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Neurotoxicity of Methylmercury:
Analysis of Molecular Mechanisms and Behavioral Alterations

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In memory of my beloved grandfather Vittorio

ABSTRACT

Methylmercury (MeHg) is a global pollutant that is toxic for the nervous system, especially during development. Contaminated fish is the main route of human exposure. Recent studies have indicated that neurological alterations in humans may occur even after exposure to fairly low levels of MeHg. Thus, the *in vitro* and *in vivo* studies reported in this thesis have focused on the neurotoxic effects of low doses of MeHg. A major aim was to characterize the intracellular pathways leading to neuronal and glial cell death. Previous reports have indicated that cerebellar granule cells (CGC) are a preferential target of MeHg and that astrocytes are also affected. Primary culture of CGC and the human astrocytoma D384 cell line were used as experimental models to investigate the cytotoxic effects of MeHg by analyzing cellular morphology and activation of pathways characteristic of apoptotic cell death. Doses of MeHg ranging from 0.1 to 1.5 μM induced apoptosis in CGC, as shown by cell shrinkage, dose-dependent nuclear condensation and high-molecular weight (HMW)-DNA fragmentation. However, MeHg did not induce activation of caspases, the major effector proteases activated during apoptosis. We detected a substantial activation of the calpains, Ca^{2+}-activated proteases. Antioxidant compounds protected CGC from MeHg damage, pointing to a key role of oxidative stress in MeHg-induced cell death. CGC undergo apoptosis when exposed to hydrogen peroxide (H_2O_2) or colchicine. While H_2O_2 did not activate caspases, colchicine induced cytochrome *c*-mediated activation of caspase 3. Together these data suggest that the activation of caspases is not always required to induce morphological changes and pattern of DNA fragmentation consistent with apoptosis. We therefore have examined possible caspase-independent pathways leading to chromatin rearrangement, focusing on the mitochondrial Apoptosis-Inducing Factor (AIF). This flavoprotein, by translocating into the nucleus, induces both chromatin condensation and HMW-DNA fragmentation in a caspase-independent manner. Translocation of AIF into the nucleus was observed in CGC exposed to MeHg for 16 h, suggesting that AIF may be responsible for the caspase-independent chromatin rearrangement induced by MeHg.

The mechanisms of MeHg-induced cell death were also investigated in glial cells. Since MeHg increases free radical generation and lysosomes are sensitive to oxidative stress, we looked at lysosome stability in D384 cells exposed to MeHg and H_2O_2. Lysosomal rupture was detected shortly after MeHg exposure in cells maintaining plasma membrane integrity. Disruption of lysosomes was also evident after exposure to H_2O_2. The lysosomal alterations induced by MeHg and H_2O_2 were persistent and preceded a decrease of the mitochondrial membrane potential and the appearance of apoptosis. Our data support the hypothesis that lysosomal membranes represent a possible target of agents causing oxidative stress, and that lysosomal hydrolytic enzymes may act as executor/regulator factors in apoptosis induced by MeHg and H_2O_2. In conclusion, our *in vitro* studies show that MeHg induces cell death by caspase-independent apoptotic pathways both in CGC and in glial D384 cells and that oxidative stress plays a key role in MeHg cytotoxicity.

Another aim of this work was to study the effects of prenatal exposure to low level of MeHg on behavior during early ontogeny in rats. For this purpose, locomotor activity, learning and memory were evaluated. Prenatal exposure to MeHg induced a slight increase in spontaneous locomotor activity in male and female rats at 14 days, but not at 21 days. When rats where stimulated with the dopamine D_2 receptor agonist U91356A, a lower dopamine-mediate locomotor activity was observed in the 21 day-old male rats exposed to MeHg, with no significant difference in the females of the same age. These results indicate that gender plays an important role in the susceptibility to MeHg, in agreement with previous observations in adult rats. Futhermore, our data show that MeHg alters dopamine receptor-mediated locomotor activity, and that dopamine D_2 receptors are involved. The gender-dependent behavioral alterations were evident at the age of 21 days, a time period crucial for the ontogeny of dopamine receptors in the rat brain. Therefore, we performed additional studies on 21 day-old male rats using apomorphine, a non-selective agonist of dopamine receptors. Apomorphine-stimulated locomotor activity was significantly higher in exposed animals. This result further confirmed that MeHg induces changes in dopaminergic transmission. MeHg-exposed rats were also analyzed with the Morris swim maze test at 2 months of age and displayed a deficit in retention as compared to control rats. Our *in vivo* studies show that prenatal exposure to MeHg impairs dopamine-mediated locomotor activity and memory functions.

LIST OF PAPERS

This thesis is based on the following publications and manuscripts, which will be referred to by their Roman numerals in the text.

I. **Daré, E.**, Götz, M.E., Zhivotovsky, B., Manzo, L., Ceccatelli, S. (2000)
Antioxidants J811 and 17β-estradiol protect cerebellar granule cells from methylmercury-induced apoptotic cell death.
Journal of Neuroscience Research, 62: 557-565.

II. **Daré, E.**, Gorman, A.M., Ahlbom, E., Götz, M., Momoi, T., Ceccatelli, S. (2001)
Apoptotic morphology does not always require caspase activity in rat cerebellar granule neurons.
Neurotoxicity Research, 3: 501-514.

III. Fonfria, E., **Daré, E.,** Benelli, M., Suñol, C., Ceccatelli, S. (2002)
Methylmercury induces release of Apoptosis-Inducing Factor (AIF) from mitochondria in cerebellar granule cells.
Submitted to European Journal of Neuroscience.

IV. **Daré, E.**, Li, W., Zhivotovsky, B., Yuan, X.M., Ceccatelli, S. (2001)
Methylmercury and H_2O_2 provoke lysosomal damage in human astrocytoma D384 cells followed by apoptosis.
Free Radical Biology & Medicine, 30: 1347-1356.

V. Giménez-Llort, L., Ahlbom, E., **Daré, E.**, Vahter, M., Ögren, S-O., Ceccatelli, S. (2001) Prenatal exposure to methylmercury changes dopamine-modulated motor activity during early ontogeny: age and gender-dependent effects.
Environmental Toxicology and Pharmacology, 9: 61-70.

VI. **Daré, E.**, Fetissov, S., Hökfelt, T., Ögren, S-O, Ceccatelli, S. (2002)
Effects of prenatal exposure to methylmercury on dopamine-mediated locomotor activity and dopamine D_1 and D_2 receptor expression.
Manuscript.

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ABBREVIATIONS

AIF	Apoptosis-Inducing Factor
AO	acridine orange
Apaf-1	Apoptotic protease activating factor-1
CGC	cerebellar granule cells
Endo G	endonuclease G
$\Delta\Psi_m$	mitochondrial membrane potential
GSH	glutathione
HMW	high molecular weight
H_2O_2	hydrogen peroxide
HSPs	heat shock proteins
IAPs	Inhibitors of apoptosis
MeHg	methylmercury
MPT	mitochondrial permeability transition
MTT	metallothioneins
PBS	phosphate-buffered saline
ROS	reactive oxygen species
SH-group	sulphydryl-group
SOD	superoxide dismutase
TMRE	tetramethylrhodamine ethyl ester

1 INTRODUCTION

Mercurial compounds are major environmental pollutants. Substantial emission of mercury occurs naturally from volcanoes, oceanic sediments, crust degassing and by forest fires. Anthropogenic sources of mercurials comprise mining, cloroalkali manufacturing and combustion of fossil fuels. Once released in the environment, these compounds enter the global cycle of mercury (Morel et al., 1998). Organic mercury originates from inorganic mercury in rivers, lakes and oceans, likely as the result of the methylating activity of sulfate-reducing bacteria (Morel et al., 1998). In the aquatic food chain methylmercury is bioaccumulated and biomagnified. The highest levels are found in predator fish, especially in the liver, brain, kidney and muscle (Clarkson, 1972; Morel et al., 1998). Thus, fish, shellfish and sea mammals are the main source of human exposure to methylmercury (EPA-US, 1990).

1.1 METHYLMERCURY POISONING

While the toxic effects of inorganic mercury were known since the antiquities (Clarkson, 1972), the devastating consequences of the exposure to organic mercury compounds were discovered only in the 19th century. In 1865, a few years after the first synthesis of methylmercury by Frankland (1850s), two chemists died and one was poisoned after spilling dimethylmercury in the laboratory floor (Clarkson, 1998). At the beginning of the 20th century, methylmercury found an important application in agriculture as a fungicide, consequently its manufacturing increased, resulting in occupational exposure. In 1938 neurological symptoms were reported among factory workers, providing the first accurate description of signs and symptoms of methylmercury poisoning (Hunter et al., 1940). The autopsy of one of these workers was the source of the first histopathological analysis (Hunter and Russell, 1954), which identified morphological changes in the brain with a striking loss of cells in the cerebellum.

In the 1950s and early 1960s two mass health disasters occurred in Japan, first in Minamata, then in Niigata. According to estimations performed in 1999, 2263 cases of methylmercury poisoning were identified in the area of Minamata Bay, and 690 cases in Niigata. In both cases people were intoxicated through seafood. Methylmercury was released

in the environment by acetaldehyde manufacturing plants and accumulated in fish and shellfish in the aquatic food chain. In the region of Minamata Bay, neurological alterations were reported predominantly among fishermen and their families. Patients presented with sensory disturbances in the distal part of extremities (parestesia of feet and hands in the "stocking and glove" areas) and periorally, followed by ataxia, bilateral concentric constriction of the visual fields, deafness and impairment of speech. Tremor, abnormal eye movement and occasionally, mental disorders and disturbances of taste and smell, were also present. In cases of severe intoxication, the patients became incapacitated and died. The syndrome caused by methylmercury poisoning was called Minamata disease (Tsubaki and Takahashi, 1986).

The histopathologic changes associated with methylmercury exposure were predominantly related to the nervous system, especially the cerebral cortices, cerebellar cortices and peripheral sensory nerves. Among Minamata patients, acute fatal cases showed loss of neurons with glial cell proliferation, microcavitation, vascular congestion and edema in the cerebral cortex (Takeuchi, 1968), whereas cases with acute onset and prolonged survival showed lesions predominantly localized in the calcarine and cerebellar cortices. The cerebellum showed characteristic lesions in the deeper portions, with loss of granule cells and relatively well-preserved Purkinje cells. The pathological lesions in the visual cortex and in the cerebellum correlated with the visual disturbances and the ataxia displayed by the patients clinically.

