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MASTERARBEIT

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„Study of the Combined Neurotoxic Effects Produced by Methylmercury and Organochlorine Pollutants in Primary Cultures of Cortical Neurons“

verfasst von

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1 INTRODUCTION

1.1 Why Environmental Pollutants Should Be Investigated

Environmental pollutants can have a big impact on human health, because humans are exposed to them in their everyday life without having a big choice to avoid exposure. They are virtually everywhere: in the air, on plants and in animals that are consumed [1, 2]. Several diseases, including reproductive and developmental issues, neurodegeneration and cancer, are results of continuous exposure to toxicants in our environment [1, 3-5]. Even some idiopathic illnesses might have their origin in chronic exposure to low-dose environmental contaminants [6, 7]. But still, not enough awareness has been addressed to this problem, as only few studies have been performed on chronic and low-dose exposure. Lacking prove of these effects results in continued ignorance of possible health risks and on-going leakage of environmental pollutants into the environment. But this happened not only in past years, even nowadays, disposal of contaminated waste or use of not yet banned pesticides increases environmental toxicant burden. Although measurements were taken since the 1970s to reduce methylmercury (MeHg) emissions, they are still reaching critical levels, especially in Asia [8]. Legally compulsory regulations for persistent organic pollutants (POPs) on a word-wide level were not achieved earlier than in 2004, when the *Stockholm Convention*, a treaty formulated by the *United Nations Environment Program* (*UNEP*) to reduce and limit the use and disposal of persistent organic pollutants (POPs), entered into force [9]. Both, MeHg and POPs, share some common properties, including long half-life, lipophilicity, volatility and toxicity [1, 10], but only POPs are regulated on a world-wide basis. Global cycling of MeHg, illustrated below (figure 1), shows quite plainly why world-wide legally binding regulations, like the *Stockholm Convention* for POPs, would be useful for MeHg too.

Mercury vapour from either anthropogenic or natural origins, described below, will be retained in the atmosphere for approximately 1 year which in turn leads to a global distribution of mercury. This atmospheric mercury will be transformed to mercuric mercury (Hg^{2+}) by oxidation. Next, Hg^{2+} gets deposited back on the earth crust by rain and enters aquatic systems in which mercury from factories is leaked too. By evaporation of the volatile mercury vapour back into the atmosphere, the cycle is completed, as can be seen in figure 1[1].

In aquatic sediments Hg^{2+} gets methylated mostly by sulphate-reducing bacteria which is thought to have a protective effect on the bacteria, as for them inorganic mercury is more toxic [11]. By consumption of these methylating bacteria by aquatic organisms, MeHg enters the food chain and accumulates in organisms. Finally, those fish will be consumed by humans and thus enter the human body [1].

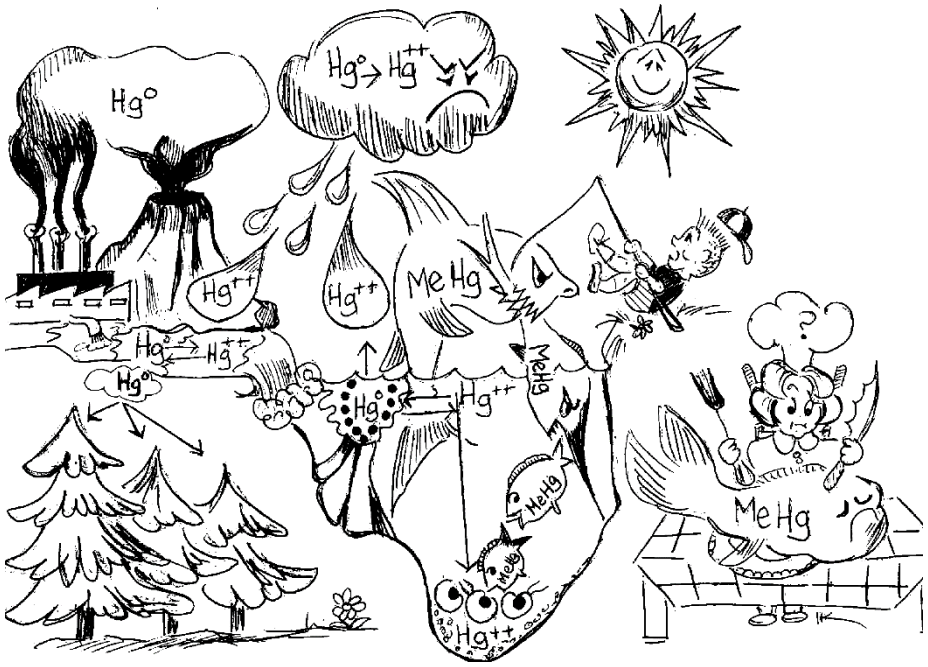


FIG. 1. Schematic drawing of the environment polluting vicious circle of MeHg. Mercury enters into the environment by various natural or anthropogenic sources, gets methylated by aquatic microorganisms which are consumed and accumulated in marine predators. By consuming sea food with high accumulated MeHg burden, MeHg enters the human body. Figure from Clarkson and Magos (2006) [(1)].

This demonstrates that mercury gets distributed throughout the whole world by displacement in the atmosphere or in oceans and is not limited to the surroundings of the MeHg emitting source. The world-wide cycling and harmful health effects are already known for POPs which lead to adopting the *Stockholm Convention*. But still many people are unaware of the health effects of low-dose (< 1 µM) MeHg exposure, and the fact that MeHg in combination with POPs might even increase the toxicity of MeHg. That's why it is necessary to investigate and highlight all harmful aspects and possible interactions with POPs to show up the urgency for a worldwide regulatory treaty of MeHg too.

1.2 Methylmercury (MeHg)

1.2.1 Sources of Mercury and MeHg

Due to various transformation processes MeHg can be generated from every form of mercury. Thus, all mercury sources contribute to the global MeHg burden [(1)]. According to the *United Nations Environment Programme's Global Mercury Assessment 2013* [(2)] 10% of the current mercury emission and re-emission into the atmosphere are due to natural sources, whereas 30% have anthropogenic origin. The final 60% are due to re-emission of previously deposited mercury. Natural sources that emit mercury into air and water include weathering of rocks that contain mercury (e.g. cinnabar), erupting volcanoes or geothermal activities. The anthropogenic source comprises a broad variety of sources such as mining, small-scale gold-mining, burning of coal and fossil fuels, metal recycling, chlor-alkali

industry to manufacture chlorine and caustic soda, production of vinyl-chloride monomers, cement production, oil refining and dental amalgam [2].

Also several products, listed in table 1, people were or are exposed to in their daily life, contain organic or inorganic mercury. Thus, they also contribute to a higher MeHg burden, not only in the environment, but through direct exposure in human bodies too.

TABLE 1: Physical and chemical appearances of mercury; routes of exposure to humans in past and present-day. Present-day exposure is marked as *. Table from Clarkson and Magos (2006) [1].

Inorganic mercury		
Hg vapour	Mercurous	Mercuric
Hg°	Hg-Hg2,+	Hg2,+
Occupational*	Laxatives	Skin creams*
Dental amalgam*	Teething powder	

Organic mercury	
Short chain alkyl	Other organics
CH3(CH2)n-Hg+	(R-C-Hg+)
MeHg in fish*	Phenyl Hg as antiseptics
Ethyl mercury in preservatives*	

Focussing on MeHg, especially consumption of fish living in saltwater that reach a high age and thus accumulate high MeHg concentrations contribute to increased MeHg levels in human bodies. So do mushrooms if they are grown in a ground with high MeHg burden. Interestingly, plants do not tend to accumulate MeHg even if their soils are contaminated with MeHg [12].

1.2.2 Adverse Health Effects of MeHg

The most common route of MeHg absorption is by ingestion. In that case the intestinal absorption rate is nearly 100% [13], [14]. Once entered the bloodstream, MeHg will be taken to every part of the body, including the brain. MeHg and can even cross the placenta and thus enter into the body of the foetus [1] who suffers from undefined and widespread brain damage due to prenatal MeHg exposure [15].

The ability of MeHg to cross the blood-brain-barrier is especially harmful, as the brain is the most susceptible organ and thus, is the mainly affected area by MeHg. Several cortical brain structures are harmed by MeHg toxicity, such as the visual centre of the occipital lobe or the primary auditory area

of the temporal lobe. Furthermore, there are MeHg-induced cortical lesions, resulting in olfactory and gustatory disturbances [16].

Additionally, brain cells are not equally susceptible to MeHg: MeHg is especially harmful for neurons. Astrocytes were found to be less susceptive than neurons, whereas all other non-neuronal cells were shown to be the most resistant [17].

1.2.3 MeHg Poisonings

Two big MeHg poisonings occurred in Japan and Iraq in the past century. The symptoms of affected people strongly point out what has been described in the paragraph above: The central nervous system is the most susceptible organ to MeHg.

Ekino et al. (2007) [16] reported in their paper about the first big epidemic MeHg poisoning that occurred in Japan at the beginning of the 1950s. An acetaldehyde plant was leaking MeHg chloride into the Minamata bay. People in this area have a very high fish consumption and thus were exposed to high MeHg concentrations over a decade of years, causing severe acute mercury poisonings, referred to as Minamata disease. Minamate disease shows symptoms such as paresthesia around the lips, seeing and hearing impairment, olfactory and gustatory disturbances, ataxic gait, dysarthria as well as somatosensory and psychiatric disorders and even death. Prenatally exposed children suffered from damage of the cerebral cortex as well, which resulted in disturbances in mental and motor development. After changing the drainage from the bay to a river which was flowing into the sea, MeHg was more diluted and widely distributed. Thus, people were exposed to a lower dose of MeHg but over a period of 20 years, resulting in the chronic Minamata disease which is characterised by starting with paraesthesia of the limbs and around the lips, steadily leading to more severe symptoms as described in the acute disease [16].

The second epidemic poisoning occurred in Iraq in the winter of 1971-1972 when people prepared bread from wheat grains treated with a MeHg fungicide. They exhibited the same symptoms as people affected by Minamata disease [18].

1.3 Persistent Organic Pollutants (POPs) and Organochlorines

Besides MeHg, another group of environmental pollutants was investigated: POPs. POPs refer to organic compounds which remain in the surroundings and persist in living beings, with extensive half-lives in ecological systems and biota. Those mostly lipophilic compounds, which concentrate in adipose tissue, gradually accumulate in the bodies of predator animals along the food chain and exhibit toxic effects in living organisms [10].

POPs are volatile compounds at ambient temperature and thus can travel long distances before deposition on the earth crust, which means that they can reach even remote areas all around the world

[10]. POPs belong to one of the most hazardous and harmful compounds discharged in the surroundings. Aware of this fact, there has been made much effort to eliminate, restrict and prevent unintentional production or use of the most detrimental environmental pollutants. They are referred to as the *Dirty Dozen* and listed in table 2 [19]. Since 2001 they are regulated on a legally-binding basis by adopting the *Stockholm Convention*. Ten years later another 10 compounds, including β-hexachlorocyclohexane, were added to the initial ones [20].

TABLE 2: List of the first 12 regulated compounds, known as *Dirty Dozen*. Table from chm.pops.int [19].

Pesticides	**Industrial chemicals**	**By-products**
Aldrin*	Hexachlorobenzene (HCB)*#	Hexachlorobenzene (HCB)*#
Chlordane*	Polychlorinated biphenyls (PCB)*#	Polychlorinated biphenyls (PCB)*#
DDT+		Polychlorinated dibenzo-p-dioxins (PCDD)#
Dieldrin*		Polychlorinated dibenzofurans (PCDF)#
Endrin*		
Heptachlor		
Hexachlorobenzen (HCB)*		
Mirex*		
Toxaphene*		

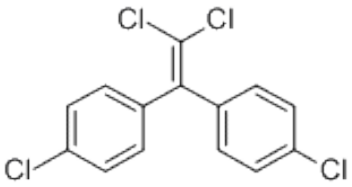
* Elimination, * Restriction, # Reduce and ultimately eliminate unintentional production

Organochlorines are a subgroup of POPs that comprise aliphatic and aromatic compounds with at least one chlorine substitute. This chlorine substitute contributes to the organochlorines' lipophilic character, increasing their uptake and storage in fatty tissue [21]. Furthermore, it facilitates the crossing of the blood-brain-barrier and placenta [22].

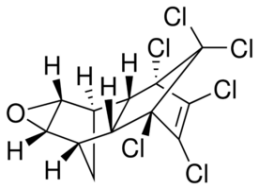
The carbon-chlorine bond exhibits big stability towards degradation due to the resistance against hydrolysis. Increasing stability comes along with increasing chlorination. The structures of the organic compounds play also an important role in the organochlorines' susceptibility towards biodegradation: aromatic rings exhibit greater stability than aliphatic structures do. Biotransformation of organochlorine pollutants (OCPs) is essential to reduce amounts of POPs in the environment and the body, as biotransformed, and hence hydrophilic forms, are secreted more easily and quickly from the body [23].

The aforementioned structure is illustrated in figure 2, where all used organochlorine compounds are shown with the structural formula. They will be briefly described in the sections below.

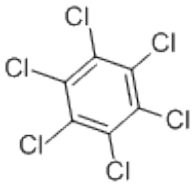
Chemical Structures of the Used Organochlorine Compounds



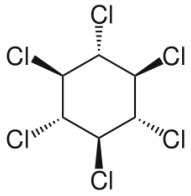
4,4'-DDE



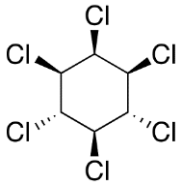
Dieldrin



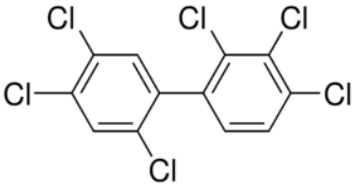
HCB



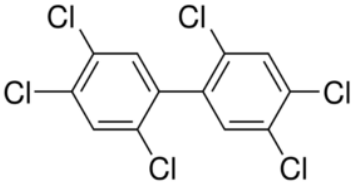
β-HCH



δ-HCH



PCB-138



PCB-153

FIG. 2. The above described POPs depicted with their chemical structure.

1.3.1 4,4´-DDE

4,4´-DDE is a metabolite of the insecticide 4,4´-DDT. The metabolite 4,4'-DDE possesses no insecticide properties and is excreted more slowly than DDT [(4)].

DDT, is maybe the most prominent organochlorine and has a long history of applications. It was used from the 1940s on in agriculture (e.g. the Colorado potato beetle plague in Europe in 1941), as anti-lice product (typhus epidemy in Naples 1943) and for combatting malaria. After health concerns on

DDT, it finally got banned in most developing countries in the 1970s [24]. Nowadays, DDT is listed in the *Stockholm Convention* which states that the use of DDT is now restricted to indoor residual spraying only and with the sole purpose of combating malaria, if there are locally no other appropriate pesticides available [25].

DDT belongs to the group of endocrine disruptors that due to their oestrogenic and anti-androgenic effects by mimicking oestrogen can lead, amongst others, to cancer [26]. According to the *International Agency for Research on Cancer* (IARC), DDT exhibits possible carcinogenicity in humans [27]. Further adverse health effects include reproductive toxicity, reduced fertility, miscarriages and neonatal deaths as well as congenital defects [26]. DDT also accounts for neurodevelopmental delays at prenatal exposure [28].

1.3.2 Dieldrin

Dieldrin describes an insecticide with the main compound called HEOD (1,2,3,4,10,10-hexachloro-6, 7-epoxy-l,4,4a,5,6, 7 ,8,8a-octahydro-endn-1 , 4-exo- 5, 8-dimethanonapthalene) and additional insecticidal compounds. It has bioaccumulative properties as it exhibits hydrophobic properties and a long half-live in the environment. Dieldrin is a metabolite of the pesticide aldrin which is biotransformed in plants or animals [29].

Dieldrin was used as insecticide against soil insects, such as termites, but was also used against moths in the textile industry [30]. Nowadays, it is banned, as regulated in the *Stockholm Convention* [25].

Dieldrin forms also part of the group of endocrine disruptors due to their interaction with the oestrogen receptor, which in turn leads to its big impact in breast cancer development [31]. Furthermore, there is evidence for an anti-androgenic effect of dieldrin, harming the male reproductivity system due to developmental anomalies which include the central nervous system too [32]. Acute neurotoxic effects include nausea, headache and convulsions [29].

1.3.3 Hexachlorbenzene (HCB)

Hexachlorbenzene's structure resembles benzene, which is an aromatic hydrocarbon. Its hydrogen atoms are substituted by chloride atoms resulting in the chemical formula C_6Cl_6. Due to its lipophilic properties it is highly bioaccumulative [33].

In history, HCB served many purposes not only in agriculture but also in industry. In agriculture it was used as fungicidal seed dressing for barley, oats, rye and wheat until the 1970s when awareness was addressed to possible harmful effects of HCB on the ambient and public health. Although in some countries its use continues, for above-mentioned purposes and additionally as anti-scabies pesticide in Tunisian sheep [34]. HCB occurred also in industry, in manufacturing pyrotechnics, pesticides and was used for preserving wood [35]. It is also present as by-product in producing chlorinated solvents [36] as

well as in chlor-alkali factories [33]. Furthermore, municipal incinerations are contributing to the elevated HCB levels in the ambient [35].

Adverse health effects include, porphyria cutanea tarda [33] (a disease affecting the synthesis of heme) and possible carcinogenicity in humans [37]. In high-doses and short-term exposure HCB accounts for symptoms such as bloated thyroid glands, scarring and infantile arthritis in offspring of exposed mothers. In experimental studies animals showed symptoms of neurotoxicity including tremors, paralysis, weakness and convulsions [33]. Furthermore, exposure to HCB can have harmful effects on the neurodevelopment of the unborn [38].

1.3.4 Hexachlorocyclohexane (HCH)

Hexachlorocyclohexane (HCH), previously called benzene hexachloride (BHC), occurs in eight isomeric forms. The most important ones, regarding environmental pollution, are alpha (α)-, beta (β)-, gamma (γ)-, delta (δ)-and epsilon (ε)-hexachlorocyclohexanes, as they are the most abundant ones in the environment. The now-banned *technical-grade HCH*, was used as pesticide and comprises all aforementioned isomers, although nearly all the insecticidal effects are due to the γ-isomer [3].

Due to non-regulated dumping of HCH waste for several years all over the world, there is still a big amount of those isomers leaking into the ambient. The more persistent forms of the above mentioned isomers include β- and δ-HCH [39].

Several adverse health effects are reported for HCH: All forms of HCH exhibit kidney and liver toxicity and possible endocrine disruptive effects and there is evidence showing that β-HCH has human carcinogenic effects, thus evoking liver cancer in animal studies. Furthermore, it can produce immunosuppression. β-HCH also accounts for neurological deficits such as behavioural changes, reduced nerve conduction velocity, seizures and even coma [3]. Prenatal exposure can lead to teratogenic, genotoxic and mutagenic effects [39].

1.3.5 Polychlorinated Biphenyls (PCBs)

There are 209 congeners of polychlorinated biphenyls (PCBs) and usually are sold as mixtures of these congeners (e.g. “Aroclor”). They differ from each other in their degree of chlorination on their biphenyl (= two linked benzenes) structure. With increasing degree of chlorination PCBs become more persistent in sediments and soil. Their lipophilic properties make them bioaccumulate in organisms, resulting in food consumption, especially fish, as major contribution to the body burden in humans. [5]

The congeners to be most quantitatively and commonly detected in humans are the numbers 138, 153, and 180 [40].

Due to their low inflammable and well insulating properties they were used as coolants and lubricants in electrical devices. Furthermore, they were heavily used in a wide range of applications, such as flame retardants, paints, inks, metal coatings, and wire insulators [5].

Toxic effects of PCBs show a broad range of symptoms affecting skin, thyroid, liver as well as the immune system. Furthermore, they cause neurodevelopmental abnormalities, which are probably due to the endocrine disrupting properties of PCBs that also affect reproductivity and can induce cancer, primarily breast and liver cancers. As the brain and the former mentioned systems are not yet fully developed in children and foetuses, the harmful effects of PCBs will have more profound consequences if human beings are exposed pre- or neonatally to them, thus rendering children and unborn more vulnerable to PCBs [5].

1.4 Molecular Mechanisms of MeHg and POPs Neurotoxicity

1.4.1 The Mitochondrial System

Mitochondria play an essential role in cell physiology as this is the place where oxidative metabolism occurs that generates adenosine triphosphate (ATP), which is crucial for providing energy to the cell. MeHg can have harmful effects on mitochondria by inhibiting the electron transport chain and thus increasing ROS [41], as illustrated in figure 3 and 4. Inhibiting or depressing effects of the respiratory chain were also demonstrated for dieldrin [42] and PCBs [43]. Literature for HCB, HCH isomers and DDE is limited on this topic.

