be suggested. There are membrane insert materials that do not require coating (coating leads to decreased permeability of the filter). When glial cells are on the opposite side of the filter, the system is not easy to handle. The response could be measured from barrier cell layer containing glia on top of the cells, since

separate glial cells did not give any additional value to the results. Further characterisation of the features of ARPE-19 cells is also needed, e.g. what kinds of intercellular junctions and transport mechanisms the cells exhibit.

Methodological considerations

The type of cells and *in vitro* exposure parameters have a great impact on the results. Throughout the study, several cell types were used (neuronal, glial, RPE and endothelial), representing functionally different brain cells. Primary cells usually exhibit characteristics that are close to their *in vivo* counterparts. That is why primary astrocytes and RPE cells were used in GFAP induction and glutamate uptake studies. In cytotoxicity studies, on the other hand, cell lines are more manageable. The basic methods for measuring cytotoxicity are based on general parameters that are valid for all cells (e.g. total ATP, mitochondrial activity). The setting-up of primary cultures always produces a mixed cell culture, whose properties may vary from one isolation time to the next. Cell line properties remain relatively similar, if passage numbers are close to each other. However, one specific characteristic of transformed cell line cells had to be taken into account: they usually do not have contact inhibition and divide continuously in culture. Therefore controlling the cell growth parameters was essential. The density of cells was determined for each cell line in a pretest, where the cell growth was analysed thoughout the exposure time. As a rule, the density chosen was such that cells were allowed to grow near to confluency but the continuance of exponential growth was not compromised. This ensured that the cells were active and were not turned to a regression phase, where spontaneous cell deterioration could have started. The growth capabilities of each cell line varied considerably, and, as expected, primary cells grew more slowly than transformed ones.

Choosing the exposure times for the study metals depended on several parameters and varied in different experimental setups. *In vitro* exposure times are normally short, and the concentrations needed to see any effects are higher than in exposures *in vivo*. Long exposure times are problematic, because there is no turnover of the cell growth medium, and the concentration of the toxin decreases over time. The growth medium has to be refreshed during the culture if the exposure lasts for many days. In toxic concentrations this can distort the results. As a reaction to the toxin, the cells tend to round and detach from the substratum. These cells, possibly still living, can be lost when the medium is changed, and thus the toxicity induced by the compound is overestimated. This is one of the reasons why the basic exposure time in the studies was 24 h (I, IV). Shorter exposure times (10-15 min, 3 min) were used when studying acute toxicity (I) or specific effects (glutamate uptake or calcium release, III). The longest exposure time, one week, was used in the GFAP induction study to allow cells to be able to synthetise GFAP proteins (II).

Yet another parameter that had to be considered in each experiment was the composition of the cell growth medium, especially its serum content. The serum contains many growth and attachment factors for cells, and cell growth is generally enhanced. Serum components, mainly albumin and other proteins, can bind various substances and buffer the toxic effects (e.g. aluminum is known to bind to transferrin in the serum). Thus the toxicity of a chemical can be under-estimated, if the exposure is carried out in serum-containing medium. That is why short exposures ($\leq$ 48h) were performed in serum-free medium (I, III). An exception was the BBB model (IV). The exposure time was 24 h, but the medium contained 1% serum. It was essential that the cells should stay attached to the filter support to ensure the integrity of the barrier. If cells lose their attachment, they can respond by turning on the apoptosis. With longer exposure times (one week, II), the need for attachment factors and growth-promoting substances necessitated the use of serum.

The concentrations chosen were based on the literature and range-finding tests in each experiment. The aim was to use relevant concentrations that could be found in the brain *in vivo*. The experimental setup sometimes necessitated the use of higher concentrations, especially when dose-response curves were determined in WST-1 studies. Over 5 mM or 10 mg/ml concentrations were avoided, because the number of molecules would then have been too high and it would have influenced intracellular physiological parameters, such as osmolality. Moreover, the toxicity would not have been specific to the study compound, as any agent is toxic when applied in excess. Blood concentrations of the test chemicals measured from animals and human beings were a starting-point in deciding exposure ranges. The normal blood concentration of aluminum is ~0.6 µM and that of mercury <25 nM (Laboratoriokäsikirja 2004). When blood mercury concentrations exceed 90-100 nM, adverse symptoms begin to appear (Mariani et al. 1980, Göthe et al. 1985, Brune et al. 1991, Grandjean et al. 1998). For aluminum the corresponding concentration is 6 µM. However, concentrations in different tissues may vary greatly (Duval et al. 1986, Duval et al. 1987, Brune et al. 1991, Yokel et al. 1999). Due to the accumulation in certain tissues, the concentration can exceed several times the blood concentration (like in the case of aluminum and mercury). The physiological state and the age of the subject also cause variation. Children are extremely vulnerable to methylmercury toxicity (Geelen et al. 1990, Grandjean et al. 1998, Myers and Davidson 1998). Furthermore, the chemical form of the metal is important, e.g. some chemical forms of aluminum precipitate with phosphates and are practically not absorbed at all. The salt forms used throughout this work are chlorides of mercury or aluminum. These are easily disintegrated to their parent ions, and the cells have efficient ways to deal with Cl$^-$ ions. Several other salt forms of mercury and aluminum are available (lactate, citrate, maltolate etc), but their disintegration adds active components to the medium, and it may direct the cellular metabolism. In *in vivo* exposures, their use may be more justified because of their better absorption qualities. In this work, the concentration ranges

varied between 0.01 µM – 100 µM for mercuric mercury and methylmercury, and 0.1-5000 µM for aluminum.