During the methylmercury outbreaks in Japan, the first cases of prenatal poisoning were reported, describing pathological findings in the brains of fetuses (Matsumoto et al., 1965; Takeuchi et al., 1964). The human prenatal brain displayed disruption of the cytoarchitecture, appearing more sensitive to methylmercury than the adult brain. Symptoms of prenatal exposure included mental retardation, ataxia, spasticity and blindness. High exposure resulted in severe mental retardation and death, whereas moderate exposure was associated with impairment of motor skill.

Another severe methylmercury outbreak took place in the early 1970s in Iraq, when seeds treated with methylmercury for agriculture purposes were used instead for baking bread. This resulted in the poisoning of thousands of people, with 459-hospital mortalities (Bakir et al., 1973). The description of symptoms and the pathology was in agreement with

previous findings reported during the poisoning in Minamata. In addition, prenatal exposure to methylmercury was found to inhibit neuronal migration (Choi et al., 1978).

In recent years, man-made methylmercury contamination has decreased considerably, due to improvements in industrial manufacturing and efforts to minimize the release of mercury in the environment. However, mercury is still a global pollutant and there are regions in the world, primarily in developing countries, where the levels of environmental contamination remain high. Mild neurological symptoms characteristic of methylmercury intoxication, such as sensory disturbance (especially glove-and-stocking type), tremor, two-point discrimination and slight balancing failure, have been reported recently among residents in fishing villages located in close proximity to gold mines in the Brazilian Amazon, consuming methylmercury-contaminated fish (Harada et al., 2001). Certain coastal areas of the Mediterranean Sea display high level of mercury in seafood, possibly in relation to the presence of enormous natural cinnabar deposits (Renzoni et al., 1998), but also as a consequence of gold mining (Horvat et al., 1999). Some lakes and coastal areas in Sweden and Finland are also contaminated, mainly as a result of the past mercury emissions from chlorine alkali industries and the earlier use of mercury as preservative in the pulp and paper industry (Skerfving et al., 1999). As a consequence, there have been recommendations in Sweden for restriction in fish consumption, especially for women in child-bearing age. Contamination of fish has resulted in fish advisories in several states in USA and in Canada (Mahaffey, 1999).

The possibility that exposure to low doses of methylmercury can have adverse consequences for the nervous system, especially during prenatal life, has given rise to a number of studies on fish-consuming populations. These investigations have produced conflicting results (Clarkson, 1998; Myers et al., 1998), but have not excluded the possibility that subtle effect in the nervous system, such as developmental delays, may be associated with exposures previously deemed safe. Studies attempting to correlate neurological symptoms and the mercury level in adult fish-consumers have given mostly negative outcomes (WHO, 1990). Neurological effects in children due to prenatal exposure, however, were reported by a study performed in Canada (McKeown-Eyssen et al., 1983), showing that maternal hair mercury levels correlated positively with abnormal muscle tone in male children. Another investigation performed in New Zealand (Kjellström et al., 1989) showed an inverse correlation between maternal mercury hair levels and IQ in their children.

Grandjean *et al.* (1997) have reported an association between mercury exposure through a fish-rich diet and neuropsychological dysfunctions in the domains of language, attention and memory. Visuospatial and motor functions were less impaired. In contrast, a parallel study performed at the Seychelles Islands did not find any correlation between exposure to methylmercury through diet and neurological alterations (Myers et al., 1995; Myers et al., 1997). The different outcomes of these studies may depend on other components present in the diet, either beneficial or toxic, ethnic differences between the two populations, the type of tests performed, and the age of children at the time of testing (Mahaffey, 1999). Above all, mercury exposure in the population of the Faroe Islands is dependent on eating fish and pilot whales, which contain rather high concentrations of methylmercury, resulting in peaks of exposure to fairly high levels of methylmercury during certain periods. For the population of Seychelles Islands, instead, sea-fish containing fairly low levels of methylmercury is the major component of the diet.

1.2 METHYLMERCURY ABSORPTION, DISTRIBUTION AND EXCRETION

Absorption rates do not vary greatly from one animal species to another. Ingested methylmercury is nearly completely absorbed in the gastrointestinal tract by both humans and experimental animals (Clarkson, 1972). Organic mercury deposits in the various organs, including blood, kidney and brain (Swensson and Ulfvarson, 1968). In humans, the brain-to-blood ratio is about 6. More than 90 % of blood methylmercury is in the red blood cells. Methylmercury appears to be bound to cysteine residues in hemoglobin (Takeda et al., 1968). The fraction of methylmercury bound to red blood cells varies depending on the species. The red cells-to-plasma ratio is about 20 in humans, 10 in monkeys and mice, and is the highest, at 300, in rats (Clarkson, 1972). This ratio correlates with the brain-to-blood concentration, possibly because the binding to the blood cells retards the diffusion of methylmercury and its entry into the brain. Thus, rats have the highest blood-to-brain ratio, while primates have the lowest. This relevant difference in brain uptake should be taken into account when choosing the dose of exposure for animal experimentation. Methylmercury distributes to all area of the brain by crossing the blood brain barrier through mechanisms not fully characterized, possibly utilizing the neutral amino acid carrier system as a methylmercury-cysteine complex (Aschner and Aschner, 1990; Mottet et al., 1997). Studies

performed with differential centrifugation have shown that methylmercury does not display a specific sub-cellular distribution, since it is present in all fractions (nuclear-, mitochondrial-, lysosomal- and soluble fraction) (Clarkson, 1972).

Methylmercury also crosses the placental barrier reaching the fetus (Vahter et al., 2000). Moreover, it is excreted in milk, and thus can reach the child during breastfeeding (Grandjean et al., 1994; Oskarsson et al., 1996). Following methylmercury exposure, mercury compounds are excreted mainly via the kidney and the gastrointestinal tract. Demethylation of methylmercury, occurring mostly in the liver, is a key step in the excretion process. Both methylmercury and the inorganic mercury formed in the liver are excreted in the bile conjugated with glutathione and related compounds (WHO, 1990). However, methylmercury is reabsorbed in the intestine, undergoing enterohepatic recirculation. The rate of methylmercury excretion is species dependent, resulting in a different body burden in animals exposed to the same level of methylmercury. The excretion rate is directly proportional to the simultaneous body burden in both humans and experimental animals. The half-time of excretion varies in different species (70 days in humans, 16 days in rats, 8 days in mice and 1000 days in fish and seafood) (Clarkson, 1972). Demethylation of methylmercury seems to also take place in the brain, as shown by the slow accumulation of inorganic mercury in lysosomes that follows methylmercury exposure (Clarkson, 1972). However, the mechanisms responsible for this process have not yet been identified (Mottet et al., 1997). The formed inorganic mercury has a very long half-life in the brain, especially in thalamus and pituitary (Vahter et al., 1995). Inorganic mercury produced by demethylation may be sequestered by metallothioneins (MTTs), a family of cysteine-rich proteins that binds to metals (Cd, Zn and Hg) with high affinity. MTTs have been found expressed in different organs, including the brain (Hidalgo et al., 2001; Nordberg and Nordberg, 2000).

Methylmercury exposure can be evaluated by measuring mercury levels in the blood or in scalp hair. After the outbreak in Niigata (Japan), a Swedish expert group identified the lowest effect levels in blood and hair, corresponding to 200 μg Hg/l of whole blood and 50 μg Hg/g of scalp hair (Swedish-Expert-Group, 1971). Moreover, studying the relationship between intake of methylmercury via fish and blood level in Sweden, they estimated that an average daily intake of 300 μg Hg as methylmercury would result in a

maximum blood level of 200 μg Hg/l. These evaluations were used by the World Health Organization (WHO) to estimate the "tolerable weekly intake of methylmercury", that is 30 μg Hg, corresponding to a blood level of 20 μg Hg/l and hair level of 5 μg Hg/g (Joint-FAO/WHO-Expert-committee, 1972). The analysis of cases of methylmercury poisoning in Iraq have shown that there is a direct correlation between the peak level of mercury in maternal scalp hair during pregnancy and psychomotor retardation in the offspring (Cox et al., 1989). The critical hair level in pregnant women for effects on the central nervous system of the fetus has been estimated to be 10-20 μg Hg/g or even lower (Mahaffey, 1999; Skerfving et al., 1999). The Committee on the Toxicological Effects of Methylmercury (National Research Council, USA) has recently stated that the current reference dose proposed by the US Environmental Protection Agency (EPA) for methylmercury, that is 0.1 μg/kg per day, is a scientifically justifiable level to protect public health (NRC, 2000). The average concentration of mercury in fresh and marine fish is approximately 0.2 mg/kg. In practical terms this means that an average person weighing 60 kg can only consume approximately 30 g of fish per day. However, a diet rich in fish has also well-known beneficial effects for human health (Hallgren et al., 2001). Thus, more effort should be focused in reducing the level of environmental pollution, by limiting mercury emission.

1.3 MECHANISMS OF METHYLMERCURY CYTOTOXICITY

Methylmercury exerts its toxic effect via three major mechanisms: i) the perturbation of intracellular Ca^{2+} levels; ii) the induction of oxidative stress; and iii) the interaction with sulphydryl (SH) groups of other mojeties.

The intracellular concentration of Ca^{2+} in the cytosol is tightly regulated (Berridge et al., 2000). A complex system of pumps and channels located in the plasma membrane and in the membrane of organelles allows the cells to keep Ca^{2+} homeostasis. Intracellular Ca^{2+} is stored mainly inside the endoplasmic reticulum. Transient and limited fluctuations of intracellular Ca^{2+} have important signaling functions, whereas prolonged and large elevations are cytotoxic and can lead to cell death (Berridge et al., 2000; Duchen, 2000). The mitochondria play an important role in regulating Ca^{2+} by taking it up during signaling. Massive release of Ca^{2+} in the cytosol due to impairment of mitochondrial functions has

catastrophic consequences for the cell, resulting in activation of Ca^{2+}-regulated proteases, e.g. calpains, and Ca^{2+}-dependent endonucleases. One route for the release of Ca^{2+} from the mitochondria is the opening of the mitochondrial permeability transition (MPT) pore (Bernardi et al., 1993). This is a megachannel formed in the inner mitochondrial membrane during adverse conditions, e.g. elevation of free radicals and increased Ca^{2+} levels in the mitochondrial matrix. The opening of the MPT pore allows the passage of Ca^{2+} and other solutes $\leq$ 1.5 kDa, and it dissipates the proton gradient across the inner mitochondrial membrane, resulting in the loss of the mitochondrial potential ($\Delta\Psi_m$).