1.4.2 Calcium (Ca^{2+}) Dyshomeostasis and Glutamate-Induced Excitotoxicity

Ca^{2+} is an important cell-signalling molecule with the ability to induce cell death pathways [41]. It can also cause mitochondrial membrane potential ($\Delta\Psi m$) loss by stimulating the opening of the mitochondrial transition pore (MTP), a pore by which molecules up to 1 500 Da can cross the impermeable inner mitochondrial membrane [44]. Both, intracellularly released Ca^{2+} of mitochondria and extracellular influx of Ca^{2+}, contribute to the induction of aforementioned target effects of Ca^{2+} signalling. It is known that MeHg has the ability to increase the intracellular calcium concentration $[Ca^{2+}]_i$ [41], amongst other mechanisms, also by releasing intramitochondrial Ca^{2+}, thus contributing to mitochondrial membrane discharge [45), (46].

Several organochlorines have been investigated regarding their impact on $[Ca^{2+}]_i$ levels: Dieldrin does not contribute to $[Ca^{2+}]_i$ increase [47], whereas β-HCH and PCBs increase $[Ca^{2+}]_i$ [48), (49]. The rise in $[Ca^{2+}]_i$ caused by PCBs is due to an increased influx of extracellular Ca^{2+} [49], whereas the mechanism of increased $[Ca^{2+}]_i$ by β-HCH is not known yet.

MeHg can increase $[Ca^{2+}]_i$ not only by intramitochondrial Ca^{2+} release, but by MeHg-induced excitotoxicity too. MeHg increases extracellular glutamate levels, which lead to increased intracellular Ca^{2+} levels [41]. This process is shown in figure 3.

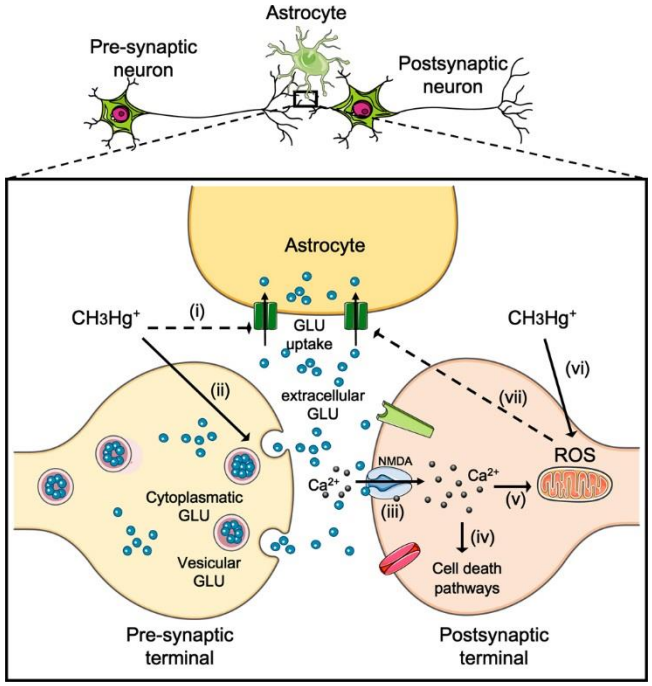


FIG.3. i&ii. MeHg exposure leads to an increase in extracellular glutamate levels which is most likely due to a MeHg-induced inhibitory effect on glutamate reuptake of astrocytes. **iii**. This increased extracellular glutamate concentration causes an *N*-methyl D-aspartate (NMDA)-type glutamate receptor hyperactivation that results in a rise in Ca^{2+} conductance into the intracellular space where **iv.** Ca^{2+} induces cell death pathways **v.** or harms mitochondria which raises reactive oxygen species production.(**i**-**iv**) [50] **vi.** Ca^{2+} release and increased oxidative stress is also caused by MeHg acting directly on mitochondria. **vii.** Free radicals in turn lower the levels of glutamate taken up by astrocytes thus again increasing oxidative stress [51]. Figure from Farina et al. (2011) [41].

1.4.3 The Glutathione System

Glutathione (*γ*-glutamyl-cysteinyl-glycine) is an enzyme which is crucial to protect against oxidative stress and is abundant in nearly every cell. Glutathione is also abbreviated as GSH, as the thiol group (-SH), the active site of the enzyme, is important in catalysing reactions. GSH is especially susceptible to MeHg as it has a high affinity for thiol groups. Due to its reducing capabilities, GSH is responsible for protecting the reduced form of important cysteine residues in proteins and enzymes. GSH is also in charge of neutralizing peroxides and thus inhibiting free radicals and oxidative stress, by using *glutathione peroxidase* which catalyses the following reaction:

$H_2O_2 + 2GSH \rightarrow GS\text{-}SG + 2H_2O$

In the following step, NADPH reduces the oxidized state of GSH (GS-SG) via the action of *glutathione reductase* to obtain reduced GSH again:

$GS\text{-}SG + NADPH + H^+ \rightarrow 2GSH + NADP^+$ [52]

MeHg interferes with the GSH system on several levels: Figure 4 illustrates the interactions of MeHg with the GSH system, including binding of MeHg to the thiol groups of GSH due to the high affinity of MeHg for thiols. Furthermore, it involves reduced *GSH reductase* activity and reduced *GSH peroxidase* activity [53].

There is also evidence for effects of some organochlorines on the GSH system. It was demonstrated that PCBs influence *GSH reductase* and *peroxidase*, but are not changing GSH levels [54]. Nor does 4,4´-DDE affect GSH levels [55], whereas for β-HCH and HCB no relevant literature was available.

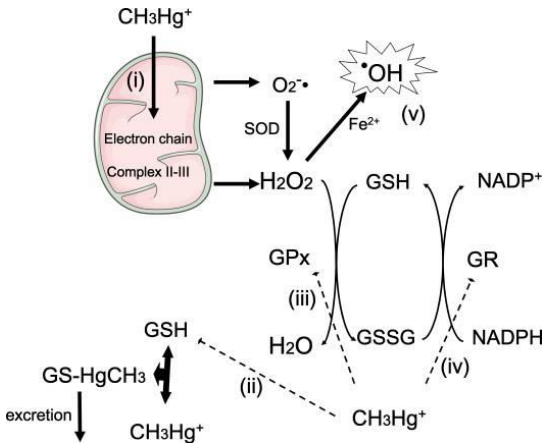


FIG. 4. i. MeHg is inhibiting the tricaboxylic acid cycle which leads to electron transport chain inhibition. This interference with the respiratory chain is responsible for increased levels of reactive oxygen species including hydrogen peroxide (H_2O_2) and superoxide anions ($O_2^{-}\cdot$). Furthermore, the inhibition leads to a discharge of the proton gradient across the mitochondrial inner membrane and subsequently decreases ATP production. [56] **ii.** Due to its high affinity for thiols, MeHg binds to the thiol group of glutathione (GSH) which hampers the enzyme's reducing abilities. Furthermore, it leads to a depletion of GSH levels because the MeHg-GSH complex is excreted as detoxifying mechanism, which in turn increases reactive oxygen levels. [41] **iii.** *GSH peroxidase* (GPx) activity is reduced by MeHg exposure. **iv.** Prenatal exposure to MeHg can also lead to reduced *GSH reductase* (GR) activity. (**iii** and **iv**)[57] Figure from Farina et al. (2011) [41].

1.4.4 Effects of MeHg and POPs on Microtubules

Playing an important role in cellular morphology and stability, consequences of microtubules affected by MeHg and organochlorines can be fatal. Microtubules are polymers of α- and β-tubulin protein dimers with each dimer containing 15 thiol groups [58]. Binding of MeHg to the thiols of the dimers leads to depolymerisation of the microtubules [59]. This depolymerisation causes a microtubule disassembly that results in neuronal degeneration. Literature on organochlorines affecting microtubules was found for PCBs only: PCBs cause a disruption of the actin cytoskeleton [60] which in turn can affect microtubules involved in branching of the axons [61]. This will result in a less dense neurite network. Interestingly, the disruption of the actin cytoskeleton could be observed before cytotoxicity was detectable [60].

1.5 The Involvement of MeHg and POPs in Neurodegenerative Diseases

The range of diseases caused by organochlorine compounds and MeHg intoxication comprises a big variety of symptoms. MeHg is known for its acute and chronic, devastating effects on the human brain and symptoms associated with this brain damage [(1)]. But its link to neurodegenerative illnesses is not very clearly established yet, which applies for OCPs too. But there is evidence that several environmental pollutants, organochlorines and MeHg included, are risk factors for neurodegenerative diseases, such as amyotrophic lateral sclerosis [(6)], Alzheimer's disease [(62), (63)] and Parkinson's disease (PD) [(62), (64), (7)] . With an increasing number of people suffering from neurodegenerative diseases [(65)], it is important to investigate potential risk factors of these illnesses, as their origin still remains unclear. The reason for the still lacking explanation of the aetiology of several neurodegenerative diseases might be due to the fact that a variety of complex mechanisms, including neurotransmitter systems, are involved in the development of these diseases.

We chose to investigate the influence of environmental pollutants with regard to PD, as cortical atrophy is present in PD patients [(66)] and thus is related to our studies of environmental toxicant neurotoxicity in cortical neurons. Although cortical atrophy can be found in Alzheimer's disease (AD) too [(62)] and AD ranges first as most common neurodegenerative disease, PD is the second most common [(67)] but with an earlier onset. The age of PD onset in patients is around 60 years with early-onset PD beginning already at the age of teenagers, compared to an average age of onset of 73 years in AD, with the youngest patients ranging around 50 years [(68)]. That means that PD is more likely to affect working people which will lead to a high economic burden, due to loss of work force and intensive costs for care. Therefore, we decided to investigate the effect of MeHg and organochlorines in combination with two neurotransmitters involved in PD pathology: acetylcholine (ACh) and dopamine (DA) [(69)].

1.6 Parkinson's Disease (PD)

With about 1% of the world´s population over 60 suffering from PD, it is one of the most frequent movement disorders worldwide. It is a neurodegenerative illness characterised by loss of dopaminergic neurons mainly in the substantia nigra and, according to recent findings, cholinergic neurons mainly in the nucleus basalis of Meynert [(70), (71)]. Symptoms of PD include akinesia (incapacity of movement) respectively bradykinesia (slower movements), tremor, rigor (muscle stiffness due to coactivation of agonist and antagonist muscles) and a postural disorder. Additional symptoms may be present including pain, depression, cognitive impairment and in severe cases dementia [(72)]. Currently, no cure for PD is available. Only symptoms can be eased by medication, which is described in more detail below. But there are studies going on that are investigating transplantation of stem or progenitor cells into various parts of the brain, including the striatum [(73)] and the cortex [(74)], to improve symptoms

caused by neuronal cell loss. Although the aetiology of PD is not known yet, there are some risk factors associated with PD: increasing probability with age, genetic predisposition as well as exposure to environmental pollutants [70]. On molecular level, several mechanisms were suggested being involved in neurodegeneration of dopaminergic and cholinergic neurons such as oxidative stress, excitotoxicity, mitochondrial dysfunction, inflammation and apoptosis [75].

1.7 The Involvement of Dopamine (DA), Acetylcholine (ACh), MeHg and POPs in PD

It has been suggested that the underlying mechanisms of PD involve a DA-ACh imbalance, which has been proposed, as medication regulating the ACh and DA balance improved symptoms of PD. Before L-DOPA (L-3,4-Dihydroxyphenylalanin) was available, PD was treated with anticholinergic drugs to improve motor symptoms [76], although the mode of action of anticholinergic drugs is not fully understood yet. Nowadays, only young patients are given anticholinergics, if they only suffer from tremor and no difficulties with gait. Usually PD is treated with L-DOPA in more severe cases but as motor fluctuations can develop in long-term use, young people with mild symptoms are preferentially treated with anticholinergic drugs first. But anticholinergics exhibit side effects too, especially in older patients where they can lead to cognitive impairment [77]. L-DOPA is used as the most powerful anti-PD medication. As DA cannot cross the blood-brain-barrier (BBB) via the large neutral amino acid transporter, the BBB-crossable L-DOPA is given that readily enters the brain. Inside the brain it is decarboxylized to DA by *DOPA-decarboxylase*. Thus, L-DOPA substitutes for the DA of the lost dopaminergic neurons. Also DA agonists can be used in PD treatment because they exhibit less side effects although their effect is not as strong as the one from Levodopa [72]. Concluding, medication that regulates ACh and DA levels provides evidence that cholinergic and dopaminergic systems are mainly affected. Further evidence for an ACh-DA imbalance is provided by a recent study by Ziegler et al. (2013) [78]. They demonstrated that the loss of dopaminergic neurons in substantia nigra occurs before cholinergic cells die in the basal forebrain. That means that there is a time span, where cholinergic neurons are still active whereas dopaminergic activity is already reduced. Thus leading to a DA-ACh imbalance and explaining, why anticholinergic drugs improve tremor in early PD stages but worsen cognitive symptoms in old PD patients with advanced cholinergic cell loss.

But not only a disturbed DA-ACh balance is associated with PD. As mentioned above, there is also a possible link between organochlorine insecticides, MeHg and PD [62], [64], [7]. A study by Richardson et al. (2011) [79] found an association between increased serum β-HCH levels and increased PD risk. Researchers in Greenland found higher DDE levels in PD patients than in the control [80]. This literature findings are in agreement with another study which demonstrates that higher incidence of PD is associated with occupational exposure to organochlorines [81]. Additionally, mercury body burden is

also linked to PD [64], [7]. Furthermore, MeHg and organochlorines are known to induce harmful effects, including cell death, oxidative stress or mitochondrial depolarization (see section 1.4) which may contribute to neuronal loss in PD too.
The various interaction of MeHg with ACh (see section 1.8) and DA (see section 1.9) might as well influence the development or severity of symptoms in PD.

1.8 ACh and its Importance in the Central Nervous System

Cholinerigc neurons synthesize ACh, an ester of acetic acid and choline, which plays an important role in sensory input regulation [82], attention [83] as well as cortical neuromodulatory effects [84]. Incorrect neuromodulation by ACh can cause mental disorders ranging from schizophrenia to depression [85] and is present in neurodegenerative diseases, including AD [86] and PD [69].

ACh-containing neurons occur in three cholinergic modulation systems: There are cholinergic neuron groups in the media septal nuclei and the nucleus basalis in the basal forebrain projecting to cortex and hippocampus. The third one is the pontomesencephalotegmental complex from where neurons are projecting to thalamus and forebrain. Furthermore, there are some cholinergic interneurons in the cortex [72].

In the cholinergic system two receptor types binding ACh are involved: the nicotinic and the muscarinic receptor type. The nicotinic receptor is an ionotropic receptor which forms an ion channel across the membrane and gets stimulated by ACh and nicotine. The muscarinic receptors forms part of the metabotropic receptor group. This receptor group is also referred to as G protein-coupled group, as metabotropic receptors use the G protein as second messenger. The subunits (α, β, γ) of the trimeric G protein are involved in cell signalling pathways. The name of the muscarinic receptor as well derives from the substance it gets stimulated from, apart from ACh: muscarine [87].
Both of them are involved in regulation of a range of cell physiological activities including proliferation, differentiation and apoptosis [88]. Muscarinic [89] as well as nicotinic [90] receptors, are present in the cortex in great number.

One of the environmental pollutants interfering with the cholinergic system is MeHg. There are three main forms of interaction between MeHg and the cholinergic system: Firstly, there is evidence that *micro*molar MeHg concentrations block ACh receptors [91]. Secondly, it has been shown that MeHg causes an activity reduction in acetylcholinesterase [92] and thirdly, has been demonstrated that MeHg increases release of ACh [93].

1.9 DA and its Importance in the Central Nervous System

The name of the neurotransmitter DA derives from 3,4-dihydroxyphenethylamine and forms part of the catecholamine group (catecholamines are molecules that have a catechol, which is a benzene ring with two hydroxyl groups). It is synthesized by an enzyme called *tyrosine-hydroxylase*, which is present in every catecholaminergic neuron. This enzyme catalyses the transformation of the amino acid tyrosine to L-DOPA. Consequently, the enzyme *dopa-decarboxylase* is the catalyst for the L-DOPA to DA reaction [87].

There are mainly two areas in the brain with dopaminergic neurons projecting to various areas of the brain. One originates in the substantia nigra in the mesencephalon projecting to the striatum, which comprises nucleus caudatus and putamen. The other dopaminergic system is located in the mesencephalon too, adjacent to the substantia nigra, and is called ventral tegmental area (VTA). The axons of the VTA are projecting to the frontal cortex and parts of the limbic system [87].
DA from the nigrostriatal area plays an important role in movement control [94] whereas dopaminergic neurons from the ventral tegmental area are essential for reward [95]. A lack or excess of DA is related to several illnesses such as restless legs syndrome [96], attention deficit hyperactivity disorder [97], schizophrenia [98] as well as PD [69].

In the dopaminergic system, there are five subtypes of DA receptors. All of them belong to the G protein-coupled receptors. Those subtypes either belong to the D_1-like group, including the subtypes D_1 and D_5, or to the D_2-like family, comprising D_2, D_3 and D_4. Those two groups differ from each other on behalf of their mode of action after activation by DA. The D_1-like receptors are coupled with the adenylate cyclase stimulating G_s protein, whereas the D_2-like family is attached to the adenylate cyclase inhibiting G_i protein, which is illustrated in figure 5. Activation of either G_i or G_s and the consequent effect on cAMP production leads to inhibition or stimulation of various cell signalling pathways that influence cell survival [99].

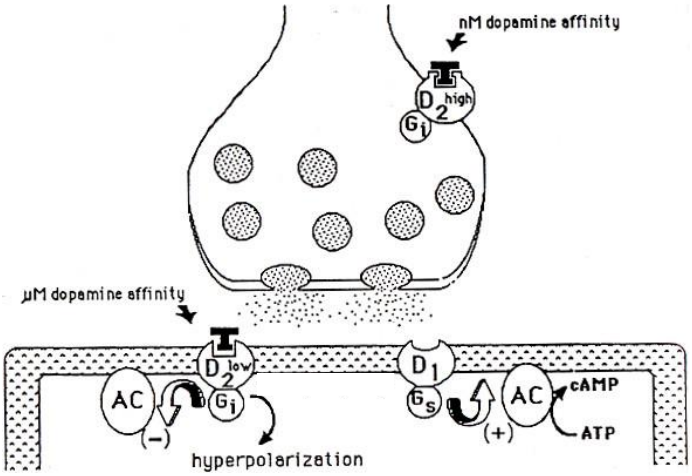


FIG. 5. Depiction of D_1 and D_2 receptors and their location in synapse and the nerve terminal. D_1 receptors are coupled to stimulating G_s proteins that increase the production of the second messenger cAMP. D_2 receptors are coupled to the inhibitory G_i protein which reduces cAMP production and can lead to hyperpolarization of the neuron. On the nerve terminal, D_2 receptors can be found as well. Those receptors are autoreceptors and are associated with G_i too and regulate the dopamine release. Activation of autoreceptors occurs only at high DA concentrations, whereas post-synaptic DA receptors are much more sensitive [(99)]. Figure from Cooper et al. (1991) [(99)].

DA receptor location in the brain and number is varying: In rat cortex, D_1 is the most numerous DA receptor and together with the less expressed D_5 receptors, occurs in virtually all parts of the brain in varying concentrations. D_2 receptors are the second most frequent DA receptors and occur in the forebrain too. D_3 and D_4 receptor presence in the cortex is limited [(100)].

In our project we focused on the effect of the D_2 receptor. Therefore, the D_2-like receptor family will be described briefly in the following: The inhibitory effects of the G_i protein of the D_2-like receptor family resemble much each other. But D_2-like receptor signalling is not limited to the $G_{\alpha i}$-subunit-induced reduction in cAMP levels. Their $G_{\beta\gamma}$-subunits is also involved in inhibiting various Ca^{2+} channels and in activating inwardly rectifying potassium channels which leads to hyperpolarization of the cell. Furthermore, a protein complex can assemble at the D_2 receptor and induce further signalling with a big variety of targets [(101)].

These signalling functions and the whole dopaminergic system can be affected by exposure to environmental pollutants, including MeHg and organochlorines. MeHg affects the dopaminergic system in several ways: There is evidence that 1µM Hg^{2+} prevents binding of a D_1 receptor antagonist [(102)]. Furthermore, it has been demonstrated that MeHg affects in *in vivo* studies the binding activity of D_2 receptors [(103)]. Additionally, a study by Zimmer et al. (2011) [(104)] showed that the exposure of developing neurons to MeHg affects neurotrophic factor levels that are important for the development of catecholaminergic cells. This leads to a reduced number of catecholaminergic neurons, including dopaminergic neurons.

Among the used organochlorine compounds PCBs and 4,4'-DDE might exert negative influence on the dopaminergic system: PCBs seem to affect the uptake of DA [105] and DA synthesis [106]. 4,4'-DDE as well inhibits uptake of DA via the DA transporter [107].