In vitro methods in neurotoxicology - future aspects

Mercury and aluminum are examples of neurotoxic compounds that have been found toxic both *in vivo* and *in vitro*. Direct comparison of the *in vivo* and *in vitro* results is often not possible or even reasonable. In *in vivo* systems, complex cellular interactions and feedbacks affect the final toxicity. In *in vitro* studies, cellular variability is usually not so great; the system is more manageable and specific effects can be measured from any cells of interest. The most important advantage is that human cells and tissues can be used, and the extrapolation from animal results to humans is not needed. In the future, the emergence of multipotent stem cell lines may enable increasing use of normal differentiated cells in neurotoxicology research.

In vitro tests usually measure specific endpoints. The ultimate and easily seen endpoint is cell death, and most cytotoxicity tests evaluate the amount of living cells. Their drawback is that they usually measure some metabolic aspect of the cells. If the compounds interfere with the metabolic pathway measured, the results do not reflect the amount of cells, but their metabolic status. This was also seen in this study (in WST-1 measurements), and it suggests that one test is not necessarily enough to reveal the whole picture of the cytotoxicity. Different exposure times and conditions, as well as additional characteristics, such as cell morphology, plasma membrane integrity, and the ability for cellular proliferation, are valuable in interpreting the results.

Cell death and morphological alterations are severe damages. Specific endpoints that measure cellular responses at subtoxic concentrations are equally important in predicting the toxicity. Initiative events, such as intracellular calcium levels, formation of ROS, gene induction and activation of enzymes are easy to measure from cell cultures and reveal data that can predict toxicity at low doses. For studying neurotoxicity, a wide range of additional parameters should be used. The effect may be highly specific to certain cell types or only to strict targets (such as ion channels and transporters). Furthermore, the interaction with neighbouring cells may cause deviations in the result. In the brain, glial cells are able to activate and repair some of the damages. Neurons are more vulnerable, and damage to them is often irreversible. By attacking glial cells, mercury and aluminum extend their effects on neurons.

Ideal *in vitro* exposure models should mimic the *in vivo* exposure as closely as possible. Explant and slice cultures resemble the structure *in vivo* and are useful in specific neurotoxicity studies. The development of complex coculture models, perfusion techniques, and computer-aided monitoring enable the handling and analysing of more sophisticated exposure schemes. In addition, novel gene array techniques reveal the exact induction patterns of genes and will

direct the study to specific endpoints active in the cells studied. In the future, the value of *in vitro* tests will increase because of the growing public pressure for developing alternative tests to replace animal studies, and because of the legislative demands to support the use of such tests.

Summary and Conclusions

1.   The cytotoxicity of mercuric mercury, methylmercury and aluminum was studied with several methods: WST-1 assay, LDH leakage, and apoptosis studies. The general outcome was that methylmercury was the most toxic compound, followed by mercuric mercury and aluminum. The most striking and novel finding was the activation of mitochondrial dehydrogenases, especially in connection with methylmercury and aluminum at low concentrations. The activation may represent a toxic response equally significant as the deactivations usually seen in cytotoxicity studies. The results with WST-1 assay and LDH leakage suggested that methylmercury toxicity may be irreversible.

All the three metals could induce apoptosis. Interestingly, mercuric mercury, not methylmercury, was the most efficient inducer of apoptosis in neuroblastoma cells. In glioblastoma cells, all the metals were clearly effective, but especially striking was the effect of aluminum. The results gave support to the presumption that apoptosis of glial cells may be one of the initial steps in mediating neurotoxic effects.

2.   Mercuric mercury was shown to decrease the glutamate uptake in primary RPE cells. Glutamate is the major excitatory amino acid in the brain and in the retina, and its accumulation in the extracellular space can be the initiating event leading to excitotoxic damages in neighbouring neuronal cells. The results gave support to the presumption that metabotrophic glutamate receptors take part in regulating the transport of glutamate with pathways involving PKC. Another marked mercuric mercury effect was that the intracellular calcium level was rapidly increased from the extracellular calcium pools. The increasing intracellular calcium may also be one of the major mechanisms in mediating mercury toxicity.

3.   GFAP measurement was applied for the first time to cell cultures to predict toxicity. The GFAP synthesis was induced with exposure to mercuric mercury, methylmercury and aluminum. Aluminum is known to induce the formation of fibrillary structures in neurones. In this study, this was also indicated in astrocytes (also seen in the photographs of glial cells). The integrity of cellular structural filaments may be a sensitive target common to many toxic metals, since mercurial compounds were also active inducers of GFAP production.

4.   An *in vitro* BBB model was constructed, able to distinguish the toxicity of methylmercury, mercuric mercury and aluminum. The results were in good accordance with the knowledge of the characteristics of the study metals, which suggests that this model may be utilised in the future in predicting neurotoxicity and passage across the BBB. The model was built on membrane filters with RPE

cells on the top side and glioblastoma cells on the opposite side. Neuroblastoma cells were the target cells on the bottom of the model system. Glial cells induced the formation of a tighter barrier, and they were beneficial for the growth of neuronal cells as well. The major advantage of the system is that it was constructed totally of human neural cells. The use of continuous cell lines makes the handling easy and will thus allow larger-scale studies in the future.

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Original communications