Exposure to methylmercury has been shown to increase intracellular Ca^{2+} in several cell types (Atchison and Hare, 1994; Graff et al., 1997; Marty and Atchison, 1997; Marty and Atchison, 1998; Sarafian, 1993). In cerebellar granule cells (CGC), the alterations appear to relate initially to Ca^{2+} mobilization from intracellular stores (first phase), followed by Ca^{2+} entry through membrane channels (second phase) (Marty and Atchison, 1997). In accordance, both Ca^{2+}-chelators and Ca^{2+}-channel blockers have been shown to ameliorate the damage caused by methylmercury (Atchison and Hare, 1994; Gasso et al., 2001; Marty and Atchison, 1997; Marty and Atchison, 1998; Sakamoto et al., 1996; Sirois and Atchison, 2000). Effects possibly due to the action of elevated Ca^{2+} as a second messenger, e.g. increased inositol phospate levels and alterations of protein phosphorylation, have also been described (Saijoh et al., 1993; Sarafian, 1993).

Uncontrolled release of Ca^{2+} from the mitochondria has been reported to occur during oxidative stress, a condition resulting from the imbalance between the production of free radicals and the counteraction by the cellular antioxidant defenses (Duchen, 2000). Many cell components, including proteins, DNA and lipids, are potential targets for free radicals, which are very reactive and, generally, short-lived moieties with one or more unpaired electrons. Oxygen radicals are constantly produced in the mitochondria through incomplete reduction of oxygen (around 2 %). Alterations of the mitochondrial electron transport system can increase the level of reactive oxygen species (ROS). The generation of free radicals can also be mediated by other cellular components, either cytoplasmatic or membrane bound. Xenobiotic compounds can give rise to free radicals by interacting with molecular machineries dispersed throughout the cells, including the mitochondria, the endoplasmic reticulum and the cytosol (Wallace, 1997). Some compounds augment the

generation of free radicals occurring during normal biological processes. Other xenobiotics, instead, are metabolized into free radicals by cellular enzymes. A wide range of mechanisms contribute to maintain free radicals at an acceptable level, e.g. quenching by the activity of enzymes (e.g., catalase, peroxidase and superoxide dismutase) and by non-enzymatic compounds (e.g., glutathione [GSH], α-tocopherol, ascorbate, retinoic acid and β-carotene).

The level of reactive oxygen species (ROS) increases after exposure to methylmercury in brain tissue (LeBel et al., 1990; Yee and Choi, 1994) and in various *in vitro* neuronal models (Mundy and Freudenrich, 2000; Sarafian and Verity, 1991). Augmented free radical generation has also been reported in other cell types (InSug et al., 1997; Shenker et al., 1999). Methylmercury exposure induces increased levels of superoxide, hydrogen peroxide and lipid peroxidation (LeBel et al., 1990; Oyama et al., 2000; Sarafian and Verity, 1991; Taylor et al., 1973; Yonaha et al., 1983). In addition, elevated levels of peroxynitrite have been found in the occipital cortex of exposed rats (Miyamoto et al., 2001). Both GSH depletion and reduction of the superoxide dismutase (SOD) have been observed following methylmercury exposure (Sarafian and Verity, 1991; Shenker et al., 1999; Yee and Choi, 1994). The binding of methylmercury to GSH, due to its high affinity for SH-groups, decreases the availability of this antioxidant factor, exposing the cells to free-radical mediated damage. In accordance with these findings, it has been shown that antioxidants, e.g. vitamin E, GSH , selenium, catalase, and the lipid peroxidation inhibitor α-lipoic acid antagonize some of the deleterious effects of methylmercury (Anuradha and Varalakshmi, 1999; Ganther, 1978; Gasso et al., 2001; Park et al., 1996; Sanfeliu et al., 2001). Moreover, thiol-resins have been used successfully to treat methylmercury-poisoned patients (Clarkson et al., 1981). The fact that deferoxamine protects from methylmercury-induced oxidative injuries indicates that iron-catalized reactions are involved in the formation of free radicals (LeBel et al., 1992; Sarafian and Verity, 1991). Ganther (1978) suggested that methylmercury by itself can generate methyl radicals via homolytic breakdown. The increased level of ROS induced by methylmercury is also likely to be related to alterations of mitochondrial functions. Methylmercury is accumulated in the mitochondria (Yoshino et al., 1966), where it decreases the rate of oxygen consumption, alters the electron transport chain by impairing complex III (Sone et

al., 1977; Yee and Choi, 1996), and induces loss of the mitochondrial membrane potential (Bondy and McKee, 1991; Castoldi et al., 2000; InSug et al., 1997; Shenker et al., 1999).

The extent of methylmercury-induced cell damage seems to depend from the intracellular level of antioxidants (Sarafian et al., 1996; Shanker and Aschner, 2001). Methylmercury affinity for SH-groups is several order of magnitude above that for other types of ligands (Clarkson, 1972). Because of this affinity, it forms complexes with various thiol-compounds, including GSH. The conjugation with GSH has been shown to be the major pathway for methylmercury efflux in astrocytes (Fujiyama et al., 1994). This suggests that cells with higher GSH levels may have an enhanced elimination of intracellular methylmercury with consequent higher resistance to its toxicity. The binding to SH-groups of peptides may have several detrimental consequences, due to modifications of structural proteins and/or inactivation of enzymes. For instance, the binding of methylmercury to tubulin and the consequent disturbances of microtubules assembly/disassembly have been proposed as possible mechanisms responsible for cytoskeletal alterations (Graff et al., 1997; Miura and Imura, 1989). Methylmercury-induced inhibition of the Na^+/K^+ pump and protein synthesis has also been observed (Atchison and Hare, 1994).

1.4 CELL DEATH INDUCED BY METHYLMERCURY

Exposure to toxicants can result in either partial damage of cellular functions or cell death, depending on the type of insult, the type of cells and the cellular defense mechanisms. The level of exposure might determine if a cell is eliminated by apoptosis, which is an active process requiring energy (ATP), or by passive cell death via necrosis (Nicotera et al., 1998). Apoptosis is a regulated process of cellular demise responsible for the removal of unwanted or supernumerary cells during normal development and adult homeostasis, but also implicated in neurodegenerative conditions and activated by many cytotoxic drugs (Gibson, 2001; Gorman et al., 1998; Jacobson et al., 1997; Robertson and Orrenius, 2000). Death by apoptosis is characterized by morphological and biochemical changes, such as cell shrinkage, condensation of chromatin, and regulated intracellular degradation processes involving cleavage of proteins and DNA at specific sites (Kerr et al., 1972; Thornberry and Lazebnik, 1998; Wyllie, 1980). At a later stage, the cell divides into smaller "apoptotic bodies" that can be cleared by phagocytosis. Plasma membrane integrity is maintained

during the entire process, avoiding the spilling of intracellular content that would result in inflammation. During necrotic cell death, instead, swelling of organelles and of the whole cell takes place, followed by cell rupture.

There are at least two pathways of apoptosis, the receptor-mediated killing and the mitochondria-mediated cell death (Gupta, 2001). In the receptor-mediated apoptosis the process is initiated by binding of ligands to "Death Receptors" (DR) located in the cell membrane. Members of this receptor family are Fas/CD95, tumor necrosis factor-α (TNF-α) receptor-1, DR4 and DR5 that bind to TNF-α-related apoptosis inducing ligand (TRAIL). Death receptors are characterized by a conserved cytoplasmic motif (death domain) capable of binding to cytoplasmic adaptor molecules. The recruitment of these molecules results in the activation of caspase-8, an "initiator caspase" that may directly cleave and activate the effector caspases, e.g. caspase 3. This pathway occurs predominantly in certain cellular models, i.e. the type I cells. In other cells (type II cells) caspase 8, instead, cleaves the cytoplasmic protein Bid generating a fragment that activates a mitochondrial pathway. Bid can also be cleaved by other enzymes, e.g. calpains, granzyme B and lysosomal enzymes, although the physiological significance of this cleavage is unclear.

Signals that activate apoptosis via the mitochondria cause the release of mitochondrial factors. These factors may trigger cell death via activation of caspases or stimulation of caspase-independent degradation events (Gupta, 2001). Upon mitochondrial injury, cytochrome *c*, which is part of the electron transport chain, can detach from the mitochondrial membrane and translocate to the cytoplasm, where it interacts with Apoptotic protease activating factor-1 (Apaf-1). The binding of cytochrome *c* to Apaf-1 in presence of dATP (or ATP) results in a conformational change that allows Apaf-1 to oligomerize into a multimeric complex, the so-called apoptosome (Green and Reed, 1998). Pro-caspase 9 is recruited to the apoptosome, becomes active by autocatalysis and proteolitically activates caspase 3, which is the main "executor caspase" (Gupta, 2001). The mitochondria can also release Second Mitochondrial Activator of Caspases (SMAC/DIABLO) that modulates apoptosis by binding and inactivating certain Inhibitors of apoptosis (IAPs) (Gupta, 2001). The anti-apoptotic activity of IAPs has been attributed, at least in part, to their ability to bind and inactivate caspases.

Many proteins may facilitate or inhibit apoptosis. The Bcl-2 family comprises proteins with structural homology that can mutually interact forming heterodimers. While some members of the family, e.g. Bcl-2, prevent apoptosis, others, e.g. Bax, induce cell death (Tsujimoto and Shimizu, 2000). The expression level of these proteins plays an important role in determining the destiny of the cell during toxic challenge. The key function of the Bcl-2 family members seems to be the regulation of the release of pro-apoptotic factors from the mitochondria. Another family of proteins that can modulate apoptosis are the heat shock proteins (HSPs). These proteins, which act as molecular chaperons in healthy cells, are induced by a variety of toxic stimuli, including heat, ischemia, toxins and heavy metals, and seem to play a role as a defense mechanism leading to cell survival. HSPs may participate in the refolding of damaged proteins and/or inhibition of apoptosis at different stages, up- or down-stream of mitochondrial events (Beere, 2001; Garrido et al., 2001; Sharp et al., 1999).