1.10 Objectives and Hypothesis

This project was about studying potential neurotoxic effects of MeHg in combination with organochlorine pollutants on developing cortical neurons derived from a primary culture. We hypothesized that we would find increased cytotoxicity when cortical neurons were exposed to a MeHg-organochlorine combination, compared to cells exposed to MeHg alone. Furthermore, we wanted to investigate underlying mechanisms of a potential cytotoxic effect. Thus, we researched mechanisms known to be involved in cell viability decreasing effects of MeHg and organochlorines in order to find interactive effects of this MeHg-organochlorine combination. These mechanisms included ROS production, GSH levels and mitochondrial membrane potential. Additionally, we extended our research on how the absence or presence of neurotransmitters involved in PD (ACh and DA) would affect neurons exposed to a MeHg-organochlorine combination. This branch of our investigation was based upon a literature finding that demonstrated that developmental exposure to MeHg decreased the number of dopaminergic neurons [104]. Loss of dopaminergic neurons is characteristic for PD, thus, an already reduced number in dopaminergic neurons might increase the probability of developing PD. Furthermore, a reduced number of cortical neurons is associated with PD [66] too. So we decided to investigate the influence of dopamine on immature cortical neurons if co-exposed to MeHg and organochlorines. We were interested in whether we could detect an interactive effect in cells exposed to the MeHg and organochlorines, if co-exposed to sub-toxic concentrations of DA. As PD is based upon a DA-ACh imbalance [78], we investigated a possible interactive effect of a sub-toxic ACh concentration with MeHg as well. We hoped that our results would help to elucidate cytotoxic mechanisms of environmental toxicants and would support previous findings related to the impact of MeHg and organochlorines on PD development.

2 MATERIALS AND METHODS

2.1 Materials

TABLE 3: Supplier of used material and further information

Animals	**Supplier**	**Reference Number**
Pregnant NMRI strain mouse at 16th day of pregnancy	Charles River Laboratories, Iffa Credo (Saint Germain-sur-l'Arbreste, France)	
Compound		
Antimycin A	Sigma Aldrich, St. Louis (MO	A8674
BSO (DL-buthionine-[S,R]-sulfoximine)		B2640
DMSO (Dimethyl sulfoxide) 99.5% GC		D5879
DNAse		D5025
H_2DCF-DA (2´,7´-Dichlorofluorescin diacetate)		D6883
L-Glutamic Acid (L-Glutamate)		G-8415
Monochlorobimane	(Fluka Analytical)	69899
Paraformaldehyde		P6148
Poly-D-lysine hydrobromide		
Rhodamine 123		R8004
Sodium dodecyl sulfate (SDS)		L4509
Resazurin sodium salt		R7017
Thiazolyl Blue Tetrazolium Bromide (MTT)		M2128
Triton 100x		X100
Uridine		
Dulbecco's MEM (10x) w/ 4.5 g/L D-Glucose	Biochrom AG	F0455
Halothane	Zeneca – Fluothane	615179
Propidium iodide	EMD Chemicals	537059
Toxicants		
Dieldrin	Sigma Aldrich (Pestanal)	33491
Hexachlorobenzene		171050
Methylmercury(II) chloride		442534
PCB No. 153	(Fluka Analytical)	35602
4,4'-DDE	Dr. Ehrenstorfer	C12041000
PCB No. 138		C200138001
Neurotransmitter and Antagonist		
Acetylcholine chloride	Sigma Aldrich	A6625
Dopamine hydrochloride		H8502
S(-)-Raclopride (+)-tartrate salt		R121
Antibodies		
	Sigma Aldrich, Israel	

Mouse monoclonal anti- Glial Fibrillary Acidic Protein (GFAP) Antibody		G3893
Rabbit polyclonal anti-Tau Antibody		080M4753
	Invitrogen, Molecular Probes, Life Technologies, Oregon, USA	
Alexa Fluor 488 Goat Anti-rabbit IgG Secondary Antibody		A11008
Alexa Fluor 594 Donkey Anti-goat IgG Secondary Antibody		A11058
Alexa Fluor 594 Goat Anti-mouse IgG Secondary Antibody		A11032
Materials		
	Thermo Scientific Nunc, Denmark	
Nunclon Delta surface 24-well plates		142475
Nunclon Delta surface 96-well plates		167008

Other material and compounds were obtained from the institution's suppliers (Sigma Aldrich and Merck).

2.2 Used Mixtures and Buffers

TABLE 4: Mixtures applied for preparations of toxicants, reagents and washing.

Mixture	Composition
Nanopure Water	Milipore miliQ synthesis A10 – 18.2 MΩ*cm; 3 ppb COT Used to make all solutions and dilutions
Hank's Buffer solution	1.3 mM $CaCl_2 \cdot 2H_2O$, 5.4 mM KCl, 0.4 mM KH_2PO_4, 0.5 mM, $MgCl_2 \cdot 6H_2O$, 0.4 mM $MgSO_4 \cdot 7H_2O$, 137 mM NaCl, 4.2 mM $NaHCO_3$, 0.3 mM $Na_2HPO_4 \cdot 2H_2O$, 8 mM HEPES, 5.5 mM Glucose-H_2O; adjusted to pH 7.4
Krebs Buffer	120.9 mM NaCl, 4.83 mM KCl, 1.22 mM KH_2PO_4, 25.5 $NaHCO_3$, 13 mM Glucose , Phenol Red (approx. 0.015 g/L)
Phosphate buffered saline (PBS) solution	135 mM NaCl, 7.5 mM $Na_2HPO_4 \cdot 2H_2O$, 1.5 mM KH_2PO_4, 2.7 mM KCl; pH 7.4

2.3 Instruments

TABLE 5: Instruments used for experimental assays and preparing primary cultures

Instrument	Model	Software
Fluorescent confocal microscope	Leica Microsystems DM5500 Q	Leica Application Suite Advanced Fluorescence (LAS AF)
Fluorometer	Molecular Devices Spectramax Gemini XS	SOFTmax PRO Software
Phase-contrast microscope	Leica Microsystems DMI4000 B Camera: Leica DFC 300 FX	Leica Application Suite V3
Spectrometer	Thermo Electron Corporation Multiskan Spectrum	SkanIt RE for MSS 2.2
Equipment in Cell Culture Laboratory	In-house service: including centrifuges, incubators, phase-	

	contrast microscope, laminar flow horizontal and vertical hoods
Equipment in General Laboratory Animal Facility	University of Barcelona, School of Medicine

2.4 Toxicants

Toxicants were used alone, in combination or as mixtures to research their impact on toxicant-induced alterations in developing cortical neurons derived from a primary culture.

2.4.1 Selection of MeHg Concentrations

The experimental concentrations of MeHg were adjusted from concentrations of previous experiments relevant to this investigational aims. The previously used MeHg concentrations ranging from 0 – 1000 nM were used at the double of the cell concentration ($1.6*10^6$ cells/ml) of what was used in this project, i.e. $8*10^5$ cells/ml. These concentrations proved to be too high for the used cell density resulting in rapid cell death. Thus, cells could not be exposed over a longer period of time to study chronic effects of MeHg. So we chose to use lower concentrations for the chronic exposure, with a maximum of 300 nM.

For acute assays (ROS, GSH levels and ΔΨm assays) higher MeHg concentrations were chosen. In acute assays shorter exposure times were used. Due to shorter exposure time, MeHg concentrations were used at higher concentrations.

2.4.2 Selection of Organochlorine Pollutants (OCPs) Concentrations

The concentration of the seven organochlorine compounds used in this project were based upon findings of Briz et al. (2010) [47] and previous relevant experiments for dieldrin, and on the INMA Project Valencia mother-infant cohort study [108], [109] for 4,4'-DDE, β-HCH, δ-HCH, HCB, PCB-138 and PCB-153. The INMA project is a study involving several research groups in Spain with the aim to investigate the impact of the environmental pollutants on children. Concentrations were used at 10 or 100 times of the geometric mean (GM) values of concentrations detected in cord blood of the INMA project and are summarized in table 6.

TABLE 6: Organochlorine toxicant concentrations found in samples of cord blood in the INMA project

OCP compound	Geometric Mean (GM) Values (ng/ml)	100x GM Values (ng/ml)	100x GM Values (nM)	10x GM Values (nM)
4,4'-DDE	0.82	82	258	25.8
HCB	0.29	29	102	10.2
β-HCH	0.12	12	41	4.1
δ-HCH	0.023	2.3	7.9	0.79
PCB-138	0.1	10	28	2.8
PCB-153	0.13	13	36	3.6

4,4'-DDE, β-HCH, HCB, PCB-138 and PCB-153 were used as mixture, while δ-HCH was not, due to its low concentration in the cord blood samples. Dieldrin was not added to the mixture as it was not investigated in the INMA study.

From now on, if a "POPs mixture" is mentioned, it is referred to a mixture of 4,4'-DDE, β-HCH, HCB, PCB-138 and PCB-153.

2.4.3 Selection of Neurotransmitter and Antagonist Concentrations

DA concentrations for determining a sub-toxic dose were chosen from 100 µM of DA downwards based on a study by Noh et al. (1999) [(110)] demonstrating that 100 µM DA cause cell mortality.

The experimental concentration of 100 µM ACh was chosen based upon previous experiments relevant to this investigational aim that demonstrated that 100 µM is a sub-toxic ACh concentration.

Raclopride concentrations were chosen following the product data sheet of Abcam suggesting an IC_{50} of D_2 receptor inhibition at 32 nM.

2.5 Stock Solutions Preparation and Toxicant Treatment

MeHg, organochlorine reagents, neurotransmitter and antagonist solids were weighed and dissolved in H_2O (MeHg and ACh), ethyl acetate (HCB) or DMSO (4,4'-DDE, β-HCH, δ-HCH, HCB, PCB-138, PCB-153 and raclopride), portioned as 50 µl aliquots and stored in the freezer at -20°C. Fresh aliquots were used for every new treatment solution and further prepared as needed.

With these solutions cortical neurons were treated and exposed over varying times. DMSO and ethyl acetate, which were used to dissolve compounds, never exceeded 0.5% per well if exposed chronically. These concentrations proved not to be cytotoxic to primary culture cortical neurons.

2.6 Cortical Neuronal Cultures

The cerebral cortex is involved in crucial functions of the human brain, including motor and sensory functions and cognition. The cytotoxic effects of MeHg are strongly exhibited in the cerebral cortex which is also one of the most susceptible brain regions to organochlorines [(111)]. Additionally, neurons are the most susceptible brain cells regarding MeHg toxicity [(17)]. That's why we chose to culture cortical neurons which were isolated from 16-day old mouse foetuses. Foetuses were used at day 16 of pregnancy, because at that time mouse neurons are not differentiated yet and are close to their last separation by mitosis, resulting in a more pure culture [(112)]. The maturation of the neurons is expressed as days in vitro (DIV), starting at the day after seeding and indicating the age of the cell culture.

Primary cultures of cortical neurons were isolated from the cerebral cortices of 16-day-old NMRI mouse foetuses. Halothane was used to anesthetize pregnant mice which were sacrificed by cervical dislocation. The foetuses were extracted by forceps and the cerebral cortices were removed in a sterile hood. The cortices were cut with a razorblade and transferred to a mixture of Krebs buffer, bovine serum albumin (1.2% w/v) and $MgSO_4$ (150 mM). Consequently, brain tissue was hydrolysed at 37°C for 20 minutes by a solution containing trypsin (0.02% w/v) to dissociate cells and DNAse (0.0075% w/v) which should reduce freely floating DNA. Soybean trypsin inhibitor (0.052% w/v) stopped trypsinization. A syringe was used to mechanically disaggregate cells to obtain a single cell suspension. The cell suspension with a density of $8*10^5$ cells/ml was prepared in adapted DMEM medium (10% fetal bovine serum, 0.2% penicillin, 26.2 mM $NaHCO_3$, 25mM glucose, 0.2 mM L-glutamine, 100 mU/l insulin, 7 µM p-aminobenzoic acid). This cell suspension was seeded at a density of $8x10^5$ cells/ml in 24- or 96-well plates previously coated with poly-D-lysine (50 mg/L). The cultivation of the plated cells took place in a humidified incubator at 37°C with a 5% CO_2/ 95% air mixture. To avoid astrocyte growth, the antimitotic compound uridine (20 µM) was added on DIV1. Animal handling was done according to standard procedures at the University of Barcelona, which were approved by the Generalitat de Catalunya, Spain and are according to EU guidelines.

2.7 Experiments for Assaying Cell Viability

By measuring the formazan product of the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the MTT assay was used to quantify cell viability. Reduction of the MTT dye to formazan occurs by reduction of mitochondrial enzymes. The MTT solution was prepared in Hank's buffer at the concentration of 5mg/ml and added to each well of cells at a 10% v/v concentration. Plates were incubated for 2.5 hours at 37°C, protected from light, before the content of the wells was discarded. Next, 100 µl of 10% sodium dodecyl sulphate (SDS) were added to lyse the cells and dissolve the formazan and consequently incubated for at least four hours in an incubator at

37°C. Spectrophotometry was used to quantify the absorbance of the formazan product in a spectrometer at 560 nm.

The resazurin assay was also used to quantify cell viability, in this case, by measuring the fluorescence of resorufin, a fluorescent product of the non-fluorescent resazurin. The reduction of resazurin occurs due to the cell´s redox potential, which the cell needs for metabolic activity [113]. The resazurin solution was prepared at a concentration of 100 µg/ml in phosphate buffered saline (PBS) and added at a 10% v/v concentration to the seeded cells. Cells were incubated for 2 hours at 37°C to allow them to convert resazurin to resorufin. The fluorescence (Ex 530 nm/Em 590 nm) of resorufin was quantified in a fluorometer.

The propidium iodide (PI) assay was the third method to assess cell viability: PI is membrane-impermeable and intercalates with DNA. Thus, PI will intercalate only with DNA of cells which have already permeabilized membranes and therefore stains damaged cells. If intercalated with DNA, the excitation and emission wavelengths of unbound PI shift to the below-mentioned excitation and emission wavelengths of bound PI. The PI solution was prepared at a concentration of 40 µg/ml in PBS and added at 2% v/v concentration to the seeded cells. 30 minutes before adding the PI solution 10% Triton-X was added to negative control cells to lyse the cells. Next, PI solution was added and incubated for 30 min at 37°C to allow PI to intercalate with intracellular DNA. The fluorescence (Ex 535 nm/Em 617nm) of intercalated PI was quantified in a fluorometer.

2.8 Immunocytochemistry for Cell Morphology

Immunocytochemistry was used to get a qualitative image of cells exposed to various toxic compounds. For immunocytochemistry cells were seeded in 24-well plates. After discarding the solutions of the plate, 4% paraformaldehyde (PFA) was added for 10-15 min to fix the cells. PFA was removed from the plate and cells were washed three times for 5-10 min with PBS. Cell membranes were permeabilized with 0.15% v/v triton-PBS for 15 min to allow antibodies to enter. Blocking unspecific binding sites was done by incubating cells with 1% w/v BSA-PBS for 1 hour. Primary antibodies were diluted in 0.1% w/v BSA-PBS, 1:500 rabbit polyclonal anti-Tau and 1:1000 mouse monoclonal anti-GFAP antibodies, and added to the plate. Incubation of the cells with the primary antibodies was done in agitation at 4°C overnight. On the following day, cells were washed three times with PBS for 5-10 min. Secondary antibodies were diluted in 0.1% w/v BSA-PBS, 1:1000 green goat anti-rabbit IgG and 1:1000 red goat anti-mouse IgG antibodies. Cells were incubated with secondary antibodies at room temperature for 1 hour protected from light. Next, cells were washed one time with PBS for 5-10 min and incubated with 5 µM bisbenzimide (nuclear dye) in PBS for 5-10 min and as

final step washed with PBS for 5-10 min. A phase-contrast fluorescence microscope was used to see the stained cells at a 200x magnification. Five fields per well were photographed with the help of Leica Application Suite V3 software of which a representative picture was chosen.

2.9 Oxidative Stress Assays

To assess production of oxidative stress in cortical neurons, the fluorescence of oxidized 2',7'-dichlorodihydrofluorescin diacetate (H_2DCF-DA) was measured. The non-fluorescent H_2DCF-DA penetrates cells and gets oxidized by reactive oxygen species (ROS) converting it to the fluorescent 2',7'-dichlorofluorescein (DCF). The H_2DCF-DA stock solution was prepared at a concentration of 2 mM (1 mg/ml) in methanol and diluted to 10 µM in Hank's buffer. In the chronic assays, cells chronically exposed to the compounds were rinsed two times with Hank's buffer and incubated with the 10 µM H_2DCF-DA solution for 20 min at 37°C protected from light. Cells were rinsed again for two times with Hank's buffer and previously prepared H_2O_2 concentrations in Hank's buffer were added. Next, fluorescence of DCF (Ex 492 nm/Em 525 nm) was measured by a fluorimeter. Measurements were taken every 5 minutes until 30 min and then every 30 min.
In the acute assays, the same procedure was performed, but instead of adding the H_2O_2 solution, toxicant solutions were added to the cells.

For assessing how GSH levels are involved in oxidative stress, quantification of GSH levels was done via measurement of fluorescent monochlorobimane (mBCl). mBCl is not fluorescent until conjugated with GSH. 10 mM DL-buthionine-[S-R]-sulfoximine prepared in PBS were added 24 hours previous to the GSH determination as negative control, to inhibit GSH synthesis and incubated at 37°C. The next day, compounds that should be tested for GSH depletion were added to cells and incubated for 60 minutes at 37°C. Next, 8 mM mBCl was added and incubated for 30 minutes at room temperature and protected from light. The fluorescence (Ex 360 nm/Em 460 nm) of the conjugated mBCl was quantified via a fluorimeter.

2.10 Mitochondrial Membrane Potential (ΔΨm)

To quantify mitochondrial membrane potential (ΔΨm) fluorescent rhodamine 123 was used. Rhodamine can enter mitochondria but its fluorescent gets quenched by mitochondrial energization. A 10 mM rhodamine 123 stock solution was prepared in dimethyl sulfoxide (DMSO) and was diluted 1:10 in Hank's buffer. Cells were rinsed two times with Hank's buffer, then, the rhodamine 123 dilution was added to the cells and incubated for 15 min at 37°C. Cells were rinsed three times with Hank´s buffer and various concentrations of the compounds to be tested, prepared in Hank's buffer, were added. As negative control, 30 µM anitmycin A, an inhibitor of the respiratory chain, were added. The

fluorescence (Ex 485 nm/Em 530 nm) of non-quenched rhodamine 123 was quantified by a fluorimeter. Measurements were taken every 5 min until 30 min and then at 60 min. As control condition, 2% triton X was added after 60 min to the cells and incubated for 15 min at 37°C to lyse cells.

2.11 Statistical Analysis of Data

Obtained data were organized and analysed with Microsoft Excel 2007 and subsequently analysed and visualized with Graph-Pad Prism version 4.0 and 6.0 software (GraphPad Software Inc., San Diego, CA). Data are represented in bar graphs with mean ± SEM. When no statistically significant difference of the basal control values (i.e. the values of the treatment groups at 0 concentration of the toxicant that is plotted at the x axis of the graph) was found, each treatment condition was normalized to their basal control values to make a comparison among the groups easier and focus on possible interactive effects. Results were analysed with a two-way ANOVA analysis followed by a Bonferroni multiple comparison post-test.

3 RESULTS

3.1 Effects of Combined Exposure of MeHg and Single Compound OCPs on Viability

To study the effects of combined MeHg and organochlorine exposure on differentiating neurons, cells were consequently exposed to the toxicants from the cortical neurons' first day in vitro (DIV1). At this stage neurons are considered to be immature [112].

The first experiments were done for two purposes. Firstly, we wanted to investigate whether OCPs in combination with MeHg exacerbate MeHg-induced effects on cortical neurons. Secondly, these experiments were also used to approve or adjust previously used MeHg working concentrations to a less dense cell culture of $8*10^5$ cells/ml, which is half as dense as the previously used cell culture density.

In the first trials, differentiating cortical neurons were exposed to 0-1000 nM MeHg alone or in combination with 60 nM dieldrin or 100 nM HCB. In the first experiments the used concentrations higher than 300 nM MeHg (600 nM and 1000 nM MeHg) proved to be too high to be exposed over a longer period of time. Hence, MeHg concentrations were changed to 0-300 nM and the same experiments were repeated. Cells were exposed to 0-300 nM MeHg in absence or presence of 60 nM dieldrin or 100 nM HCB for 6-8 days (DIV1 to DIV6-8), depending on the culture's susceptibility to MeHg.

In this experiment no statistically significant change in cell viability by dieldrin and HCB could be observed, thus every value of the MeHg-dieldrin condition was normalized to the value of 0 nM MeHg and 60 nM dieldrin. The same applied for HCB, where values were normalized to 0 nM and 100 nM HCB. This was done to facilitate direct comparison in-between groups to focus on interactive effects. Furthermore, there is no statistically significant difference in cell viability when cells exposed to MeHg alone are compared to the combination of MeHg and dieldrin or MeHg and HCB, neither at sub-toxic or toxic MeHg concentrations, as can be seen in figure 6. No toxic effects of MeHg are observable until 100 nM MeHg. Beginning at 200 nM MeHg, cell viability decreases by 30% and is further reduced at 300 nM, reaching 60% of viability.

The same pattern was found, when viability curves were evaluated by means of a resazurin assay (data not shown).

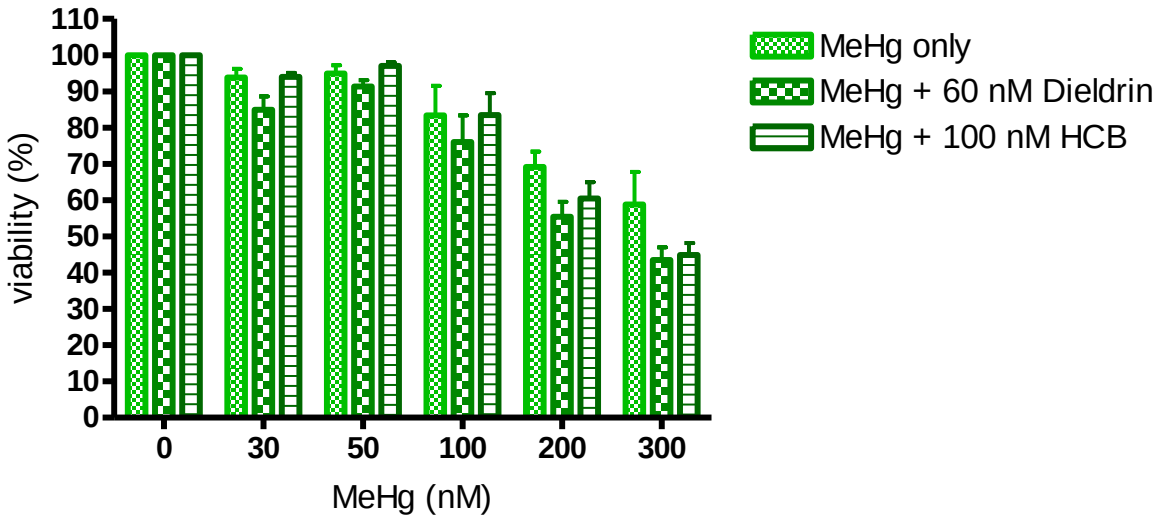


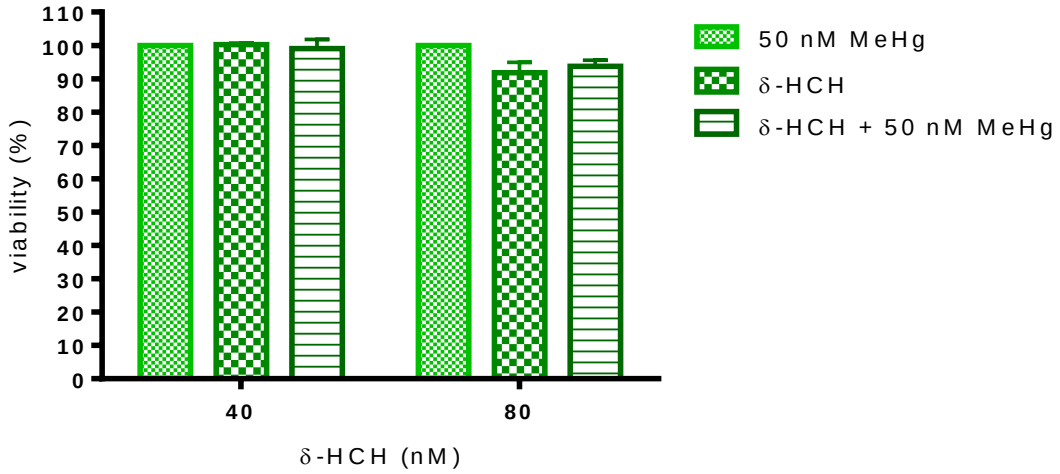
FIG. 6. Viability curve of cortical neurons after exposure to different MeHg concentrations alone or in combination with dieldrin or HCB. Primary culture cortical neurons were exposed to 0-300 nM MeHg in absence or presence of 60 nM dieldrin or 100 nM HBC from their first day in vitro (DIV1) to DIV 6-8. Viability was evaluated via MTT assay. The bars are expressed as a percentage of the MeHg-free basal control values. The data are represented as mean ± SEM and were obtained in three independent trials, using triplicates per treatment condition.

As there were no significant interactive cytotoxic effects of dieldrin or HCB with MeHg at any concentration observable, we decided to screen some OCPs for interactive cytotoxic effects with MeHg at a sub-toxic concentration. We chose δ-HCH, 4,4'-DDE, PCB-138 and PCB-153, as this selection comprises a representative variety of different compound groups that belong to the compounds with the highest levels detected in infant cord blood in the INMA project [(108), (109)].

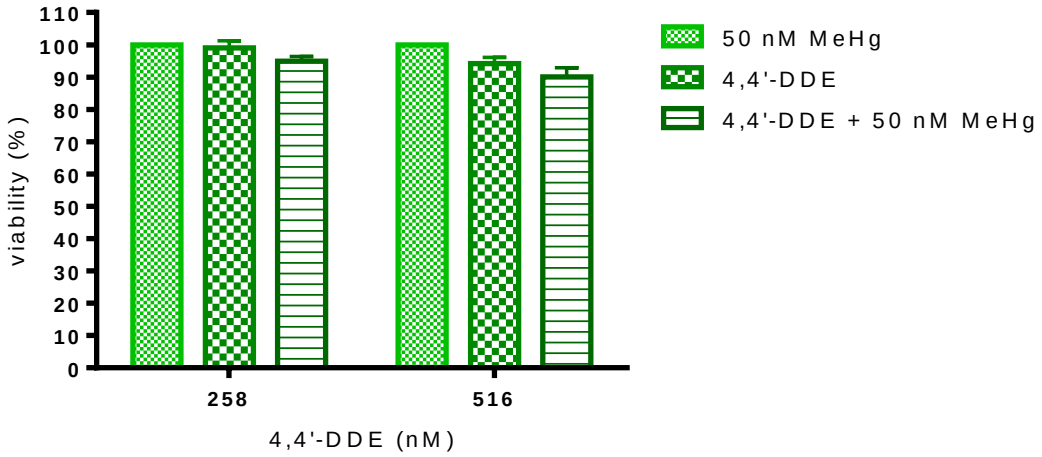
Primary culture cortical neurons were exposed for 8 or 9 days (DIV1 to DIV8/9) to 100 or 200 times the geometric mean (GM) of each of the abovementioned organochlorines, in absence or presence of 50 nM MeHg. By using 100x GM we compensated for the long-term exposure over months in real life, whereas by using 200x GM we wanted to determine whether the double of our working concentration would exhibits toxic effects.

Neither, there was a statistically significant toxic effect of an OCP compound alone, nor was a statistically significant interactive toxic effect of one of the four OCPs with 50 nM MeHg observable. No effect was observed at 100x GM, nor at 200xGM concentrations, as shown in figure 7.

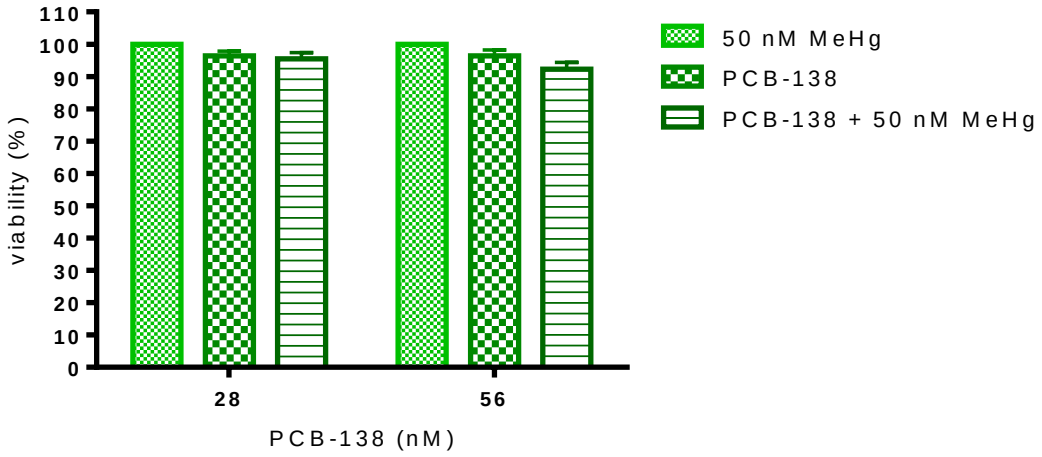
A


viability (%)
110
100
90
80
70
60
50
40
30
20
10
0
40
80
δ-HCH (nM)
50 nM MeHg
δ-HCH
δ-HCH + 50 nM MeHg

B


viability (%)
110
100
90
80
70
60
50
40
30
20
10
0
258
516
4,4'-DDE (nM)
50 nM MeHg
4,4'-DDE
4,4'-DDE + 50 nM MeHg

C


viability (%)
110
100
90
80
70
60
50
40
30
20
10
0
28
56
PCB-138 (nM)
50 nM MeHg
PCB-138
PCB-138 + 50 nM MeHg

D

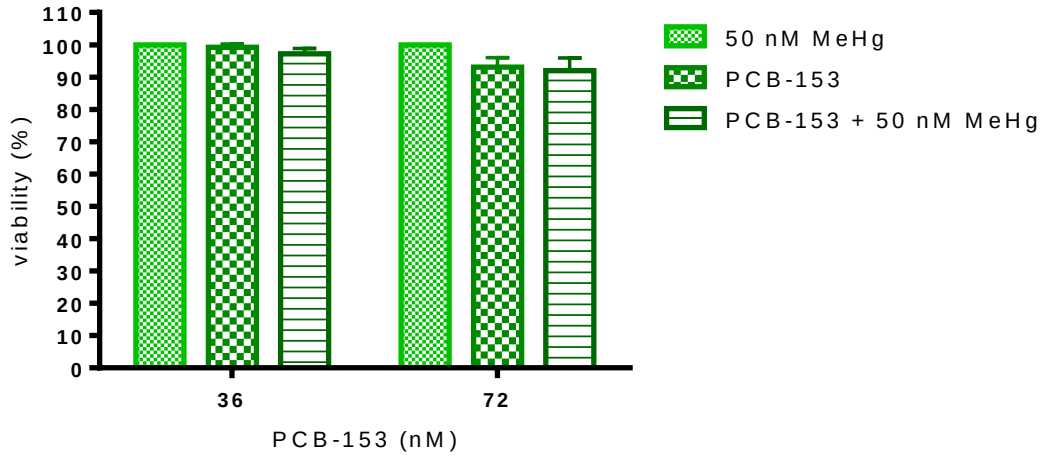


FIG. 7. Viability of cortical neurons after exposure to MeHg alone or in combination with an organochlorine compound at different concentrations. Cells were intoxicated either in absence or presence of 50 nM MeHg with 100 or 200 times the geometric mean (GM) of δ-HCH (A), 4,4'-DDE (B), PCB-138 (C) and PCB-153 (D) on DIV1 and exposed until DIV8-9. The viability was evaluated via MTT assay. The bars are expressed as percentage of the value of 50 nM MeHg. The data are represented as mean ± SEM and were obtained in three independent trials, using triplicates per treatment condition.

3.2 Effects of Combined Exposure of MeHg and POPs mixture on Viability

Our next experiment was done to evaluate a possible interactive toxicity of a mixture of representative organochlorines, found at high levels in infant cord blood in the INMA project [(108), (109)], and MeHg. In this experiment sub-toxic and toxic MeHg concentrations in combination with a POPs mixture at different GM concentrations were used. The mixture comprises 4,4'-DDE, HCB, β-HCH, PCB138 and PCB153. From now on, the mixture comprising 4,4'-DDE, HCB, β-HCH, PCB138 and PCB153 will be referred to as "POPs mixture".

In this experiment cells were exposed consequently for 8-9 days (DIV1 to DIV8/9) to MeHg concentrations ranging from 0-300 nM and the mixture of OCPs at 10x GM and 100x GM concentrations. Neither, statistically significant toxic effects of the POPs mixture at 10x or 100x GM, nor statistically significant interactive cytotoxic effects between the different treatment conditions were found, as illustrated in figure 8.

The results are supported by data which were obtained by evaluating the same experiments by means of a PI assay (data not shown). The obtained graph showed the same viability pattern as the assay done by MTT.

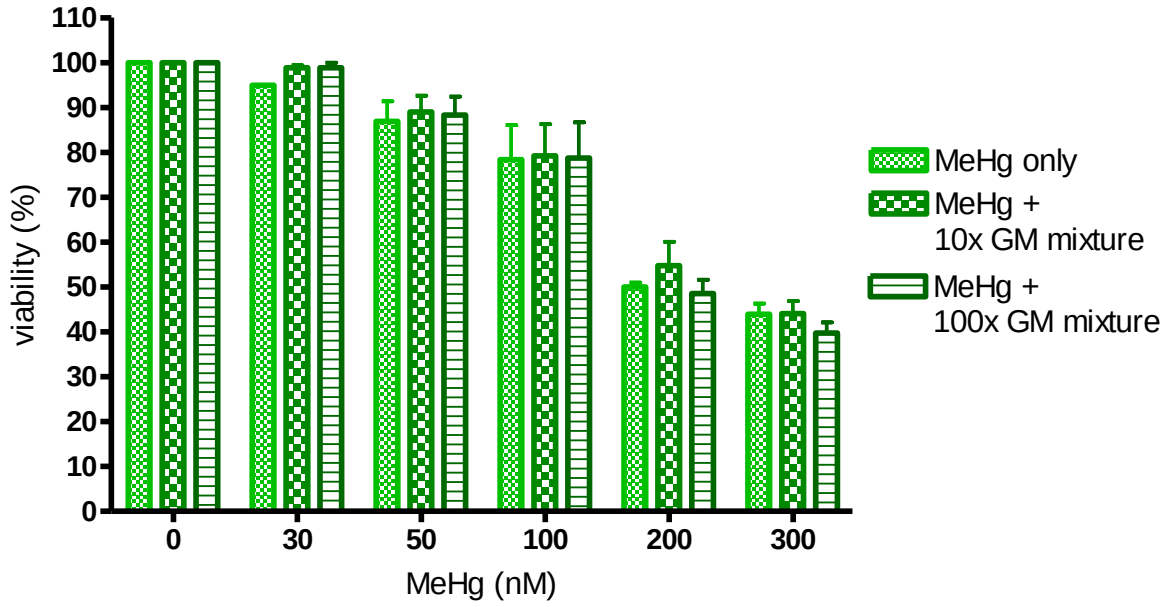


FIG. 8. Viability of cortical neurons after exposure to the POPs mixture and MeHg at different concentrations. Cells were intoxicated with MeHg concentrations in a range from 0-300 nM and the POPs mixture at 10x or 100x GM of HCB, β-HCH, 4,4'-DDE, PCB-138 and PCB-153 on DIV1 and exposed until DIV8-9. The viability was evaluated via MTT assay. The bars are expressed as a percentage of the MeHg-free basal control values. The data are represented as mean ± SEM and were obtained in three independent trials, using triplicates per treatment condition.

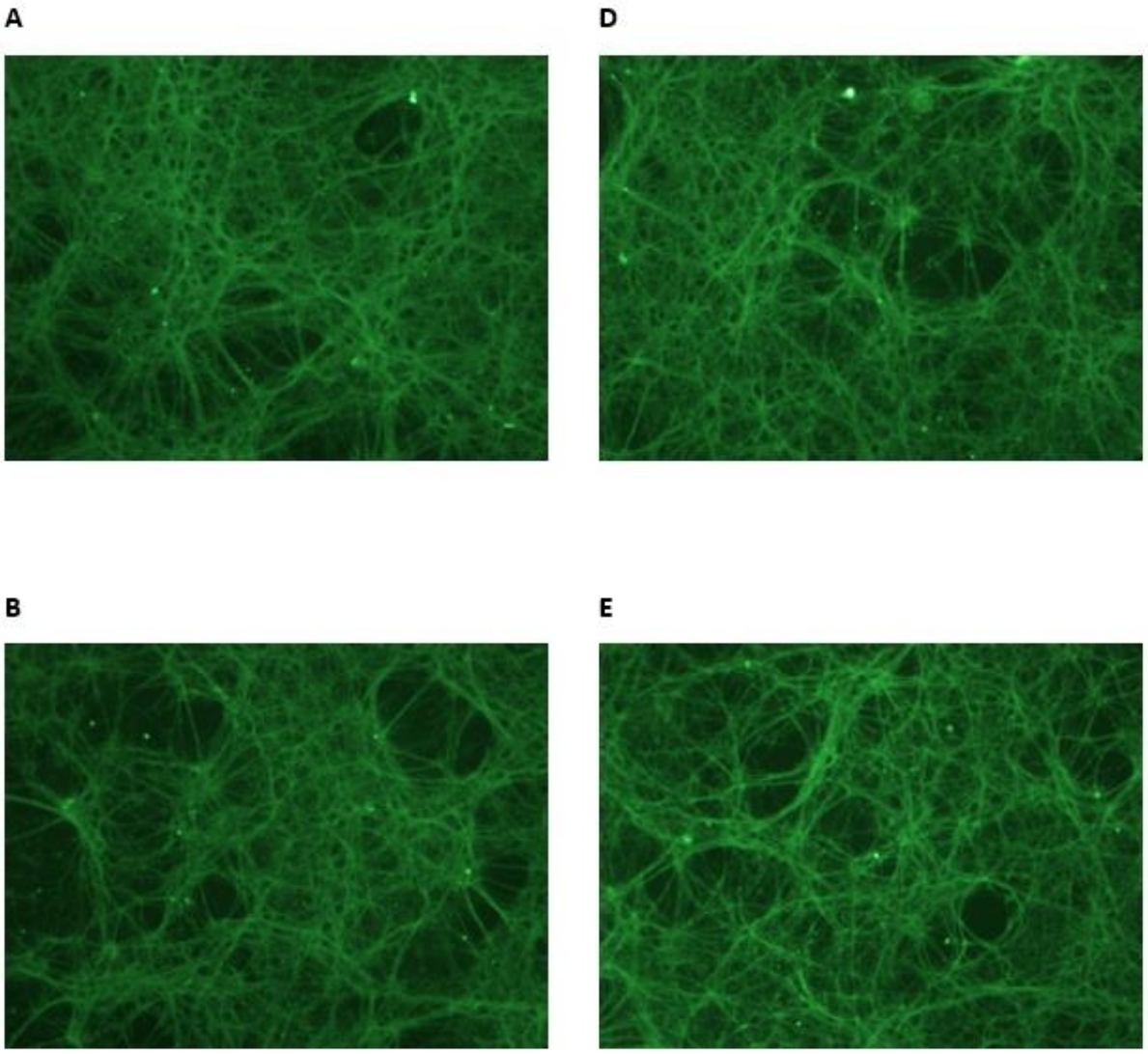
To investigate whether the MeHg concentrations of acute assays influence cell viability, cells were exposed on DIV7/8 for 24h (which is longer than actually needed for acute assays) to MeHg (up to 1 µM) and the 100x GM POPs mixture. No statistically significant decrease in cell viability could be found in any of the treatment conditions. The experiments were evaluated by PI and MTT assay and were realized in two independent trials, using triplicates per treatment condition (data not shown).

We were not only interested in qualitative viability assays but we also wanted to investigate the quality of the neuronal culture exposed to toxicants. Immunostains can provide interesting qualitative information about cell morphology. We were interested in possible differences in cell morphology, comparing cells with different treatment conditions (MeHg alone and in combination with the POPs mixture). Although we did not find any statistically significant difference in cell viability, we decided to make cell morphology visible by immunocytochemistry, as MeHg [59] and PCBs [60], [61] are supposed to have the ability to interfere with microtubules and thus change cell morphology.

Cortical neurons from primary cultures were exposed to MeHg at 0, 50 and 100 nM alone or in combination with the POPs mixture at 100x GM concentrations for 8 days (DIV1-8) and consequently fixed. To make neurons and astrocytes visible, we stained the cells with antibodies for Tau and GFAP.

Tau is a protein that is bound to the microtubules of neurons and GFAP occurs specifically in astrocytes. The fluorescent stain bisbenzimide was used to stain the nuclei of the cells.