A very important role in signaling and perpetuation of apoptosis is played by caspases, a family of cysteine proteases that are present in cells as zymogens, requiring proteolytic processing to generate the two active subunits. An active enzyme consists of a tetramer composed of two large and two small subunits (Thornberry and Lazebnik, 1998). Caspases are divided into three groups, based on their substrate specificity. The capability of certain caspases to cleave other members of the family creates the opportunity for sequential activation of these enzymes, resulting in a cascade of proteolytic events. Moreover, caspase activation causes degradation of cytoskeletal and nuclear proteins critical for maintenance of cell structure, cleavage of other enzymes necessary for cell survival and DNA fragmentation (Enari et al., 1998; Thornberry and Lazebnik, 1998).

Beside caspases, another family of cysteine proteases, the calpains, has also been found activated in several models of apoptosis (Chan and Mattson, 1999). Elevation of Ca^{2+} is the main signal to activate these proteases (Sorimachi et al., 1997). Calpain involvement in apoptosis may relate to pathological alteration of intracellular Ca^{2+} that occurs, for example, in cells with mitochondrial dysfunction (Nicotera and Orrenius, 1998). Calpains are also regulated by the endogenous inhibitor calpastatin. Activated caspases can cleave calpastatin, resulting in calpain activation (Pörn-Ares et al., 1998). As mentioned above, calpains can also cleave Bid and activate mitochondria-mediated pathways (Chen et al., 2001).

Recent work has shown that other factors released from the mitochondria participate in apoptosis in a caspase-independent manner. For instance, apoptosis can be promoted by the translocation from the mitochondria to the nucleus of Apoptosis-Inducing Factor (AIF), a flavoprotein normally confined to the mitochondrial intermembrane space (Susin et al., 1999). AIF is capable of inducing chromatin condensation and large-scale DNA degradation into high-molecular weight fragments, which is one of the hallmarks of apoptosis (Oberhammer et al., 1994; Zhivotovsky et al., 1994). Mitochondrial endonuclease G (Endo G) can also be released, contributing to chromatin rearrangement (Gupta, 2001). Thus, the release of AIF and Endo G exemplifies caspase-independent pathways of cell death.

Apoptosis is often a consequence of oxidative stress-related events (Chandra et al., 2000). Lysosomes are intracellular organelles where degradation processes take place. The lysosomal membrane keeps apart a large variety of enzymes potentially harmful to the cell. Lysosomes are very sensitive to oxidative stress (Ollinger and Brunk, 1995) and their labilization may result in apoptosis (Brunk et al., 2001; Ollinger and Brunk, 1995), possibly via intralysosomal iron-catalized oxidative processes (Antunes et al., 2001). Lysosomal proteases and endonucleases have been indicated as potential regulators/effectors in apoptosis, even if the precise role of these enzymes is unclear (Kagedal et al., 2001; Stoka et al., 2001).

Morphological and biochemical analysis (DNA fragmentation) of the rat cerebellum after methylmercury intoxication have shown that methylmercury induces apoptotic cell death *in vivo* (Nagashima, 1997; Nagashima et al., 1996). Different hallmarks of apoptosis have also been described in cells exposed to methylmercury *in vitro*, e.g., rat cerebellar granule neurones (Bulleit and Cui, 1998; Castoldi et al., 2000; Kunimoto, 1994), rat cerebral microglial cells (Nishioku et al., 2000), human T-cells (Shenker et al., 1997; Shenker et al., 1999), human monocytes (InSug et al., 1997), myogenic cells (Usuki and Ishiura, 1998), rat pheochromocytoma PC12 cells and mouse neuroblastoma cells (Miura et al., 1999). It is possible that methylmercury induces cell death by apoptosis *in vivo* not only in the cerebellar granule layer but also in other brain locations. For instance, disappearance of astrocytes has been reported in the brain of monkeys exposed chronically to low level of methylmercury (Charleston et al., 1996). Apoptosis may also be involved in the process of degeneration observed at postnatal day 1 and 3 in the brain of rats exposed to relatively low doses of methylmercury *in utero* (Kakita et al., 2000).

2 THE PRESENT STUDY

2.1 SPECIFIC AIMS

The aims of this work can be summarized as follows:

- to characterize the intracellular pathways leading to cell death in neuronal and glial cells exposed to methylmercury *in vitro*;

- to evaluate the effects of prenatal exposure to low levels of methylmercury on behavior during early ontogeny in rats.

2.2 THE *IN VITRO* STUDIES

In vitro cellular models have proven to be useful tools in neurotoxicology to identify intracellular targets of a neurotoxicant and to dissect the pathways of toxicity. As previously mentioned, neurons, in particular, cerebellar granule cells (CGC), are the cell type most affected by methylmercury exposure *in vivo* both in humans and in experimental animals (Clarkson, 1972). However, glial cells are also a target for methylmercury, as shown by the disappearance of astrocytes from the thalamus of monkeys exposed chronically to low-levels of methylmercury (Charleston et al., 1996). In addition, cell swelling, alterations of the K^+ and amino acid transport, mitochondrial dysfunction and accumulation of free radicals have been described in primary cultures of rat astrocytes exposed to methylmercury *in vitro* (Aschner et al., 1996; Yee and Choi, 1996).

In the present studies, primary culture of rat CGC were employed to characterize the cell death pathways activated by methylmercury in neuronal cells. The human astrocytoma cell line D384 was chosen for analyzing the methylmercury cytotoxicity in glial cells. The studies were focused on the exposure to low doses, i.e. below the estimated brain concentrations that produce neuropathological effects in developing human and mammalian nervous system (Bulleit and Cui, 1998; Burbacher et al., 1990). The cytotoxic effects of methylmercury were evaluated by analyzing modifications of cellular morphology and

activation of pathways characteristic of apoptosis, e.g. alterations of the mitochondria, release of mitochondrial factors, DNA fragmentation, activation of caspases and calpains. Moreover, the role of oxidative stress in methylmercury toxicity was examined by studying the protective effect of antioxidants in neuronal cells and by exploring the role of lysosomes in the glial model. The effects of the oxidative stress-inducer hydrogen peroxide and the microtubule-disrupting agent colchicine were analyzed in parallel to methylmercury, with the purpose of comparing the pathways activated in the same cell by different neurotoxic stimuli.

2.3 THE *IN VIVO* STUDIES

The analysis of behavior is a powerful tool to evaluate the consequences of prolonged exposure to low doses of toxicants that do not produce gross morphological alterations detectable by neuropathological examination. The behavioral approach allows for the identification of functional modifications, and drug challenges may be used to uncover alterations. Several tests in sequential order can be performed in the same animal at different ages, and biochemical and molecular analysis of brain tissue can be performed after the behavioral tests. Both primates and rodents have been employed for behavioral analysis to study the effect of methylmercury poisoning.

In this thesis we have analyzed the behavior of the offspring of female rats exposed to methylmercury at low-levels through the drinking water during pregnancy and lactation. This exposure is known to give a level of mercury in the newborn rat brains comparable to the mercury levels detected in infant brains from fish-eating populations (Cernichiari et al., 1995; Rossi et al., 1997). Using the same experimental model, Rossi *et al.* (1997) have previously shown that the male offspring displayed a decrease in spontaneous locomotor activity (motility and rearing) at six moths of age, whereas females where unaffected. This study suggested that methylmercury altered the dopamine mediated motor activity in the adult rat, therefore we have further investigated the effect of prenatal exposure to methylmercury focusing on rats at prepubertal age. Rats were examined at 14 and 21 days, since these time points are relevant for the development of the dopaminergic system and its receptors (Gerfen and Wilson, 1996). Locomotor activity was analyzed after stimulation with the preferential dopamine D_2 receptor agonist U91356A in both female and male rats.

In addition, stimulation with the dopamine receptor agonist apomorphine was employed to study the interaction between dopamine D_1 and D_2 receptors. Alterations of cerebellar function were evaluated with the Rotarod test. Furthermore, the effect of methylmercury on spatial learning and memory was studied in the rats at the age of two months using the Morris swim maze.

3 MATERIALS AND METHODS

3.1 MATERIALS

A detailed description of the materials, including the list of antibodies and their working dilution, can be found in the methodological sections of the papers.

3.2 ANIMALS (PAPER I, II, III, V, VI)

For the preparation of CGC, pregnant Sprague-Dawley rats (B&K, Stockholm, Sweden) were housed singly and checked twice daily. Animals were kept in air-conditioned quarters, with a controlled photoperiod and free access to food and tap water. Procedures used in animal experimentation complied with the Karolinska Institute guidelines in the care and use of laboratory animals. For the prenatal exposure, primigravid Sprague-Dawley female rats were administered 0.5 mg/kg/day of methylmercury hydroxide in the drinking water from day 7 of pregnancy until day 7 of lactation. During this period the food and water consumption, the daily intake of methylmercury and the weight of the animals were monitored every 3rd day. At the end of the treatment, the mean of daily methylmercury intake (mg/kg) was calculated to control the actual level of exposure.

3.3 CELL CULTURE AND EXPOSURE PROCEDURES (PAPER I, II, III, IV)

Primary cultures of CGC were used as a model for neuronal cells and the human D384 cell line was used as a model for glial cells. CGC were prepared from rat pups on postnatal day 7

as previously described (Schousboe et al., 1989). In brief, the cerebella from rat pups were dissected, minced with a McIlwain tissue chopper (Histo-Lab, Gothenburg, Sweden), dissociated with trypsin and seeded on dishes (or glass coverslips for microscopic analysis) coated with poly-L-lysine at a density of 500,000 cells/cm^2. Cells were maintained in basal Eagle's medium supplemented with 10 % FCS, 25 mM potassium chloride and 0.5% (v/v) penicillin-streptomycin. To prevent growth of glial cells 10 μM cytosine arabinoside was added to the cultures 40 h after seeding. The cells were left for 7 days in culture to differentiate before exposure to the neurotoxic compound. The toxic agents (methylmercury, hydrogen peroxide and colchicine) were directly added to the conditioned medium. The caspase inhibitor z-VAD-fmk was added to the medium 30 min before the toxic agents and left in the culture for the entire time. J811 and 17β-estradiol were added either 30 min before or 30 min after the neurotoxic compounds. For harvesting, floating cells collected from the medium by centrifugation were pooled together with cells detached by scraping.