Neuronal cell bodies and a dense neurite network are well observable due to the Tau immunofluorescence. Cell density and neurite network density decreases with increasing MeHg concentrations, showing no difference between treatment conditions except for cells at 100 nM MeHg, where a slight difference between the two different treatment conditions is observable. The cells exposed to MeHg alone at 100 nM seem to be less susceptible than those where the additional 100x GM POPs mixture was added which can be seen in figure 9.



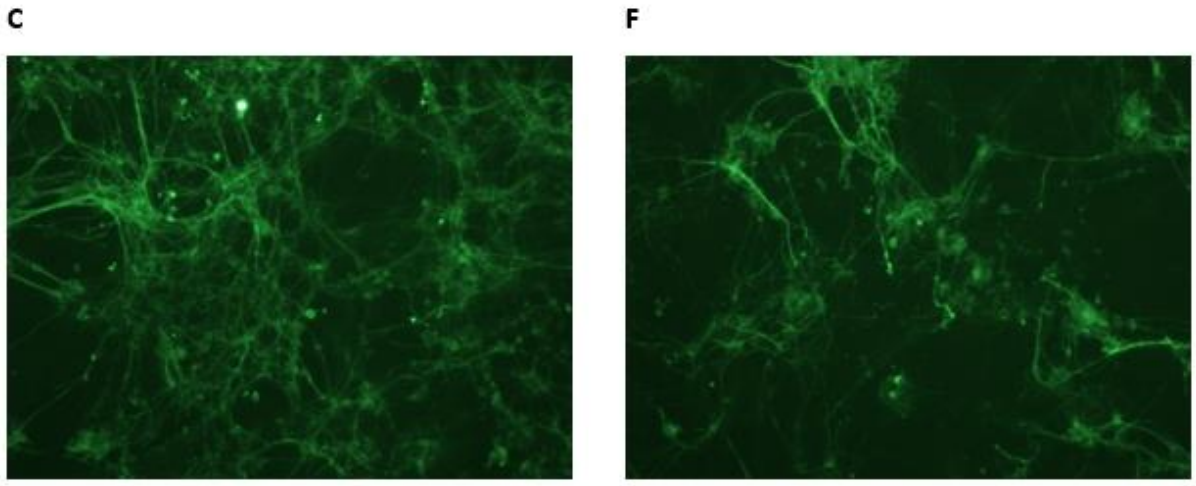


FIG. 9. Fluorescence microscopy images of Tau-immunostained cortical neurons from primary cortical neuronal cultures. Only neurons (at DIV8) immunostained with Tau are depicted here, as astrocytes, stained with GFAP, and nuclei, stained with bisbenzimide, are not shown for clarity. Neurons were either left untreated (A), exposed to 50 nM MeHg (B), 100 nM MeHg (C), 100x GM POPs mixture (D), 50 nM MeHg and 100x GM POPs mixture (E) or 100 nM MeHg and 100x GM POPs mixture (F). Decreasing cell body density and neurite density with increasing MeHg concentrations are observable. One image was chosen from five regions per well, with one well per condition. The images were taken at a 200x magnification.

3.3 Effects of Combined Exposure of MeHg and POPs on Oxidative Stress

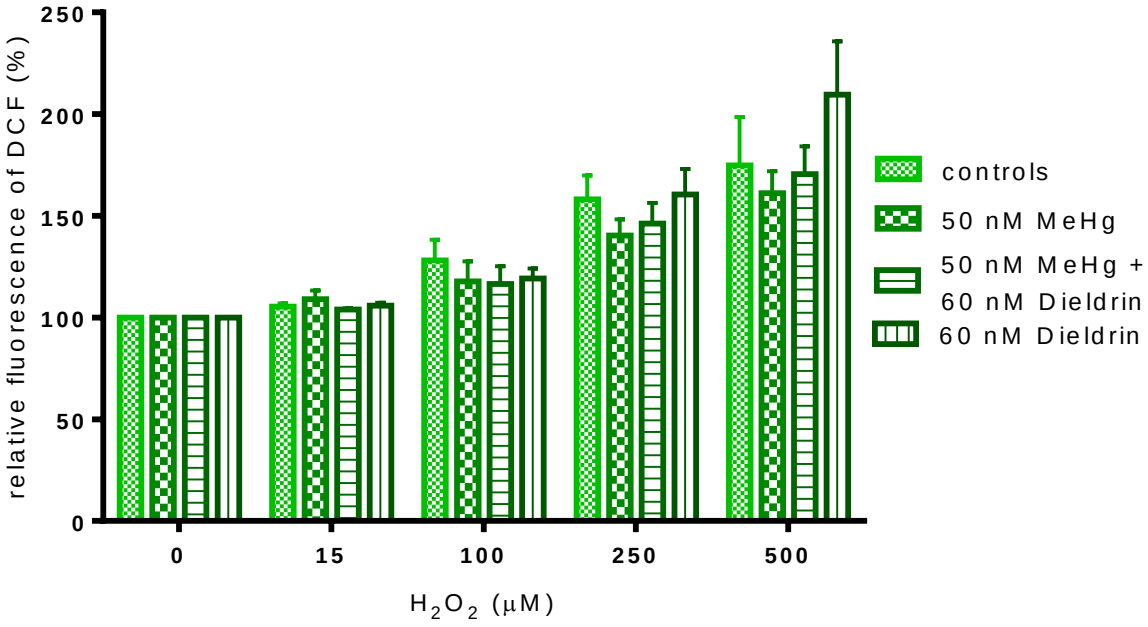
Studies by Francoa et al. (2009) [53] and Shinyashiki et al. (1996) [114] have demonstrated that enzymes important for protection against oxidative stress can be inhibited by MeHg, including the ROS-neutralizing enzymes *glutathione peroxidase* and *superoxide dismutase*. In this experiment we wanted to investigate whether the chronic and combined exposure of MeHg and dieldrin or MeHg and HCB would exhibit a difference in ROS production if compared to cells exposed to MeHg only. In this assay we used a sub-toxic MeHg concentration to study the influence on ROS production in the cells.

Cortical neurons were left alone or exposed to sub-toxic 50 nM MeHg and 60 nM dieldrin (each compound alone and in combination) for 6 to 8 days, depending on the culture's susceptibility. On the last day of exposure the assay was performed by adding increasing hydrogen peroxide (H_2O_2) concentrations to induce oxidative stress and investigate if cells exposed to the toxicant combination would exhibit increased ROS production compared to untreated cells or cells exposed to single compounds. The same experiment was also performed with 100 nM HCB, instead of 60 nM dieldrin.

In this experiment, it has been demonstrated that there is no statistically significant difference between cells exposed to different treatment conditions. As there was no statistically significant difference between the treatment groups without H_2O_2, their values were normalized to each of their H_2O_2-free condition to facilitate in-between comparison among groups.

The same results were obtained when the experiment was realized with 100 nM HCB instead of dieldrin. Both graphs are shown in figure 10.

A



B

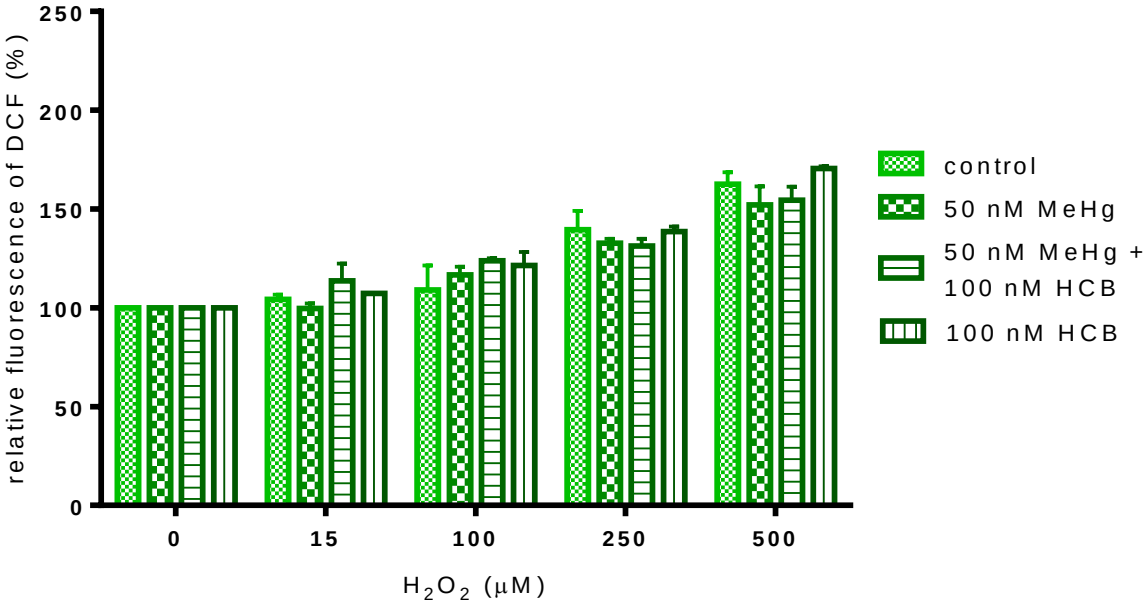


FIG. 10. Effect of MeHg alone or in combination with organochlorine pesticides dieldrin or HCB on ROS production. Cells were intoxicated with 50 nM MeHg and 60 nM dieldrin (A) or 100 nM HCB (B) on DIV1 and exposed until DIV6-8 when H_2O_2 at different concentrations was used to induce oxidative stress in mature cells. Reactive oxygen species were measured via DCF fluorescence at 30 minutes after adding hydrogen peroxide. The bars are expressed as a percentage of the H_2O_2-free basal control values. The data are represented as mean ± SEM and were obtained in three independent trials using triplicates per treatment condition.

Next, we investigated the acute effects of chronically toxic MeHg concentrations (up to 1 µM) in combination with the 100x GM POPs mixture on oxidative stress. We used a ROS assay (on DIV6/7), to investigate a possible induction or increase of oxidative stress, and measured GSH levels (DIV2 and DIV8), as GSH plays an important role in neutralizing ROS. In both experiments we could not find any observable, statistically significant effects.
Both experiments were realized in two independent trials, using triplicates per treatment condition (data not shown).

3.4 Effects of Combined Exposure of MeHg and POPs mixture on ΔΨm

Because of studies suggesting the involvement of the mitochondria as one possible mechanism for the neurotoxicity of MeHg [46] and for the harmful effects of POPs on mitochondria [42], [43], we decided to do an experiment investigating mitochondrial function at combined exposure of MeHg and the POPs mixture.

Primary cultures of cortical neurons were exposed at DIV 7-8 to the 100x GM POPs mixture in absence or presence of MeHg concentrations ranging from 0 to 1000 nM. 30 µM of antimycin A were used as positive control, leading to mitochondrial membrane depolarization of nearly 200%.

We found a tendency that increasing MeHg concentrations lead to an increased level of depolarized mitochondrial membranes, as well as cells exposed to the 100x GM POPs mixture alone, tend to depolarize the inner mitochondrial membrane too. When primary cultures were exposed to both, MeHg and the mixture, the depolarization by the POPs mixture on its own is statistically significant different ($p < 0.05$) from the control value without MeHg and POPs mixture. Furthermore, statistically significant ($p < 0.01$) interaction between the two treatments was found. The tendency of the POPs mixture to depolarize the mitochondrial membrane seems to be increasingly counteracted by increasing MeHg concentrations. In combination, the depolarizing effect of the POPs mixture seems to be negatively correlated with the MeHg concentrations, as can be seen in figure 11.

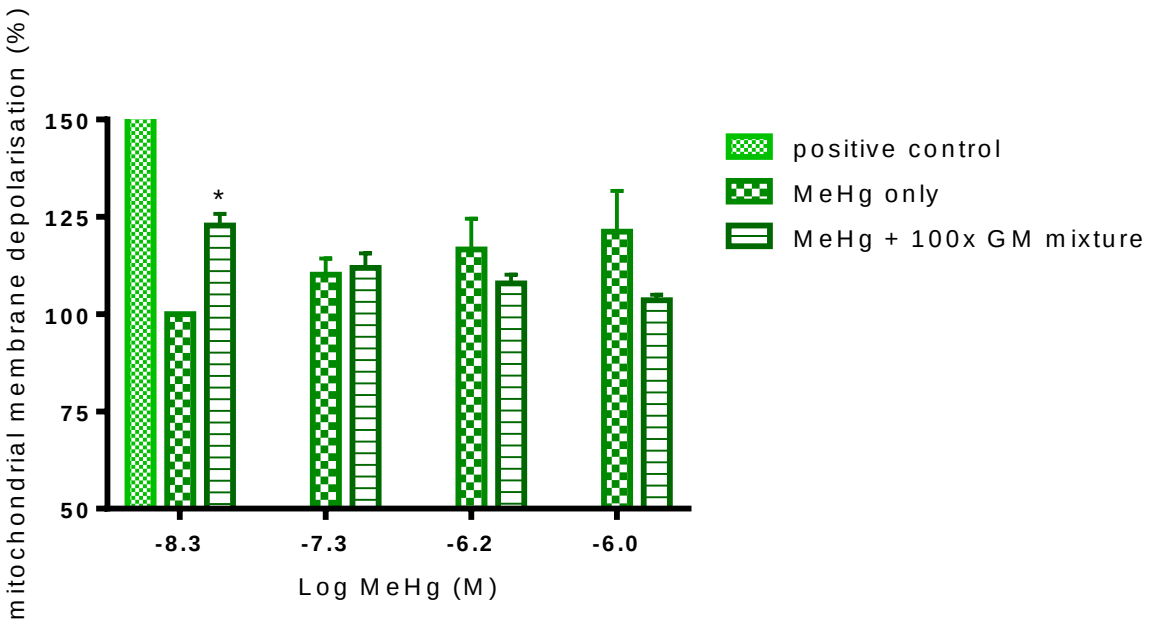


FIG. 11. Mitochondrial membrane depolarization in cortical neurons after MeHg and 100x GM POPs mixture exposure. On DIV 7-8 cells were exposed to MeHg concentrations at 0, 50, 600 and 1000 nM in absence or presence of the POPs mixture at concentration 100x GM, and to 30µM antimycin A as positive control. Levels were measured via rhodamine 123 fluorescence at 60 minutes after addition of the reagents. The bars are expressed as a percentage of the control basal value. The data are represented as mean ± SEM and were obtained in three independent trials using triplicates per treatment condition. Statistical significance marked as * ($p < 0.05$) compares the difference between cell exposed to MeHg only and cells exposed to MeHg and the OCP 100x mixture. Statistical significant ($p < 0.01$) interaction was found between the two treatments.

3.5 Effects of Combined MeHg-ACh Exposure on Viability

We used the sub-toxic ACh concentration of 100 µM, to investigate possible interactive effects with sub-toxic and toxic concentrations of MeHg. The sub-toxic ACh concentration was determined in previous experiments relevant to this investigational aim.

Neuronal cells from the primary culture were exposed from DIV1 for 5-6 days to MeHg alone at concentrations ranging from 0-300 nM or in combination with 100 µM ACh.

In this experiment it could be proven that 100 µM do not exhibit any toxic effect on the used cell culture. For this all MeHg-100 µM ACh values were normalized to the ACh only-value to facilitate in-between treatment comparison. But, as seen in figure 12, ACh was found to statistically significant increase cell mortality in combination with 100 nM MeHg.

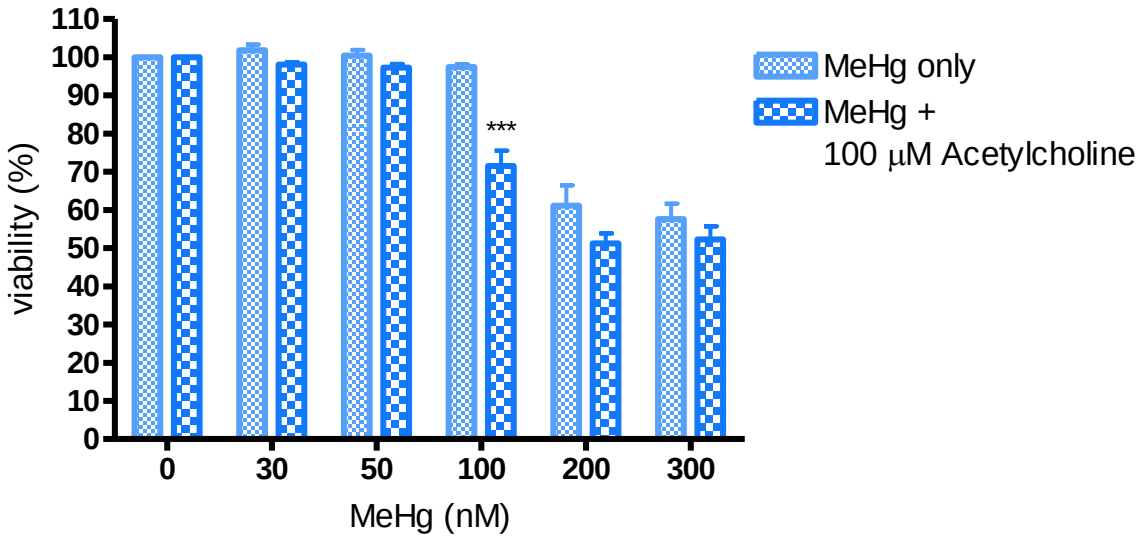


FIG. 12. Concentration dependent viability of cortical neurons after exposure to MeHg alone at different concentrations or in combination with ACh. Cells were exposed to MeHg concentrations in a range from 0-300 nM and 100 μM ACh from DIV1 to DIV5-6. The viability was evaluated via MTT assay. Bars are represented as percentage of MeHg-free basal control values. The data are represented as mean ± SEM and were obtained in three independent trials, using triplicates per treatment condition. Statistical significance is marked as *** ($p < 0.001$).

3.6 Effects of Combined MeHg-DA Exposure on Viability

Cortical neurons were exposed for 5-7 days (DIV1-5/7) to DA at concentrations ranging from 10-100 μM, in presence or absence of 50 nM MeHg.

Figure 13 shows that concentrations up to 10 μM DA, in combination with MeHg or not, do not exhibit any statistically significant effect on cell viability, whereas from 30 μM on, cell mortality increases from 40% at 30 μM DA up to 80% at 100 μM DA.

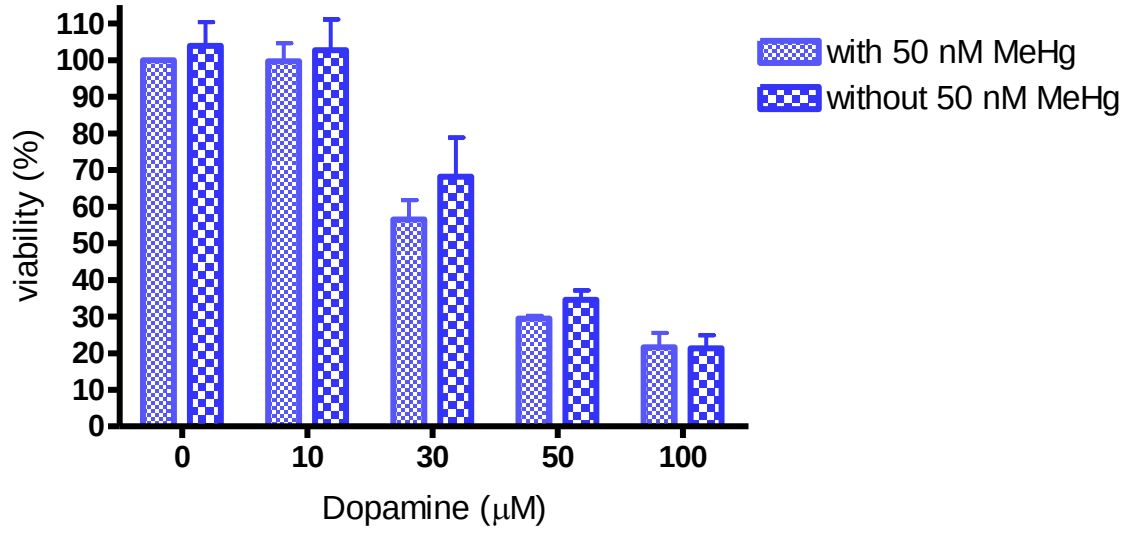


FIG. 13. Concentration dependent viability of cortical neurons after exposure to different concentrations of DA in absence or presence of 50 nM MeHg. Cells were exposed to DA concentrations in a range from 0-100 µM alone or in presence of 50 nM MeHg from DIV1 to DIV5-7. The viability was evaluated via MTT fluorescence. Bars are represented as percentage of the MeHg- and DA-free basal control value. The data are represented as mean ± SEM and were obtained in three independent trials, using triplicates per treatment condition.

Based on the previous finding the working concentration was set to the sub-toxic concentration of 10 µM DA and was used to further investigate the effect of this DA concentration on various MeHg concentrations, including toxic ones.

Cortical neurons were exposed for 6-8 days (DIV1 - DIV6/8) to 10 µM DA in presence or absence of various MeHg concentrations ranging from 0-300 nM.

As already proven above, 10 µM proved not to be toxic alone, hence MeHg-DA values were normalized to the DA alone-value. As can be seen in figure 14, 10 µM DA statistically significant increased cell viability at toxic MeHg concentrations. As shown in figure 16, at 100, 200 and 300 nM a viability increasing effect of DA could be observed. At 200 nM MeHg viability was increased up to 30%.

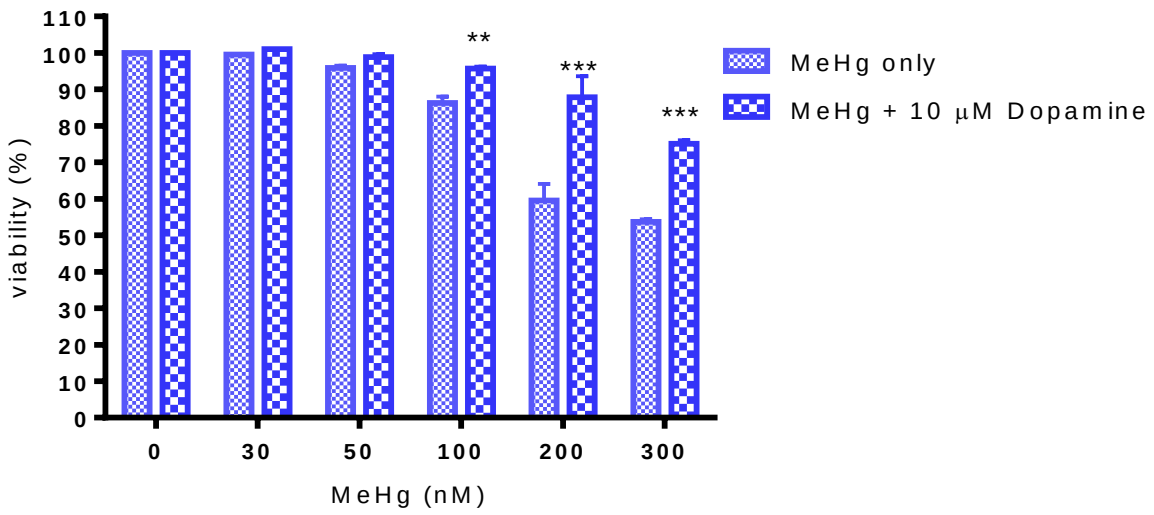


FIG. 14. Concentration dependent viability of cortical neurons after exposure to different concentrations of MeHg alone or in combination with 10 µM DA. Cells were exposed to MeHg concentrations in a range from 0-300 nM and 10 µM DA from DIV1 until DIV6-8. The viability was evaluated via MTT assay. The bars are represented as percentage of the MeHg-free basal control values. The data are represented as mean ± SEM and were obtained in three independent trials, using triplicates per treatment condition. Statistical significance is marked as ** ($p < 0.01$) and *** ($p < 0.001$).

3.7 Mechanisms involved in the Protective Effect of DA against MeHg toxicity

As MeHg and organochlorines compounds are suspected to be involved in inducing excitotoxicity (see section 1.4.2), we investigated a possibly increased susceptibility to glutamate-induced excitotoxicity.

Primary culture cortical neurons were exposed for 5 days (DIV1-5) to 50 nM MeHg in absence or presence of 10 µM DA. On DIV5 glutamate concentrations (0-600 µM) were added and exposed for another 2-4 days, depending on the culture's vulnerability.

As none of the treatment conditions exhibited toxic effects at 0 µM glutamate they were normalized to each of their basal control values. No statistically significant differences could be observed regarding the treatment: Cells exposed to MeHg were not more susceptible to glutamate-induced excitotoxicity than naïve ones, nor did 10 µM DA statistically significant increase cell viability, regardless of the presence of 50 nM MeHg. After 2-4 days of exposure to glutamate, cell-viability was reduced up to 40% at 600 µM glutamate, as can be seen in figure 15.

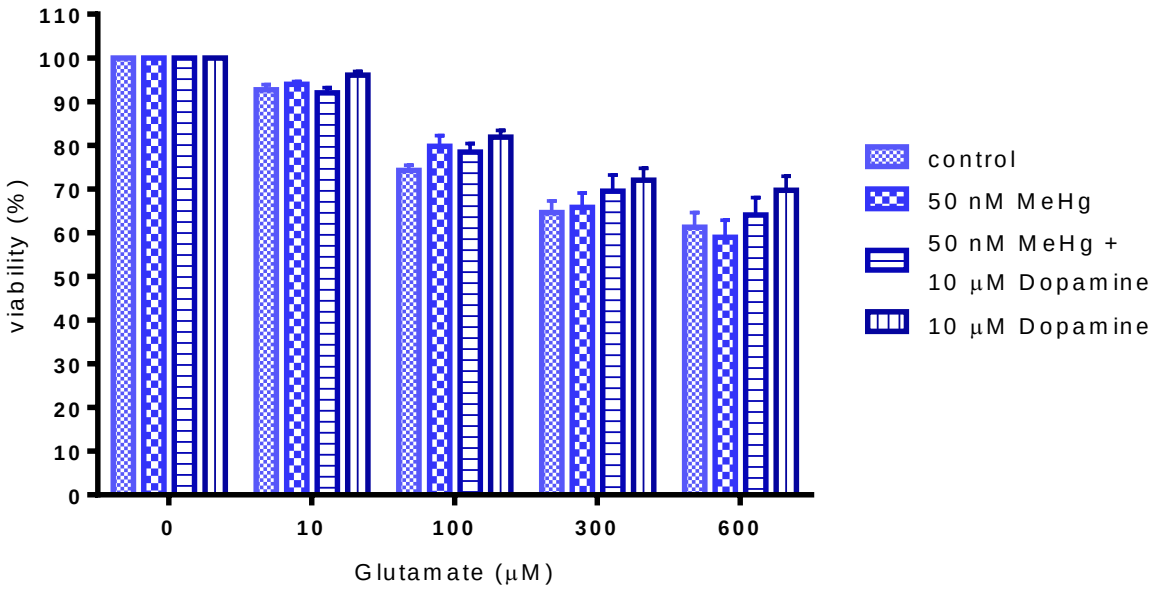


FIG. 15. Concentration dependent viability of cortical neurons after exposure to 50 nM MeHg alone or in combination with 10 µM DA and consequent exposure to different glutamate concentrations. Cells were exposed to 50 nM MeHg in absence or presence of 10 µM DA or DA alone from DIV1 until DIV5. On DIV5 glutamate (0-600 µM) was added and exposed for another 2-4 days (DIV7-9). The viability was evaluated via MTT assay. The bars are represented as percentage of the glutamate-free basal control values. The data are represented as mean ± SEM and were obtained in three independent trials, using triplicates per treatment condition.

To study the protective effect of DA against MeHg toxicity, we did an experiment to investigate the role of DA receptors involved in this mechanism. A study by Coccini et al. (2011) [115] showed that exposure to MeHg leads to alterations in D_1 and D_2 receptors. We were interested in whether blocking the D_2 receptor with 2 µM of the D_2 receptor antagonist raclopride could prevent the protective effect of 10 µM DA.

Using an MTT assay, we could not find any statistical significant difference between cells exposed to the MeHg-DA combination and the MeHg-DA-raclopride combination. The protective effect was not reduced by the additional exposure of the D_2 receptor antagonist raclopride.

Experiments were realized in two independent trials, using duplicates per treatment condition (data not shown).

3.8 Effects of Combined MeHg-POPs Exposure on the Protective Effect of DA

Due to the interesting effect of 10 µM DA, we wanted to investigate whether the same viability increasing effect could also be observed, if cells were exposed not only to MeHg but additionally to the 100x GM POPs mixture.

Primary cortical neuronal cultures were exposed for 6-8 days (DIV1 to DIV6-8) to 10 µM DA in absence or presence of the 100x GM POPs mixture and MeHg at different concentrations (0-300 nM).

As no statistical difference at 0 nM MeHg between the two treatments was observable, values were normalized to the corresponding control value. In this experiment we also found the viability increasing effect of 10 µM DA, on cells exposed to MeHg and the POPs mixture at 100x GM concentrations. Figure 16 shows that statistically significant cell viability increase was found at MeHg concentrations of 100, 200 and 300 nM, with the biggest increase of 20% at 200 nM MeHg and POPs mixture.

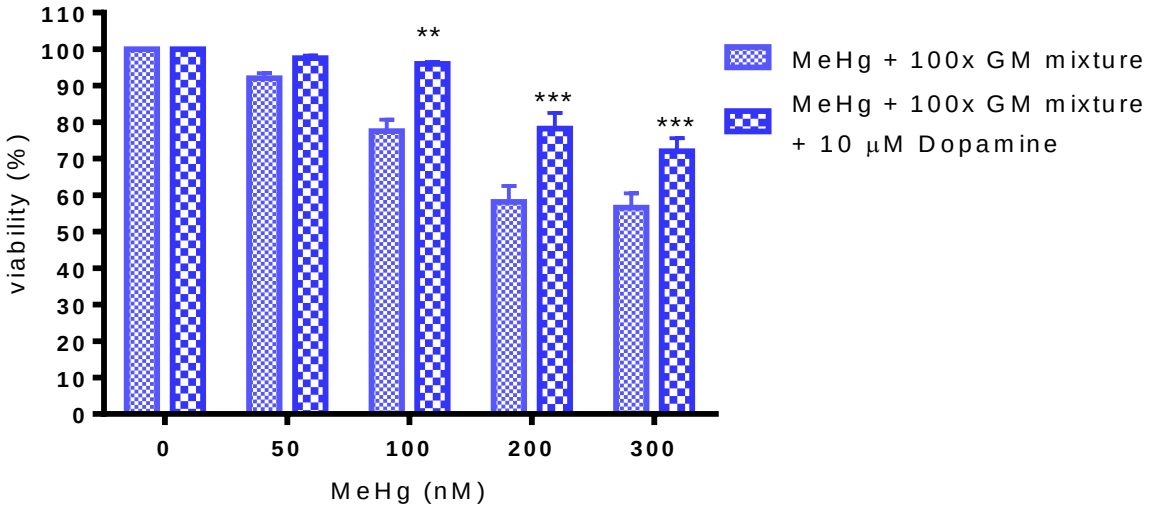


FIG. 16. Concentration dependent viability of cortical neurons after exposure to different concentrations of MeHg and POPs mixture in absence or presence of 10 µM DA. Cells were exposed to the POPs mixture at 100x GM concentrations and MeHg concentrations in a range from 0-300 nM and 10 µM DA from DIV1 until DIV6-8. The viability was evaluated via MTT assay. The bars are represented as percentage of the MeHg-free basal control values. The data are represented as mean ± SEM and were obtained in three independent trials, using triplicates per treatment condition. Statistical significance is marked as ** ($p < 0.01$) and *** ($p < 0.001$).

4 DISCUSSION

4.1 Effects of Combined Exposure of MeHg and Single Compound OCPs on Viability

Our findings demonstrate that exposure of differentiating cortical neurons to MeHg in presence of the organochlorine pesticides dieldrin, HCB, β-HCH, δ-HCH, 4,4'-DDE, PCB-138 and PCB-153 at 100x GM concentrations do not increase the cytotoxic effect of MeHg.

The literature on interactive toxicity of the seven used OCPs with MeHg is limited, only one study by Omara et al. (1997) [(116)] reports about no interactive toxicity if cells were exposed to MeHg and PCB mixtures concentrations in the same range as the used concentrations.

Along our experiments we experienced difficulties with the 100 nM MeHg concentration, as depending on the cell culture, weaker cultures exhibited an increase in mortality at already 100 nM of MeHg, whereas less susceptible neurons of other primary cultures did not show any toxic effects at this concentration. It was interesting to observe that 100 nM of MeHg seemed to be a threshold value for MeHg toxicity.

Four different assays were used to evaluate cell viability: MTT, resazurin and PI as quantitative assay, and immunostains as a qualitative method. We compared the three of them at the beginning of this project to find the optimal method. Although they showed the same pattern of cell viability in the same experiment, there were some quantitative differences between resazurin and MTT assay. MTT and PI assay exhibited the same quantitative results. Comparing resazurin with MTT assay, MTT results were more in accordance with the observations made via the phase-contrast microscope. Resazurin assays possibly would have needed adjusted reagent concentrations which would have cost time and material. The PI assay is much more time-consuming if several plates have to be treated, as the small volume of reagent cannot be pipetted with the multi-step or repetition pipet. So we chose to do further viability evaluation by means of MTT assays only.

The fourth technique, the immunostains, shows a qualitative image of the different conditions cells were exposed to. A less dense neuronal network was observed with increasing MeHg concentration, with no big difference regarding treatment condition, apart from cells exposed at 100 nM MeHg. Cells intoxicated with 100 nM MeHg in combination with POPs mixture seemed to be more susceptible. The decreased cellular morphology in the well exposed to MeHg and organochlorines could be explained by PCBs' ability to depolymerize actin filaments that are interacting with microtubules [(60), (61)]. At 100 nM MeHg, the effect of the PCBs might have become observable in combination with the increased microtubule depolarizing activity of MeHg. The decreasing cell morphology due to MeHg exposure are

in accordance with a study by Miura et al. (1998) [59] that reports about the ability of MeHg to depolymerize microtubules ($5*10^{-6}$ M MeHg/3 hours; Miura et al. (1998) [59]).

The conflicting decrease in cell number seen at 100 nM MeHg, compared to no observable or just little toxic effect if evaluated by MTT, may be due to the fact that this culture might have been one of the more susceptible cultures. Furthermore, we have to take into account that despite constant cell density among the immunostains and MTT assay experiments, there was a difference in surface area of the culture wells. This possibly influences neuronal distribution patterns and exposure physics. Lower concentrations of OCPs and MeHg might be necessary.

4.2 Effects of Combined Exposure of MeHg and OCPs on Oxidative Stress

As increased ROS production can render cells more susceptible and contribute to cell death [117], it seemed interesting to investigate the effect of MeHg and OCPs on ROS production in a chronic assay. But investigating ROS generation in cells exposed to MeHg over a longer period of time proved not to be easy, as MeHg concentrations higher than 50 nM would kill the cells and therefore, could not be compared to non-treated cells regarding their ROS levels. So we chose to first investigate ROS protective mechanisms at sub-toxic pollutant concentrations. Chronic exposure of cells to sub-toxic 50 nM MeHg in combination with 100 nM HCB or 60 nM dieldrin did not seem to have any observable influence on ROS levels. Neither did single exposure of the compounds. Although studies by Francoa et al. (2009) [53] and Shinyashiki et al. (1996) [114] demonstrate inhibition in two important anti-oxidant enzymes, *glutathione peroxidase* (GPx) and *superoxide dismutase*, low dose MeHg concentrations did not show increased oxidative stress levels. This might be due to the fact that low dose MeHg concentrations do not cause enough inhibition of ROS neutralizing enzymes (25% GPx activity reduction at 1µM MeHg; Francoa et al. (2009) [53]) to influence oxidative stress levels.

As we could not find any difference in oxidative stress levels at sub-toxic toxicant level, we investigated if MeHg, at concentrations that proved to be toxic in chronic exposure, and combined with various organochlorines, could induce oxidative stress. As we performed an acute assay, we used higher MeHg concentrations and the POPs mixture at 100x GM concentrations.

We did not find any increase in ROS levels if cells were exposed to MeHg-POPs mixture combination, neither, if cells were exposed to MeHg or the mixture alone. Francoa et al. (2009) [53] demonstrated an increase in ROS levels at 1µM MeHg. The non-existing effect in our experiment might be due to the fact that cells in the study were exposed for 24 hours instead of 3 hours in our experiment. Furthermore, we were hypothesizing that the method might not have been sensitive enough. This, as well as the generally non-toxic concentration of PCBs in our experiment, could also be a valid

explanation for apparently no effects by PCBs, although there is evidence that PCBs principally have the ability to affect mitochondrial function and thus increase ROS [43].

ROS and GSH are closely related, as GSH is important for neutralizing peroxides and thus reduces ROS levels. But GSH gets bound by MeHg due to its high affinity for thiol groups which in turn leads to increased ROS levels, as less free GSH is available [41]. We were interested in how the combination of MeHg and POPs would affect GSH levels and if our previous ROS-results would be supported by the GSH assay. This is why we investigated the ability of MeHg and organochlorine pesticides of affecting GSH levels in primary culture cortical neurons. The combination of both of them did not alter GSH levels.
A study by Gatti et al. (2004) [118] reports that 5µM MeHg depleted GSH levels of 15%. We might have seen no increase because we were using lower MeHg concentrations (maximum of 1µM). Studies investigating PCBs' and 4,4'-DDE's effect on GSH levels, are in agreement with our findings, as both didn't show any influence on GSH levels [54], [55].

Concluding on oxidative stress, our findings suggest that low MeHg and organochlorine concentrations do not affect cellular oxidative stress levels. In the experiments of literature findings, always higher (*micro*molar) MeHg concentrations were used. They are reported to increase statistically significant ROS production in a rat synaptosome [119] and cerebellar neurons [120], as well as GSH depletion [118]. As the highest MeHg concentration we used was 1µM, we may conclude that at low MeHg (*nano*molar) concentrations neither ROS generating mechanisms, nor ROS protective mechanisms or GSH levels are affected.

4.3 Effects of Combined Exposure of MeHg and POPs mixture on ΔΨm

Another cell parameter closely related with cell survival is the ΔΨm which as well is known to be affected by MeHg and organochlorines. Our experiment, investigating mitochondrial membrane depolarization, demonstrates that the 100x GM POPs mixture causes a loss of ΔΨm and that depolarization increases with increasing MeHg concentrations. But interestingly, in combination with the POPs mixture, increasing MeHg concentrations do not lead to an increased depolarization, but to reduced mitochondrial membrane depolarization, comparable to untreated cells.

This result is supported by several literature findings. According to a study by Tofighi et al. (2011) [121], MeHg can lead to mitochondrial membrane depolarization in hippocampal neurons and mitochondrial dysfunction [122]. The antagonistic effect of organochlorines and MeHg is in agreement with a study by Vettori et al. (2006) [123] that reports about antagonistic effects of PCB-153 and MeHg on lipid

peroxidation which is associated with mitochondrial membrane stability. A study supporting the ability of OCPs to depolarize the mitochondrial membrane is based upon the fact that mitochondrial calcium sequestration, which requires a membrane potential, is inhibited by PCBs [124), 125]. There is also evidence that MeHg has the ability to induce release of inner mitochondrial calcium stores [45), 46]. This occurs via the opening of the mitochondrial transition pore (MTP) and results in a membrane potential decrease [126]. This again is supported by a study of Limke and Atchison (2002) [46] demonstrating the ability of MeHg to open the MTP.

The opening of the MTP and consequent mitochondrial membrane depolarization occurs at intramitochondrial increased Ca^{2+} levels which will result in increased $[Ca^{2+}]_i$ levels. It is known that β-HCH and PCBs increase $[Ca^{2+}]_i$ [48), 49], although it is not clear whether the increase in $[Ca^{2+}]_i$ is a result of intramitochondrial calcium release or extracellular Ca^{2+} influx. In that case, β-HCH and PCBs might be responsible for the POPs mixture's ability to depolarize the inner mitochondrial membrane.

4.4 Effects of Combined MeHg-ACh and MeHg-DA Exposure on Cell Viability

The fact that MeHg and organochlorines affect cell physiology, like ΔΨm, and their increased presence in people with PD [62), 64), 7] , led to the idea of studying how neurotransmitter involved in PD pathology would influence cell viability in combination with environmental pollutants. It has been suggested that PD is based upon a DA-ACh imbalance, which is the result of a loss of dopaminergic neurons [70], while cholinergic neurons remain unaffected [78]. A similar situation occurs at prenatal exposure to MeHg: Developmental MeHg exposure is associated with a decreased number in dopaminergic neurons [104], while cholinergic neurons are not affected. As PD, associated with low dopamine levels, is linked to cortical atrophy too [66], we were interested in how DA and ACh would affect immature cortical neurons exposed to environmental pollutants.

Our results demonstrate that cells exposed to MeHg in presence of 100 µM ACh, show an increase in cell mortality at the "threshold value" of 100 µM MeHg.