The D384 clonal cell line has been established from a human astrocytoma (Balmforth et al., 1986). Cells were routinely seeded at the density of 10,000 cells/cm^2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37 °C in humidified air with 5 % CO_2. The cells were cultured in complete medium for a period of 24 h before adding the treatments. The monolayers were exposed to methylmercury or hydrogen peroxide by adding directly the compounds to the conditioned medium. Cells were collected either by scraping or by treatment with trypsin.

3.4 ANALYSIS OF CELL MORPHOLOGY AND VIABILITY (PAPER I, II, III , IV)

Cells were observed and photographed at the phase contrast microscope to analyze alteration of the cell morphology, e.g cell shrinkage. Moreover, nuclear stainings were used to visualize alterations of chromatin, which is a hallmark of apoptosis. For this analysis, cells grown on coverslips were fixed in ice-cold methanol/water (8/2 = v /v), at –20 °C for 30 min, washed with phosphate-buffered saline (PBS) and stained with propidium iodide (2.5 μg/ml in PBS)

for 4 min. The coverslips were mounted onto glass slides with PBS/glycerol (1/9 = v/v) containing 0.1 % (w/v) phenylenediamine. Stained cells were analyzed at the fluorescence microscope (Olympus BX60 equipped with the photosystem PM 20) or at the LSM 510 laser scanning confocal microscope (Carl Zeiss, Jena, Germany). At least 300 cells were examined in each coverslip. The smaller size, irregular shape and higher intensity of chromatin staining identified condensed nuclei. Alterations of the plasma membrane permeability were analyzed with the Trypan blue exclusion test (**PAPER IV**). Upon staining with the dye Trypan blue, cells with damaged cell membrane stain blue (dead cells), while cells with plasma membrane integrity prevent the dye entry remaining unstained (healthy cells and apoptotic cells). Cells were harvested with trypsin and then an aliquot of the cell suspension was mixed with an equal volume of 0.4 % Trypan blue in PBS. Cells were scored at the phase contrast microscope using a Neubauer improved counting chamber. The experiments were performed in triplicates and repeated twice. In other experiments (**PAPER II**) cells exposed to the stimuli inducing apoptosis were incubated with Hoechst 33342 and propidium iodide (= 0.5 µg/ml each in culture medium) for 10 min prior examination under a fluorescence microscope. A total of at least 200 cells were examined. Uptake of propidium iodide indicates membrane damage, a feature characteristic of necrosis, while Hoechst 33342 readily traverse the membrane of intact cells. Consequently, nuclear condensation visualised with Hoechst 33342, in the absence of propidium iodide uptake, is characteristic of apoptotic cells. However, in cell culture systems where phagocytosis of apoptotic cells does not occur, there is a delayed lysis of the plasma membrane of apoptotic cells (secondary necrosis), and some cells show condensed nuclei in addition to membrane permeability.

3.5 MTT ASSAY (PAPER II)

This assay is useful for determining cell survival and function of mitochondrial enzymes (Ankarcrona et al., 1995; Mosmann, 1983). It is based on the capacity of mitochondrial enzymes to transform the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a blue formazan product. CGC were treated with the toxic agents for 24 h, then the medium was removed and the cells were covered with MTT tetrazolium dissolved in serum free culture medium without phenol red to a final concentration of 0.3 mg/ml. After incubation at 37 °C for 1 h, the medium was removed and isopropanol was

added to extract the formazan crystals. The absorbance of the samples was measured at $\lambda = 592$ nm on a Multiscan reader.

3.6 LYSOSOMAL INTEGRITY MEASUREMENTS (PAPER IV)

Damage of the lysosomes was measured using the lysosomotropic weak base acridine orange (AO), according to a protocol previously described (Yuan et al., 1997). At the low pH of the lysosomes, the vital staining AO becomes charged (AOH^+) and consequently membrane impermeable, thus it is retained into these organelles. The AO concentrated in the lysosomes has a monochromatic red fluorescence when activated with green light. The amount of red fluorescence per cell is indicative of lysosomes with high AO-concentration and intact proton gradients. D384 cells were grown on coverslips and exposed to the toxic agents, then incubated with AO-medium (5 µg/ml AO in complete medium pre-equilibrated at 37^0C for 15 min) and washed with fresh medium before the measurements. The coverslips were mounted on a well-slide and the red fluorescence was measured in single cells by static cytofluorometer using green light excitation (Nikon B-2A filter cube and an extra 630 nm barrier filter in a Nikon p102 photometer linked to a computer system). At least 50 cells from one dish were measured in each experiment and the mean value was used for further statistical analysis. The experiments were repeated 4 times. Pictures of AO-stained cells were taken with the LSM 410 inverted confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) using the 488 nm argon laser as light source.

3.7 MEASUREMENTS OF THE MITOCHONDRIAL MEMBRANE POTENTIAL (PAPER IV)

Evaluations of the mitochondrial membrane potential were performed using tetramethylrhodamine ethyl ester (TMRE), a cationic, lipophilic dye that partitions to the negatively charged mitochondrial matrix according to the Nernst equation. Decreases in the mitochondrial membrane potentials are paralleled by a reduction of the fluorescence emitted by TMRE, which acts as a voltage sensitive probe (Ehrenberg et al., 1988; Krohn et al., 1998). For this analysis, cells grown on coverslips were exposed to the toxic agents, then incubated with 20 nM TMRE for 30 min at room temperature and washed with PBS before

further analysis. The fluorescence emitted by single cells was measured by static cytofluorometer using green light excitation. At least 50 cells were measured in each experiment and the mean was calculated and used for statistical analysis. The experiments were repeated 3-4 times.

3.8 IMMUNOBLOTTING (PAPER I, II , V)

This method was used both to evaluate specific cleavage of proteins during apoptosis (**PAPER I and II**) and to evaluate the level of dopamine D_2 receptor expression (**PAPER V**). For the extraction of proteins from primary cultures, the cells were scraped, centrifuged and washed with PBS. The cells were suspended in gel loading buffer (0.4% SDS, 4% glycerol, 1% β-mercaptoethanol, 12.5 mM Tris-HCl, pH 6.8) and boiled for 5 min. These extracts were used for the separation by electrophoresis. For the analysis of proteins in the rat stiatum, the tissue was dissected according to the method described by Palkovits and Brownstein (1988), then homogenized in RIPA buffer (1% Nonidet P-40, 0.1 % SDS, 0.5 % deoxycolate, 27 µg/ml aprotinin, 10 mg/ml PMSF, in PBS), on ice for 1 min. The extracts were then centrifuged at 14 000 × *g* for 20 min and the supernatant was collected for further analysis. After determining the protein concentration with the micro BCA assay (Bio-Rad, Hercules, CA, USA), the samples were mixed with 2× gel loading buffer and boiled for 5 min. Proteins were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (Laemmli, 1970) in Tris-glycine buffer, pH 8.3, loading equivalent amounts of proteins in the wells. The proteins were then electroblotted onto nitrocellulose membranes at 75 V for 1-2 h using as transfer buffer 20% (v/v) methanol, 186 mM glycine, 26 mM Tris, pH 8.3. In order to verify equal loading, the proteins transferred on the membranes were visualized with 2% red Ponceau-S, 3% trichloroacetic acid. The blots were blocked with a solution containing 5% non-fat dry milk, 1% BSA in HSB (500 mM NaCl, 50 mM Tris-HCl, pH 7.5), at 4 °C overnight. The membranes were then incubated with the primary antibody diluted in a solution containing 1% BSA in HSBT (0.05 % Tween-20 in HSB) at room temperature for 1 h. After washing 3 times for 15 minutes with HSBT, the blots were incubated with the proper secondary antibody, diluted 1/10,000 in 1% BSA in HSBT, at room temperature for 1 h. They were then washed again 3 times as described above, rinsed

twice for 5 min with 150 mM NaCl, 50 mM Tris, pH 7.5, incubated with ECL reagents for chemiluminescence (Amersham, Little Chalfont, Bucks, UK) and exposed to X-ray autoradiography films (Fuji, Japan).

3.9 IMMUNOCYTOCHEMISTRY (PAPER II, III)

The use of immunocytochemistry to detect polypeptide expression presents the advantage of allowing the analysis of individual cells and the identification of the intracellular localization. Moreover, it is possible to perform simultaneous analysis of different proteins and/or of other parameters, e.g. the nuclear morphology. For the immunocytochemistry, cells were grown on coverslips, exposed to the chemicals and then fixed with 4% paraformaldehyde for 1 h at 4 °C. The coverslips were incubated with the primary antibody diluted in PBS supplemented with 0.3% Triton-X100 and 0.5% BSA, in a humid chamber at 4°C overnight. The cells were then rinsed with PBS, and incubated with a FITC-conjugated secondary antibody diluted in PBS containing 0.3% Triton-X100 for 30 min at room temperature. After 3 rinses with PBS, coverslips were mounted in glycerol-PBS containing 0.1% phenylenediamine. The cells were then examined with the fluorescence microscope Olympus BX60 and images were also collected with the C4742-95-10sc digital camera (Hamamatsu, Japan). In some experiments images were collected with the Bio-Rad 1024 laser scanning confocal equipment (Bio-Rad Laboratories, Hemel-Hempstead, UK) attached to a Nikon Eclipse 800 upright microscope (Nikon Japan).

3.10 DETECTION OF AIF TRANSLOCATION TO THE NUCLEUS (PAPER III)

The translocation of the mitochondrial factor AIF to the nucleus was evaluated by co-staining the cells with an antibody specific for AIF and with propidium iodide, which binds to DNA. The immunocytochemistry with the AIF antibody was performed as described in the previous section. At the end of the procedure, the cells were stained with propidium iodide (2.5 μg/ml in PBS) at room temperature for 5 minutes, then washed with PBS and mounted onto glass slides with PBS/glycerol (1/9 = v/v) containing 0.1 % (w/v) phenylenediamine. The samples were analyzed in a Bio-Rad 1024 laser scanning confocal equipment. The merging and quantification of merged area vs. propidium iodide staining

(percentage of merging) was performed with the Bio-Rad Image Pro Plus software (Bio-Rad Laboratories, Hemel-Hempstead, UK), calculating the percentage of co-localized fluorescence signal versus the red nuclear signal.

3.11 CASPASE ACTIVITY ASSAY (PAPER I, II , IV)

Caspases are divided into 3 classes, based on the substrate-specificity. A fluorogenic assay which evaluates cleavage of the substrate DEVD can be used to evaluate the activity of class II caspases (2, 3 and 7), which are regarded as main executioner caspases. For this purpose, cells were harvested by scraping. The capability of cell extracts to cleave the substrate DEVD-MCA, leading to the release of free 4-methyl-coumaryl-7-amide (excitation 355 nm, emission 460 nm), was monitored at 37 °C using a Fluoroskan II (Labsystem AB, Stockholm, Sweden), as previously described (Nicholson et al., 1995). Fluorescent units were converted to pmoles of 4-methyl-coumaryl-7-amide released using a standard curve generated with 4-methyl-coumaryl-7-amide and subsequently related to protein content. The measurements were performed in triplicates and the experiments were repeated 2 times.

3.12 DETECTION OF HIGH-MOLECULAR WEIGHT DNA FRAGMENTS BY PULSE FIELD GEL ELECTROPHORESIS (PAPER I , IV)

Degradation of chromatin into large-molecular weight fragments is a hallmark of apoptosis. To monitor the formation of high-molecular weight DNA fragments, field inversion gel electrophoresis (FIGE) was performed as described previously (Zhivotovsky et al., 1994). Cells were immobilized into agarose plugs and subjected to proteinase K digestion prior to loading into agarose gels (Götz et al., 1999). Two sets of DNA pulse markers were used for determination of molecular weights: i) chromosomes from *Saccharomyces cerevisiae* (225-2200 kbp); and ii) a mixture of λ-DNA, λ-Hind III fragments, and λ-DNA concatemers (0.1-200 kbp) purchased from Sigma (St. Louis, MO). The gels were stained with ethidium bromide (50 μg/L) to visualize the DNA and photographed on a 305 nm UV-transilluminator with Polaroid 665 positive/negative films.

3.13 STAINING WITH TUNEL (PAPER II, IV)

Besides analyzing DNA degradation by pulse field electrophoresis, we have also detected chromatin fragmentation in individual cells by using the TUNEL (Terminal Deoxyribonucleotide Transferase [TdT]-Mediated dUTP Nick End Labeling) method. This procedure allows the identification of DNA fragments with 3′-hydroxyl ends by fluorescence labeling. Cells were grown on coverslips, treated with the toxic agents and then fixed with methanol as described above. Cells were then covered with reaction mixture [20 µM dUTP-biotinylated, 0.25 U/µl terminal deoxyribonucleotide transferase, 1 mM $CoCl_2$, 25 mg/ml BSA (bovine serum albumin), 200 mM potassium cacodylate, 25 mM Tris-HCl pH 6.6 (Boehringer Mannheim, Bromma, Sweden)] and incubated at 37 °C for 1 h. Coverslips were then incubated in 300 mM NaCl, 30 mM sodium citrate at room temperature for 15 minutes. After washing twice with PBS, the samples were blocked with 2% BSA in PBS for 30 min at room temperature, then rinsed with PBS twice, covered with Extravidine-FITC (fluorescein isothiocyanate) (Sigma, St. Louis, MO) diluted 1/100 in PBS and incubated at 37 °C for 30 min in the dark. After 2 washes with PBS, the coverslips were stained with propidium iodide (2.5 µg/ml in PBS) and mounted onto slides as described above for microscopic analysis. Images were collected with a C4742-95-10sc digital camera.

3.14 MEASUREMENTS OF MERCURY CONTENT IN BRAINS (PAPER V)

The content of both organic and inorganic mercury was determined in the brain of pups exposed to methylmercury and controls, since it has been shown that inorganic mercury can be produced from methylmercury by demethylation (Vahter et al., 1995). At the end of the behavioral analysis, the brains were collected and analyzed using a cold vapor atomic absorption technique according to Magos (1971).

3.15 *IN SITU* HYBRIDIZATION (PAPER VI)

This method was used to analyze the level of the dopamine receptor mRNAs in the striatum. Groups of 21 day-old rats that did not undergo behavioral testing were used, in order to avoid possible alteration in gene expression due to the treatment with dopamine receptor

agonists. The rats were sacrificed by decapitation, brains were dissected, immersed in ice-cold phosphate-buffered saline, immediately frozen, cut at 14 μm sections using a cryostat (Microm, Heidelberg, Germany), and thaw-mounted onto "Probe On" slides (Fischer Scientific, Pittsburgh, PA, USA). The rostral part of the corpus striatum, which included the nucleus accumbens, was cut serially. Every fourth section was selected and included in a series. A series of 10 sections was then analyzed by *in situ* hybridization with the use of specific probes.

Antisense oligoprobes were synthesized by CyberGene AB (Huddinge, Sweden). Oligoprobes for rat D_1 and D_2 dopamine receptor mRNAs were designed using Primer Premier 5.0 software (Biosoft Itl, Palo Alto, CA, USA) based on published sequences (Monsma et al., 1990; Rao et al., 1990). Primers for D_2 receptors were designed based on an alignment analysis of D_2 short (D_2S) and D_2 long (D_2L) mRNA sequences (GeneBank accession numbers: X53278 and AF293964, respectively), corresponding to two isoforms of the receptor due to alternative splicing (the region encoding amino acid 242-272 in the third cytoplasmic domain is missing in the D_2S). The non-isoform specific D_2 oligoprobes were complementary to both D_2S and D_2L dopamine receptor mRNAs, whereas D_2L oligoprobes were complementary to mRNA fragments that are missing in the spliced D_2S mRNA. A mixture of 2 or 3 oligoprobes was used for each receptor mRNA.

Oligoprobes complementary to D_1-mRNA:

5'-CGCAGAGGTTCAGAATGGACGCCGTAGAGCACA-3';

5'-ACAGGCCGTGAGGATGCGAAAGGAGAAA-3';

5'-GGGTTCCCGTTACCTGCGGTGGTCTGGC-3'.

Oligoprobes complementary to D_2-mRNA (isoform non-specific):

5'-TGCGTGATGAAGAAGGGCAGCCAGCAGATGA-3';

5'-TGAGCTGGTGGTGACTGGGAGGGATGGGG-3';

5'-TGAGTCGGAAGAGCAGTGGGCAGGAGATGGTG-3'.

Oligoprobes complementary to D_2L-mRNA (isoform specific):

5'-GAGTTTCATGTCCTCAGGGTGGGTACAGTT-3';

5'-ATTCTCCGCCTGTTCACTGGGAAACTCC-3'.

The oligonucleotides were labeled at the 3'- end using terminal deoxynucleotidyltransferase (Amersham, Buckinghamshire, UK) with [α-^{35}S]dATP (NEN,

Boston, MA, USA) to a specific activity of 1-4×10^7 cpm/ml. Probes were purified through QIA quick spin columns (Qiagen/GmbH, Hilden, Germany). Dithiothreitol was added to a final concentration of 10 mM.

The sections were air dried and hybridized as described previously (Dagerlind et al., 1992; Schalling et al., 1988). Briefly, sections were incubated with a hybridization buffer [50% formamide, 4×SSC, 1xDenhardt's solution (1% sarcosyl, 0.02 M phosphate buffer, 10% dextran sulfate), 500 µg/ml heat-denatured salmon sperm DNA, 200 mM dithiothreitol, 1x10^7 cpm/ml of labeled probe] in a humidified chamber 16-18 hr at 42 °C. For specificity control of *in situ* hybridization, adjacent sections were incubated with a 100-fold excess of unlabelled probe or labeled sense probes. No hybridization signal was observed in these sections. After hybridization, the slides were washed in 1×SSC 4 times for 15 min at 55 °C and one time for 30 min at room temperature. Air-dried sections and autoradiographic [^{14}C]Micro-Scale standards (Amersham) were exposed in a same X-ray film cassette to BioMax MR X-ray film (Kodak, Rochester, NY, USA) for 3 days at -20 °C. Films were developed in LX 24 and fixed in AL 4 (Kodak). Then sections were dipped in Ilford K5 nuclear emulsion (Ilford, Mobberly, UK) diluted 1:1 with water. After exposure at 4 °C for 3 weeks, slides were developed in D19 and fixed in Unifix (Kodak). Sections were mounted in glycerol-phosphate buffer and analyzed with a Nikon Microphot-FX microscope equipped with a dark-field condenser. Neuronal localization of hybridization signal was also verified by microscopy. Sections were counter stained with cresyl violet and photographed using a DC 300 (Leica Microsystems, Heerbrugg, Switzerland) digital camera.

For the quantification, autoradiographs and autoradiographic standards were scanned with a resolution 800 dpi using UMAX Power Look 3000 Scanner (Umax Systems GmbH, Willich, Germany). The intensity of mRNA labeling was measured from the scanned images using Scion Image 4.0 which is based on NIH Image by Wayne Rasband (NIH, Bethesda, MD, USA). Each image was calibrated to nCi/g values of the autoradiographic standards using third degree polynomial function. At least 10 sections cut through the striatum were examined from each animal. The anatomical levels were established by comparison to the rat brain atlas by Paxinos and Watson (1986). Sampling of film images was done with manual outlining. Areas were measured in the caudate putamen and nucleus accumbens

(core and shell). Background level was measured from every slide from an outside section area and was ultimately subtracted. The average values for each animal in the controls and the methulmercury treated groups were used to calculate group means ± SEM.