Literature findings show that MeHg concentrations could increase ACh toxicity by increasing ACh concentrations which might be due to an inhibition of *ACh esterase* and an increase in ACh release [92), 93]. The toxicity may be a result of a raise in $[Ca^{2+}]_i$ which occurs after stimulation of nicotinic ACh receptors [127]. The increase in $[Ca^{2+}]_i$ is in particular toxic for undifferentiated neurons because they can't buffer big amounts of calcium yet [128]. We hypothesize that if MeHg inhibits *ACh esterase* and increases ACh release, the total ACh concentration and hence, the stimulation of nicotinic ACh receptors is increased. This results in increased, toxic $[Ca^{2+}]_i$ levels. As cells at 100 µM MeHg, the "threshold value", are in general more vulnerable, increased ACh concentration may lead to increased

cell death, compared to cells exposed to MeHg only. The fact that we do not see any increased cell mortality in MeHg concentrations higher than 100 nM, might be due to the toxicity of MeHg that seems to exceed ACh toxicity. The exact mode of action is not known, but may involve ACh receptors, as they can influence cell physiology with a broad variety of mechanisms, as explained in section 1.8.

The interactive toxicity of MeHg and ACh should also be taken in account as additional risk of smoking mothers who are exposed to mercury not only by fish consumption, but by cigarettes [(129)] too. Increased nicotine levels are found in foetuses of smoking mothers [(130)] that may also lead to increased ACh release [(131)]. This effect would even be worsened in combination with the mercury of the cigarettes and could lead to increased cell mortality.

Our experiments investigating DA show that cells exposed to toxic MeHg concentrations exhibit less mortality if co-exposed to 10 µM DA. We found the same effect if MeHg was combined with the POPs mixture.

On the look for possible mechanisms involved in this protective effect of 10 µM DA, we started by investigating glutamate-induced excitotoxicity, which can be elicited by MeHg exposure. This hypothesis was based upon a study by Vaarman et al. (2013) [(132)], who showed a protective effect of DA against glutamate-induced excitotoxicity. Although effective concentrations against glutamate-induced excitotoxicity were smaller than 10 µM DA, we wanted to study if 10 µM DA, which proved to be protective against MeHg in our case, would also protect against glutamate-induced excitotoxicity in combination with MeHg. Furthermore, we wanted to investigate the possible ability of a sub-toxic MeHg concentration to promote excitoxicity. But our results demonstrated that sub-toxic MeHg concentrations do not induce excitotoxicity, nor do 10 µM DA protect against glutamate-induced excitotoxicity. So we may conclude that 10 µM DA do not protect against glutamate-induced excitotoxicity by MeHg. Furthermore, the findings that MeHg and the POPs mixture do not induce excitotoxicity are in agreement with our findings that MeHg and POPs mixture do not induce ROS production: Excitotoxicity is known to increase ROS levels [(51)]. Therefore, no increase in excitotoxicity by MeHg and POPs mixture is in consonance with no rise in ROS production due to MeHg and POPs mixture.

Although we could not demonstrate any increase in excitotoxicity, Petroni et al. (2013) [(133)] provide evidence for an increase in toxicity in SH-SY5Y neuroblastoma cells if they were exposed to 50 nM MeHg in combination with 1 mM glutamate compared to glutamate only. But these cells seem to be generally more susceptible to MeHg: In this study 50 nM MeHg alone cause already a viability decrease

of 30%. Furthermore, 600 µM glutamate were highest glutamate concentration, compared to 1 mM in the previously mentioned study.

Therefore, our search for a possible explanation for the protective effect of DA continued and we focused on the D_2 receptor. Literature findings suggest that the D_2 receptor is responsible for the protective effect of DA [132], [134]. Despite this fact, we did not find any D_2 receptor-mediated protective effect, as we used the D_2 receptor antagonist raclopride at 2 µM to investigate a possible D_2 receptor effect. Although the IC_{50} of raclopride is 32 nM, we used a higher concentration to be sure that it would block efficiently in presence of 10 µM DA. But no effect was observed.

Concluding on the mechanisms of action of DA, we can say that the protective effect of 10 µM DA against MeHg-induced toxicity was not affected by additional OCP exposure. Furthermore, this protective effect is not due to effects involving mechanisms against excitotoxicity or D_2 receptor-mediated actions. Regarding the protective effect of DA, it should be further investigated whether other DA receptors or transporters are involved in this mechanism.

In conclusion, the results of our experiments, investigating the impact of DA and ACh in cortical neurons exposed to MeHg and organochlorines, highlight the link between prenatal MeHg exposure, DA-ACh imbalance and cortical atrophy in PD: Decreased number of dopaminergic neurons by prenatal MeHg exposure [104] leads in total to a loss of projections to the cortex resulting in a lack of DA there. This renders cortical neurons more susceptible to the toxic effects of MeHg, as the protective effect of DA is lacking. In turn, the presence of ACh, in combination with MeHg and organochlorines, might even contribute to the loss of cortical neurons, if the number of dopaminergic neurons, hence the dopamine concentration, is reduced. The reduced number of dopaminergic neurons, in turn, might increase the risk of developing PD and of cortical atrophy induced by low-dose MeHg.

4.5 Conclusions

Generally, our results are in agreement with relevant literature. Our findings demonstrate that at environmental concentrations, MeHg seems to be a greater risk for a developing nervous system than organochlorines. The mitochondrial membrane depolarizing effect of the OCPs and MeHg alone, which is counteracted by the combination of both, provides evidence for possible interactive effects of MeHg and organochlorine pollutants without affecting cell viability. Additionally, the cell mortality-increasing effect of combined MeHg and ACh exposure should be taken into account in further investigations on the topic of environmental pollutant-induced PD. This finding addresses also more awareness to the harmful effects of smoking while pregnancy. Foetuses of smoking mothers are exposed to both

compounds and thus are more vulnerable to neuronal degeneration. The beneficial effect of DA against MeHg neurotoxicity and in PD treatment suggests that the harmful effect of low-dose MeHg concentrations might be increased in PD patients as protecting DA levels are reduced.

Concluding, our experiments and literature provide evidence that MeHg alone or in combination with organochlorine pollutants or ACh can have harmful effects on human health and therefore should be further investigated. Interactions of MeHg with DA could provide useful information for the development of nutritional supplements for pregnant women in regions with a high mercury burden and could be a hint for further PD and PD progenitor cell transplant research.

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REFERENCES

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1. Clarkson TW, Magos L. The toxicology of mercury and its chemical compounds. Crit Rev Toxicol. 2006 Sep;36(8):609-62.

2. The global atmospheric mercury assessment: sources, emissions and transport. UNEP. 2013. Available at: http://www.unep.org/PDF/PressReleases/GlobalMercuryAssessment2013.pdf. Acessed September 9, 2014.

3. Toxicological Profile for Alpha-, Beta-, Gamma-, and Delta-Hexachlorocyclohexane. ATSDR. 2005. Available at: http://www.atsdr.cdc.gov/toxprofiles/tp43.pdf. Accessed September 5, 2014.

4. Toxicological Profile for DDT, DDE and DDE. ATSDR. 2002. Available at: http://www.atsdr.cdc.gov/toxprofiles/tp35.pdf. Accessed September 5, 2014.

5. Toxicological Profile for Polychlorinated Biphenyls (PCBs). ATSDR. 2000. Available at: http://www.atsdr.cdc.gov/toxprofiles/tp17.pdf. Accessed September 5, 2014.

6. Vinceti M, Bottecchi I, Fan A, Finkelstein Y, Mandrioli J. Are environmental exposures to selenium, heavy metals, and pesticides risk factors for amyotrophic lateral sclerosis? Rev Environ Health. 2012;27(1):19-41.

7. Dantzig PI. Parkinson's disease, macular degeneration and cutaneous signs of mercury toxicity. J Occup Environ Med. 2006 Jul;48(7):656.

8. Selin NE, Selin E. Global Politics of Mercury Pollution: The Need for Multi-Scale Governance. RECIEL. 2006 Nov;15(3):258–269.

9. History of the negotiations of the Stockholm Convention. Stock Conv. 2008. Available at: http://chm.pops.int/TheConvention/Overview/History/Overview/tabid/3549/Default.aspx. Accessed September 5, 2014.

10. What are POPs? Stock Conv. 2008. Available at: http://chm.pops.int/TheConvention/ThePOPs/tabid/673/Default.aspx. Accessed September 9, 2014.

11. Gilmour CC, Podar M, Bullock AL, Graham AM, Brown SD, Somenahally AC, Johs A, Hurt RA Jr, Bailey KL, Elias DA. Mercury methylation by novel microorganisms from new environments. Environ Sci Technol. 2013 Oct 15;47(20):11810-20.

12. Public Health Statement for Mercury. ATSDR. 1999. Available at: http://www.atsdr.cdc.gov/ToxProfiles/tp46-c1-b.pdf. Accessed September 9, 2014.

13. Syversen T, Kaur P. The toxicology of mercury and its compounds. J Trace Elem Med Biol. 2012 Oct;26(4):215-26.

14. Aberg B, Ekman L, Falk R, Greitz U, Persson G, Snihs JO. Metabolism of methyl mercury (203Hg) compounds in man. Arch Environ Health. 1969 Oct;19(4):478-84.

15. Choi BH, Lapham LW, Amin-Zaki L, Saleem T. Abnormal neuronal migration, deranged cerebral cortical organization, and diffuse white matter astrocytosis of human fetal brain: a major effect of methylmercury poisoning in utero. J Neuropathol Exp Neurol. 1978 Nov-Dec;37(6):719-33.

16. Ekino S, Susa M, Ninomiya T, Imamura K, Kitamura T. Minamata disease revisited: an update on the acute and chronic manifestations of methyl mercury poisoning. J Neurol Sci. 2007 Nov 15;262(1-2):131-44.

17. Ceccatelli S, Aschner M. Methylmercury and Neurotoxicity. Springer. New York i.a. 2012.

18. Skerfving SB, Copplestone JF. Poisening caused by the consumption of organo-mercury dressed seed in Iraq. Bull World Health Organ. 1976;54(1):101-12.

19. The 12 initial POPs under the Stockholm Convention. Stock Conv. 2001. Available at: http://chm.pops.int/TheConvention/ThePOPs/The12InitialPOPs/tabid/296/Default.aspx. Accessed September 9, 2014.

20. The new POPs under the Stockholm Convention. Stock Conv. 2009. Available at: http://chm.pops.int/TheConvention/ThePOPs/TheNewPOPs/tabid/2511/Default.aspx. Accessed September 9, 2014.

21. Chapter 22: Exposure and effects of airborne contamination for the Great Waters Program. UNEP. 1992. Available at: http://nepis.epa.gov/Exe/ZyPDF.cgi/91011NIN.PDF?Dockey=91011NIN.PDF. Accessed September 5, 2014.

22. Recognition and Management of Pesticide Poisonings.EPA. 2013. Available at: http://www2.epa.gov/sites/production/files/documents/rmpp_6thed_final_lowresopt.pdf. Accessed September 5, 2014.

23. Kannan N, Tanabe S, Tatsukawa R. Toxic potential of non-ortho and mono-ortho coplanar PCBs in commercial PCB preparations: "2,3,7,8-T4 CDD toxicity equivalence factors approach. Bull Environ Contam Toxicol. 1988 Aug;41(2):267-76.

24. Jarman WM, Ballschimtter K. From coal to DDT: the history of the development of the pesticide DDT from synthetic dyes till Silent Spring. Endeavour. 2012 Dec;36(4):131-42.

25. Stockholm Convention on Peristent Organic Pollutants (POPs) - as ammended in 2009 and 2011. Stock Conv. 2011. Available at: http://chm.pops.int/TheConvention/Overview/tabid/3351/Default.aspx. Accessed September 5, 2014.

26. Orris P, Chary LK, Perry K, Asbury J. Persistent organicoPollutants (POPs) and human health. WFPHA. 2000. Available at: http://www.wfpha.org/tl_files/doc/about/POPs%20WFPHA%202000.pdf. Accessed September 5, 2014.

27. Occupational Exposures in Insecticide Application and Some Pesticides. IARC. 1999. Available at: http://monographs.iarc.fr/ENG/Monographs/vol53/volume53.pdf. Accessed September 5, 2014.

28. Eskenazi B, Marks AR, Bradman A, Fenster L, Johnson C, Barr DB, Jewell NP. In utero exposure to dichlorodiphenyultrichloroethane (DDT) and dichlorodiphenyldichloroethylene (DDE) and neurodevelopment among young Mexican American children. Pediatrics. 2006 Jul;118(1):233-41.

29. A Review of Selected Persistent Organic Pollutants. WHO. 1995. Available at: http://www.who.int/ipcs/assessment/en/pcs_95_39_2004_05_13.pdf. Accessed September 5, 2014.

30. Jorgenson JL. Aldrin and dieldrin: a review of research on their production, environmental deposition and fate, bioaccumulation, toxicology, and epidemiology in the United States. Environ Health Perspect. Mar 2001; 109(Suppl 1): 113–139.

31. Briz V, Molina-Molina JM, Sánchez-Redondo S, Fernández MF, Grimalt JO, Olea N, Rodríguez-Farré E, Suñol C. Differential estrogenic effects of the persistent organochlorine pesticides dieldrin, endosulfan, and lindane in primary neuronal cultures. Toxicol Sci. 2011Apr;120(2):413-27.

32. Mably TA, Moore RW, Peterson RE. In utero and lactational exposure of male rats to 2,3,7,8-tetrachlorodibenzo-p-dioxin. 1. Effects on androgenic status. Toxicol Appl Pharmacol. 1992 May;114(1):97-107.

33. Hexachlorobenzene (Environmental Health Criteria 195). UNEP. 1997. Available at: http://www.inchem.org/documents/ehc/ehc/ehc195.htm. Accessed September 5, 2014.

34. Jemaa Z, Sabbah S, Driss MR, Bouguerra ML. Hexachlorobenzene in Tunisian mothers' milk, cord blood and foodstuffs. IARC Sci Publ, 1986;(77):139-42.

35. Exposure Assessment for Hexachlorbenzen. EPA. 1986. Available at: http://nepis.epa.gov/Exe/ZyPDF.cgi/9100AMPP.PDF?Dockey=9100AMPP.PDF. Accessed September 5, 2014.

36. Hung H, Kallenborn R, Breivik K, Su Y, Brorström-Lundén E, Olafsdottir K, Thorlacius JM, Leppänen S, Bossi R, Skov H, Manø S, Patton GW, Stern G, Sverko E, Fellin P. Atmospheric monitoring of organic pollutants in the Arctic under the Arctic Monitoring and Assessment Programme (AMAP): 1993-2006. Sci Total Environ. 2010 Jul 1;408(15). S. 2854-73.

37. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. IARC. 1987; suppl 7:219-220. Available at: http://monographs.iarc.fr/ENG/Monographs/suppl7/Suppl7.pdf. Accessed September 9, 2014.

38. Korrick SA, Sagiv SK. Polychlorinated biphenyls, organochlorine pesticides and neurodevelopment. Curr Opin Pediatr. 2008 Apr;20(2):198-204.

39. Nayyar N, Sangwan N, Kohli P, Verma H, Kumar R, Negi V, Oldach P, Mahato NK. Hexachlorocyclohexane: persistence, toxicity and decontamination. Rev Environ Health. 2014;29(1-2):49-52.

40. Hansen LG. Stepping backward to improve assessment of PCB congener toxicities. Environ Health Perspect. 1998 Feb;106 Suppl 1:171-89.

41. Farina M, Rocha JB, Aschner M. Mechanisms of methyl mercury-induced neurotoxicity: Evidence from experimental studies. Life Sci. 2011 Oct 10;89(15-16):555-63.

42. Bergen W. The in vitro effect of dieldrin on respiration of rat liver mitochondria. Proc Soc Exp Biol Med. 1971 Mar;136(3):732-5.

43. Pardini RS. Polychlorinated biphenyls (PCB): effect on mitochondrial enzyme systems. Bull Environ Contam Toxicol. 1971 Nov-Dec;6(6):539-45.

44. Akopova OV, Sagach VF. [Effect of Ca2+ on induction of the mitochondrial pore opening in the rat myocardium]. Ukr Biokhim Zh. 2004 Jan-Feb;76(1):48-55.

45. Chávez E, Holguín JA. Mitochondrial calcium release as induced by Hg2+. J Biol Chem. 1988 Mar 15;263(8):3582-7.

46. Limke TL, Atchison WD. Acute exposure to methylmercury opens the mitochondrial permeability transition pore in rat cerebellar granule cells. Toxicol Appl Pharmacol. 2002 Jan 1;178(1):52-61.

47. Briz V, Galofré M, Suñol C. Reduction of glutamatergic neurotransmission by prolonged exposure to dieldrin involves NMDA receptor internalization and metabotropic glutamate receptor 5 downregulation. Toxicol Sci.2010 Jan;113(1):138-49.

48. Joy RM, Burns VW. Exposure to lindane and two other hexachlorocyclohexane isomers increases free intracellular calcium levels in neurohybridoma cells. Neurotoxicology. 1988 Winter;9(4):637-43.

49. Magi S, Castaldo P, Carrieri G, Scorziello A, Di Renzo G, Amoroso S. Involvement of Na+-Ca2+ exchanger in intracellular Ca2+ increase and neuronal injury induced by polychlorinated biphenyls in human neuroblastoma SH-SY5Y cells. J Pharmacol Exp Ther. 2005 Oct;315(1):291-6.

50. Hidalgo C, Donoso P. Crosstalk between calcium and redox signaling: from molecular mechanisms to health implications. Antioxid Redox Signal. 2008 Jul;10(7):1275-312.

51. Allen JW, Mutkus LA, Aschner M. Methylmercury-mediated inhibition of 3H-D-aspartate transport in cultured astrocytes is reversed by the antioxidant catalase. Brain Res. 2001;902(1):92–100.

52. Elliot WH, Elliot D. Biochemistry of Molecular Biology. Oxford University Press. New York. 2001.

53. Franco JL, Posser T, Dunkley PR, Dickson PW, Mattos JJ, Martins R, Bainy AC, Marques MR, Dafre AL, Farina M. Methylmercury neurotoxicity is associated with inhibition of the antioxidant enzyme glutathione peroxidase. Free Radic Biol Med. 2009 Aug 15;47(4):449-57.

54. Makary M, Kim HL, Safe S, Womack J, Ivie GW. Constitutive and Aroclor 1254-induced hepatic glutathione S-transferase, peroxidase and reductase activities in genetically inbred mice. Comp Biochem Physiol C. 1988;91(2):425-9.

55. Simonetti J, Berner J, Williams K. Effects of p,p'-DDE on immature cells in culture at concentrations relevant to the Alaskan environment. Toxicol In Vitro. 2001 Apr;15(2):169-79.

56. Atchison WD, Hare MF. Mechanisms of methylmercury-induced neurotoxicity. FASEB J. 1994 Jun;8(9):622-9.

57. Stringari J, Nunes AK, Franco JL, Bohrer D, Garcia SC, Dafre AL, Milatovic D, Souza DO, Rocha JB, Aschner M, Farina M. Prenatal methylmercury exposure hampers glutathione antioxidant system ontogenesis and causes long-lasting oxidative stress in the mouse brain. Toxicol Appl Pharmacol. 2008 Feb 15;227(1):147-54.

58. Vogel DG, Margolis RL, Mottet NK. The effects of methyl mercury binding to microtubules. Toxicol Appl Pharmacol. 1985 Sep 30;80(3):473-86.

59. Miura K, Kobayashi Y, Toyoda H, Imura N. Methylmercury-induced microtubule depolymerizytion leads to inhibition of tubulin synthesis. J Toxicol Sci. 1998 Dec;23(5):379-88.

60. Tang F, Yan C, Wu S, Li F, Yu Y, Gao Y, Jin X, Shen X. Polychlorinated biphenyls disrupt the actin cytoskeleton in hippocampal neurons. Environ Toxicol Pharmacol. 2007 Mar;23(2):140-6.

61. Dent EW, Kalil K. Axon Branching Requires Interactions between Dynamic. J Neurosci. 2001 Dec 15;21(24):9757-69.

62. Parrón T, Requena M, Hernández AF, Alarcón R. Association between environmental exposure to pesticides and neurodegenerative diseases. Toxicol Appl Pharmacol. 2011 Nov 1;256(3):379-85.

63. Mutter J, Naumann J, Schneider R, Walach H. [Mercury and Alzheimer's disease]. Fortschr Neurol Psychiatr. 2007 Sep;75(9):528-38.

64. Ngim CH, Devathasan G. Epidemiologic study on the association between body burden mercury level and idiopathic Parkinson's disease. Neuroepidemiology. 1989;8(3):128-41.

65. Kondo K. Rising prevalence of neurodegenerative diseases worldwide. Intern Med. 1996 Apr;35(4):238.

66. Song SK, Lee JE, Park HJ, Sohn YH, Lee JD, Lee PH. The pattern of cortical atrophy in patients with Parkinson's disease according to cognitive status. Mov Disord. 2011 Feb 1;26(2):289-96.

67. Differences between subacute and chronic MPTP mice models: investigation of dopaminergic neuronal degeneration and alpha-synuclein inclusions. Gibrat C, Saint-Pierre M, Bousquet M, Lévesque D, Rouillard C, Cicchetti F. J Neurochem. 2009 Jun;109(5):1469-82.