3.16 LOCOMOTOR ACTIVITY TEST (PAPER V, VI)

The behavioral tests for motor functions were performed at 14 and 21 days of age. A computerized motion detection system employing beams of infrared and red lights in combination with vertical and horizontal photocell arrays was used to detect movement (Ögren et al., 1979). The experimental chamber (Motron activity apparatus, Stockholm, Sweden) contained two horizontal and one vertical array of photocell detectors per cage. Three parameters of motor activity were measured: locomotion, motility and rearing. Locomotion was measured by counting the number of times the animal moved from one side of the box to the other, a distance of at least 32 cm (8 photocells). Motility was measured by counting all movements beyond a distance of 4 cm from the point of origin, representing a measurement of general activity. Rearing was measured by counting the number of times the animal stood on its hindlegs and interrupted the infrared beams passing horizontally at a height of 7 cm above the cage floor. Measurements of motor activity were performed during the first part of the light cycle between 09.00-15.00. Prior to the test, all animals were allowed a period of 45-60 min in a room adjacent to the behavioral testing room. In one set of experiments (**PAPER V**), spontaneous activity was recorded for 60 min, then the animals were injected s.c. with 0.1 mg/kg of U91356A, and returned to the test cage. Motor activity was recorded for an additional 60 min period. In another set of experiments (**PAPER VI**) the rats were injected with apomorphine (0.1 or 1 mg/kg, s.c.) and immediately placed in individual activity boxes. The motor activity was recorded for 60 min.

3.17 ROTAROD (PAPER VI)

Alteration of cerebellar function, i.e. balance and coordination, were evaluated with an accelerating Rotarod (Panlab s.l., Barcelona, Spain by) in 20 day-old rats. The animals were habituated on the rotating drum at a constant speed (4 r.p.m.) for at least 30 s, and then the

rotarod accelerated from 4 to 40 r.p.m. over a 5 min period. The time until the rat fell off the drum was recorded in 4 consecutive sessions.

3.18 MORRIS SWIM MAZE (PAPER VI)

The Morris swim maze test was used to analyze the effect of prenatal exposure to mercury on learning and memory. The test was performed in 2 month old rats. The swim maze was a circular tank (1.8 m in diameter, thermostatically controlled at 22 °C) that during acquisition training had a circular platform (15 cm in diameter) 1 cm below the water surface (Ögren et al., 1996). Each animal was given four trials per training session with four different starting positions. The following parameters were recorded: (i) the swim path monitored by a video camera; (ii) the time to reach the platform (escape latency); (iii) the swim distance to reach the platform; and (iv) the swim speed. The platform was located in the middle of one of the four quadrants and remained in the same position throughout the training. If the rat did not find the platform within 65 s it was led by hand to the platform. The rats were allowed to rest on the platform for 30 s (inter-trial interval) before the start of the next trial. The retention test was performed 24 h after the last training session. The test was identical to the training sessions, with the exception that the platform was removed (probe trial).

3.19 STATISTICAL ANALYSIS (PAPER I, II, III, IV, V, VI)

Depending on the specific experimental design, data were analyzed with the Student *t*-test or the analysis of variance (ANOVA), as described in the papers.

4 RESULTS AND DISCUSSION

4.1 THE *IN VITRO* STUDIES

4.1.1 PATHWAYS ACTIVATED BY METHYLMERCURY AND OTHER NEUROTOXIC AGENTS IN CGC

The cytotoxic effects of methylmercury exposure were evaluated in neuronal cells by analyzing several morphological and biochemical parameters hallmarks of apoptosis.
Primary culture of CGC differentiated *in vitro* for 7 days were exposed to methylmercury for 24 h. Concentrations ranging from 0.1 μM to 1.5 μM caused cell shrinkage, loss of neurites, dose-dependent chromatin condensation and formation of high molecular weight-DNA fragments of 700-, 300- and 50-Kbp (**PAPER I**). These results indicated that the cells died by apoptosis. Nevertheless, caspase-3-like activity was not significantly induced, as monitored by the fluorogenic DEVDase assay over a time course of 24 h (**PAPER I**). In accordance, immunocytochemistry revealed that cells positive for the active caspase 3-fragment (p17) appeared only sporadically (**PAPER II**). In addition, the broad caspase inhibitor z-VAD-fmk was not capable of protecting the cells (**PAPER I**). These results pointed to a minor role of caspases in the intracellular pathways leading to methylmercury-induced cell death in CGC.

In contrast, the exposure to methylmercury, caused a substantial activation of the Ca^{2+}-activated proteases calpains, since proteolytic fragments obtained by specific calpain cleavage of procaspase-3 and α-fodrin were increased (**PAPER I**). These results are consistent with the reported elevation of intracellular Ca^{2+} levels in CGC exposed to methylmercury (Marty and Atchison, 1997; 1998) and with the described capability of Ca^{2+}-channel blockers and Ca^{2+}-chelators to partially diminish methylmercury-induced damage (Gasso et al., 2001; Sirois and Atchison, 2000). The presence of active calpains may explain the lack of caspase activity, since calpains have been shown to inhibit caspases (Lankiewicz et al., 2000). Alternatively, it is possible that the presence of ROS may block the activation of caspases that require a reducing environment for their activity (Hampton and Orrenius, 1997). In contrast to our result, a small increase in caspase activity was found

in methylmercury-exposed rat cerebral microglia (Nishioku et al., 2000) and human lymphoid cells (Shenker et al., 2000). Perhaps the different outcomes of methylmercury exposure depend on the modulation of its cytotoxic effects by cellular antioxidant defenses. In this context it should be mention that the brain has relatively low levels of free-radical eliminating enzymes, and the antioxidant systems are primarily in glial rather than neuronal cells (Philbert et al., 1991; Trenga et al., 1991).

Since free-radical generation is a well-known consequence of methylmercury exposure, we tried to protect exposed neurons by treating them with estrogen-like compounds displaying antioxidants activity. These properties are independent from the estrogenic activity and are likely based on the presence of a phenolic A-ring in the structure of the compound (Behl et al., 1997; Mooradian, 1993). The scavestrogen J811 (10 μM), which is a synthetic derivative of 17α-estradiol with low estrogenic activity, and 17β-estradiol (10 μM) protected CGC from methylmercury-induced damage, preventing morphological alterations, chromatin fragmentation and activation of calpains (**PAPER I**). Antioxidants were protective even if administered 30 min after methylmercury. Moreover, the protection exerted by J811 was long lasting (48 h). Our data confirm that free radicals play a key role in methylmercury neurotoxicity and suggest that oxidative stress takes place up-stream of calpain activation and is required for Ca^{2+}-induced damage. In accordance, a recent study has shown that antioxidants, e.g. propyl gallate, are more protective than Ca^{2+}-channel blockers against methylmercury (Gasso et al., 2001). More importantly, Usuki *et al.* (2001) have recently demonstrated that TROLOX, a water soluble derivative of vitamin E, protects the rat cerebellum from methylmercury induced apoptosis. Oxidative stress emerges as one of the most important mechanisms of methylmercury toxicity (Eto, 2000) thus, antioxidants, including estrogen-like compounds, may prove to be a valuable therapeutic tool for methylmercury intoxication.

Beside characterizing the molecular mechanisms of methylmercury cytotoxicity, we have also studied the effects of the oxidative stressor hydrogen peroxide and the microtubule-disrupting agent colchicine. The purpose was to compare the intracellular pathways activated by different neurotoxic compounds in CGC. Morphological and biochemical features induced by methylmercury in CGC (cell shrinkage, chromatin condensation and DNA degradation) were also observed following exposure to hydrogen

peroxide and colchicine (**PAPER II**). We found that hydrogen peroxide, similarly to methylmercury, did not induce caspase 3-like activity, and that the broad caspase inhibitor z-VAD-fmk could not protect from the toxic effect (**PAPER II**). Also in this case, the scavestrogen J811 could prevent chromatin condensation (Götz et al., 1999). We conclude that the processes of chromatin fragmentation and DNA degradation induced by methylmercury and hydrogen peroxide are related to events triggered by oxidative stress and most probably are not mediated by caspases. In contrast, as shown previously, colchicine caused apoptosis (Bonfoco et al., 1995) with release of cytochrome *c* and activation of caspase 3 (Gorman et al., 1999 **; PAPER II**). These data are in accordance with the observation that colchicine toxicity was partially reduced by the caspase inhibitor z-VAD-fmk (Gorman et al., 1999 **; PAPER II**), but not by antioxidants (Götz et al., 1999). The results of this study suggest that although the three neurotoxic stimuli caused apoptosis in CGC, as indicated by morphological changes and biochemical alterations, they activated different pathways, since caspase activity was increased by colchicine but not by methylmercury or hydrogen peroxide.

Consequently, we formulated the hypothesis that methylmercury and hydrogen peroxide caused cell death via alterations of the mitochondria, releasing mitochondrial factors capable of inducing apoptosis in a caspase-independent manner. In support of this hypothesis, other studies have established that both methylmercury and hydrogen peroxide induce damage of the mitochondria, causing MPT and release of mitochondrial factors (Close et al., 1999; InSug et al., 1997; Takeyama et al., 2002). We focused our analysis on the possible release of AIF from the mitochondria, since this factor is capable of inducing chromatin rearrangement, e.g. condensation of chromatin and large-scale DNA fragmentation, in absence of caspase activity. Analysis of CGC exposed to either methylmercury or hydrogen peroxide for 16 h revealed that AIF was translocated from the mitochondria to the nucleus (**PAPER III**). These findings confirm that methylmercury and hydrogen peroxide induce release of mitochondrial factors, and indicate that AIF translocation to the nucleus is a crucial step in apoptosis induced by these neurotoxicants. In addition, AIF translocation to the nucleus was observed also following exposure to colchicine (**PAPER III**), suggesting that colchicine exerts its toxic effects through two parallel pathways, one dependent on cytochrome *c* release with consequent activation of caspases, and the other on AIF translocation. In agreement, the caspase inhibitor

z-VAD-fmk protected only partially from colchicine-induced chromatin condensation (Gorman et al., 1999). The existence of redundant pathways has been reported also in other cell models (Susin et al., 2000).