68. Age at Onset in Two Common Neurodegenerative Diseases Is Genetically Controlled. Li YJ, Scott WK, Hedges DJ, Zhang F, Gaskell PC, Nance MA, Watts RL, Hubble JP, Koller WC, Pahwa R, Stern MB, Hiner BC, Jankovic J, Allen FA Jr, Goetz CG, Mastaglia F, Stajich JM, Gibson RA, Middleton LT, Saunders AM, Scott BL, Small GW, Nicodemus KK, Ree. Am J Hum Genet. 2002 Apr;70(4):985-93.

69. Spehlmann R, Stahl SM. Dopamine acetylcholine imbalance in Parkinson's disease. Possible regenerative overgrowth of cholinergic axon terminals. Lancet. 1976 Apr 3;1(7962):724-6.

70. Samii A, Nutt JG, Ransom BR. Parkinson's disease. Lancet. 2004 May 29;363(9423):1783-93.

71. Nakano I, Hirano A. Parkinson's disease: neuron loss in the nucleus basalis without concomitant Alzheimer's disease. Ann Neurol. 1984 May;15(5):415-8.

72. Poeck K, Hacke W. Neurologie. Springer Medizin. Heidelberg. 2006.

73. Svendsen CN, Caldwell MA, Shen J, ter Borg MG, Rosser AE, Tyers P, Karmiol S, Dunnett SB. Long-term survival of human central nervous system progenitor cells transplanted into a rat model of Parkinson's disease. Exp Neurol. 1997 Nov;148(1):135-46.

74. Wang Q, Matsumoto Y, Shindo T, Miyake K, Shindo A, Kawanishi M, Kawai N, Tamiya T, Nagao S. Neural stem cells transplantation in cortex in a mouse model of Alzheimer's disease. J Med Invest. 2006 Feb;53(1-2):61-9.

75. Samii A, Calne DB. Research into the etiology of Parkinson's disease. In Oertel W, LeWitt PA, eds. Parkinson's disease: the treatment options. Martin Dunitz Publishers. London. 1999.

76. Calabresi P, Picconi B, Parnetti L, Di Filippo M. A convergent model for cognitive dysfunctions in Parkinson's disease: the critical dopamine-acetylcholine synaptic balance. Lancet Neurol. 2006 Nov;5(11):974-83.

77. Schapira A, Olanow C. Principles of Treatment in Parkinson's Disease. Butterworth Heinemann. 2005.

78. Ziegler DA, Wonderlick JS, Ashourian P, Hansen LA, Young JC, Murphy AJ, Koppuzha CK, Growdon JH, Corkin S. Substantia nigra volume loss before basal forebrain degeneration in early Parkinson disease. JAMA Neurol. 2013 Feb;70(2):241-7.

79. Richardson JR, Roy A, Shalat SL, Buckley B, Winnik B, Gearing M, Levey AI, Factor SA, O'Suilleabhain P, German DC. β-Hexachlorocyclohexane levels in serum and risk of Parkinson's disease. Neurotoxicology. 2011 Oct;32(5):640-5.

80. Koldkjaer OG, Wermuth L, Bjerregaard P. Parkinson's disease among Inuit in Greenland: organochlorines as risk factors. Int J Circumpolar Health. 2004;63 Suppl 2:366-8.

81. Elbaz A, Clavel J, Rathouz PJ, Moisan F, Galanaud JP, Delemotte B, Alpérovitch A, Tzourio C. Professional exposure to pesticides and Parkinson disease. Ann Neurol. 2009 Oct;66(4):494-504.

82. Eggermann E, Feldmeyer D. Cholinergic filtering in the recurrent excitatory microcircuit of cortical layer 4. Proc Natl Acad Sci U S A. 2009 Jul 14;106(28):11753-8.

83. McGaughy J, Kaiser T, Sarter M. Behavioral vigilance following infusions of 192 IgG-saporin into the basal forebrain: selectivity of the behavioral impairment and relation to cortical AChE-positive fiber density. Behav Neurosci. 1996 Apr;110(2):247-65.

84. Gil Z, Connors BW, Amitai Y. Differential Regulation of Neocortical Synapses by Neuromodulators and Activity. Neuron. 1997 Sep;19(3):679-86.

85. Higley MJ, Picciotto MR. Neuromodulation by acetylcholine: examples from schizophrenia and depression. Curr Opin Neurobiol. 2014 Jun 28;29C:88-95.

86. Anand P, Singh B. A review on cholinesterase inhibitors for Alzheimer's disease. Arch Pharm Res. 2013 Apr;36(4):375-99.

87. Bear MF, Connors BW, Paradiso MA. Neurowissenschaften-Ein grundlegendes Lehrbuch für Biologie, Medizin und Psychologie. Springer. Heidelberg. 2008.

88. Resende RR, Adhikari A. Cholinergic receptor pathways involved in apoptosis, cell proliferation and neuronal differentiation. Cell Commun Signal. 2009 Aug 27;7:20.

89. Rotter A, Birdsall NJ, Burgen AS, Field PM, Hulme EC, Raisman G. Muscarinic receptors in the central nervous system of the rat. I. Technique for autoradiographic localization of the binding of [3H]propylbenzilylcholine mustard and its distribution in the forebrain. Brain Res. 1979;180(2):141-65.

90. Alkondon M, Pereira EF, Eisenberg HM, Albuquerque EX. Nicotinic receptor activation in human cerebral cortical interneurons: a mechanism for inhibition and disinhibition of neuronal networks. J Neurosci. 2000 Jan 1;20(1):66-75.

91. Eldefrawi ME, Mansour NA, Eldefrawi AT. Interactions of acetylcholine receptors with organic mercury compounds. Adv Exp Med Biol. 1977;84:449-63.

92. Suresh A, Sivaramakrishna B, Victoriamma PC, Radhakrishnaiah K. Comparative study on the inhibition of acetylcholinesterase activity in the freshwater fish Cyprinus carpio by mercury and zinc. Biochem Int. 1992 Feb;26(2):367-75.

93. Saijoh K, Inoue Y, Sumino K. Stimulating effect of methylmercury chloride on [(3)H]acetylcholine release from guinea-pig striatal slices. Toxicol In Vitro. 1987;1(4):233-7.

94. Cenci MA. Dopamine dysregulation of movement control in L-DOPA-induced dyskinesia. Trends Neurosci. 2007 May;30(5):236-43.

95. Ranaldi R. Dopamine and reward seeking: the role of ventral tegmental area. Rev Neurosci. 2014 May 28.

96. Garcia-Borreguero D, Williams AM. An update on restless legs syndrome (Willis-Ekbom disease): clinical features, pathogenesis and treatment. Curr Opin Neurol. 2014 Aug;27(4).

97. Koutsilieri E, Riederer P, du Plessis S, Scheller C. A short review on the relation between the dopamine transporter 10/10-repeat allele and ADHD: implications for HIV infection. Atten Defic Hyperact Disord. 2014 Apr 3.

98. Brisch R, Saniotis A, Wolf R, Bielau H, Bernstein HG, Steiner J, Bogerts B, Braun AK, Jankowski Z, Kumaritlake J, Henneberg M, Gos T. The role of dopamine in schizophrenia from a neurobiological and evolutionary perspective: old fashioned, but still in vogue. Front Psychiatry. 2014 May 19;5:47.

99. Cooper J, Bloom F, Roth R. The Biochemical Basis of Neuropharmacology. Oxford University Press. New York. 1991.

100. Dunnett SB, Bentivoglio M, Björklund A, Hökfelt T. Dopamine. Elsevier Science. Amsterdam i.a. 2005.

101. Beaulieu JM, Gainetdinov RR. The physiology, signaling, and pharmacology of dopamine receptors. Pharmacol Rev. 2011 Mar;63(1):182-217.

102. Braestrup C, Andersen PH. Effects of heavy metal cations and other sulfhydryl reagents on brain dopamine D1 receptors: evidence for involvement of a thiol group in the conformation of the active site. J Neurochem. 1987 Jun;48(6):1667-72.

103. Daré E, Fetissov S, Hökfelt T, Hall H, Ogren SO, Ceccatelli S. Effects of prenatal exposure to methylmercury on dopamine-mediated locomotor activity and dopamine D2 receptor binding. Naunyn Schmiedebergs Arch Pharmacol. 2003 May;367(5):500-8.

104. Bastian Zimmer, Stefan Schildknecht1, Philipp B. Kuegler1, Vivek Tanavde2, Suzanne Kadereit1 and Marcel Leist1. Sensitivity of dopaminergic neuron differentiation from stem cells to chronic low-dose methylmercury exposure. Toxicol Sci. 2011 Jun;121(2):357-67.

105. Mariussen E, Fonnum F. The effect of polychlorinated biphenyls on the high affinity uptake of the neurotransmitters, dopamine, serotonin, glutamate and GABA, into rat brain synaptosomes. Toxicology. 2001 Feb 21;159(1-2):11-21.

106. Choksi NY, Kodavanti PR, Tilson HA, Booth RG. Effects of polychlorinated biphenyls (PCBs) on brain tyrosine hydroxylase activity and dopamine synthesis in rats. Fundam Appl Toxicol. 1997 Sep;39(1):76-80.

107. Hatcher JM, Delea KC, Richardson JR, Pennell KD, Miller GW. Disruption of dopamine transport by DDT and its metabolites. Neurotoxicology. 2008 Jul;29(4):682-90.

108. Guxens M, Ballester F, Espada M, Fernández MF, Grimalt JO, Ibarluzea J, Olea N, Rebagliato M, Tardón A, Torrent M, Vioque J, Vrijheid M, Sunyer J. INMA. Cohort Profile: the INMA--INfancia y Medio Ambiente--(Environment and Childhood) Project. Int J Epidemiol. 2012 Aug;41(4):930-40.

109. Vizcaino E, Grimalt JO, Lopez-Espinosa MJ, Llop S, Rebagliato M, Ballester F. Maternal origin and other determinants of cord serum organochlorine compound concentrations in infants from the general population. Environ Sci Technol. 2010 Aug 15;44(16):6488-95.

110. Noh JS, Kim EY, Kang JS, Kim HR, Oh YJ, Gwag BJ. Neurotoxic and neuroprotective actions of catecholamines in cortical neurons. Exp Neurol. 1999 Sep;159(1):217-24.

111. Parent AS, Naveau E, Gerard A, Bourguignon JP, Westbrook GL. Early developmental actions of endocrine disruptors on the hypothalamus, hippocampus, and cerebral cortex. J Toxicol Environ Health B Crit Rev. 2011;14(5-7):328-45.

112. Suñol C, Babot Z, Fonfría E, Galofré M, García D, Herrera N, Iraola S, Vendrell I. Studies with neuronal cells: From basic studies of mechanisms of neurotoxicity to the prediction of chemical toxicity. Toxicol In Vitro. 2008 Aug;22(5):1350-5.

113. White MJ, DiCaprio MJ, Greenberg DA. Assessment of neuronal viability with Alamar blue in cortical and granule cell cultures. J Neurosci Methods. 1996 Dec 28.

114. Shinyashiki M, Kumagai Y, Homma-Takeda S, Nagafune J, Takasawa N, Suzuki J, Matsuzaki I, Satoh S, Sagai M, Shimojo N. Selective inhibition of the mouse brain Mn-SOD by methylmercury. Environ Toxicol Pharmacol. 1996 Dec 20;2(4):359-66.

115. Coccini T, Roda E, Castoldi AF, Poli D, Goldoni M, Vettori MV, Mutti A, Manzo L. Developmental exposure to methylmercury and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153) affects cerebral dopamine D1-like and D2-like receptors of weanling and pubertal rats. Arch Toxicol. 2011 Oct;85(10).

116. Omara FO, Brochu C, Flipo D, Denizeau F, Fournier M. Immunotoxicity of environmentally relevant mixtures of polychlorinated aromatic hydrocarbons with methyl mercury on rat lymphocytes in vitro. Environ Toxicol Chem. 1997 March;16(3):576–581.

117. Buttke TM, Sandstrom PA. Oxidative stress as a mediator of apoptosis. Immunol Today. 1994 Jan;15(1):7-10.

118. Gatti R, Belletti S, Uggeri J, Vettori MV, Mutti A, Scandroglio R, Orlandini G. Methylmercury cytotoxicity in PC12 cells is mediated by primary glutathione depletion independent of excess reactive oxygen species generation. Toxicology. 2004 Nov 15;204(2-3):175-85.

119. Ali SF, LeBel CP, Bondy SC. Reactive oxygen species formation as a biomarker of methylmercury and trimethyltin neurotoxicity. Neurotoxicology. 1992 Fall;13(3):637-48.

120. Kaur P, Aschner M, Syversen T. Glutathione modulation influences methyl mercury induced neurotoxicity in primary cell cultures of neurons and astrocytes. Neurotoxicology. 2006 Jul;27(4):492-500.

121. Tofighi R, Johansson C, Goldoni M, Ibrahim WN, Gogvadze V, Mutti A, Ceccatelli S. Hippocampal Neurons Exposed to the Environmental Contaminants Methylmercury and Polychlorinated Biphenyls Undergo Cell Death via Parallel Activation of Calpains and Lysosomal Proteases. Neurotox Res. 2011 Jan;19(1):183-94.

122. Carocci A, Rovito N, Sinicropi MS, Genchi G. Mercury toxicity and neurodegenerative effects. Rev Environ Contam Toxicol. 2014;229:1-18.

123. Vettori MV, Goldoni M, Caglieri A, Poli D, Folesani G, Ceccatelli S, Mutti A. Antagonistic effects of methyl-mercury and PCB153 on PC12 cells after a combined and simultaneous exposure. Food Chem Toxicol. 2006 Sep;44(9):1505-12.

124. Rottenberg H, Scarpa A. Calcium uptake and membrane potential in mitochondria. Biochemistry. 1974 Nov 5;13(23):4811-7.

125. Kodavanti PR, Ward TR, McKinney JD, Tilson HA. Inhibition of microsomal and mitochondrial Ca2+-sequestration in rat cerebellum by polychlorinated biphenyl mixtures and congeners. Arch Toxicol. 1996;70(3-4):150-7.

126. Hüser J, Blatter LA. Fluctuations in mitochondrial membrane potential caused by repetitive gating of the permeability transition pore. Biochem J. 1999 Oct 15;343 Pt 2:311-7.

127. Dajas-Bailador F, Wonnacott S. Nicotinic acetylcholine receptors and the regulation of neuronal signalling. Trends Pharmacol Sci. 2004 Jun;25(6):317-24.

128. Berger F, Gage FH, Vijayaraghavan S. Nicotinic Receptor-Induced Apoptotic Cell Death of Hippocampal Progenitor Cells. J Neurosci. 1998 Sep 1;18(17):6871-81.

129. Suzuki T, Shishido S, Urushiyama K. Mercury in cigarettes. Tohoku J Exp Med, 1976 Aug;119(4):353-6.
130. Lambers DS, Clark KE. The maternal and fetal physiologic effects of nicotine. Semin Perinatol. 1996 Apr;20(2):115-26.

131. Armitage AK, Hall GH, Sellers CM. Effects of nicotine on electrocortical activity and acetylcholine release from the cat cerebral cortex. Br J Pharmacol. 1969 Jan;35(1):152-60.

132. Vaarmann A, Kovac S, Holmström KM, Gandhi S, Abramov AY. Dopamine protects neurons against glutamate-induced excitotoxicity. Cell Death Dis. 2013 Jan 10;4.

133. Petroni D, Tsai J, Mondal D, George W. Attenuation of low dose methylmercury and glutamate induced-cytotoxicity and tau phosphorylation by an N-methyl-D-aspartate antagonist in human neuroblastoma (SHSY5Y) cells. Environ Toxicol. 2013 Dec;28(12):700-6.

134. Bozzi Y, Borrelli E. Dopamine in neurotoxicity and neuroprotection: what do D2 receptors have to do with it? Trends Neurosci. 2006 Mar;29(3):167-74.

ABSTRACT

Methylmercury's (MeHg) devastating health effects were demonstrated in the Minamata disease outbreak in Japan when people were exposed over decades to low MeHg concentrations, which resulted in severe brain damage and long-term effects affecting the central nervous system. But MeHg is not the only perfidious compound causing harm to the human brain at chronic low-dose exposure. Organochlorine compounds, used as pesticides or in industry, and present in daily life have a big potential to cause neurological damage too. Exposure to both groups of toxicants, MeHg and organochlorine compounds, are linked to neurodegenerative diseases, including Parkinson's disease. We wanted to investigate cytotoxic effects of MeHg in combination with various organochlorine compounds and neurotransmitters involved in Parkinson's disease pathology: acetylcholine and dopamine.

We did not find any interactive cytotoxic effect between MeHg and organochlorines. Furthermore, there was also no evidence for interactive effects of MeHg and organochlorines involved in oxidative stress. But measurements of the mitochondrial membrane potential showed that a mixture of various organochlorine compounds and MeHg, depolarizes mitochondrial membrane, but in combination counteract mitochondrial membrane depolarization. By researching MeHg in combination with Acetylcholine we could show that 100 µM acetylcholine can increase toxicity at MeHg concentrations of little toxicity. Furthermore, dopamine seems to have a protective effect against MeHg toxicity, which is not diminished by additional exposure to a mixture of numerous organochlorines. Moreover, we found that neither D_2-receptor mediated actions, nor protection against glutamate-induced excitotoxicty is the mode of action in dopamine's protective actions.

We hypothesize that the mitochondrial membrane depolarization occurs due to opening of the mitochondrial membrane transition pore induced by intramitochondrial rise in Ca^{2+} which can be induced, according to literature findings, by MeHg and various organochlorines. The interactive harmful effect of acetylcholine and interactive protective effect of dopamine in combination with environmental pollutants provide useful information for neuronal progenitor cell transplantations, a potential cure for neurodegenerative diseases, in people with high environmental pollutant burden.

ABSTRACT (DEUTSCH)

Methylquecksilbers (MeHg) verheerende Auswirkungen auf die Gesundheit wurden bei Ausbruch der Minamata-Krankheit in Japan deutlich, als die lokale Bevölkerung über Jahrzehnte hinweg niedrigen Dosen von MeHg ausgesetzt war, was zu schweren Hirnschäden und Schädigungen des Zentralen Nervensystems führte. Aber MeHg ist nicht das einzige Umweltgift, das dem menschlichen Gehirn, sogar in sehr geringen Dosen, Schaden zufügen kann. Organochlorine, die als Pestizide oder in der Industrie Verwendung finden, und denen man im täglichen Leben ausgesetzt ist, haben auch großes Potential neurologische Erkrankungen hervorzurufen. Belastung mit Umweltgiften, wie MeHg und Organochlorine, sind mit neurodegenerativen Erkrankungen assoziiert, unter anderem Parkinson. Wir wollten mögliche cytotoxische Effekte von MeHg in Kombination mit diversen Organochlorinen und Neurotransmittern, die in der Pathologie von Parkinson eine Rolles spielen, nämlich Acetylcholin und Dopamin, erforschen.

Wir konnten keine interaktiven cytotoxischen Effekte zwischen MeHg und Organochlorinen finden, ebenso wenig wie interaktive Effekte zwischen MeHg und Organochlorinen in Bezug auf oxidativen Stress. Allerdings konnten wir zeigen, dass MeHg und ein Mix aus Organochlorinen die innnere Mitochondrienmembran teilweise depolarisieren kann. Aber in Kombination von MeHg und Organochlorin-Mix, kommt es zu einem der Depolarisation entgegengesetzten Effekt. Außerdem wurde gezeigt, dass 100 µM Ach -alleine ein sub-toxische Konzentration- in Kombination mit 100 nM MeHg zu vermehrtem Zellensterben führt. Außerdem haben unsere Experimente gezeigt, dass Dopamin schützend gegen die toxischen Effekte von MeHg wirkt. Dieser Effekt wird nicht von dem Organochlorin-Mix beeinflusst. Wir fanden heraus, dass der schützende Effekt gegen MeHg nicht auf einem Schutz vor Exzitotoxizität oder Mechanismen auf Grund einer Dopamin D_2-Rezeptor-Aktivierung beruht.

Wir denken, dass die Depolarisation der mitochondriellen Membran auf der Öffnung der „mitochondrial transition pore“ durch Zunahme an intramitochondriellen Kalziumkonzentration basiert. Die Zunahme intramitochondrieller Kalziumkonzentrationen kann, laut Literatur, durch MeHg oder Organochlorine hervorgerufen werden. Der interaktive schädliche Effekt von Acetylcholin und der interaktive schützende Effekt von Dopamin in Kombination mit Umweltgiften liefern nützliche Informationen für die Erforschung eine mögliche Therapie für Parkinson-Patienten, die mit hohen körperlichen Konzentrationen an Umweltgiften belastet sind.

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