4.1.2 METHYLMERCURY AND HYDROGEN PEROXIDE INDUCE LYSOSOMAL DAMAGE AND ACTIVATION OF APOPTOSIS IN ASTROCYTES

The human D384 astrocytoma cell line was used to characterize the cytotoxic effects caused by methylmercury in glial cells. Generation of ROS has been shown to occur in rat astrocytes exposed to methylmercury *in vitro* (Yee and Choi, 1996). Since lysosomes are sensitive targets of free radicals, and they may trigger apoptosis (Brunk et al., 2001), we have analyzed the stability of lysosomes in human astrocytoma D384 cells exposed to methylmercury (1 μM) or hydrogen peroxide (100 μM). Furthermore, a possible activation of mitochondrial pathways leading to apoptosis was evaluated. Significant lysosome disruption was found in exposed cells at an early time point (1 h) (**PAPER IV**). These alterations were persistent and preceded the loss of mitochondrial membrane potential, which was detected after exposure for 8 h. Both methylmercury and hydrogen peroxide induced hallmarks of apoptosis, e.g. cell shrinkage, chromatin condensation and large-scale DNA degradation (**PAPER IV**). However, they activated also divergent pathways, since only hydrogen peroxide increased caspase 3-like activity (**PAPER IV**). DNA fragmentation occurring in D384 cells exposed to hydrogen peroxide was not prevented by incubation with the caspase inhibitor z-VAD-fmk. Hence, both neurotoxicants caused degradation of chromatin in a caspase-independent manner. The results of this study point to both lysosomes and mitochondria as early targets of methylmercury toxicity in astrocytes. It can be envisaged that lysosomal enzymes e.g. cathepsins and DNases, may act as executor factors participating in the process of chromatin rearrangement. In addition, AIF translocation to the nucleus may also contribute to the observed alterations. It has been recently shown that lysosomal extracts can cleave Bid (Stoka et al., 2001). The resulting truncated-Bid is capable of inducing cytochrome *c* release from the mitochondria, triggering caspase activation. This pathway may be operating in D384 cells exposed to hydrogen peroxide, where a significant increase in caspase 3-like activity was detected. The different

effect of the two toxic stimuli could be due to a direct inhibition of caspase activity by methylmercury, which may bind with high affinity to the SH-groups present in the active site of caspases. In addition, methylmercury is characterized by a higher stability as compared to hydrogen peroxide that is rapidly degraded. Thus, exposure to methylmercury may result in prolonged oxidative stress, with consequent inhibition of caspases (Hampton et al., 1998).

The endosomal/lysosomal compartment has been found involved also in cell death caused by methylmercury in rat cerebral microglia (Nishioku et al., 2000). However, in this cell type methylmercury induced also a modest increase in caspase activity. To summarize, our work suggests that methylmercury and hydrogen peroxide cause apoptosis in D384 cells. In addition, it identifies lysosomes as an early target of methylmercury toxicity. The contribution of lysosomes to oxidative stress-activated pathways induced by methylmercury in neural cells may be of importance and needs further investigation.

4.2 THE *IN VIVO* STUDIES

4.2.1 METHYLMERCURY ALTERS DOPAMINE-DEPENDENT LOCOMOTOR ACTIVITY IN THE RAT

Behavioral tests were used to study the effect of prenatal exposure to methylmercury in rats during early ontogeny, i.e. at the age of 14 and 21 days. The type of exposure that we employed resulted in brain levels of mercury comparable to those found in fish-eating populations (Cernichiari et al., 1995). In addition, mercury levels in 21day-old rats were about three-fold lower than in 14-days old rats. In the first study rats were analyzed using a test for locomotor activity (**PAPER V**). Spontaneous locomotor activity was increased in the 14 day-old methylmercury treated rats as compared to control, i.e. motility was higher in treated males and locomotion was higher in treated females. At 21 days of age the methylmercury-treated rats showed no difference in motility and locomotion, but exhibited lower rearing.

To further investigate possible alteration of functions mediated by the dopamine system, the rats were stimulated with the dopamine D_2 receptor agonist U913456A, which

caused an increase of locomotor activity (**PAPER V**). No differences in locomotor activity were observed in the methylmercury treated group at 14 days of age as compared to controls. In addition, motility, locomotion, and rearing were significantly lower in methylmercury-treated male rats at the age of 21 days, whereas the females did not differ from the controls. These data indicate impairment of the dopaminergic system during a time crucial for the ontogeny of dopamine receptors (Gerfen and Wilson, 1996). The gender dependent effect of methylmercury on locomotor function at the age of 21 days is in line with the previous observation that male adult rats are more affected (Rossi et al., 1997). Interestingly, a study performed in Canada has shown that mild neurological abnormalities correlated with higher maternal hair mercury level only in male children. Moreover, Sakamoto *et al.* (2001) have reported a decreased male-female ratio at birth and an increase in the proportion of male stillborn fetuses after the Minamata outbreak, suggesting that male fetuses might have been more susceptible to methylmercury poisoning. It is possible to envisage that sexual hormones influence methylmercury toxicity, for example by modulating the antioxidants defenses (Hirayama et al., 1987).

Since our results pointed to a gender- and age-dependent effect of prenatal exposure to methylmercury, we extended the analysis focusing on male rats at the age of 21 days. The prenatal exposure did not determine prominent cerebellar alterations, as evaluated by the Rotarod test (**PAPER VI**). Apomorphine was used to study a possible effect of methylmercury on the interaction between D_1 and D_2 receptors. Apormophine induced stimulation of locomotor activity at both the doses tested (0.1 mg/kg and 1 mg/kg). The 1 mg/kg dose caused a more prolonged stimulation of the locomotor activity, but the maximal increase of activity was observed at the lower dose (**PAPER VI**). Rats exposed to methylmercury during prenatal life showed a higher motility and locomotion than the control group after stimulation with apomorphine (**PAPER VI**). No difference was observed in rearing. The data confirm that methylmercury at low doses determines subtle alterations of dopamine-mediated functions in 21 day-old rats. It should be pointed out that both dopamine D_1 and D_2 receptors are involved in the locomotor stimulation by apomorphine, which is a full agonist of D_1 and D_2 receptors with a similar intrinsic activity as dopamine (Creese et al., 1983; Ögren, 1996; Seeman and Van Tol, 1994). This may explain why locomotor activity was increased in methylmercury-treated rats stimulated with apomorphine (**PAPER VI**), whereas it was decreased in rats stimulated with the specific

dopamine D_2 receptor agonist U913456A (**PAPER V**). Several studies have reported that methylmercury may interfere with the release of neurotransmitters (Mottet et al., 1997). However, published data are still controversial. The difference that we have observed in the exposed rats may relate to mechanisms at the neuronal system level, e.g. interactions via the direct and indirect projections (Gerfen, 2000; Walters et al., 2000). Alternatively, methylmercury may exert its effect by interfering with the cooperative synergy between D_1 and D_2 receptors at the signal transduction level, e.g. by altering phosphorylation (Yagame et al., 1994), or by binding to SH-group of receptors impairing their function.

Another possible cause of the alterations in dopamine-mediated functions observed in methylmercury-treated rats is a change in the levels of dopamine receptors in the striatum. However, the analysis of the D_2 receptor expression in 21 day-old rats by immunoblotting showed similar levels in methylmercury-treated rats and controls (**PAPER V**). Furthermore, we investigated the levels of D_1 and D_2 receptor mRNAs in striatal sections by *in situ* hybridization. Quantification of the signal in the caudate/putamen and in the nucleus accumbens did not reveal significant differences between the two groups of animals (**PAPER VI**). Thus, based on our data, the behavioral alterations observed in rats exposed to methylmercury cannot be attributed to changes in dopamine D_1 and D_2 receptor levels.

We have also studied possible effects of prenatal exposure to methylmercury on learning capabilities and memory. To address this issue, rats were tested in the Morris swim maze at the age of 2 months. No differences were observed between methylmercury-treated animals and controls in acquisition of the spatial task (**PAPER VI**). In contrast, methylmercury-exposed rats displayed a significant impairment in memory (retention). The retention deficit that we have observed appears not to be due to a motivational deficit, since swim speed did not differ between control and treated rats at the day of the retention test. The rats may be unable to process sensory input, e.g. spatial stimuli, in an adequate manner, rather than having a deficit in the actual consolidation of memory. A memory deficit in 22 day-old rats after a single exposure to a higher dose of methylmercury (8 mg/kg/day) during prenatal life has been previously reported (Cuomo et al., 1984). Our results further point to persistence of methylmercury-induced neurotoxic effects until adult life.

5 CONCLUSIONS

The results of our *in vitro* and *in vivo* studies are in agreement with previous epidemiological and experimental investigations showing that methylmercury is neurotoxic at relatively low doses that are environmentally relevant. Whereas the detrimental consequences of exposure to high levels of methylmercury on the nervous system are well known, there is still a need for information on the potential adverse effects of low doses, and to reveal the mechanisms involved.

It clearly appears from our *in vitro* work that the response to toxic insults is cell specific, and that different intracellular pathways may be activated in parallel. In general terms, in view of the complexity of the nervous system and the wide range of intracellular mechanisms that can be affected by toxic events, it is advisable to use multiple cellular models when *in vitro* studies are performed for identifying neurotoxic effects.

A major aim of this thesis was to identify the mechanisms leading to methylmercury induced neural cell death. The morphological and biochemical evaluations performed have characterized the type of death as apoptosis. We have identified a number of relevant end-points, such as nuclear condensation, high molecular weight DNA fragmentation, calpain activity, mitochondrial and lysosomal damage that are useful to further explore the effects of methylmercury in different systems. Interestingly, estrogen-like compounds with antioxidant properties protect neuronal cells from methylmercury cell death, pointing to oxidative stress being a crucial event in methylmercury toxicity.

Another major aim of this thesis was the investigation of the neurotoxic effects of prenatal exposure to low doses of methylmercury. A full understanding of the action of neurotoxic compounds requires knowledge of their impact on neural function across level of analysis, since the nervous system operates not only as many individual units, but also as a highly organized communication and information processing network. Among the various types of behavior, motor activity, learning and memory have been proven to be very sensitive indicators for exposure to methylmercury. Our studies show that prenatal exposure to methylmercury induces locomotor and cognitive behavioral alterations in prepubertal and adult rats in a gender dependent manner. The effects were seen at doses that apparently do not affect the cerebellum, a preferential target of methylmercury. This confirms that the

analysis of behavior is a powerful approach to detect subtle modifications induced by methylmercury *in vivo*, offering the possibility to evaluate the same animals at different stages of development.

The combination of *in vitro* and *in vivo* experimental approaches used in this thesis should be regarded as a valuable strategy to study the effects of other environmental neurotoxicants and to investigate the exposures to multiple factors.

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